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Selective inhibition of meningeal nociceptors by botulinum neurotoxin type A: Therapeutic implications for migraine and other pains

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Abstract

Background: Meningeal and other trigeminal nociceptors are thought to play important roles in the initiation of migraine headache. Currently, the only approved peripherally administered chronic migraine prophylactic drug is onabotulinumtoxinA. The purpose of this study was to determine how botulinum neurotoxin type A (BoNT-A) affects naïve and sensitized meningeal nociceptors.

Material and methods: Using electrophysiological techniques, we identified 43 C- and 36 Aδ-meningeal nociceptors, and measured their spontaneous and evoked firing before and after BoNT-A administration to intracranial dura and extracranial suture-receptive fields.

Results: As a rule, BoNT-A inhibited C- but not Aδ-meningeal nociceptors. When applied to nonsensitized C-units, BoNT-A inhibited responses to mechanical stimulation of the dura with suprathreshold forces. When applied to sensitized units, BoNT-A reversed mechanical hypersensitivity. When applied before sensitization, BoNT-A prevented development of mechanical hypersensitivity. When applied extracranially to suture branches of intracranial meningeal nociceptors, BoNT-A inhibited the mechanical responsiveness of the suture branch but not dural axon. In contrast, BoNT-A did not inhibit C-unit responses to mechanical stimulation of the dura with threshold forces, or their spontaneous activity.

Discussion: The study provides evidence for the ability of BoNT-A to inhibit mechanical nociception in peripheral trigeminovascular neurons. These findings suggest that BoNT-A interferes with neuronal surface expression of high-threshold mechanosensitive ion channels linked preferentially to mechanical pain by preventing their fusion into the nerve terminal membrane.

Keywords

Pain, nociception, headache, TRPV1, TRPA1, high-threshold mechanosensitive ion channels

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Introduction

Migraine

Migraine is a complex neurological disorder involving multiple sensory pathways, emotional networks, autonomic systems and cortical functions. The pain-related symptoms are similar to those experienced in other pain states and thus fit into a scientific framework generally consistent with the current understanding of chronic pain states. Under normal conditions, pain-sensing organs (and pathways) are designed to be sufficiently sensitive and function as an early warning mechanism for avoidance of potentially harmful stimuli (1). Under pathological conditions, however, pain-processing systems can change their state in a way

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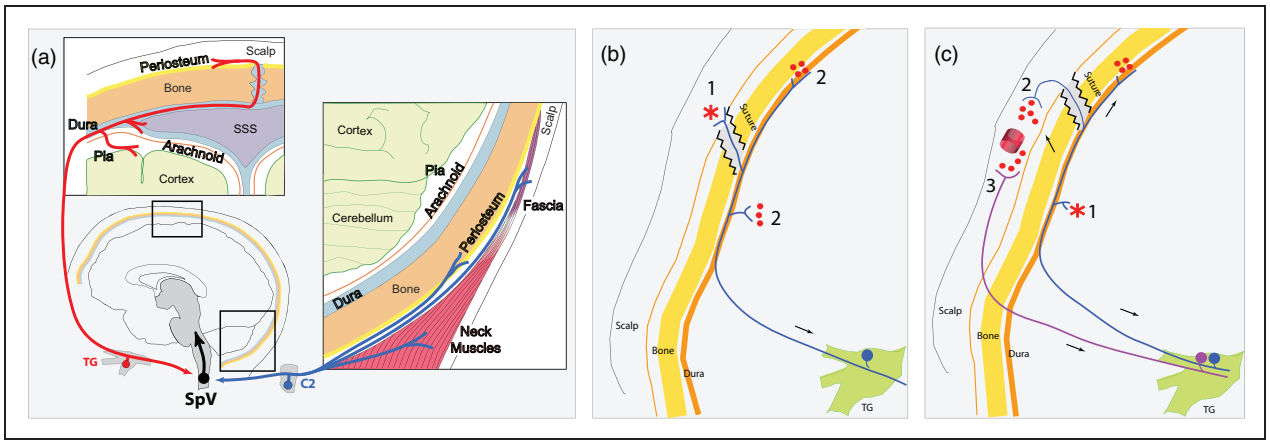

Figure 1. Peripheral innervation of intracranial and extracranial structures relevant to migraine. (a) Schematic illustration of peripheral nerves that carry sensory/nociceptive information from pericranial muscles, fascia and periosteum through the greater occipital nerve (blue) and from the intracranial dura and pia and extracranial periosteum through meningeal nociceptors that travel along intracranial nerves (red, such as the tentorial nerve). (b) Possible scenario of extracranial origin of intracranial pain. In this scenario, action potentials generated at extracranial collaterals of meningeal pain fibers (1) spread antidromically to collaterals that terminate inside the cranium, resulting in local release of proinflammatory neuropeptides and activation of neighboring meningeal nociceptors (2). (c) Possible scenario of intracranial origin of extracranial pain. In this scenario, action potentials generated at intracranial meningeal pain fibers (1) spread antidromically to collaterals that terminate outside the cranium (2), resulting in local release of proinflammatory neuropeptides in the scalp and activation of neighboring somatic nociceptors (3). Asterisk marks original site of activation. Red dots represent local release of inflammatory neuropeptides. Red cylinder depicts a blood vessel. SpV: spinal trigeminal nucleus; SSS: superior sagittal sinus; TG: trigeminal ganglion.
Source: Panels (b) and (c) adapted with permission from Kosaras B, Jakubowski M, Kainz V, et al. Sensory innervation of the calvarial bones of the mouse. *J Comp Neurol* 2009; 515: 331–348 (4).

that leads to the perception of pain in the absence of a noxious stimulus and consequently provide no benefit to the individual. While central nervous system (CNS) pathologies that predispose an individual to migraine attacks, and factors leading to attack initiation, are not fully elucidated, it is generally believed that some aspects of migraine and some of its associated symptoms reflect abnormal excitability in both central and peripheral neurons.

The trigeminovascular system

The heavily studied trigeminovascular pathway is thought to play a key role in the headache phase of a migraine attack. It consists of (a) first-order sensory neurons of the trigeminal nerve whose axons convey nociceptive information from the face, skull, meninges and intracranial blood vessels; (b) second-order neurons of the spinal trigeminal nucleus, extending between the obex and the upper three segments of the cervical spinal cord, that receive input from the intracranial meninges, occipital region of the head and neck muscles; (c) third-order thalamic neurons; and (d) higher-order neurons in cortical regions that process this information. Relevant to the current study is the notion that intrinsic neuronal hypersensitivity in localized regions of the spinal trigeminal nucleus reflects sensory and nociceptive signals that come from any one of the many organs supplied by meningeal and ophthalmic branches of the trigeminal nerve, and branches of the greater and lesser occipital nerves. These organs include the intracranial meninges and blood vessels, head and neck muscles, and the different layers of the scalp (Figure 1). Physiologically, as in other pain conditions, the clinical manifestation of intrinsic neuronal hyperexcitability include (a) allodynia whereby pain thresholds are lowered so that normally innocuous stimuli become painful, (b) hyperalgesia whereby noxious stimuli produce exaggerated and prolonged pain, and (c) pain that persists long after injury or pain stimulus. Current knowledge supports the view that, like in other chronic pain states, the pathophysiology of chronic migraine and some of its associated clinical symptoms involve peripheral and central sensitization.

Peripheral and central sensitization

Peripheral sensitization is a term used to define a state in which primary afferent nociceptive neurons exhibit increased responsiveness to external mechanical or thermal stimuli at the site of inflammation or injury (2). The functional manifestation of peripheral sensitization includes novel responses to previously

subthreshold stimulus intensities, increased response magnitude to suprathreshold stimuli, and increased level of ongoing discharge in the absence of externally applied stimuli (3,5,6).

Central sensitization is a term used to define a condition in which nociceptive neurons in the spinal and medullary dorsal horn exhibit increased excitability, increased synaptic strength, and enlargement of their receptive fields beyond the original site of inflammation or injury (7–9). Central sensitization is triggered by sensory inputs arriving from sensitized nociceptors. Once initiated, the sensitization of central neurons may remain dependent on incoming input or become self-sufficient altogether.

Possible scenarios for 'peripheral' activation of the trigeminovascular system

Possible scenarios for activation of the trigeminovascular pathway through its peripheral limb include activation of the nociceptors by cortical spreading depression (10,11), mast cell degranulation, which may be in proximity with peptide-containing nerve fibers (12), neurogenic inflammation (13,14), hydrogen ions (15), adenosine triphosphate (ATP) release from intracranial endothelial blood vessels (16), and mild trauma to the head (17–19). Receptor-wise, activation/upregulation of meningeal nociceptors by each of the above-mentioned scenarios may involve one or more of the many receptors found in the dura. These include receptors such as the transient receptor potential (TRP) channels TRPV1, TRPA1 and TRPM8 (20), ATP-gated P2X3 receptors (21), dopaminergic D1 and D2 receptors (22), serotonergic 5HT1b/1d receptors (23,24), the calcitonin gene-related peptide (CGRP) receptor calcitonin receptor-like receptor/receptor activity-modifying protein-1 (CRLR/RAMP1) (25), tumor necrosis factor (TNF)-α (26), and acid-sensing ion channel 3 (ASIC3) (15). Collectively, these receptors can promote mechanical and chemical activation and/or sensitization of meningeal nociceptors. Once activated and sensitized, meningeal nociceptors are thought to bombard the second-order trigeminovascular neurons with a barrage of action potentials, resulting in a massive release of glutamate and the development of central sensitization.

Anatomical substrate of extracranial/intracranial interactions

The anatomical ballpark in which activation of peripheral nociceptors may contribute to the pathophysiology of chronic migraine is conceived based on the following: (a) Trigeminal sensory fibers are pseudounipolar cells (27), capable of conducting action potentials in both orthodromic (toward the cell body—away from the nerve ending) and antidromic (away from the cell body—toward the nerve ending) directions. (b) Intracranial meningeal nociceptors (supplying the dura and pia) that issue collateral branches that cross the calvarial sutures from inside to outside the head (4,28,29) convey to the spinal trigeminal nucleus nociceptive signals that originate in the pia (cortical surface), dura, calvarial periosteum and potentially pericranial muscles (Figure 1). (c) Sensory fibers of the six extracranial nerves that supply the head (greater occipital, lesser occipital, auriculotemporal, zygomaticotemporal, supraorbital, supratrochlear) are also capable of conveying sensory and nociceptive signals from the calvarial periosteum, pericranial fascia and head and neck muscles to the spinal trigeminal nucleus.

Based on these anatomical data, it is now reasonable to conceive three scenarios. According to scenario (a) extracranial pathologies such as muscle tenderness or mild head trauma can activate extracranial branches of meningeal nociceptors and consequently, through bidirectional action potential propagation, produce local release of proinflammatory neuropeptides and activation of intracranial meningeal nociceptors constituting the peripheral limb of the trigeminovascular pathway. According to scenario (b) similar pericranial pathologies can also activate the trigeminovascular pathways through pericranial nerves that converge on second-order neurons in the spinal trigeminal nucleus. According to scenario (c) cortical and intracranial pathologies such as aura, meningitis or subarachnoid bleed can generate action potentials in intracranial meningeal nociceptors and consequently, through bidirectional action potential propagation, produce local release of proinflammatory neuropeptides in the dura (30) and different scalp tissues (4).

BoNT-A role in sensory and nociceptive transmission

The anatomical substrate described above may also provide a framework for using the trigeminovascular pathway to study sensory and analgesic effects of botulinum neurotoxin type A (BoNT-A) in the context of migraine headache. Given that the headache phase of migraine involves activation of peripheral and central trigeminovascular neurons, that the prophylactic treatment of migraine with BoNT-A is achieved through its administration to peripheral sites outside the calvaria, and that the sites of BoNT-A administration include structures whose sensory and nociceptive signals can activate extracranial branches of intracranial meningeal nociceptors, in the current study we attempted to determine whether BoNT-A can alter the processing of sensory and/or nociceptive signals by meningeal nociceptors.

Materials and methods

Animal preparation

All experiments were approved by the standing committee on animals at Harvard Medical School and Beth Israel Deaconess Medical Center in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Male Sprague-Dawley rats weighing 250–350 g were deeply anesthetized with urethane (1.8 g/kg intraperitoneally (i.p.)) and mounted on a stereotaxic frame. Core temperature was kept at 37°C using a heating blanket. End-tidal CO_2 was continuously monitored and kept within a physiological range of 3.5%–4.5%.

Recording and identification of meningeal nociceptors

Single-unit activity of meningeal nociceptors (one unit/rat) was recorded in the trigeminal ganglion as described in detail previously (31,32). A platinum-coated tungsten microelectrode (impedance 50 kΩ; FHC Inc, Bowdoinham, ME) was lowered toward the left trigeminal ganglion through an incision made in the dura, approximately 2 mm caudal to the Bregma suture and 2 mm lateral to the midline. Included in the study were meningeal nociceptors that exhibited discrete bursts of activity in response to mechanical stimulation of the dura overlying the ipsilateral transverse sinus (indentation with von Frey monofilaments), plus consistent response latencies to repeated electrical stimulation of the dura (0.5 ms pulse, 5 mA, 0.5 Hz).

The dura over the left hemisphere was exposed between Bregma and 2 mm caudal to Lambda. The tip of the stimulating electrode was moved to different sites within the dural receptive field to find a point at which electrical pulses yielded the shortest neuronal response latency. This response latency was divided by the average distance between the dural receptive field and the trigeminal ganglion (12.5 mm), yielding the conduction velocity (CV) of the neuron under study. Slow conduction velocities (CV $\leq$1.5 m/sec) were attributed to C-units; fast conduction velocities (CV $>$1.5 m/sec) were attributed to Aδ-units. Throughout the experiment the exposed dura was kept moist using modified synthetic interstitial fluid (SIF, pH 7.2) containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES.

Real-time waveform discriminator was used to create and store a template for the action potential evoked in the neuron under study by electrical pulses on the dura; spikes of activity matching the template waveform were acquired and analyzed online and offline using Spike 2 software (CED, Cambridge, UK).

Mechanical stimulation of meningeal nociceptors

Mechanical receptive fields of meningeal nociceptors were initially mapped using a series of calibrated von Frey monofilaments exerting pressure stimuli in the range of 38–443 kPa. Mechanically evoked neuronal responses were then determined quantitatively using a servo force-controlled stimulator (Series 300B, Aurora Scientific, Aurora, ON, Canada) fitted with a flat-ended plastic cylinder. One of three probe diameters (0.5, 0.8, or 1.1 mm) was chosen for each neuron, depending on the sensitivity for the neuron. Stimulus intensity is reported in units of pressure or force per area (kPa, where 1 kPa = 1 mN/mm^2). Stimulus trials for testing changes in mechanical sensitivity consisted of graded isosceles trapezoid-wave stimuli (100-ms rise time, 2-s width, 60-s inter-stimulus interval) delivered in ascending order, which included threshold and suprathreshold stimuli. Neuronal responses to mechanical stimulation of the dura as well as ongoing spontaneous activity were recorded every 15 minutes throughout the experiment. Baseline measurements of spontaneous and mechanically evoked activity were obtained in at least three consecutive trials prior to drug administration. Only units that exhibited consistent responses were tested further.

Recording and identification of extracranial suture branches of meningeal nociceptors

In this set of experiments we first identified meningeal nociceptors (described above) and then searched for extracranial suture branches by applying electrical and mechanical stimuli to different areas along the superior sagittal and lambdoid sutures. Neurons were initially classified based on their conduction velocity between the dura and the trigeminal ganglion. They were then classified based on the conduction velocity between the intracranial dura and extracranial suture.

Mechanical stimulation of extracranial trigeminal nociceptors

Because of lack of elasticity in bony structures, it is not possible to use a servo force-controlled stimulator for quantitative assessments of changes in mechanosensitivity. Instead, suture-receptive fields were stimulated mechanically with a series of von Frey monofilaments (five seconds duration) and changes in mechanosensitivity were determined using the smallest force

(threshold) that was required to activate the meningeal nociceptor from its suture receptive field.

Experimental paradigm

BoNT-A (onabotulinumtoxinA, BOTOX®) effects on meningeal nociceptors were studied using four different experimental paradigms. In the first paradigm, we applied four Units (U) of BoNT-A to the dural receptive field of naïve (nonsensitized) meningeal nociceptors (21 C- and 17 Aδ-units). Spontaneous activity and mechanosensitivity were recorded before (baseline) and hourly after administration of BoNT-A (baseline vs. BoNT-A). In the second paradigm, we sensitized meningeal nociceptors (six C- and six Aδ-units) by applying inflammatory soup (IS) to their dural receptive fields and 30 minutes later applied BoNT-A (four U) to determine whether it can reverse the sensitization. Spontaneous activity and mechanosensitivity were recorded at baseline, after IS administration, and hourly after BoNT-A administration (IS vs. BoNT-A). In the third paradigm, we applied BoNT-A (four U) to the dural receptive field of 13 C- and 10 Aδ-meningeal nociceptors and three hours later attempted to sensitize the neurons by IS. Spontaneous activity and mechanosensitivity were recorded at baseline, after BoNT-A administration (BoNT-A), and after IS (BoNT-A vs. IS). In the fourth paradigm, we applied BoNT-A (four U) to the extracranial receptive field of the suture branch of naïve meningeal nociceptors and tested its effects on mechanosensitivity of three C- and two Aδ-meningeal nociceptors, and on spontaneous activity of 10 C- and six Aδ-units.

Data analysis

Data are presented as mean ± SEM. Data were analyzed by nonparametric statistics, using two-tailed level of significance set at ≤0.05. The Friedman test was used to analyze repeated measures within individual groups of neurons. The Wilcoxon matched pairs signed rank test was used to compare a pair of repeated measures within a given group of neurons.

Results

BoNT-A effects on naïve meningeal nociceptors (baseline—BoNT-A)

Spontaneous activity. BoNT-A treatment had no effect on the spontaneous activity of nonsensitized meningeal nociceptors (Figure 2). Neuronal firing rate of

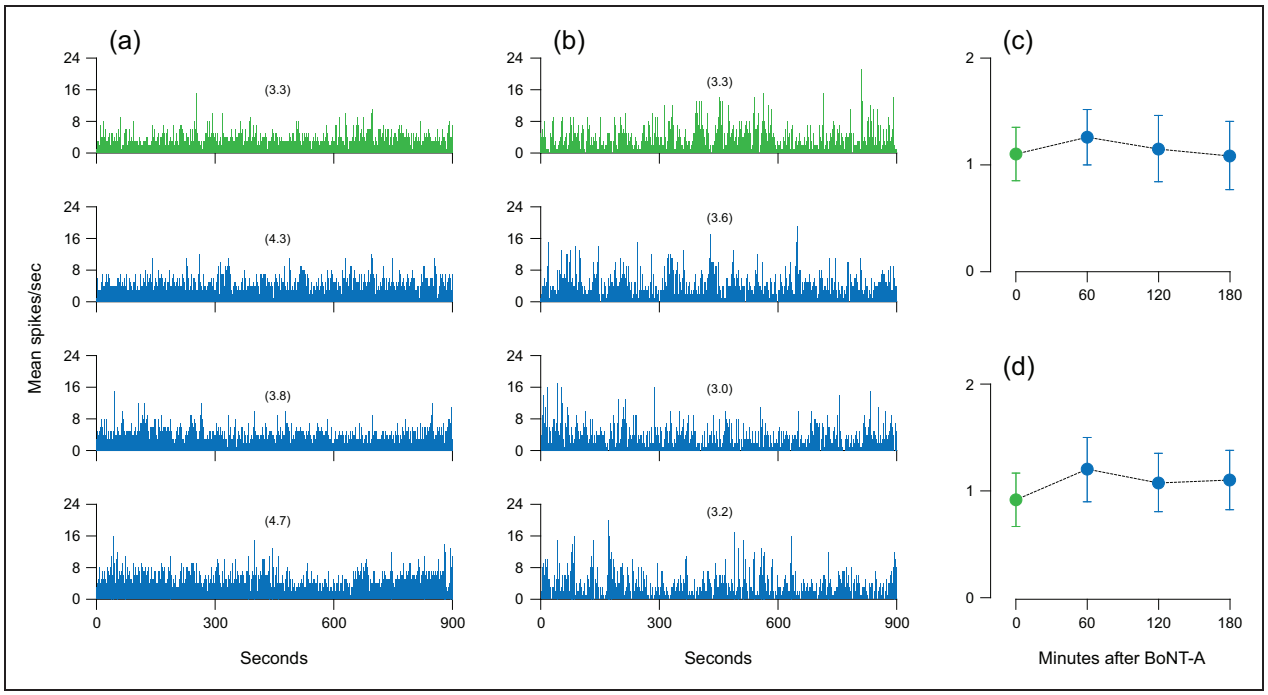

Figure 2. BoNT-A does not reduce the spontaneous activity of naïve meningeal nociceptors. (a) Peristimulus-time histograms showing firing rate of an Aδ unit before (green) and one, two and three hours after (blue) BoNT-A administration to the dural receptive field. (b) Peristimulus-time histograms showing firing rate of a C-unit before and one, two and three hours after BoNT-A administration to the dural receptive field. (c) Mean firing rate of 17 Aδ-units. (d) Mean firing rate of 21 C-units. Numbers in parentheses depict mean spikes per second for the illustrated 900 seconds. Bars represent SEM. BoNT-A: botulinum neurotoxin type A.

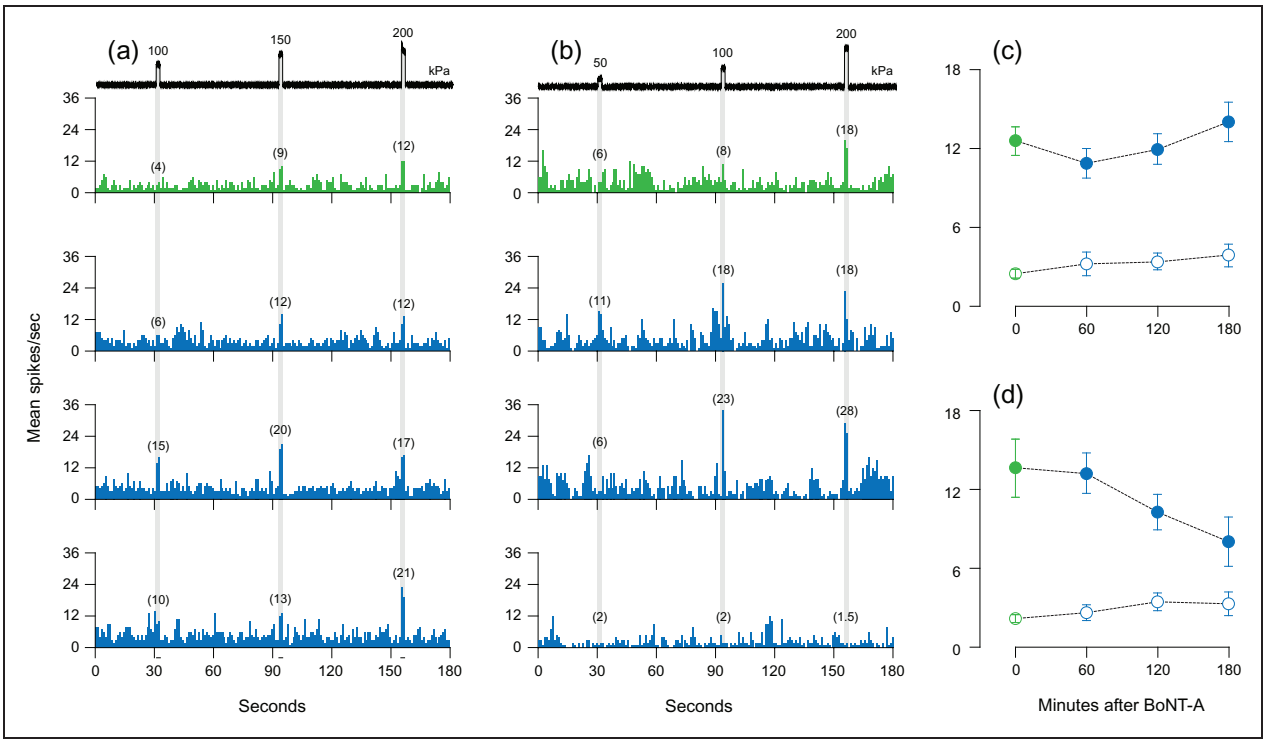

Figure 3. BoNT-A suppresses mechanical nociception in naïve C- but not Aδ-units. (a) Peristimulus-time histograms showing individual Aδ unit responses to mechanical dural indentation with threshold and suprathreshold forces before (green) and one, two and three hours after (blue) BoNT-A administration to the dural receptive field. (b) Peristimulus-time histograms showing individual C-unit responses to mechanical dural indentation with threshold and suprathreshold forces before and one, two and three hours after BoNT-A administration to the dural receptive field. Black traces on top show timing and force of each mechanical stimulus. (c) Mean response to threshold (open circles) and suprathreshold (filled circles) mechanical stimuli of 17 Aδ-units. (d) Mean response to threshold (open circles) and suprathreshold (filled circles) mechanical stimuli of 21 C-units. Note that BoNT-A suppresses responses only in C-units and only to dural stimulation with suprathreshold mechanical forces. Numbers in parentheses indicate mean spikes per sec for the duration of the stimulus. BoNT-A: botulinum neurotoxin type A.

Aδ-units was 1.1 ± 0.3 spikes/sec (mean ± SEM) at baseline, and remained that way one, two and three hours after treatment ($p = 0.63$) (Figure 2(a), (c)). Neuronal firing rate of C-units was 0.9 ± 0.3 spikes/sec at baseline, and 1.1 ± 0.3 spikes/sec one, two and three hours after treatment ($p = 0.18$) (Figure 2(b), (d)).

Mechanosensitivity. BoNT-A treatment did not change the mechanosensitivity of Aδ–meningeal nociceptors (Figure 3(a), (c)). Dural indentation with threshold and suprathreshold forces induced 2.5 ± 0.3 and 12.6 ± 0.3 spikes/sec at baseline, and 3.9 ± 0.8 and 14.0 ± 1.5 spikes/sec three hours after treatment, respectively ($p = 0.20$; $p = 0.11$). In contrast, BoNT-A treatment selectively reduced the mechanosensitivity of C-meningeal nociceptors to suprathreshold, but not threshold forces (Figure 3(b), (d)). Dural indentation with suprathreshold force was reduced by about 40% (from 13.6 ± 2.2 spikes/sec at baseline to 8.0 ± 1.9 spikes/sec three hours after treatment; $p = 0.052$), whereas dural indentation with threshold forces remained unchanged (2.2 ± 0.3 spikes/sec at baseline vs. 3.3 ± 0.9 spikes/sec three hours after treatment; $p = 0.70$).

Individual cell analysis showed that BoNT-A inhibited responses to suprathreshold mechanical stimulation in 10/21 (48%) C-units (Figure 4) and two of 17 (12%) Aδ-units. In contrast, BoNT-A reduced spontaneous activity in only three of 21 (12%) C-units and three of 17 (17%) Aδ-units.

Reversal of sensitization (baseline—IS—BoNT-A)

Spontaneous activity. BoNT-A treatment reduced IS-induced firing both in C- and Aδ-units (Figure 5). The firing rate of Aδ-units increased 37% by the IS (from baseline level of 1.2 ± 0.4 to 1.9 ± 0.5 spikes/sec after IS), and decreased 19% by the BoNT-A treatment (from 1.9 ± 0.5 to 1.6 ± 0.7 spikes/sec; $p = 0.22$) (Figure 5(a), (c)). The firing rate of C-units increased 32% by the IS (from baseline level of 1.5 ± 0.7 to 2.2 ± 0.7 spikes/sec after IS), and decreased 32% by the

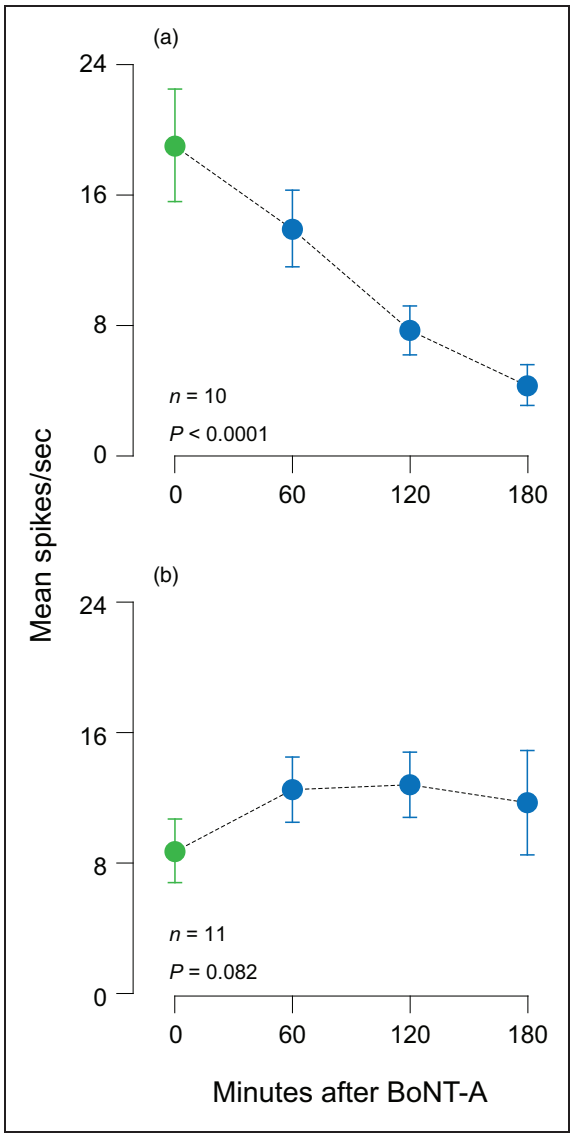

Figure 4. Selective suppression of naïve C-unit meningeal nociceptors by BoNT-A. (a) C-units exhibiting reduced response magnitude to suprathreshold mechanical stimulation of the dura following BoNT-A administration. (b) C-units exhibiting no reduction in response magnitude to suprathreshold stimulation of the dura following administration of BoNT-A. BoNT-A: botulinum neurotoxin type A.

BoNT-A treatment (2.2 ± 0.7 to 1.5 ± 0.7 spikes/sec; $p = 0.18$) (Figure 5(b), (d)).

Mechanosensitivity. BoNT-A treatment reversed the IS-induced increase in mechanosensitivity of C- but not Aδ-meningeal nociceptors (Figure 6). In the Aδ-units (Figure 6(a), (c), open circles), dural indentation with threshold forces induced 1.7 ± 0.7 spikes/sec at baseline, 5.0 ± 1.7 spikes/sec after IS, and 6.2 ± 2.6 spikes/sec after BoNT-A treatment. In contrast, in the C-units (Figure 6(b), (d), open circles), dural indentation with threshold forces induced 2.5 ± 0.4 spikes/sec at baseline, 4.2 ± 1.1 spikes/sec after IS, and 2.1 ± 0.8 spikes/sec after BoNT-A treatment. The drop in activity after BoNT-A treatment was significant ($p = 0.042$). Similar findings were recorded in the responses to suprathreshold mechanical stimulation. In the Aδ-units (Figure 6(a), (c), filled circles), dural indentation with suprathreshold forces induced 11.9 ± 2.5 spikes/sec at baseline, 17.2 ± 1.4 spikes/sec after IS, and 14.0 ± 2.8 spikes/sec after BoNT-A treatment. In contrast, in the C-units (Figure 6(b), (d), filled circles), dural indentation with suprathreshold forces induced 6.7 ± 2.0 spikes/sec at baseline, 9.9 ± 2.6 spikes/sec after IS, and 6.9 ± 2.4 spikes/sec after BoNT-A treatment. The drop in activity after BoNT-A treatment was significant ($p = 0.049$).

Prevention of sensitization (baseline—BoNT-A—IS)

Spontaneous activity. Pre-treatment with BoNT-A prevented IS-induced increased firing in C- but not Aδ-units (Figure 7). In the Aδ-units, firing rate was 1.2 ± 0.3 spikes/sec at baseline, 1.3 ± 0.4 spikes/sec after BoNT-A, and 2.2 ± 0.5 spikes/sec after IS (Figure 7(a), (c)). The rise in activity of Aδ-units after the IS administration fell short of the level of significance ($p = 0.059$). In contrast, the firing rate of C-units remained unchanged (1.3 ± 0.3, 1.4 ± 0.4, and 1.5 ± 0.3 spikes/sec at baseline, after BoNT-A and after IS, respectively) (Figure 7(b), (d)).

Mechanosensitivity. Pre-treatment of C- and Aδ-units with BoNT-A did not prevent the IS-induced increase in responses to dural stimulation with threshold forces (Figure 8(c), (d) open circles). It did, however, prevent the IS-induced increase in responsiveness to mechanical stimulation of the dura with suprathreshold forces (Figure 8(c), (d) filled circles). In the Aδ-units responses to threshold mechanical stimulation increased significantly (baseline $= 2.9 \pm 0.4$ spikes/sec, after BoNT-A $= 3.6 \pm 1.6$, after IS $= 7.3 \pm 1.8$; $p = 0.002$) whereas responses to suprathreshold forces did not (baseline $= 11.7 \pm 1.1$ spikes/sec, after BoNT-A $= 13.9 \pm 1.8$, after IS $= 16.6 \pm 2.3$; $p = 0.091$) (Figure 8(a), (c)). In the C-unit, responses to threshold stimulation increased significantly (baseline $= 2.3 \pm 0.4$ spikes/sec, after BoNT-A $= 3.3 \pm 0.7$, after IS $= 7.1 \pm 1.5$; $p = 0.002$) (Figure 8(d) open circles) whereas responses to suprathreshold stimulation remained unchanged (baseline $= 9.7 \pm 1.4$ spikes/sec, after BoNT-A $= 8.2 \pm 1.2$, after IS $= 11.9 \pm 2.0$; $p = 0.10$) (Figure 8(d) filled circles).

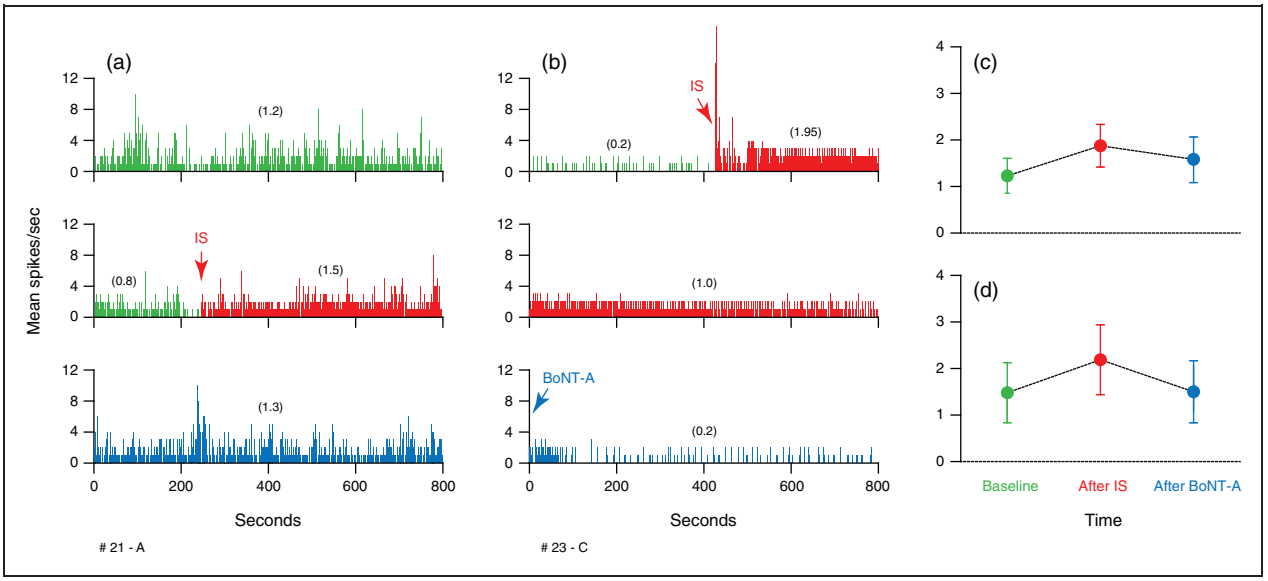

Figure 5. IS-mediated increased spontaneous activity is partially decreased (though not significantly) by delayed administration of BoNT-A. (a) Peristimulus-time histograms showing firing rate of an Aδ unit before (green) and after (red) sensitization, and two hours after BoNT-A administration (blue) to the dural receptive field. (b) Peristimulus-time histograms showing firing rate of a C-unit before (green) and after (red) sensitization, and two hours after BoNT-A administration (blue) to the dural receptive field. (c) Mean spontaneous firing rate of six Aδ-units. (d) Mean spontaneous firing rate of six C-units. Numbers in parentheses depict mean spikes per second for the illustrated 800 seconds. Bars represent SEM. IS: inflammatory soup; BoNT-A: botulinum neurotoxin type A.

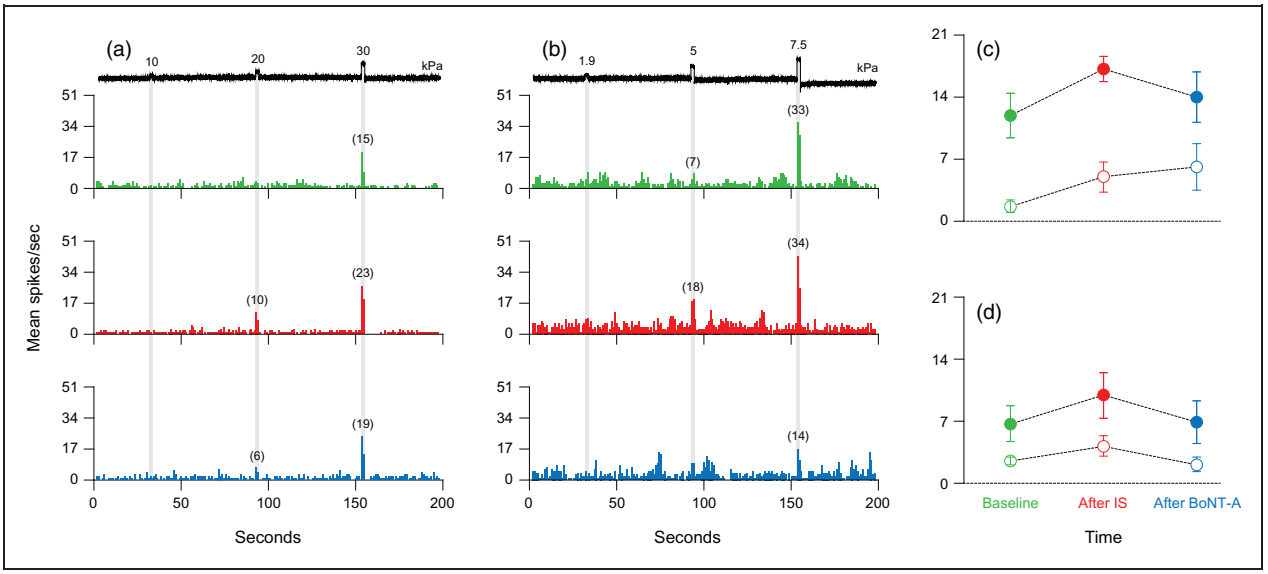

Figure 6. BoNT-A reverses mechanical nociception in sensitized C- but not Aδ-units. (a) Peristimulus-time histograms showing individual Aδ unit responses to mechanical dural indentation with threshold and suprathreshold forces, before (green) and after (red) sensitization, and three hours after BoNT-A administration (blue) to the dural receptive field. (b) Peristimulus-time histograms showing individual C unit responses to mechanical dural indentation with threshold and suprathreshold forces, before (green) and after (red) sensitization, and three hours after BoNT-A administration (blue) to the dural receptive field. (c) Mean response to threshold (open circles) and suprathreshold (filled circles) mechanical stimuli of six Aδ-units. (d) Mean response to threshold (open circles) and suprathreshold (filled circles) mechanical stimuli of six C-units. Black traces on top ((a), (b)) show timing and force of each mechanical stimulus. Numbers in parentheses indicate mean spikes per second for the duration of the stimulus. Note that BoNT-A significantly suppressed mechanical sensitization in C- but not Aδ-nociceptors. BoNT-A: botulinum neurotoxin type A.

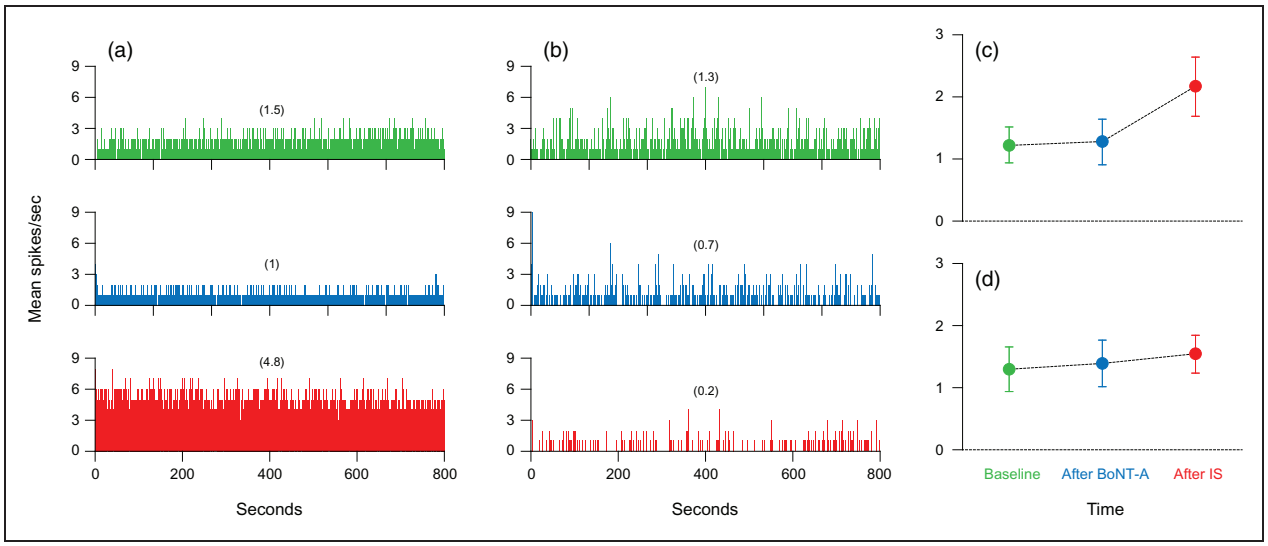

Figure 7. When given early, BoNT-A prevents IS-induced increase in spontaneous activity of C- but not Aδ-meningeal nociceptors. (a) Peristimulus-time histograms showing firing rate of an Aδ unit before (green) and three hours after (blue) BoNT-A administration, and 30 minutes after IS. (b) Peristimulus-time histograms showing firing rate of C unit before (green) and three hours after (blue) BoNT-A administration, and 30 minutes after IS. (c) Mean firing rate of 10 Aδ units. (d) Mean firing rate of 13 C units. BoNT-A: botulinum neurotoxin type A; IS: inflammatory soup.

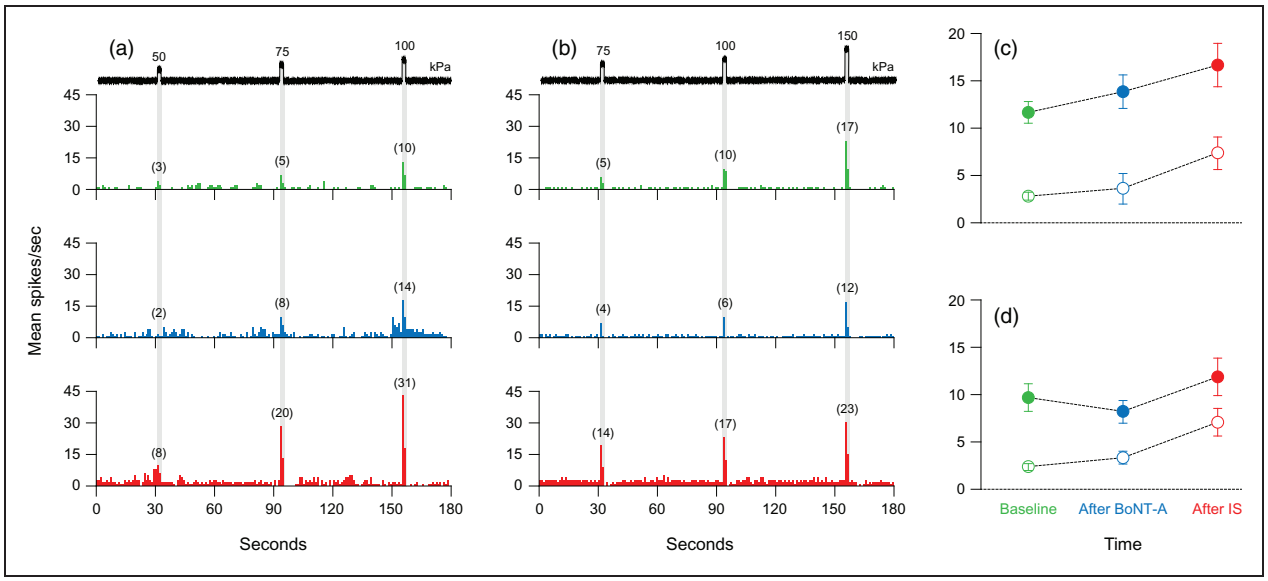

Figure 8. When given early, BoNT-A prevents IS-induced increase in C-unit responses to suprathreshold mechanical stimulation. (a) Peristimulus-time histograms showing individual Aδ-meningeal nociceptor responses to threshold and suprathreshold stimuli of the dura before (green) and three hours after (blue) BoNT-A administration, and 30 minutes after IS. Note that BoNT-A did not prevent the IS-induced increase in response magnitude. (b) Peristimulus-time histograms showing individual C meningeal nociceptor responses to threshold and suprathreshold stimuli of the dura before (green) and three hours after (blue) BoNT-A administration, and 30 minutes after IS. Note that BoNT-A prevented the IS-induced increase in response magnitude to suprathreshold, but not threshold mechanical stimulation. (c) Mean responses of 10 Aδ units to threshold (open circles) and suprathreshold (filled circles) mechanical stimulation of the dura. (d) Mean responses of 13 C units threshold (open circles) and suprathreshold (filled circles) mechanical stimulation of the dura. Black traces on top ((a), (b)) show timing and force of each mechanical stimulus. Numbers in parentheses indicate mean spikes per sec for the duration of the stimulus. IS: inflammatory soup; BoNT-A: botulinum neurotoxin type A.

BoNT-A effects on extracranial suture branches of naïve meningeal nociceptors (baseline—extracranial BoNT-A)

Spontaneous activity. Topical application of BoNT-A to the suture-receptive field of both C- and Aδ-units (Figure 9(a)) had no effect on their spontaneous activity (Figure 9(b), (c)). Neuronal firing rate of Aδ-units was 1.0 ± 0.5 spikes/sec at baseline and 0.8 ± 0.3 spikes/sec three hours after treatment ($p = 0.30$) (Figure 9(b)). Neuronal firing rate of C-units was 0.3 ± 0.1 spikes/sec at baseline, and 0.5 ± 0.3 spikes/sec three hours after treatment ($p = 0.88$) (Figure 9(c)).

Mechanosensitivity. Topical application of BoNT-A to the suture-receptive field of C-units increased the threshold for their activation by mechanical stimulation of the suture or rendered them mechanically insensitive (Figure 9(d), (f)). The decrease in three C-unit mechanosensitivity was reflected by the increase in the minimal force required to activate them from baseline to three hours after BoNT-A administration (from 7 to 30, from 3 to 7.5 and from 1 to 100 g). Application of BoNT-A to the suture, however, did not change neuronal mechanosensitivity to stimulation of dural receptive field (Figure 9(e), (g)), judged by application of pressure to the bone overlying the dural receptive field (Figure 9(a)). Topical application of BoNT-A to the suture-receptive field of Aδ-units had no effect on the threshold for their activation by mechanical stimulation of the suture or the dura.

Discussion

The study provides direct evidence for the ability of BoNT-A to inhibit mechanical nociception in peripheral trigeminovascular neurons. Most significant were the findings that BoNT-A (a) inhibited C- but not Aδ-type meningeal nociceptors, (b) inhibited naïve C-type meningeal nociceptor responses to suprathreshold (i.e. nociceptive) but not threshold (non-nociceptive) mechanical stimulation, (c) reversed (when given after) and prevented (when given before) IS-induced mechanical hypersensitivity, and (d) inhibited the mechanical sensitivity of the suture branches of intracranial meningeal nociceptors when administered extracranially. The preferential suppression of responses to suprathreshold mechanical simulation suggests that BoNT-A inhibits high-threshold mechanosensitive ion channels linked preferentially to mechanical pain. As outlined below, in the context of migraine, inhibition of the flow of mechanical pain signals from meningeal and other trigeminovascular nociceptors to the spinal trigeminal nucleus may be most critical for the ability of BoNT-A to act as a migraine prophylactic.

Clinical evidence for abnormal mechanical pain processing in migraine includes worsening of the headache by physical activities that momentarily increase intracranial pressure (33,34). These activities include bending over, coughing, sneezing, and even the relatively small exertion associated with bowel movement (35). Preclinical evidence for abnormal mechanical nociception in meningeal nociceptors, the afferent limb of the trigeminovascular pathway, includes their responsiveness to dural indentation and their remarkable ability to develop mechanical hypersensitivity (i.e. peripheral sensitization) when exposed briefly to inflammatory molecules thought to appear in the meninges during migraine (13,30,31). Other evidence for abnormal mechanical pain processing in migraineurs include the development of mechanical allodynia—commonly described by patients as wanting to avoid physical contact with the scalp and face (35–39), and muscle tenderness—commonly described by patients as exacerbated by neck flexion (40,41). Preclinical studies suggest that this so-called extracranial cephalic mechanical hypersensitivity is mediated by sensitization of central trigeminovascular neurons in the spinal trigeminal nucleus (42). Judging by the high incidence of mechanical, relative to thermal (i.e. heat, cold), allodynia in migraine (36,43–47), it is reasonable to conclude that mechanical pain contributes more than thermal pain (i.e. hot and cold) to the pathophysiology of migraine. Conceptually, therefore, we propose that BoNT-A prophylactic effects in migraine should take into consideration its ability to reduce the overall flow of sensory signals that originate in mechano-nociceptors supplying peripheral structures involved in the initiation of migraine.

Further support for the preferential effects of BoNT-A on mechanical pain processing comes from recent clinical and preclinical studies that showed that BoNT-A reduced mechanical allodynia in patients diagnosed with focal neuropathic pain (48) and in animal models of neuropathic pain induced by chronic constriction injury (49), and increased mechanical pressure pain threshold in several models of experimentally induced pain in human subjects and animals (50–53). In contrast, BoNT-A injections did not change pain threshold and tolerance to heat and electrical stimuli (54,55).

The findings of the current and previous studies call attention to the possibility that BoNT-A interferes with the function of high-threshold mechanosensitive ion channels required for the generation of mechanical pain. To do so, the toxin must be able to interfere with functions of mechanosensitive channels that transduce high-intensity noxious pressure applied to peripheral terminals of sensory nociceptive neurons. To date, little is known about the molecular substrate of mechanical transduction in mammals. Although putative mechanosensitive ion channels have been proposed,

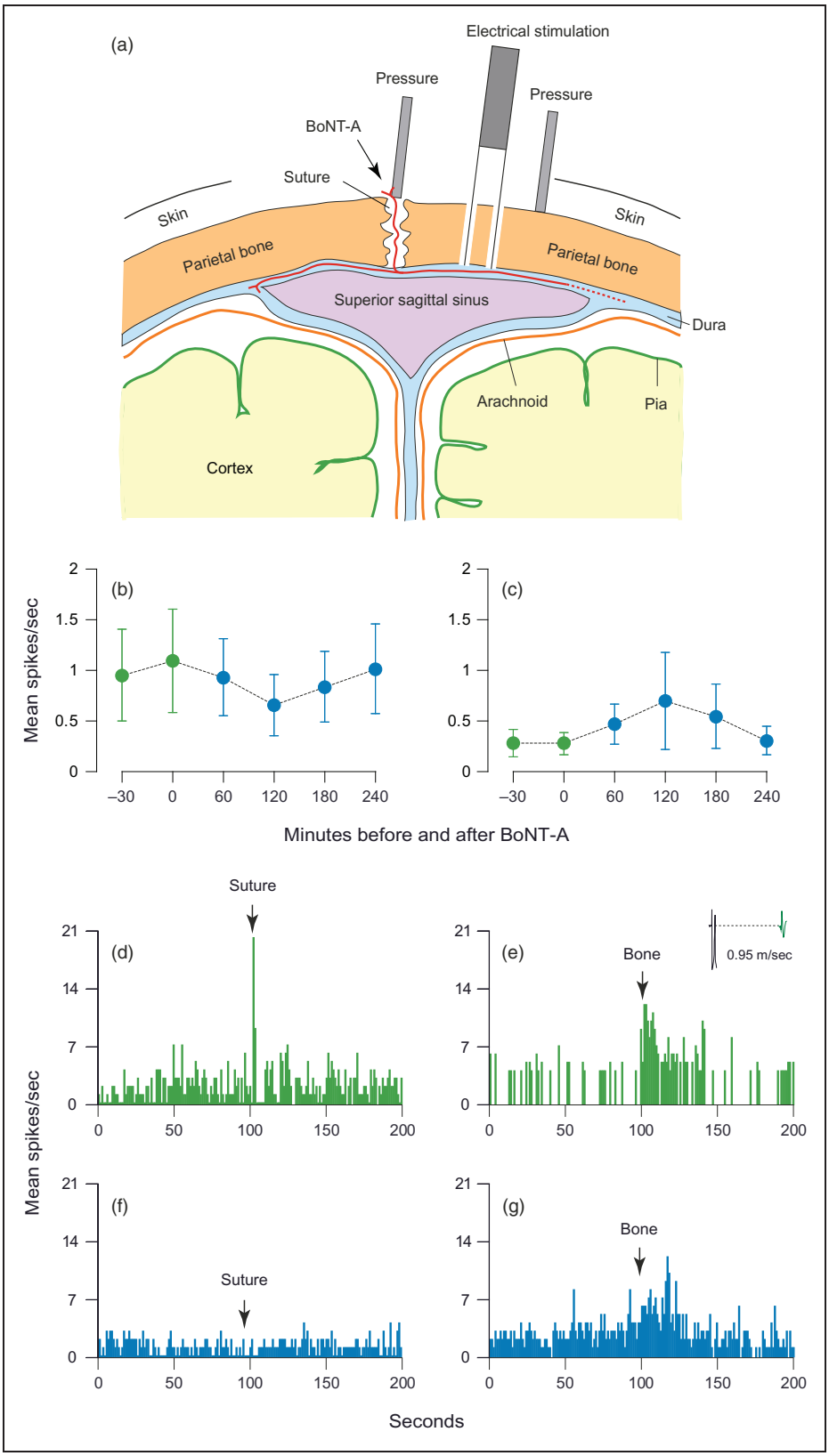

Figure 9. Selective inhibition of mechanosensitivity of suture branches of C- but not Aδ-units. (a) Experimental setup. Initial identification of a meningeal nociceptor was conducted by stimulating the dura electrically through two small holes in the skull. Identification of a suture branch was performed by applying mechanical pressure (using VFH) along the superior sagittal and transverse

(Continued)

none have been identified as essential for pain or touch. Most well-studied candidates include the nonselective cation transient receptor potential (TRP) channels (56). In this superfamily of ion channels, TRPA1 is thought to amplify mechanical transduction (especially to suprathreshold firing), rather than being the mechanical transducer itself, and as for the TRPV1, there is no evidence that it is directly involved in mechanotransduction or that it amplifies a mechanical response in naïve, uninjured afferents (57–62). Although not fully understood, recent studies provide reasonably convincing evidence for a selective role in transduction of mechanical force by noxious mechanosensation blocker 1 (NMB-1), a conopeptide analog shown to bind to nociceptive sensory neurons, to inhibit slowly adapting mechanically activated current with slow activation kinetics, and when blocked, to inhibit behavioral responses to painful mechanical stimulation (63). Critical to its mechanical transduction selectivity, this conopeptide shows no activity at ASICs, TRPV1, TRPA1 and voltage-gated sodium, potassium or calcium channels.

In the context of our study, we propose a scenario in which BoNT-A either inhibits mechanical pain by interacting with toxin-sensitive slowly adapting ionic channels that are preferentially expressed on C-type meningeal nociceptors or reduces the neuronal surface expression of these channels or associated receptors during chronic pain. In support of this scenario, Paterson and colleagues (64) showed recently that BoNT-A decreases mechanical pain sensitivity in human subjects and the proportion of neurons expressing slowly-adapting mechano-gated currents without affecting neuronal excitability or rapidly and intermediately adapting mechanically activated inward currents and concluded that the toxin selectively inhibits the transduction of mechanical pain.

Alternatively, consideration should also be given to the possibility that the selective inhibition of C-fiber meningeal nociceptors by BoNT-A inhibition may be mediated through potential interaction with the Piezo2 protein, a pore-forming subunit of the mechanically activated channel found in dorsal root ganglion (DRG) neurons expressing TRPV1 and shown to be involved in slowly adapting C-fiber mechanical responses (65–68). Mechanistically, if BoNT-A can bind directly and selectively to the ionic channel that mediates mechanical pain, one would have expected the toxin to have an almost immediate effect on mechanical transduction and thus on the pain. Given the remarkable delay in BoNT-A onset of action, a more logical explanation for its ability to reduce responses to noxious mechanical stimuli is that the toxin interferes with the process by which newly synthesized high-threshold mechanosensitive ion channels are inserted into the membrane at the nerve terminal site. We propose that blockade of vesicle trafficking by BoNT-A can play a critical role in this scenario—providing that it acts selectively on a subpopulation of mechanosensitive C-type meningeal nociceptors. This mechanism would be similar to the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE)-mediated delivery of TRPV1, TRPA1 and P2X3 to the neuronal surface (see below).

BoNT-A and chronic migraine

An understanding of the mechanism of BoNT-A in migraine prophylaxis is derived from principles of (a) effect on vesicular trafficking, (b) release of neurotransmitters and inflammatory peptides in the peripheral nervous system, and (c) mechanism of cell surface membrane expression of relevant ion channels and receptors. As reviewed below, we propose that in the context of chronic migraine, BoNT-A modulates sensory transmission and interferes with the neuronal surface expression of relevant membrane-bound ion channels and/or receptors.

Synaptic vessel membrane delivery: BoNT-A's effect on nociceptor receptor trafficking and stimulated delivery to plasma membranes

Cell surface proteins are delivered to the plasma membrane by either a constitutive or a regulated vesicle pathway (69) involving trafficking proteins (e.g. vesicle-associated membrane protein (VAMP)/synaptobrevin),

Figure 9. Continued.
sutures. Neuronal spontaneous activity and mechanosensitivity were determined before and after application of BoNT-A to the extracranial suture. To determine mechanosensitivity of the suture branch, suprathreshold mechanical stimuli were applied to the most sensitive site along the suture (defined as the site from which the smallest force induced the largest number of spikes). To determine mechanosensitivity of the dural parent axon, suprathreshold forces were applied to the bone overlying the dural receptive field. (a) Mean spontaneous firing rate of six Aδ units before (green circles) and up to four hours after (blue circles) administration of BoNT-A to the suture. (c) Mean spontaneous firing rate of 10 C-units before (green circles) and up to four hours after (blue circles) administration of BoNT-A to the suture. Note that suture application of BoNT-A did not reduce the spontaneous firing of the nociceptors in the four-hour period in which it was measured. (d), (e) Peristimulus-time histograms showing C-unit responses to mechanical stimulation of the suture (d) and bone overlying the dural receptive field of the unit. (f), (g) Peristimulus-time histograms showing loss of mechanosensitivity in the suture branch of the meningeal nociceptor (f) but not in the parent axon in the dura (g). Trace in (e) (upper right) identifies the studied neuron as a C-type meningeal nociceptor (conduction velocity = 0.95 m/sec). VFH: von Frey hair; BoNT-A: botulinum neurotoxin type A.

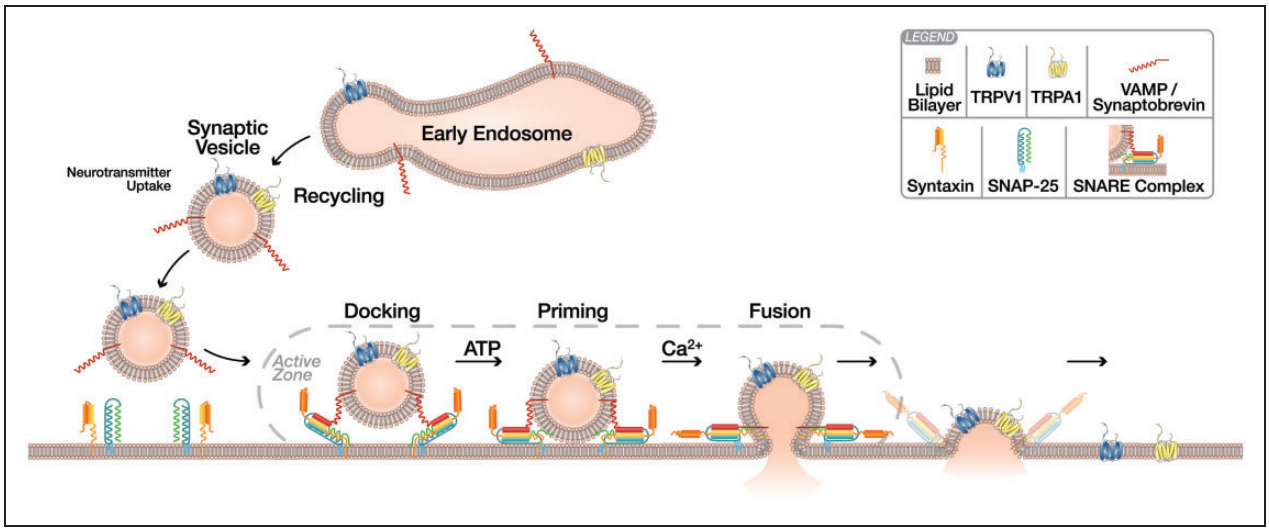

Figure 10. Synaptic vesicle membrane delivery of ion channels and receptors. Cell surface proteins are delivered to the plasma membrane by either a constitutive or regulated synaptic vesicle (SV) pathway whereby proteins channels, receptors and transporters associated with the SV lipid bilayer are inserted into the nerve terminal. SVs form a reserve pool at the nerve terminal and may be filled with neurotransmitter(s). Most SVs are decorated with multiple proteins (70); delivery of membrane-associated protein receptors TRPV1 and TRPA1 are depicted. SVs dock adjacent to the nerve terminal inner membrane "active zone," and undergo an adenosine triphosphate (ATP)-dependent "priming" step that enables responding to the Ca^{++} signal that triggers fusion, exocytosis and consequent delivery of not only SV contents into the extracellular space but also lipid membrane and associated proteins into the cell surface. Successful fusion requires an interaction between the vesicle-associated membrane protein (VAMP)/synaptobrevin with those on the internal membrane surface, namely synaptosomal-associated protein of molecular weight 25 kDa (SNAP-25) and syntaxin, which together form the soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) complex; other associated proteins (e.g. Munc 18, Rab) are also involved (not depicted) (85). The SV membrane may fully fuse into the terminal membrane ("full collapse fusion"), thus delivering the protein receptors, e.g. TRPV1 or TRPA1, into the cell surface. Excess terminal membrane is recycled through one of the endocytosis pathways (86). BoNT-A cleaves SNAP-25, impairing SV fusion and the regulated delivery of receptors TRPV1 or TRPA1 to the terminal membrane, thus down-regulating receptor activity. BoNT-A: botulinum neurotoxin type A; TRPV1: transient receptor potential cation channel vanilloid subfamily, member 1; TRPA1: transient receptor potential cation channel ankyrin subfamily, member 1.

channels, receptors and transporters (70), and forms, in part, the mechanism of up- and down-regulation (Figure 10). Critical to the regulation of surface expression of mechanosensitive ion channels in sensory nerve endings is the extent (duration, intensity, frequency) of the receptor's activation. By this principle, it is now believed that inflammation-mediated facilitation of trafficking of TRPV1 receptor channels play an important role in the development and maintenance of inflammatory hyperalgesia. If chronic pain may in fact regulate itself through its ability to increase surface expression of relevant ion channels, a logical therapeutic approach for pain management may then be pharmacological intervention that aims at inhibiting axonal/synaptic receptor channels trafficking. At the molecular level, it has been shown that protein kinase C (PKC) activation regulates axonal trafficking and membrane translocation of TRPV1 receptor channels in DRG cells, and that BoNT-A administration inhibits this process (71). Mechanistically, the findings that BoNT-A can block the PKC-mediated membrane translocation of TRPV1 suggest that the magnitude of the surface expression of this mechanosensitive channel receptor could be regulated, in part, by SNARE-dependent exocytosis (72,73). Along this line, we propose that, in addition to TRPV1 and P2X3 (74), BoNT-A-regulated SNARE-dependent cell-surface expression of TRPA1 receptors, which mediate different aspects of pain sensation (75–80). This is a novel ballpark for conceptualizing about the mechanisms of action of BoNT-A. Support for this proposal comes from clinical studies showing that the expression of P2X3 and TRPV1 in urinary bladder epithelial cells decreased significantly (without any loss of fiber density) four weeks after BoNT-A treatment of neurogenic detrusor overactivity in patients suffering idiopathic overactive bladder (81,82). In this case, improvements in patients' sensation of urgency and urodynamic physiology parameters were correlated with the temporal change in P2X3 immunoreactivity (83,84).

In summary, this study provides evidence for the ability of BoNT-A to inhibit mechanical nociception in peripheral trigeminovascular neurons. Recognizing that mechanical pain is an important component of many painful disorders, our findings may expand our understanding of reported clinical outcomes when

BoNT-A has been tested in other conditions, such as low back pain (87) and osteoarthritis (88). Reflecting on the role in improvement of chronic migraine, future studies will focus on identifying which channel receptors are expressed lower in cranial tissues following BoNT-A injections, and which of these are correlated with the delayed improvement in migraine frequency.

Clinical implications

- Migraine is a complex neurosensory disorder for which the pathophysiology is under active investigation; botulinum neurotoxin type A (BoNT-A) has a defined intracellular mechanism of action, impairing the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE)-mediated synaptic vesicle fusion to nerve terminals.
- Utilizing a preclinical cranial pain model, this research demonstrates that BoNT-A selectively inhibits C- but not Aδ-trigeminal meningeal nociceptors.
- BoNT-A reduced sensory transduction of suprathreshold mechanical stimuli associated with processing mechanical pain, but not sensory transduction of threshold tactile mechanosensitivity.
- Extracranial application of BoNT-A inhibits mechanical transduction in suture branches of meningeal nociceptors.
- These findings suggest that in the prophylactic treatment of chronic migraine, BoNT-A interferes with neuronal surface expression of high-threshold mechanosensitive ion channels by preventing their fusion into the nerve terminal membrane.

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Conflicts of interest

Dr Burstein has received research funds from Allergan. He is also a consultant to Allergan. Drs Brin and Aoki are employees of Allergan Inc and receive stock. Drs Levy and Zhang have nothing to declare.

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