Chapter 5
Basic Science of Pain and Botulinum Toxin

Zdravko Lacković, Ivica Matak, and Lidija Bach-Rojecky

Abstract The use of botulinum toxin type A (BoNT-A) in pain conditions is continuously growing largely because of its long-lasting effect after local application and safety profile. These unique features distinguish BoNT-A from other conventional and adjuvant analgesic drugs. Furthermore, BoNT-A diminishes only the pathological pain, without affecting the normal pain threshold. Preclinical data from several complex pain models suggested the central site of its action on pain after retrograde axonal transport from the peripheral site of application. Further investigations of the mechanism of BoNT-A antinociceptive action are ongoing as well as experiments on new recombinant BoNTs with higher selectivity for nociceptive neurons.

Keywords Botulinum toxins · Pain · CNS · Experimental models of pain · Recombinant toxins

> Clinicians… loathe chronic pain, perhaps the symptom that brings more patients into our practices than any other but also the symptom most likely to make us feel helpless as healers.
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Over the last decades, our understanding of botulinum toxin mechanism of action has changed. Intensive research has shown that peripherally administered botulinum toxin type A (BoNT-A) reaches the central nervous system (CNS) by axonal transport. Major molecular mechanism is prevention of neurotransmitter release: synaptic silencing. Such effect is long-lasting but reversible. This action might

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occur at central synapse of the first sensory neuron. Whether there is occurrence of transcytosis is not yet known. Events after first sensory neuron are just at the beginning of intensive research. There are influences on other neurons and glial cells in the CNS. Unique characteristic of BoNT-A is lack of analgesic action on acute nociceptive pain that has important warning function; in humans, analgesic activity usually is monthslong. In spite of some still missing pieces of the puzzle, there is increasing evidence that botulinum toxin, especially type A (BoNT-A), is preventing pain in a growing range of disorders. In the absence of unexpected findings, or an increase in the uncontrolled use of illicit preparations by uneducated persons, BoNT-A is emerging as a new long-lasting and relatively safe analgesic. BoNT-A is not devoid of side effect – even fatalities occurred; however, in the usage of registered product by well-trained professionals, side effects are mild and rare.

Basic Science of Pain

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [2].

Classification of pain is complex and a matter of debate [3, 4]. Classification could be based on localization (somatic or visceral; organ or body part) and cause (nociception, inflammation, tumors, neurogenic, psychogenic). According to mechanism, pain can be divided into nociceptive (peripheral and central), reflexive and nonreflexive, neuropathic (also peripheral and central), and psychogenic; according to duration, pain is commonly divided into acute and chronic. There is no unified definition of chronic pain. Chronicity depends on disease, for example, migraine is considered chronic if there are more than 15 days of attack per month, while in some other disorders chronic pain should last more longer, usually 3 months. Pain that is caused by the presence of a painful stimulus on nociceptors is called nociceptive pain. Nociceptive pain in its acute form usually serves an important biological (or evolutionary) function as it warns the organism of impending danger and informs the organism of tissue damage or injury.

Neuropathic pain as experimental prototype of chronic pain is caused by a primary lesion or dysfunction in the nervous system and could be peripheral or central. However, the pain is projected into the region supplied by the nerve ("projected pain"). Some of the most baffling types of chronic pain, such as diabetic neuropathy, phantom limb pain, and postherpetic neuralgia, are neuropathic in origin. A significant proportion of patients suffering from chronic low back pain or cancer pain have, in addition to a nociceptive part, also a neuropathic component.

Psychogenic pain is caused by the mental processes of the sufferer rather than by immediate physiological causes. Purely psychogenic pain is rare, and its incidence is often overestimated. Nevertheless, chronic pain frequently has a secondary psychological component resulting in a mixed presentation (e.g., psychosomatic pain) (Fig. 5.1).

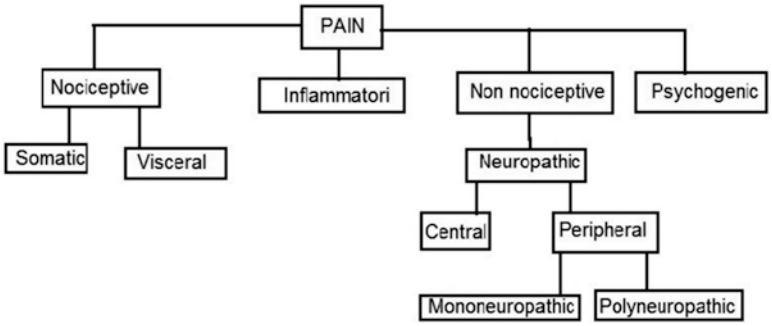

Fig. 5.1 Compilation of attempts to present classification of pain

Anatomy of Pain Classical anatomy of pain is well known: Shortly painful information travels from peripheral pain receptors (nociceptors) to the spinal cord through primary afferent neurons or "first-order" sensory neurons consisting of A-delta and C fibers. Pain transmitted by A-delta fibers is described as sharp and is felt first. This is followed by a duller pain carried by the C fibers. Cell bodies of unipolar neurons in sensory dorsal root ganglia have central projections that reach dorsal column of the spinal cord. Besides different interneurons, A-delta and C fibers innervate "second-order" nerve fibers in laminae II and III of the dorsal horns. They form spinothalamic tract and reach thalamus and finally somatosensory cortex. In cranial nerves (i.e., *n. trigeminus*, n. *facialis*, etc.), first-order neurons innervate second-order neurons in their nuclei in the brainstem.

In addition to the described ascendant system, there is also a complex descending pain modulatory system that influences nociceptive input from the spinal cord or the brainstem sensory nuclei. This descendant system is under influence of cortical, subcortical, and brainstem structures that can modulate perception of pain. Accordingly, perception of pain in humans is influenced by experience, emotions, cultural social factors, etc. Such influence in a more simple way exists in experimental animals as well and can influence results of pharmacological research in rodents [5]. It is a common knowledge that different individuals, humans but some higher animals as well, have very different reactions to pain. Consequently, measurement of pain can be considered as a prototype for the quantitative study of subjective responses [6].

In vitro experiments are basis to elucidate molecular mechanism of physiological functions including sensory system and pain. The hope of in vitro experiments is that they reflect the biology of the intact organism. Investigators doing in vitro work must be careful to avoid overinterpretation of their results, which can lead to erroneous conclusions [7].

Measuring pain and analgesia in experimental animals in vivo is the mainstream of study of pain and analgesic drug assessment and development. There are a number of tests developed to measure reflexive pain and evaluate behavioral, withdrawal responses after the application of painful stimuli like heat (like tail flick or

hot plate test), cold (acetone, etc.), mechanical (like pinprick, Randall-Selitto test), and electrical stimuli. These tests activate nociceptors at the site of testing and trigger localized, motor responses and could exist even in animals without the pain as many of these responses can occur in the absence of supraspinal activation; however, in higher animals and humans, they are modified by descending pain control system.

Classical criticism to behavioral assessment of pain, usually in rodents, is that most of them measure withdrawal responses to evoked painful stimuli instead of the more clinically important spontaneous pain [8].

Nonreflexive pain tests record spontaneous pain behavior [9]. The most common example is formalin test, which refers to the quantification of pain behavior, such as time spent licking chemically injured part of the body (usually paw pad or vibrissal pad in the face). Additional pain behavior could include, for example, paw elevation and smoothing. Application of other irritant substances (capsaicin, mustard oil, carrageenan, etc.) can also be used. Similarly, quantification of writhing behaviors after an intraperitoneal injection of acetic acid can be useful to quantify visceral pain.

Ultrasonic vocalization was used to measure pain intensity in chronic cancer pain and neuropathic pain models in mice. Mice and rats communicate by ultrasound; thus, distinguishing pain and normal ultrasound communication might not be easy [10].

Grimace scale is the most recent test that records and measures pain-induced facial expression. It is described both, in mice [11] and rats [10]. Based on orbital tightening, nose bulge, cheek bulge, ear position, and whisker change, a score on a 0–2 scale for their prominence in still photographs allows quantification of spontaneous pain (Fig. 5.2). There are reports that facial grimace scale in rats and mice

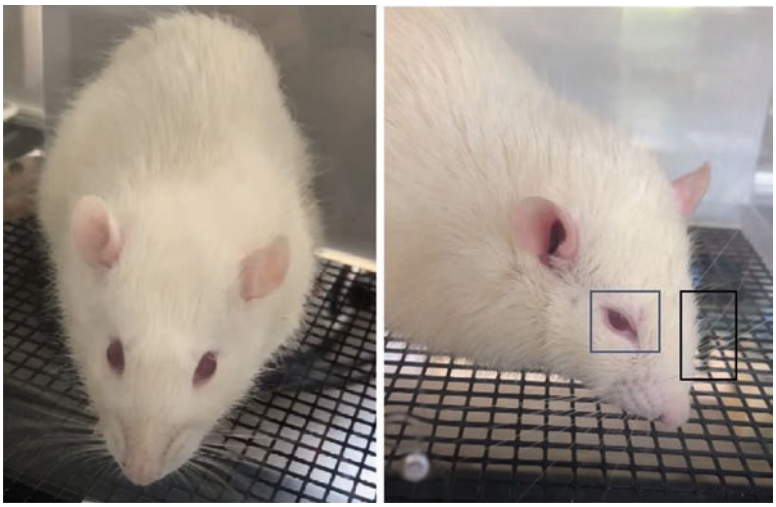

Fig. 5.2 Rat grimace in normal rat (left) and a rat feeling modest pain (right). Nose/cheek flattening and ear changes are not visible

after infraorbital nerve constriction injury remains high for 10 days or more [12]. Fentanyl reversed the changes in rat grimace scale scores, suggesting that these scores reflect pain perception [12].

In animals with chronic pain, usually behavioral responses to additional painful stimuli (mechanical or thermal) reflect hypersensitivity to pain and allodynia using additional pain test, often von Frey filaments. Thus, what is measured is not "basic," "tonic" spontaneous chronic pain but rather a reaction to the additional stimulation. Therefore, instead of spontaneous pain, supersensitivity to pain and allodynia are measured.

A review of tests to measure pain in experimental animals shows that they are all movement-related. They are based on avoidance or the reduction of painful stimuli, that is, the movements of the experimental animals. Because BoNT-A reduces movement due to its effect on muscles, this can significantly affect the results of behavioral experiments. This is probably why in behavioral tests no one has so far shown an acceptable relationship between BoNT-A dose and effect. A detailed analysis shows that such research often yields yes/no results. In conclusion, by investigating the effect of botulinum toxin on pain, we obtain a response that represents a balance between the analgesic and paralytic effects of BoNT-A.

The Studies of Pain in Humans In assessment of pain in patients, some mechanical tests are sometimes applied like pinprick, von Frey filaments, but most common assessments are based on subjective feeling by a particular patient. To standardize patient rating of pain feeling, numerous rating scales have been developed.

Haefeli and Elfering describe and discuss most commonly used pain measurement scales [13]. All of them are subjective and based on patient assessment of intensity of pain, for example, on the scale of 1–10. Best known are the visual analogue scale, numerical rating scale, verbal rating scale, pain drawing, etc.

Besides rating scales, clinical drug testing on a larger group of patients have many methodological requirements to make results more reliable. Those requirements are described in many documents on Good Clinical Practice and are also a part of the American Academy of Neurology (AAN) criteria for evaluation of new drugs.

Structural and functional neuroimaging clearly demonstrated central nervous system contributors to chronic pain in humans. There is a belief that brain imaging could provide objective biomarkers of chronic pain and guide treatment for personalized pain management; however, before that, there is a need for standardization and validation [14].

Synaptic Silencing: The Main Molecular Effect of BoNT-A

As described many times, BoNTs are produced primarily by bacteria of the genus *Clostridium* and have been classified as eight distinct types (A–G and X) [15], while over 40 subtypes are known, five for BoNT-A (BoNT-A1–5). BoNT-A1 is only one

commercially available in the USA and Europe (Botox® and Botox Cosmetic® *onabotulinumtoxinA* by Allergan; Dysport® *abobotulinumtoxinA* by Ipsen; Xeomin® *incobotulinumtoxinA* by Merz; and only one BoNT-B1 preparation Myobloc® *rimabotulinumtoxinB*, by Solstice Neurosciences) [16]. BoNTs contain two core subunits responsible for toxic and therapeutic activity: light chain (50 kDa) and heavy chain (100 kDa), linked by disulfide bond. The light chain is Zn^{2+} metalloprotease that represents the actual toxic domain of the holoprotein [17]. This enzyme specifically cleaves the particular proteins responsible for the fusion of synaptic vesicles with the plasma membrane: synaptosomal N-ethylmaleimide-sensitive attachment protein receptors (SNAREs) containing several different proteins: syntaxin, synaptobrevin (VAMP), and synaptosomal-associated protein 25 (SNAP-25). BoNT-A, BoNT-E, and BoNT-C cleave SNAP-25, and BoNT-B, BoNT-D, BoNT-F, and BoNT-G cleave VAMP, while BoNT-C cleaves both SNAP-25 and syntaxin [17, 18]. Consequently, function of synaptic vesicles is prevented, and the result is neuronal silencing. Silencing of neuromuscular junction causes flaccid paralysis as a main sign of botulism. As could be expected, heterozygous missense mutation in the SNAP-25 gene causes *congenital myasthenic syndrome-18* with myasthenia, cortical hyperexcitability, ataxia, and intellectual disability [19]. Less predictable is association of SNAP-25 polymorphisms with *attention-deficit disorder* [20].

Molecular action of BoNTs consists of several steps [21–23]:

(a) Binding of BoNTs to the presynaptic membrane, mediated with gangliosides (polysialogangliosides, PSG) and synaptic vesicle protein 2 (SV2)
(b) Internalization of BoNTs, via endocytosis of the BoNTs-acceptor complex (PSG and SV2) inside the neurons
(c) Translocation of BoNTs' light chain from the endocytosed vesicle to the neuronal cytosol and release of the light chain in the cytosol by reduction of the interchain disulfide bond.
(d) Cleavage of protein target by Zn^{2+}-endopeptidase blocking the activity of specific SNARE proteins (Fig. 5.3)
(e) Axonal transport (retrograde and anterograde) to the place of enzymatic action [24, 25]
(f) Cell-to-cell, transsynaptic transport to remote place of action [24, 26].

The high potency and neurospecificity of the BoNTs is associated with binding two acceptors ganglioside and SV2 [23]. Dual acceptor binding is probably responsible for high neurospecificity of BoNT, including higher affinity to block the release of acetylcholine and then the release of other neurotransmitters. As could be expected, transgenic mice and cell lines devoid of PSG are largely resistant to BoNTs [27]. Interestingly, BoNT-s is not toxic for insects that are devoid of PSG. This makes insects an excellent vector to spread botulism among birds and fishes [28].

Pharmacologically unique characteristic of BoNT-A is long-lasting effect. Following i.m. injection of radioiodinated BoNT-A, the radioactivity returned to control value within 12 h. In vitro in neuronal culture, enzymatic activity of BoNT-A persists for up to 1 year; in humans, the effect can last 3–6 months and in

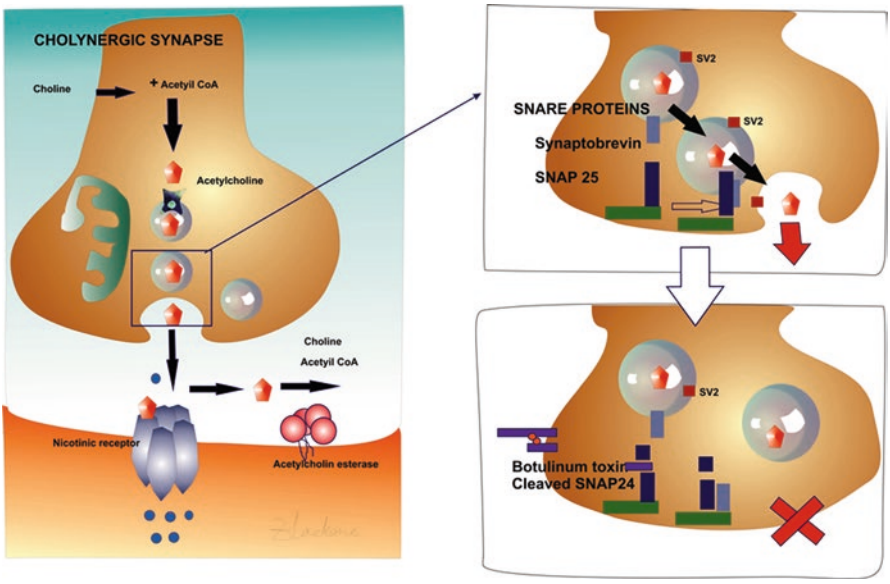

Fig. 5.3 Synapse silencing by BoNT-A

experimental animals usually up to 30 days. Turnover of SNARE proteins is estimated to be 4–5 days. However, BoNT-A duration of action is much longer than turnover rate of SNARE. There are several theories that attempt to explain the length of the BoNT-A effect, but a definitive answer is still to be expected.

Fifteen Years' Debate: Controversies About Botulinum Toxin A Site of Analgesic Action

The question whether BoNT-A affects only peripheral nerve endings or is it axonally transported to the CNS was a matter of debate lasting over 15 years. BoNT-A and even BoNT-B have a remarkable similarity to tetanus toxin (TeNT). Molecular structure is similar, molecular target in both cases is SNARE protein complex, and final results of BoNTs and tetanus toxin are neuronal silencing. Difference is in central target(s) that are known for TeNT. Clinical difference is remarkable as well: spastic vs. flaccid paralysis occurs. This makes the debate if BoNTs are axonally transported or not from periphery toward CNS fundamentally important. Most important arguments demonstrated the existence of axonal transport, and central effects of peripherally applied BoNT-A are shortly discussed in the following text.

Out of many behavioral experiments (review Matak and Lackovic [29]), most convincing arguments showing central effects of peripherally applied BoNT-A are obtained in studies of mirror pain.

"Mirror pain," typically presented as mechanical allodynia (pain in response to light innocuous mechanical stimuli), is a phenomenon where the pain is perceived in an uninjured area contralateral to the actual site of injury/inflammation. Although the exact mechanisms for the contralateral spread of pain are still a matter of debate, it is accepted to be centrally mediated. Mirror image pain (MP) can be experimentally induced by different types of tissue injury. For example, acidic saline-induced mirror pain is developed to study chronic, widespread, and neuronally mediated musculoskeletal pain.

When applied peripherally, BoNT-A reduced pain on both sides. This bilateral effect was prevented with ipsilateral colchicine that blocks axonal transport, thus suggesting retrograde axonal transport as a prerequisite for the central antihyperalgesic effect of BoNT-A. The bilateral effect was elicited only if BoNT-A was applied on the side of injury, not on the contralateral side, thus suggesting that the toxin is not transported from the site of application to the contralateral side [30].

Bilateral long-lasting effect of unilateral peripheral toxin application was demonstrated in the models of streptozotocin- and paclitaxel-induced polyneuropathy [31, 32], as well. Thus, it was unequivocally shown that bilateral toxin effect after peripheral application is not just a phenomenological finding after specific type of injury but is a feature that distinguishes BoNT-A from other locally applied analgesic drugs.

The quantities of BoNT-A that might come into the CNS structure are extremely low, and up to now it was not possible to detect functionally active toxin in the spinal cord or the brain. However, light chain of BoNT-A is a Zn^{2+} endopeptidase cleaving SNAP-25. In series of immunohistochemical experiments using specific antibody against cleaved SNAP-25, Matak et al. were able to identify the presence of cleaved SNAP-25, clear footstep of the enzymatic activity of BoNT-A (Fig. 5.4), in dorsal horn of the spinal cord and trigeminal nuclei in the brainstem [25, 33, 34]. It is important that those immunohistochemical experiments were performed at the end of behavioral experiments showing antinociceptive action of peripherally applied BoNT-A.

All mentioned experiments clearly demonstrate the existence of axonal transport of peripherally applied BoNT-A and enzymatic action within CNS. Some experiments like those on bilateral and mirror pain cannot be explained differently other by central action of BoNT-A. However, this does not exclude the participation of peripheral endings of sensory neurons in some actions of BoNT-A.

Botulinum Toxin Beyond First Sensory Neuron: Mechanism of Analgesic Effect

The exact mechanism of BoNT-A action on pain in the dorsal horn of the spinal cord or brain nuclei is not completely elucidated. There are two general possibilities for the central action of BoNT-A:

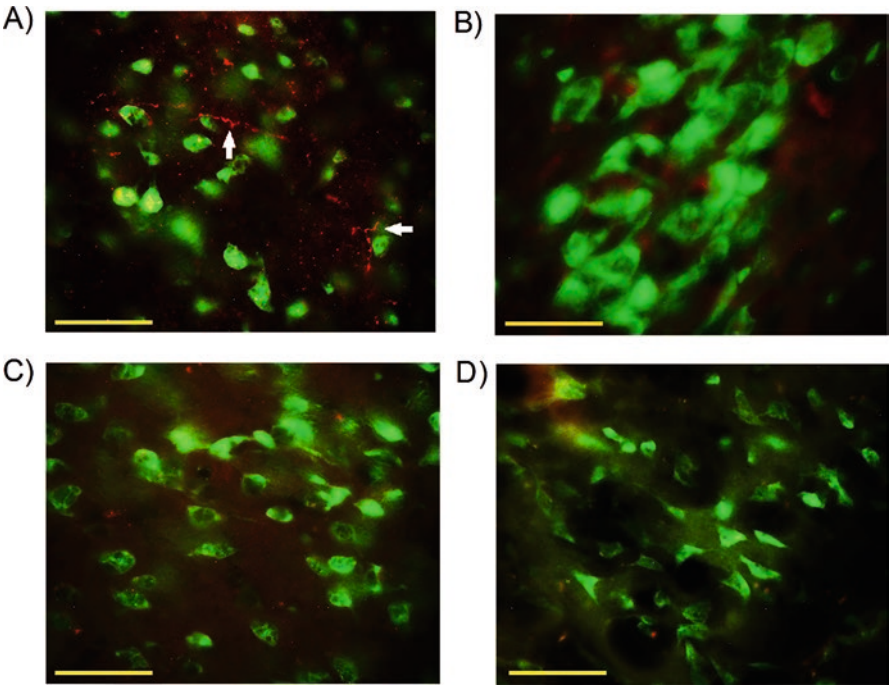

Fig. 5.4 Presence of BoNT-A-cleaved SNAP-25 occurrence in the trigeminal nucleus caudalis (TNC) and the lack of detectable action in sensory regions upstream from TNC. Cleaved SNAP-25 was examined 6 days after peripheral BoNT-A injection into the rat whisker pad (5 U/kg). SNAP-25 immunoreactivity (red) was visible in TNC (**a**). Cleaved SNAP-25 (red) was not visible in ipsilateral locus coeruleus (**b**), periaqueductal gray (**c**), or contralateral ventral posteromedial nucleus of thalamus (**d**). NeuN (green) represents neuronal counterstaining. Scale bar = 100 μm. (From Matak I. PhD thesis)

1. The activity ends by silencing primary sensory neuron, thereby stopping the pain information further in the CNS.
2. Or thereafter, indirectly or transsynaptically, BoNT-A modulates smaller or larger neural loops which participate in the forming of memory of pain in the CNS that could explain bilateral effects after unilateral peripheral administration, similar effect in mirror image allodynia, and the like.

Investigation of pain in the area of trigeminal innervation provided additional important insights into the central mechanisms of BoNT-A action on pain. BoNT-A unilateral peripheral application significantly reduced bilateral mechanical allodynia induced after unilateral infraorbital nerve injury and temporomandibular joint inflammation. After intraganglionic application, colchicine also prevented BoNT-A bilateral effect on pain. Additionally, it was shown that peripherally injected BoNT-A reaches trigeminal nucleus caudalis where it inhibits the expression of TRPA1, TRPV1, and TRPV2 that was induced after infraorbital nerve injury. Furthermore, enzymatic activity of BoNT-A (cl-SNAP-25) in ipsilateral dura mater

and colocalization with CGRP in intracranial dural nerve endings was demonstrated after peripheral toxin application. Based on these results, it was suggested that after entering extracranial trigeminal afferents and upon retrograde axonal transport to the trigeminal ganglion, BoNT-A is transcytosed to meningeal afferents and anterogradely transported to dura mater [35–37].

Neuronal events after BoNT-A reaches CNS are only partially investigated. Other neurons and also glial cell could be affected.

Experimental data propose the interaction with opioid and GABA inhibitory systems that have a role in the attenuation of sensory input to the spinal dorsal horn. Involvement of these two systems was demonstrated in the model of carrageenan-induced mirror pain, as well. Namely, when applied at the level of the lumbar spinal cord, opioid antagonist naloxonazine and GABA antagonist bicuculline abolished toxin's bilateral effect on pain. Since opioid and GABA antagonists didn't affect the BoNT-A action on pain if injected either in cisterna magna or cerebral ventricles, it was logical to conclude that BoNT-A reduces pain primarily at the level of the spinal cord [38–40].

Effects on Astroglia and Microglia (Neuroinflammation) While searching for an explanation for the central mechanism of the long-term effect of BoNT-A on chronic pain, investigation of the involvement of glial cells in the antinociceptive action of BoNT-A seemed the logical next step, keeping in mind important role of glial cells in the induction and persistence of chronic pain.

In 2011, Mika et al. showed that a single intraplantar administration of BoNT-A, after chronic constriction nerve injury in rat, diminished the injury-induced ipsilateral spinal and dorsal root ganglia upregulation of microglial C1q mRNA (measured by RT-PCR). These results suggested that reduction in neuroimmune interactions between microglia and neurons is connected and according to authors could be the key to the long-lasting BoNT-A effect on neuropathic pain [41]. Furthermore, in the same model in mice, it was demonstrated that intraplantar BoNT-A (15 pg/paw) injection reduced microglia activation but also astrocyte number and the percentage of activated astrocytes in both the dorsal and ventral horns of the spinal cord [42]. Similarly, Finocchiaro et al. when investigating the analgesic effect of BoNT-B found reduced abundance and activation of astrocytes in the ipsilateral dorsal but not ventral horn of the spinal cord after the constriction injury of the mice sciatic nerve. In contrast to BoNT-A, BoNT-B did not change the expression of activated microglia, thus suggesting different effects of BoNT-A and BoNT-B in neuropathic pain [43].

Using colocalization experiments of cl-SNAP-25 (a marker of enzymatic activity of BoNT-A) with markers of either microglia or astrocyte, it was shown that after peripheral injection of BoNT-A, its enzymatic product cl-SNAP-25 colocalized with glial fibrillary acidic protein (GFAP), a protein marker expressed in non-myelinating Schwann cells, and in spinal cord astrocytes, but not with the marker of microglial activation [44]. This was an indication that BoNT-A may be transcytosed from nociceptive fibers in spinal cord and may enter into glial cells. The absence of

cl-SNAP-25 in microglia can be explained with predominant expression of SNAP-23 in these cells, in contrast to astrocyte which expresses both proteins.

Additionally, in satellite glial cells (SGCs) of rat trigeminal ganglion expressing both SNAP-23 and SNAP-25, BoNT-A in a concentration of 100 pM blocked ionomycin-stimulated glutamate release. These findings demonstrate the existence of vesicular glutamate release from SGCs, which could potentially play a role in the trigeminal sensory transmission and additionally suggested interaction of BoNTA with non-neuronal cells at the level of TG [45].

Except in the models of neuropathic pain, mostly induced by nerve injury, glial cell activation was demonstrated in models of chronic inflammation. Specific glial cell populations become activated in both the trigeminal ganglia and the CNS following induction of temporomandibular joint inflammation using complete Freund's adjuvant (CFA) intra-articular injection. Seventy-two hours after CFA injection, activated microglial cells can be observed in the ipsilateral trigeminal subnucleus caudalis and the cervical dorsal horn, with a significant upregulation of ionized calcium binding adaptor molecule (Iba1) immunoreactivity but with no signs of reactive astrogliosis in the same areas [46].

In the CFA-induced monoarthritis model, significant elevation of microglial activation markers Iba-1 and phosphorylation of P38MAPK (P-p38MAPK) was detected in the lumbar spinal cord even 21 days after induction of ankle joint inflammation, thus suggesting the role of microglia not just in induction but in maintenance of chronic hyperalgesia as well, at least in this chronic pain model. The intra-articular administration of a single effective dose of BoNT-A (5 U/ankle) on day 21 after CFA injection significantly decreased protein overexpression and immunoreactivity for Iba-1 and P-p38MAPK in CFA-induced rat. It additionally inhibited the increase in TNF-α mRNA and P2X4R mRNA expression induced by CFA injection. These results suggested that BoNT-A can modulate neuroinflammation in chronic inflammatory pain by reducing the activation of microglial cells and the release of microglia-derived TNF-α, possibly by inhibiting the activation of the P2X4R-P38MAPK signaling pathways in spinal microglial cells [47].

The emerging results provide novel insights into the potential mechanism of BoNT-A action on chronic pain at the level of the spinal cord, with the reduction of neuroinflammation in its center.

Antinociceptive Effects of Other BoNT Serotypes

In humans, naturally occurring botulism is caused by serotypes A, B, E, and F, while intoxication with other serotypes is also possible. Thus, theoretically other BoNT serotypes could be employed for the treatment of neurological disorders, particularly in case of a developed immune resistance to BoNT-A. Apart from BoNT-A, BoNT-B (rimabotulinumtoxinA) is the only clinically used serotype registered for treatment of cervical dystonia. BoNT-B cleaves synaptotagmin part of SNARE

proteins and also prevents neurotransmitter release the same as BoNT-A. BoNT-B reduces pain associated with cervical dystonia [48]. Case reports or retrospective studies have reported possible efficacy in the treatment of migraine headache [49, 50], but there are no placebo-controlled clinical studies.

In the formalin test, BoNT-B injected intrathecally (0.5 U) or intraplantarly (1 U) reduced nocifensive behavior, c-Fos activation, and neurokinin-1 (NK1) receptor internalization (indicative of substance P release) in intraplantar formalin-evoked pain. Intrathecally or intraplantarly injected BoNT-B reduces the experimental mononeuropathic pain evoked by spinal nerve ligation or constriction of sciatic nerve and polyneuropathic pain evoked by cisplatin [43, 51–53]. Interestingly, BoNT-B did not induce a regenerative effect upon sciatic nerve injury comparable to BoNT-A [43].

The effect of peripherally injected BoNT-B was associated with lowered VAMP-1 expression in dorsal root or trigeminal ganglia, a possible indication of toxin's retrograde axonal transport and cleavage of the synaptic protein. In addition, unilateral reduction of otherwise bilateral increase of NK1 receptor internalization induced by intrathecal injection of TRPV1 activator capsaicin suggests the BoNT-B action at the level of central afferent terminals. Blockade of c-Fos expression after intrathecal substance P injection was interpreted as a possible transsynaptic cell-to-cell traffic of the toxin within the dorsal horn [52]; however, this has not been definitively confirmed. Antinociceptive effect upon dural stimulation with capsaicin and reduction of VAMP-1 expression in trigeminovascular neurons innervating the dura after facial BoNT-B injection suggest the toxin transcytosis within trigeminal neurons innervating different intracranial and extracranial targets [54].

Up to now, other toxin serotypes have not been investigated for analgesic efficacy in humans or preclinically in pain models; however, some insights into their actions have been obtained by employing cultured sensory neurons. BoNT-E was shown not to affect the evoked CGRP release, due to the possibility that BoNT-E heavy chain lacks acceptor binding activity on rat sensory neurons [55]. BoNT-B, on the other hand, did not prevent the evoked neurotransmitter release most likely due to the mutated VAMP-1 which is resistant to the proteolytic activity of the toxin, which was also reported in vivo [51]. Unlike BoNT-B, BoNT-D was able to cleave VAMP isoforms and prevent the neurotransmitter release [56]. BoNT-C1 prevents the capsaicin-evoked CGRP release most likely due to its effect on both, SNAP-25 and syntaxin1, compared to BoNT-A which cleaves SNAP-25 only and has no effect on CGRP release in vitro [56, 57].

Antinociceptive Effects of Recombinant BoNT-A-Based Molecules

Considerable efforts have been made in designing new recombinant BoNT-A-based toxins with higher selectivity for nociceptive neurons and, supposedly, reduced risk for potential side effects mediated by native holotoxin's nonspecific action in other

types of neurons. One of the common strategies has been to develop a chimeric molecule which retains the enzymatic light chain (L) and heavy chain (HC) translocation domains of the native toxin molecule and to exchange the acceptor binding domain of HC for another domain that targets primarily first-order or second-order sensory nociceptive neurons [58].

First of the studies employing these strategies used a plant-derived lectin that recognizes sensory neurons by binding to the glycoproteins residing on their neuronal surface. Duggan et al. reported that such construct reduces the glutamate and substance P release from embryonic dorsal root ganglion neurons [59]. Despite being successfully retargeted to block the neurotransmitter release from sensory neurons and shown to have improved toxicity profiles, lectin containing construct exhibited much lower in vitro potency on substance P release.

A more recent study (Maiarù et al. 2018) reported the use of L-HN construct linked to substance P or endogenous opioid dermorphin, applying the so-called "protein stapling" technique [60]. Stapling technique employed for connection of L-HN to native HC of BoNT-A produces a recombinant toxin termed BiTOX with larger size and lowered paralytic potency, supposedly due to a larger size compared to native toxin. In rats, BiTOX injected intraplantarly reduces CFA, capsaicin, or neuropathic pain-evoked mechanical hyperalgesia [61]. Mentioned results suggest reduced spectrum of antinociceptive effects of BiTOX compared to native holotoxin.

One of the major hurdles in employing such high doses of recombinant retargeted toxins could be the development of immunological resistance upon repeated use, already considered a major problem even at low doses of BoNT-A native toxin-based preparations used. In line with that possibility, it was reported that recombinant BoNT-A with lower potency compared to native toxin, already after second injection (200 ng dose), exhibits lower reduction of toe spreading reflex – indicative of reduced response to BoNT-A suggested to be due to immune response [62].

Dolly and collaborators conducted a series of studies by combining the BoNT-A with light chains of other serotypes into functional chimeras. Based on findings that, unlike BoNT-A itself, BoNT-E light chain coupled with BoNT-A heavy chain prevents capsaicin-evoked CGRP release under certain experimental conditions, it was hypothesized that BoNT-E protease could be more efficacious sensory neurotransmitter release blocker [55, 63]. More recent studies employing a similar chimera demonstrated a prolonged activity in neuropathic pain models [64]. Moreover, repeated injection of the recombinant toxin reproduced the unchanged analgesic efficacy, suggesting the lack of immune response. The overall efficacy of L(E)-BoNT-A against neuropathic pain was higher compared to native BoNT-A, which is, thus, the first observation of a recombinant molecule with improved efficacy compared to BoNT-A holotoxin. This could be of clinical benefit since, due to the lack of dose-response relation within the safe non-paralytic range, employing higher BoNT-A doses does not lead to improved antinociceptive efficacy.

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