Chapter 3
Analgesic Effects of Botulinum Neurotoxins: Data from Animal Studies Volunteers

Introduction

Over the past 20 years, a large volume of literature has been published in the field of pain based on investigations conducted on animals and asymptomatic human volunteers. These data have defined new pain receptors, expanded our knowledge on pain mediators/modulators, and refined our understanding of pain pathophysiology. Moreover, new data derived from experiments on animals and human volunteers have provided important information on how BoNTs influence pain mechanisms and alleviate pain by altering and modifying the function of nerve endings, 0-), and spinal and brain stem neurons. In this chapter, the pathophysiology of pain based on this novel data is presented and discussed.

Pathophysiology of Pain at Peripheral and Central Levels

Pain is an unpleasant and annoying sensation which is usually provoked by a noxious stimulus contacting skin, bone, or muscles; a less common form of pain, central pain originates from the disturbance of structures mostly at the level of spinal cord or thalamus. Specialized structures and pathways of the somatosensory system participate in conveying the pain signals to the sensory cortex (Fig. 3.1). Final perception of pain at the cortical level requires four sequential processes: transduction, transmission, modulation, and perception. During the transduction phase, noncapsular, nociceptive nerve endings are stimulated by pain-inducing agents (heat, cold, chemical, and mechanical). Stimulation of free nerve endings by noxious stimuli opens sodium channels which are present on the sensory nerves in abundance. With sodium influx, the negative charge inside of the cell (−70 uV) moves toward positivity, and when it reaches +40, it generates an action potential that travels along

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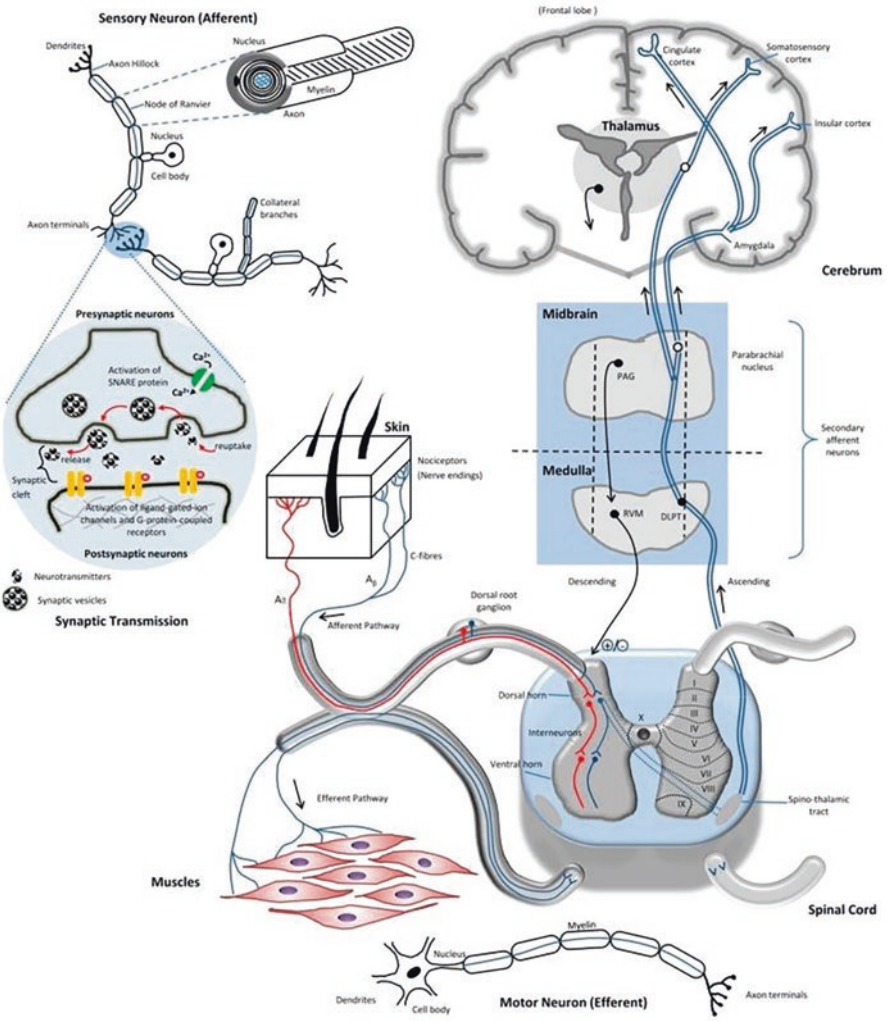

Fig. 3.1 Pain pathways. (From Yam et al. Int J mol Sci. Reproduced under Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). Publisher MPDI)

sensory pain axons conveying nociceptive information to dorsal root ganglion (DRG) and to the central nervous system. The sensory fibers that convey pain signals are unmyelinated C and small, myelinated Aδ fibers. C fibers are very thin with a diameter of less than 2 micrometers and a conduction velocity of about 2–5 meters/second. They are activated by poorly localized stimuli and are polymodal, that is, they respond to a variety of stimuli, such as chemical agents, heat, and cold. Aδ fibers are the smallest myelinated fibers with a diameter of 2–5 μm and with faster conduction of 30 meters/second compared to C fibers [1]; they respond to tactile and temperature stimulation.

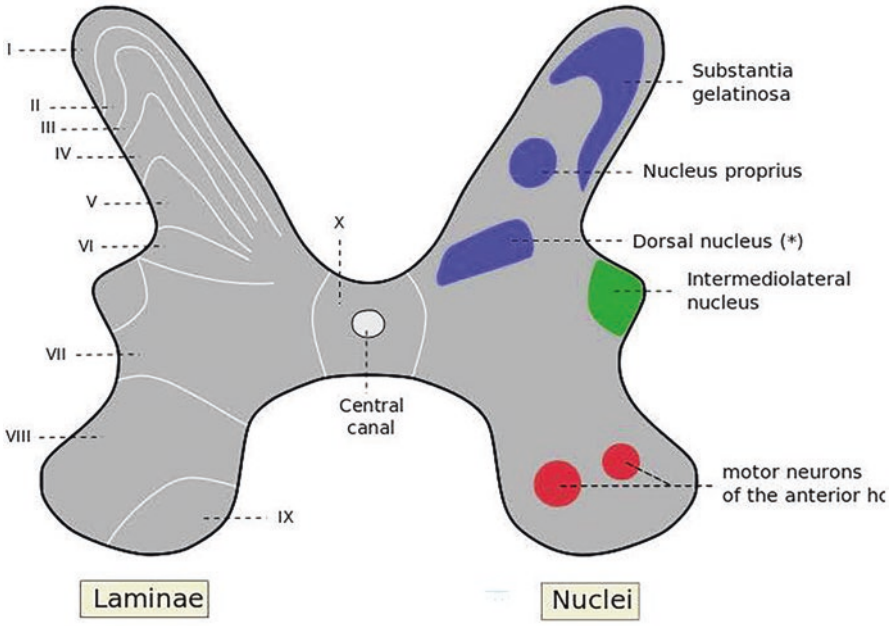

Fig. 3.2 Anatomical location of Rexed lamina including substantia gelatinosa (lamina II)

The C fibers are peptidergic or nonpeptidergic. The peptidergic fibers use Substance P (SP) and calcitonin gene-related peptide (CGRP) as pain signal transmitters. The second nociceptive neurons are located in the gray matter of the posterior horn of the spinal cord which has a laminar structure (Rexed lamina), numbered from layers I to VII (Fig. 3.2). The peptidergic C fibers end in Rexed lamina I and outer part of Rexed lamina II (substantia gelatinosa) which are the superficial layers of the spinal dorsal horn, whereas nonpeptidergic fibers terminate in the inner part of lamina II [2].

Dorsal root ganglia (DRG), which are located in dorsal roots, contain thousands of specialized, bipolar sensory cells that encode and transmit sensory information (received from periphery) to the spinal cord sensory neurons. Peptidergic neurons of DRG contain substance P (SP), CGRP, and somatostatin. The most common neurotransmitter made by DRG cells is glutamate, but many cells also express SP, a major nociceptive transmitter [3]. After injury, C fibers may alter DRG sensitivity by changing the intracellular calcium level affecting N-methyl-aspartate receptors of DRG neurons. The resultant plastic reorganization of DRG is believed to play an important role in the development of peripheral sensitization and process of pain chronicity [4].

The C and Aδ fibers enter the spinal cord via posterior roots and, after traveling a few segments in the spinothalamic tract, synapse with ipsilateral sensory neurons (mainly in Rexed areas I and II). The axons of these neurons then cross the spinal

gray matter in the anterior decussation and travel in the contralateral spinothalamic tract rostrally (Fig. 3.1).

At the spinal cord level, lamina I carries both nociceptive and wide dynamic range neurons. The nociceptive neurons of this lamina express a variety of peptide transmitters such as SP, CGRP, serotonin, and enkephalin and respond to noxious stimuli. Lamina II is rich in inhibitory interneurons that release GABA [4]. These neurons arborize to the other lamina of the posterior horn including lamina I and II. It is believed that the neurons of lamina II (substantia gelatinosa) have a modulatory effect on incoming pain signals [5].

In the thalamus, the posterior–inferior segment of ventralis posterior (VP) nucleus is a major site of transmission of the nociceptive signals to the cortex; this region is the main target of deep brain stimulation (DBS) for relieving intractable pain [6]. The central nuclei of the thalamus (intralaminar, central medial, and parafasciculus) also receive nociceptive input from the spinothalamic tract as well as input from the brainstem reticular formation.

At the cortical level, primary and secondary somatosensory cortices (SI and SII) are the main recipients of the nociceptive information (Fig. 3.1). SI, due to its graded and proportional response to pain signals, is considered to be the main site for the appreciation of the discriminative quality of pain. SII receives nociceptive information from both contralateral and ipsilateral spinothalamic tracts. Nociceptive signals end primarily on cortical levels III and IV. Other cortical areas which receive pain signals include the insular, dorsolateral, prefrontal, and cingular cortices as well as the amygdala. The anterior cingular cortex which receives information from intralaminar nuclei of the thalamus is believed to be involved in motivational and emotional responses to pain [7].

Pain Modulation

The traveling pain signals to the cortex are modulated both on the way to the cortex and by specific descending tracts which influence spinal sensory neurons. One purpose of this pain modulation is protecting the cortex from excessive nociceptive stimuli. As already mentioned, on the ascending arm of the pain system, substantia gelatinosa at the spinal level and central nuclei of the thalamus, which receive input from the reticular formation, are all involved in pain modulation. A descending and better-studied system for pain modulation exists that includes neurons of periaqueductal gray region in the midbrain as well as the neurons of rostroventromedial (RVM) medulla which are a part of the medullary reticular formation. These neurons exert their antinociceptive effects on sensory neurons of lamina I and II of dorsal horn using noradrenalin and serotonin as neurotransmitters.

In addition, the descending endogenous opioid pain-modulating system also reduces pain transmission. Activation of mu-opioid receptors blocks pain both centrally and via activating abovementioned descending modulating systems. It does this through changing membrane conductivity and the state of protein

phosphorylation. Dynorphin, an opioid peptide, is present in periaqueductal gray (PAG), midbrain reticular formation, and the laminae I to IV of spinal dorsal horn [4, 8].

Data from Animal Studies

Data from animal studies indicate that BoNTs can reduce or block pain transmission in peripheral nerves and at the level of DRG, spinal cord, and midbrain. Convincing data for the thalamic and cortical levels are not available, however. BoNTs block pain transmission through influencing the function of a variety of pain receptors, pain transmitters, and modulators such as substance P and CGRP as well as opioid receptors.

One of the first studies investigating the analgesic effect of BoNTs was published by Cui and coworkers in 2004 [9]. The authors used the formalin pain model for their experiments. In this model, subcutaneous injection of formalin in rat's paw produces a biphasic pain response. The first peak of pain that develops within 5 min of injection is caused by the direct chemical effect of formalin upon C fibers. The second peak occurs within 15–60 min of injection and is a more intense pain induced by local tissue inflammation [10] during which there is local accumulation of inflammatory agents (neuropeptides, kinins) and pain mediators (glutamate, substance P, and CGRP) at the injection site. It is believed that, unlike the first peak, the second peak of pain is not related to the irritation of C fibers, but rather it represents pain related to central sensitization of the pain pathways. Cui et al. pretreated rats with onaA for 2–12 days in order to observe the timeframe of onaA's effect on formalin-induced pain. Four groups of rats that received 3.5, 7, 15, and 30 μ/kg of onaA diluted in 0.9% saline (22 ml bolus) into the hind paw subcutaneously. For control rats, the same volume of 0.9% saline was injected into the hind paw. The rats were then injected with 50 ml of 5% formalin in the same paw, and their pain behavior (lifting/licking) was recorded within 5 minutes post injection and, again, at 15–30 min post injection corresponding to the first and second peaks of formalin-induced pain. Pretreatment with onaA (at doses mentioned above) 5 days prior to formalin injection significantly reduced the level of formalin-induced pain in a dose-dependent manner (Fig. 3.3).

As evident in Fig. 3.3, the second peak of pain (inflammatory peak) was the one most affected by the onaA pretreatment. The largest dose (30 units/kg) used in this experiment affected both peaks, but rendered the animals too lethargic to make a reliable assessment. The authors also noticed significant reduction in paw edema in onaA-treated animals. Furthermore, the animals pretreated with onaA demonstrated significant reduction of accumulated tissue glutamate (after formalin injection) compared to animals who received saline only; the mean tissue glutamate level was 280.2 ng/ml for those injected with saline versus a mean of 208.4 ng/ml for those treated with 15 μ/kg of toxin ($P < 0.05$). These results demonstrated that

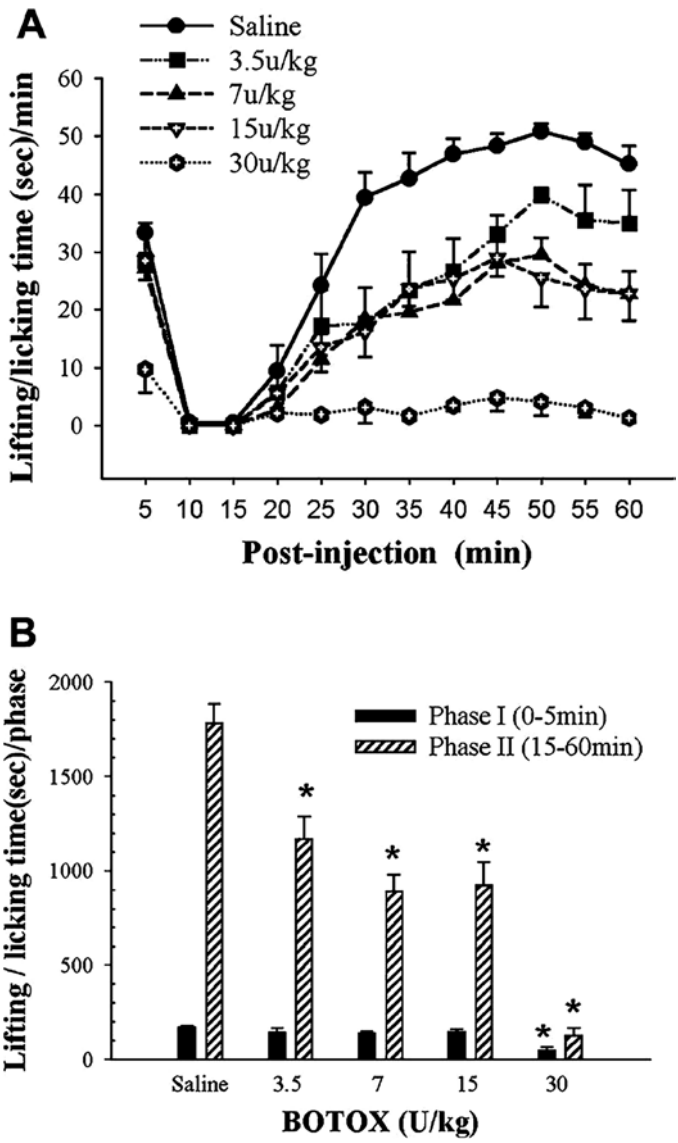

Fig. 3.3 Pretreatment with BoNT-A reduces formalin injection-induced paw pain in rats in a dose-dependent manner. (Cui et al. [9], with permission from the journal *Pain* and publisher)

onabotulinumtoxinA exerts both analgesic and anti-inflammatory effects in formalin-induced model of pain.

Ten years later, Marino et al. [11], in a similar experiment, investigated the effect of botulinum toxin-B (rimabotulinumtoxinB, rimaB/Myobloc) in formalin pain model. One unit of BoNT-B or a similar volume of saline was injected into

intraplantar region unilaterally in mice. Pretreatment of mice with BoNT-B before saline injection reduced intraplantar formalin-evoked flinching, capsaicin-evoked plasma extravasation in the hind paw, formalin-evoked dorsal horn substance P (SP) release, formalin-evoked dorsal horn neuronal activation (c-fos) as well as ipsilateral dorsal root ganglion (DRG) vesicle-associated membrane protein (VAMP) and ipsilateral SP release otherwise evoked bilaterally by intrathecal capsaicin administration. This study showed that injection of BoNT-B affected and reduced the release of SP both by DRG neurons and by spinal cord sensory neurons of the dorsal horn.

In another experiment, Welch et al. [12] studied the effect of botulinum toxins A, B, C, and F on SP release from DRG neurons that had been exposed to elevated extracellular potassium in order to enhance their calcium-dependent SP release. All toxins exerted some degree of SP release inhibition, but this effect was most prominent for BoNT-A and least notable for BoNT-B. BoNT-A cleaved the SNAP 25 within 2 h, but inhibited SP release at 4th hour. In another study [13], researchers demonstrated that acute bladder injury after exposure to HCL resulted in marked release of SP and CGRP into the injured bladder tissue (1235 and 1655 pg/g, respectively, compared to 183 and 449 pg/g for controls, respectively) ($P < 0.001$). The levels of SP and CGRP dropped to 870 and 1033 pg/g, respectively, following BoNT-A injection ($P < 0.05$ and <0.01). Similar results with BoNT-A administration were observed on elevated levels of SP and CGRP after chronic exposure to cyclophosphamide.

In trigeminal neurons, the release of CGRP was blocked after exposure to BoNT-A [14]. In these neurons, SNAP25 and CGRP were noted to be colocalized. A, C, and D botulinum toxins (but not B) also blocked calcium-dependent SP release from the same neurons [14]. BoNTs, however, failed to block capsaicin-induced elevation of CGRP from trigeminal neurons. In a later experiment, the same group of researchers studied and noted that an A/E chimera of BoNT which specifically targets the sensory cells can subdue capsaicin activation of TRPV1 nociceptive channel as well as the rise of CGRP that results from capsaicin exposure [15].

Matak et al. [16] demonstrated the role of SP in the analgesic effect of BoNT-A by studying knockout mice lacking encodement of SP-neurokinin gene. In this model, injection of BoNT-A before formalin in mice showed no analgesic effect. In another study [17], investigators found that injection of botulinum toxin-A reduced the number of immunoreactive substance P (SP-IR) and calcitonin gene reactive protein (CGRP) in the sensory neurons of dorsal root ganglia innervating pig's bladder. In a recent experiment, special delivery of a genetically engineered BoNT-D protease (light chain) to the sensory cells prevented release of substance P from sensory neurons [18]. Further support for effect of BoNTs on CGPR release was provided in another recent study where authors delivered the light chain (active moiety) of several BoNTs to rat's DRG cells using engineered herpes simplex virus as a vector. They noted a marked decrease in CGRP release from DRG cells; this effect was most noticeable for Type D and A toxins.

Effect of Botulinum Toxins on Pain Channels and Receptors

Effect on Sodium Channels

As mentioned above, activation of Na + channels in response to a noxious stimulus generates a propagating action potential in the peripheral nerve. Sodium channels are present in abundance on pain receptors, C fibers, and DRG, and hence play a pivotal role in transmitting nociceptive signals. Among a variety of known Na + channels, Na1.7, Na1.8, and Na1.9 are most relevant to pain. The Na + channels are classified as tetrodotoxin sensitive (TTX-S) with a fast activation/inactivation nature, whereas tetrodotoxin-resistant (TTX-R) channels have slow activation/inactivation. Na1.7 is a TTX-S channel, whereas Na 1.8 and 1.9 are TTX-R type of sodium channels. Sodium channel mutations are associated with some of the most severe forms of human pain such as the pain experienced in erythromelalgia [19]. Shin et al. [20] have shown that injection of botulinum neurotoxin A2 significantly inhibits neuronal Na channel in rats. Unlike tetrodotoxin (TTX), local anesthetics, and antiepileptic drugs, BoNT completely inhibited Na channels in a concentration-dependent manner. The authors concluded that type A neurotoxins inhibit membrane Na (+) channel activity in CNS neurons and also in both TTX-sensitive and -insensitive peripheral dorsal ganglion cells (40% more than controls). Based on their results, they suggested that BoNT-A2 has a potential for treatment of epilepsy and several types of pain.

Effect on Transient Receptor Potentials (TRP) Channels

One of the major areas of progress in understanding the molecular physiology of pain is the discovery of transient receptor potential channels (TRPs) [21]. TRPs are expressed specifically on sensory nociceptive neurons. These receptors which are made of vanilloid protein (TRPV) are cation-gated calcium channels. Produced by DRG neurons, these protein channels are then transferred by axonal transport peripherally to the nerve endings and centrally to dorsal horn neurons (Rexed lamina II-substantia gelatinosa). There are several types of TRP channels designated as TRP1, TRP2, TRP3, TRP4, and TRP8, but TRPV1 plays the dominant role in neuropathic and nociceptive pain. The influx of cations, especially calcium, opens the TRPV1 channel leading to hyperexcitability of the peripheral and central neurons enhancing pain. Heat of over 42 °C, chemicals such as capsaicin, and low pH of <5.9 directly stimulate and open the TRPV1 channel. A large number of other agents also activate TRPV1 indirectly including inflammatory mediators such as prostaglandin E2, proteases, and nerve growth factor (NGF) [22]. The function of TRPV1 channel seems to be different in peripheral (DRG) and central (dorsal horn of spinal cord) neurons. While TRPV1 in DRG neurons receives pain signals from periphery and conducts the information to spinal cord sensory neurons, activation of

TRPV1 in spinal cord neurons releases glutamate locally and promotes central excitability of the sensory neurons [23].

Inflammatory hyperalgesia is absent in TRPV1 knockout mouse [24], and TRPV1 expression is markedly enhanced in neuropathic pain and inflammatory hyperalgesia. Intrathecal injection of TRPV1 antagonist AS1928370 alleviates the neuropathic pain in the mouse model [25]. Another TRPV channel, TRPA1, is also upregulated in DRG and dorsal horn neurons by peripheral inflammation and is implicated in cold hyperalgesia caused by inflammation and nerve injury [26].

Several studies have shown that BoNTs can reduce or block the activity of TRP channels. In one study, injection of an engineered A/E botulinum toxin chimera reduced the function of TRP1 channel and improved capsaicin-induced hyperalgesia [15]. Xiao et al. [27] demonstrated that rats with neuropathic pain, when injected with botulinum toxin type A, showed reduction of clinical hyperalgesia and TRPV1 expression after BoNT exposure. Subcutaneous BoNT-A injection (0.25, 0.5, or 5 ng/kg) into the face close to the ophthalmic division of the trigeminal ganglion neurons decreased TRPV1-immunoreactive neurons in the trigeminal ganglion and TRPV1-immunoreactive fibers in rat trigeminal nerve terminals [28]. The authors believed that the mechanism by which BoNT-A reduced TRPV1 expression was inhibition of TRPV1 plasma membrane trafficking and proteasome-mediated degradation in the cytoplasm. Further information on the effect BoNTs on TRP channels comes from the recent works of Zhang et al. [29] and Nuget et al. [30]. The former authors studied the effect of subcutaneous facial injection of BoNT-A on TRP4 expression in rat's trigeminal neuralgia induced by chronic constriction injury to the infraorbital nerve. Four days after BoNT-A injection, rats injected with 3 and 10 units of BoNT-A demonstrated significantly higher pain threshold compared to control rats who had not received toxin injections. Additionally, rats injected with this toxin showed significant reduction of expression of TRP4 compared to controls ($P < 0.5$). Nuget et al. [30] have also observed decreased expression of TRP1 in neonatal rats' dorsal root ganglion using an E/A neurotoxin chimera in their experiment; EA is an engineered toxin linking C chain of E to full chains of A toxin.

Effect on Purinergic Channels

Purinergic receptors are ligand-gated Ca++ channels that respond to adenosine triphosphate (ATP) stimulation. The P2x3-ATP-responsive receptor channel is specifically expressed in sensory nociceptive neurons. Purinergic channels have both chemical and mechanical sensitivity. ATP applied to a blister base causes pain in humans and also induces pain behavior in animals [31].

Apostolidis et al. [32] studied immunoreactivity of P2X3 and TRPV1 channels in the bladder biopsies of 38 patients with bladder overactivity (22 neurogenic type) after intravesical BoNT-A injection. Immunoreactivity of both channels was significantly decreased at 4 and 16 weeks after BoNT injection; this finding was associated with improvement of the patients' urinary urgency. Xiao et al. [33] assessed the

effects of BoNT-A or saline injection on P2X3 receptors in DRG neurons of rats experiencing neuropathic pain after L5 ventral root transection. Subcutaneous injection of BoNT-A into the rat's left hind paw significantly reduced expression of P2X3 and pain behavior on days 4, 8, and 16 after surgery. Liu et al. [34], retrospectively, evaluated the results of intravesical BoNT-A injection (200 units) in 27 patients with overactive bladder (OAB) both clinically (6 patients) and in regard to its effect on tissue P2X3. BoNT-A injection cleaved SNAP 25 and effectively decreased the frequency of urgency episodes in patients with OAB. Liposome-encapsulated BoNT-A injections decreased urothelial P2X3 expression in the five responders ($p = 0.04$). These data suggest that purinergic P2X3, like TRPV receptor, plays an important role in nociception, and reducing the function of P2X3 receptor has a potential to alleviate pain.

The Role of Nerve Growth Factor (NGF) in Pain

Emerging data in the literature indicate that nerve growth factor is a major factor in nociception [31]. Development of peripheral nerve endings, C fibers, DRG neurons, and nociceptive sensory spinal neurons is highly dependent on NGF. A specific NGF receptor, Tyrosine receptor kinase A (TrkA), is expressed in abundance on nociceptive neurons. Long-term exposure to NGF increases production of SP and CGRP as well as expression of Na+, P2X3, and TRPV1 channels [35]. NGF antagonists have been shown to exert analgesic effects [36]. In human, botulinum toxin injection into the overactive bladder has been shown to reduce the level of urinary NGF levels [36], but the effects of BoNT on NGF have not been properly studied yet in animal pain models.

The Effects of Botulinum Toxins on Inflammation

As stated earlier in this chapter, exposure to chemicals, high or low temperature, and following nerve injury, pain mediators such as glutamate accumulate locally in the injured tissue [9, 11]. This would lead to vasodilation and development of inflammation in the injured area. Inflammation, which is usually associated with lower tissue pH, starts a cascade of events leading to enhancement of pain through influencing the function of pain receptors via a variety of mechanisms. Inflammatory cells can activate local production of NGF which enhances pain (see above). It has been shown that in acute inflammation, macrophages can directly invade DRG neurons and interrupt the function of DRG's sensory neurons [37]. Low pH caused by inflammation also triggers the acid-sensing sodium channels, resulting in hyperexcitability of the neural tissue. Furthermore, lowered tissue pH activates ATP

production, which in turn opens the purinergic channels and TRPV1 channels leading to more excitation of nerve terminals. The resultant effects are mechanical hyperalgesia and thermal hyperalgesia due to stimulation of dermal nociceptors along with heightened and sustained excitability of nociceptive nerve terminals (peripheral sensitization) [38].

The literature pertaining to the presence of local inflammation in the peripheral nervous system, as observed in experimental pain models, is controversial. While some studies have shown clear evidence of local inflammation at the site of peripheral pain, others have failed to do so. Three controlled studies have demonstrated that local injection of BoNTs reduces accumulation of glutamate and local edema caused by local injection of pain-inducing agents [9, 11, 39]. Two of these studies injected BoNT-A and -B before formalin injection into rat's paw [9, 11]. One study was performed in asymptomatic human volunteers in whom increased tissue glutamate release was measured by dermal microdialysis [39].

In contrast to above-mentioned studies, Attal and coworkers did not find increased tissue accumulation of SP and CGRP in biopsy specimens of patients with neuropathic pain [40]; however, the normal values used in their laboratory were not provided. Furthermore, in a study of capsaicin- and carrageenan-induced neuropathic pain (injected intratarsally), investigators did not find that pretreatment with BoNTs reduces focal edema or protein extravasation caused by these two agents [41].

Despite this controversial data reported on the presence of inflammation in experimental pain models (which may be related to the type of pain model and to technical issues), substantial literature indicates that BoNTs exert their antinociceptive effect through subduing inflammatory processes; they do so by both affecting production and release of inflammatory agents and also by affecting major cellular players such as microglia in the inflammatory cascade.

Intra-articular injection of Botulinum toxin-A reduces expression of proinflammatory cytokines in the synovial tissue as well as reducing cartilage degeneration and local infiltration of inflammatory cells [42]. In a study using Freund adjuvant (FA) to inflame and destroy a joint, authors demonstrated that intra-articular injection of BoNT-A before FA injection reduces the number of inflammatory cells around the articular cartilage and synovial membrane of the involved joint [43]. In cyclophosphamide-induced cystitis, intravesical injection of BoNT-A decreased inflammatory cell accumulation and levels of SP and CGRP as well of bladder sensitivity and pain behavior [44]. In animal model of capsaicin-induced prostatitis, injection of BoNT-A into bladder wall decreased inflammatory cells and the expression of cyclooxygenase 2 (COX2) in the bladder and spinal cord [45]. BoNT-A inhibits a family of G proteins including Rho guanosine triphosphatase which is essential for activation of interleukin-1, an important proinflammatory cytokine [46]. Intraprostatic injection of BoNT type A inhibits cyclo-oxygenase-2 expression and suppresses capsaicin-induced prostatitis in animal models [47].

In a rat constrictive injury model, injection of BoNT-A into metatarsal joint alleviated the neurogenic pain. The pain relief was associated with suppression of

inflammatory cytokine release from microglia; it was attributed to targeting and cleavage of a newly discovered SNARE protein, SNAP23 [48]. In another animal study of Freund's adjuvant-induced arthritis, intra-articular injection of BoNT-A reduced pain and subdued the release of inflammatory agents released by activated spinal cord microglia [49]. Injection of BoNT-A into temporomandibular joint of rats affected by antigen (Freund's adjuvant) alleviated joint pain and subdued the microglial P2X7 pain pathway activated by this antigen [50]. In the sciatic nerve injury model, intraplantar injection of BoNT-A activated microglia in the lumbar spinal cord ipsilateral to the injury along with improvement of thermal and mechanical hypersensitivity [51]. In another study, using the same technique of injection and same toxin, examination of the spinal cord demonstrated activation of microglia and astrocytes in dorsal and ventral cords [52]. In another study, injection of BoNT-A into the paw of the rats with pain related to sciatic nerve injury alleviated pain behavior, decreased the level of pro-inflammatory cytokines, increased the level of anti-inflammatory interleukins, and decreased the activity of microglia in DRG and spinal cord [53].

Spinal Cord Gabaergic Neurons and Pain: Effects of Botulinum Toxins

The activity of both superficial and deep laminae of the spinal cord's dorsal horn is controlled by two inhibitory neurotransmitters, gamma-aminobutyric acid (GABA) and glycine. The interneurons of dorsal horn and inhibitory descending fibers act on GABA-A (ionotropic) and GABA-B (metatropic) receptors; activation of these receptors reduces excitation of spinal sensory neurons via hyperpolarization of the postsynaptic membrane and/or activation of a shunting conductance. Additionally, GABA can directly decrease glutamate release from primary sensory afferent fibers [54]. Therefore, enhanced function of Gabaergic neurons can reduce central sensitization which results from hyperexcitability of spinal cord sensory neurons in chronic pain disorders.

Drinovac et al. [55] studied the role of the Gabaergic system on the analgesic effect of BoNT-A in the formalin model of inflammatory pain and in mechanical allodynia. In their experiment, intrathecal (1 ug) or intraperiteoneal 0.6–0.8 mg injection of bicuculline (GABA-A antagonist) prevented antinociceptive effect of onabotulinumtoxinA (5–7 units) in rats. The authors noted that their results provided evidence for a central mode of action for botulinum toxin-A in this pain model. They also demonstrated that intraperitoneal injection of bicuculline ($P < 0.05$) reversed the reduction of mechanical pain induced by BoNT-A. Since injection of bicuculline into cisterna magna did not reverse the effect of botulinum toxin-A, authors concluded that the effect of botulinum toxin must be at the spinal level (not supraspinal), and is partly mediated by inhibition of GABA effect centrally.

Effects of Botulinum Toxins on Opioid Channels

Opioid Receptors

Opioid receptors are present in abundance in the brain and spinal cord and play a major role in pain modulation. Endogenous opioids include dynorphins, enkephalins, endomorphins, and nociceptin. Among several described kinds of opioid receptors, μ opioid receptors are most widely distributed in the peripheral and central nervous systems (brain, brain stem, and spinal cord).

Drinovac et al. [56] studied the potential role of opioid receptors in BTX-A's antinociceptive activity in rat's formalin pain model. As described previously in this chapter, pretreatment with BoNTs-A and B in this model alleviates local pain and reduces local accumulation of glutamate. To assess the effect of the opioid system on BoNT's antinociceptive role in this model, the authors injected opioid antagonist naltrexone subcutaneously (0.02–2 mg/kg) or intrathecally (0.07 μg/10 μl–350 μg/10 μl) in some rats, while other rats received selective μ-antagonist naloxonazine intraperitoneally (5 mg/kg). The influence of naltrexone (2 mg/kg s.c.) on BoNT-A antinociceptive activity was also additionally examined in partial sciatic nerve transection induced experimental painful neuropathy. The authors found that antinociceptive effects of BoNT-A in formalin and sciatic nerve transection-induced pain were prevented by nonselective opioid antagonist naltrexone. Additionally, the pain-reducing effect of BoNT-A in this model was abolished by low dose of intrathecal naltrexone and by selective μ-antagonist naloxonazine. The decrease in dorsal horn's c-Fos expression caused by BoNT-A injection was also prevented by injection of naltrexone. Prevention of BoNT-A effects on pain and c-Fos expression by opioid antagonists suggested to the authors that the central antinociceptive action of BoNT-A might be associated with the activity of endogenous opioid system (involving μ-opioid receptor).

BoNT Effects on Nerve Regeneration and Nerve Recovery

Mice suffering from neuropathic pain and allodynia secondary to sciatic nerve ligation demonstrate quicker recovery of walking pattern after intraplantar, intrathecal, or intraperitoneal injection of 15 pg/kg of onabotulinumtoxinA [57]. In this experiment, authors used expression of S100β protein and glial fibrillary acidic protein (GFAP) by immunofluorescence to illustrate the changes in the sciatic nerve; there was evidence for structural modification such as expression of cell division cycle 2 and growth-associated protein 43 (GAP-43) regeneration-associated proteins which suggested treatment with onabotulinumtoxinA facilitates nerve recovery. Lima et al. [58] also found that when compared with controls, transected tibial nerve also recovered faster in rats injected with BoNT-A into the gastrocnemius muscle. In

another study [59], using a similar mouse model of peripheral nerve injury, authors noted that injection of BoNT-B improved pain behavior but failed to promote functional recovery. Cobianchi et al. [60] assessed the effect of low-dose (15 pg) intraplantar injection of BoNT-A in mice after inducing chronic constriction injury (CCI) of the sciatic nerve. They noted regrowing myelinated axons and increase in reinnervation of gastrocnemius and plantar muscles of the injected mouse compared to controls. Franz et al. [61] tested the effects of BoNT-A in mouse model of tibial nerve injury and human stem cells. Injection of BoNT into triceps surae of the mouse, 1 week before afflicting injury to the animal, resulted in significantly enhanced outgrowth of murine motor axons as well as the human motor neuron neurites tested in vitro (upon exposure to BoNT).

In a recent publication, Vacca et al. [62] reported on the effect of spinal injection of BoNT-A (between L4-L5 vertebrae) on the motor function of mice after induced traumatic spinal cord injury at T10–T11 level. BoNT-A was injected 1 h after induced injury. The dose of injected BoNT-A was 15 pg/5 μL corresponding to 7.5 μ/kg of Botox (onabotulinumtoxinA). The results from toxin-injected mice were compared with a group of mice injected with saline at the same level. Motor recovery was assessed by the Bosco Mouse Scale (BMS) and spinal reflex by tail-flick test.

All animals demonstrated absence of hind limb movements at day one after cord injury. On day four, gradual return of function was noted only in the BoNT-treated mice. At day 30 post injury, all subjects in the BoNT-injected group demonstrated complete recovery from paralysis, whereas all mice in the saline-injected group remained still paralyzed ($P < 0.000$). Mice injected with BoNT-A regained thermal sensitivity at day 20 post injection, but the saline-injected mice totally lost thermal sensitivity in the hind limb. The mechanical threshold for neuropathic pain was reduced in mice after injury and remained reduced only in the saline group ($P = 0.007$). Examination of the tissue showed less scar formation and considerably less spinal cord atrophy in the toxin-injected group ($P < 0001$). In the toxin-injected group, 30 days after injury, motor neurons were preserved in the spinal cord below the level of injury and survived, whereas they did not in the saline-injected group ($P = 0.0036$). Vesicular transporter of glutamate 1, a marker of neural excitability, was found significantly decreased in the toxin-injected group ($P = 0.0013$). The authors suggested that retrograde transfer of the toxin from the site of injection to the site of injury accounted to the BoNT-A's protective and regenerative action. In the authors' words, "their study demonstrated an extraordinary ability for BoNT-A for neuroprotection and CNS regeneration." The experiment also showed the BoNT-A's potential for reducing neuropathic pain after spinal cord injury. The figure from their article demonstrates the site of injection and the cascade of events that followed at the cellular level following BoNT-A injection leading to the toxin's effect (Fig. 3.4). Luvisetto [63] has recently reviewed the emerging literature on the role of BoNT's injection on enhancing the regeneration and recovery of injured nerves and emphasized the potential of BoNTs in treatment of pain caused by central or peripheral trauma.

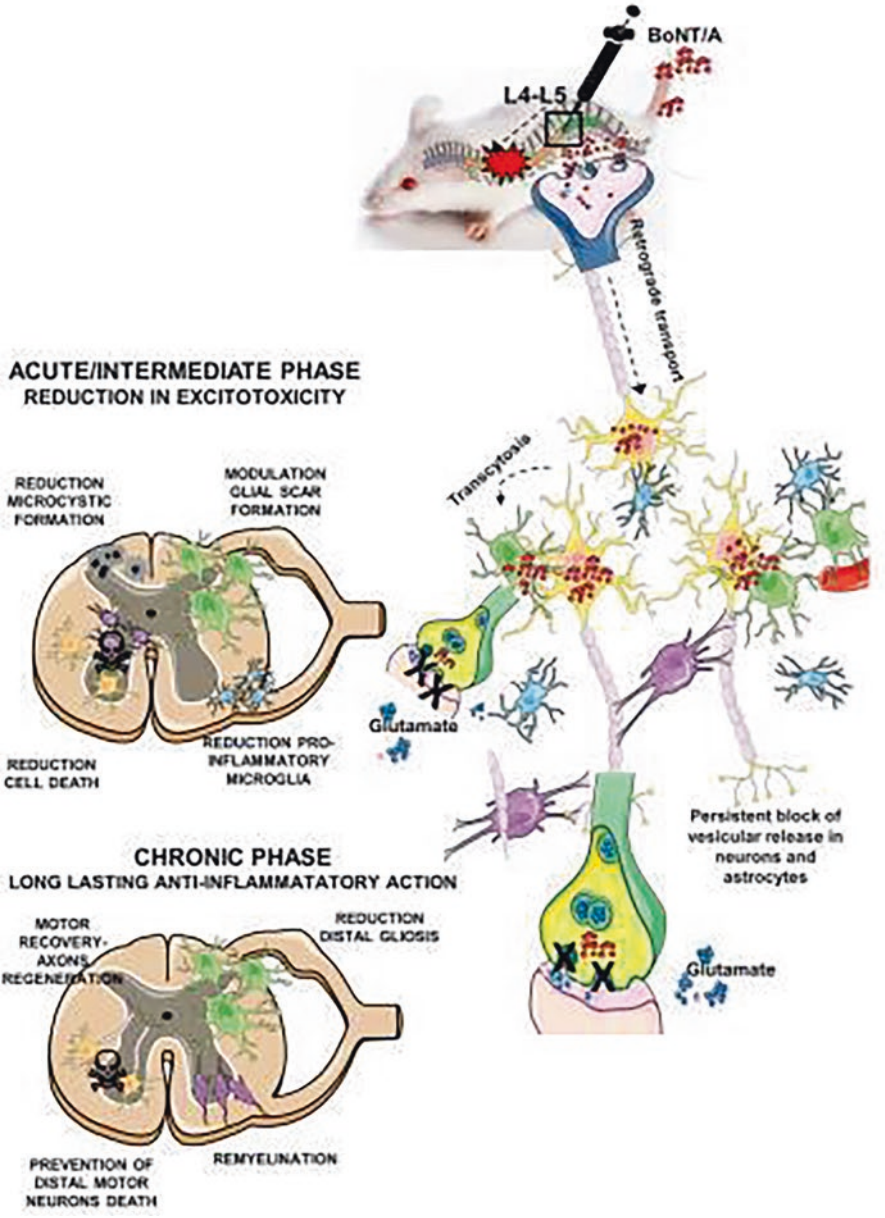

Fig. 3.4 Acute and chronic phase of nerve injury from Vacca et al. [62]. Published in Toxins. Reproduced with permission from Publisher PMC

Evidence for Central Analgesic Effect of Botulinum Neurotoxins

Emerging data from in vitro and in vivo studies suggest that analgesic effect of BoNTs is, at least partly, related to their role on central nociceptive pathways [64–66]. This information comes mainly from two lines of evidence:

1. Data suggesting that the active moiety of peripherally injected BoNT travels along the peripheral nerve to the central nervous system.

This process has been demonstrated convincingly for motor neurons by Caleo and his colleagues in a recent experiment [67]. They have shown that catalytically active BoNT-A was transported to the facial nucleus (FN) in the pons after injection into the nasolabial musculature of rats and mice. BoNT-A-mediated cleavage of SNAP-25 in the FN was prevented by intraventricular delivery of antitoxin antibodies, indicating that BoNT-A physically left the motor neurons to enter second-order neurons. Analysis of nerve terminals within the FN showed that BoNT-A was transcytosed preferentially into cholinergic synapses.

In the sensory system, Matak et al. [68] have found cleaved SNAP-25 in spinal trigeminal nucleus caudalis and oralis after injecting BoNT-A into the rats' whisker pad. In the optic system, after injection of BoNT-A into the rat's eye, catalytically active, cleaved SNAP 25 appears in abundance in the superior colliculus (upper brain stem) and is transcytosed into the tectal synapses [69]. This transfer of toxin, after peripheral injection into the central nervous system, appears to be an active and energy-dependent process [64–69]. Indirect evidence for central effect of BoNT has been provided by the studies that have shown enhanced expression of c-fos and production of pain transmitters such as substance P and CGRP following peripheral injection of BoNT-B into spinal sensory neurons [11].

2. Data from animal and human (asymptomatic volunteers) studies demonstrating improvement of bilateral limb pain after unilateral injection of BoNT.

Much of the data have been provided by the investigators of the Department of Pharmacology in the University of Zagreb in Croatia. Development of bilateral pain (mirror pain), after development of unilateral pain caused by exposure to noxious agents, is a curious phenomenon which has been shown to develop after unilateral injection/exposure of a nociceptive agent such as acidic saline [70]. Back-Rojecky and Lackovic [71] have shown that in the acid saline model of bilateral pain, unilateral injection of 5 units of BoNT-A into the sciatic nerve on the side of saline injection improved pain bilaterally. This effect was blocked by injection of colchicine which prevents axonal transport. In another study, the same group of investigators [72] showed that unilateral injection of BoNT-A can reduce pain behavior bilaterally in rats with bilateral painful diabetic neuropathy. Similar bilateral effect after unilateral injection was also induced by abobotulinumtoxinA (Dysport) injection in the case of bilateral carrageenan-induced peripheral neuropathy [73, 74]. The

above-mentioned data in mirror pain support the notion that the analgesic effects of BoNTs are partly conducted through a central effect.

Additional Mechanisms Potentially Promoting the Analgesic Effects of BoNTs in Pain

Fillipi et al. [75] have shown that injection of BoNT-A into jaw muscles of the rat substantially reduces the discharge of muscle spindles. Since muscle spindles provide a major sensory input into the spinal cord, and inhibiting this input can reduce the central sensitization caused by chronic pain. Local injection of BoNTs also impairs sympathetic transmission which is believed to contribute to pain chronicity and maintenance (sympathetically maintained pain) [76].

Conclusion

Botulinum neurotoxins exert an analgesic effect through a myriad of different mechanisms**.** These include inhibitory actions upon pain receptors and pain transmitters as well interference with inflammatory cascades that are at work in several pain states. In addition to their well-known peripheral analgesic effect, the emerging data have shown that after peripheral injection, the active moiety of the botulinum toxin is transported to the central nervous system suggesting an additional central analgesic effect. The animal data demonstrating the role of BoNT injection in neural regeneration and protection after spinal cord injury have major implications in human subjects both for recovery after injury and reduction of posttraumatic pain.

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