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Botox (onabotulinumtoxinA) mechanism of action

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Abstract

Studies in the 1920s found that botulinum neurotoxin type A (BoNT/A) inhibited the activity of motor and parasympathetic nerve endings, confirmed several decades later to be due to decreased acetylcholine release. The 1970s were marked by studies of cellular mechanisms aided by use of neutralizing antibodies as pharmacologic tools: BoNT/A disappeared from accessibility to neutralizing antibodies within minutes, although it took several hours for onset of muscle weakness. The multi-step mechanism was experimentally confirmed and is now recognized to consist broadly of binding to nerve terminals, internalization, and lysis or cleavage of a protein (SNAP-25: synaptosomal associated protein-25 kDa) that is part of the SNARE (Soluble NSF Attachment protein REceptor) complex needed for synaptic vesicle docking and fusion. Clinical use of the BoNT/A product onabotulinumtoxinA was based on its ability to reduce muscle contractions via inhibition of acetylcholine from motor terminals. Sensory mechanisms of onabotulinumtoxinA have now been identified, supporting its successful treatment of chronic migraine and urgency in overactive bladder. Exploration into migraine mechanisms led to anatomical studies documenting pain fibers that send axons through sutures of the skull to outside the head—a potential route by which extracranial injections could affect intracranial processes. Several clinical studies have also identified benefits of onabotulinumtoxinA in major depression, which have been attributed to central responses induced by feedback from facial muscle and skin movement. Overall, the history of BoNT/A is distinguished by basic science studies that stimulated clinical use and, conversely, clinical observations that spurred basic research into novel mechanisms of action.

Abbreviations: ATP = adenosine triphosphate, BoNT = botulinum neurotoxin, CGRP = calcitonin gene related peptide, FAERS = Food and Drug Administration Adverse Event Reporting System, FDA = Food and Drug Administration, FGFR3 = fibroblast growth factor receptor 3, fMRI = functional magnetic resonance imaging, NGF = nerve growth factor, P2X = purinergic ionotropic receptor, PSG = polysialiogangliosides, SNAP-25 = synaptosomal associated protein-25 kDa, SNARE = Soluble NSF attachment protein receptor, SP = substance P, SV2 = synaptic vesicle 2, TRPA1 = transient receptor potential ankyrin 1, TRPV1 = transient receptor potential cation channel subfamily V member 1, VAMP = vesicle associated membrane protein.

Keywords: botulinum toxin, chronic migraine, depression, overactive bladder, skin quality, SNAP-25, TRPV1

1. Early Observations

The first systematic description of the neurological effects of botulinum neurotoxin (BoNT) was published by the German physician Justinus Kerner in the 1800s, who described symptoms exhibited by individuals following ingestion of improperly preserved food.[1] In 1897, Belgian microbiologist van Ermengem reported that the symptoms were caused by bacteria that would eventually become known as *Clostridium botulinum*.[2] In 1905, Tchitchikine found that the bacteria produced a neuroactive substance,[3] setting the stage for mechanism of action studies. Dickson and Shevky subsequently established that BoNT acted on parasympathetic and motor nerve endings,[4,5] which led to the discovery in the late 1940s and early 1950s that BoNT serotype A (BoNT/A) inhibited the release of acetylcholine from motor nerve terminals.[6,7]

Studies on the synthesis and structure of BoNTs aided understanding of mechanism of action. Attempts at purification began with Hermann Sommer in the 1920s[8] and culminated with the crystallization of BoNT/A by Carl Lamanna in the 1940s.[9,10] Both neurotoxin and neurotoxin associated proteins (NAPs) were later identified in the crystalline toxin,[11,12] leading to the conclusion that BoNTs were produced as progenitor toxin complexes. The neurotoxin portion of the complex was found to be a ~150 kDa protein synthesized as a single chain that must be proteolytically cleaved to exert its activity.[13] The resulting di-chain molecule was found to consist of an ~100 kDa heavy chain and ~50 kDa light chain.

2. Multi-Step Mechanism of Acetylcholine Inhibition

Studies in the late 1940s and early 1950s found that BoNTs acted presynaptically to block the release of acetylcholine from motor nerve terminals.[6,7] This action depended on the presence

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of calcium ions, leading to the conclusion that the mechanism was related to the process of neurotransmitter release.[14] Research conducted throughout the 1970s established the key steps in the mechanism of action: binding, internalization, translocation, and interruption of neurotransmitter release.[15,16] However, the final step in the process, then called the lytic step, took another decade to elucidate.

In 1992, separate research teams led by Südhof and Montecucco reported that the light chains of tetanus toxin[17,18] and BoNT/B[18] were zinc-dependent metalloproteases that cleaved the protein synaptobrevin present in synaptic vesicle membranes. In 1993 these groups reported that BoNT/A and BoNT/E selectively cleaved synaptosomal associated protein-25 kD (SNAP-25),[19,20] and that type C1 also cleaved syntaxin.[21] A year later, these authors observed that the three synaptic proteins cleaved by different BoNT serotypes formed a stable complex that mediated synaptic vesicle exocytosis[22]—subsequently known as the SNARE complex (Soluble NSF Attachment protein REceptor). Discovery of the lytic step in the action of BoNTs played a key role in understanding the machinery regulating vesicle traffic, for which the 2013 Nobel Prize in Physiology or Medicine was awarded to James Rothman, Randy Schekman, and Thomas Südhof.[23] The mechanisms of BoNT/A action at the synapse are shown in Figure 1.

Even though it was known that BoNTs interacted with and were internalized into nerve terminals, the binding mechanisms took decades to establish. Studies in the 1970s and 80s documented interactions between BoNT and gangliosides on neuronal membranes.[24,25] However, due to their low affinity binding and localization on non-neuronal cells, gangliosides were not believed to be the sole binding mechanism.[26] In 1994, Nishiki and colleagues identified a protein receptor for BoNT/B located in synaptic vesicle membranes—synaptotagmin.[27] Another vesicle membrane protein, SV2, was found to be the protein receptor for BoNT/A.[28,29] This dual binding mechanism and the localization of the two receptors (and their various subtypes/isoforms) in the nervous system may be responsible for the selective action of BoNTs, although the in vivo interactions of BoNTs on non-cholinergic neurons are not yet well understood.[26] More recently, studies have shown that BoNT/A binds to fibroblast growth factor receptor 3 (FGFR3) in motor neurons,[30] and the neurotoxin heavy chain increases dimerization of FGFR3 receptors.[31] The contribution of FGFR3 binding to the actions of BoNT/A requires further investigation.

Clinical studies throughout the 1980s using the BoNT/A product onabotulinumtoxinA confirmed that the actions were transient and reversible, with most patients treated for blepharospasm receiving reinjection after approximately 3–4 months.[32] Following BoNT/A injection, nerve terminal sprouts appear and release acetylcholine,[33] although they are not as efficient as the parent terminals.[34] Sprouting is followed by a second phase in which vesicular release returns to the original terminals and the sprouts are eliminated.[34] The BoNT/A light chain remains catalytically active inside cells for months after injection, which may be due to its subcellular localization and reduced accessibility to proteinases[35] and/or influence on the ubiquitination process.[36] Other research indicates that the BoNT/A light chain interacts with septins—cytoskeletal proteins at the plasma membrane—that protect it from intracellular degradation.[37]

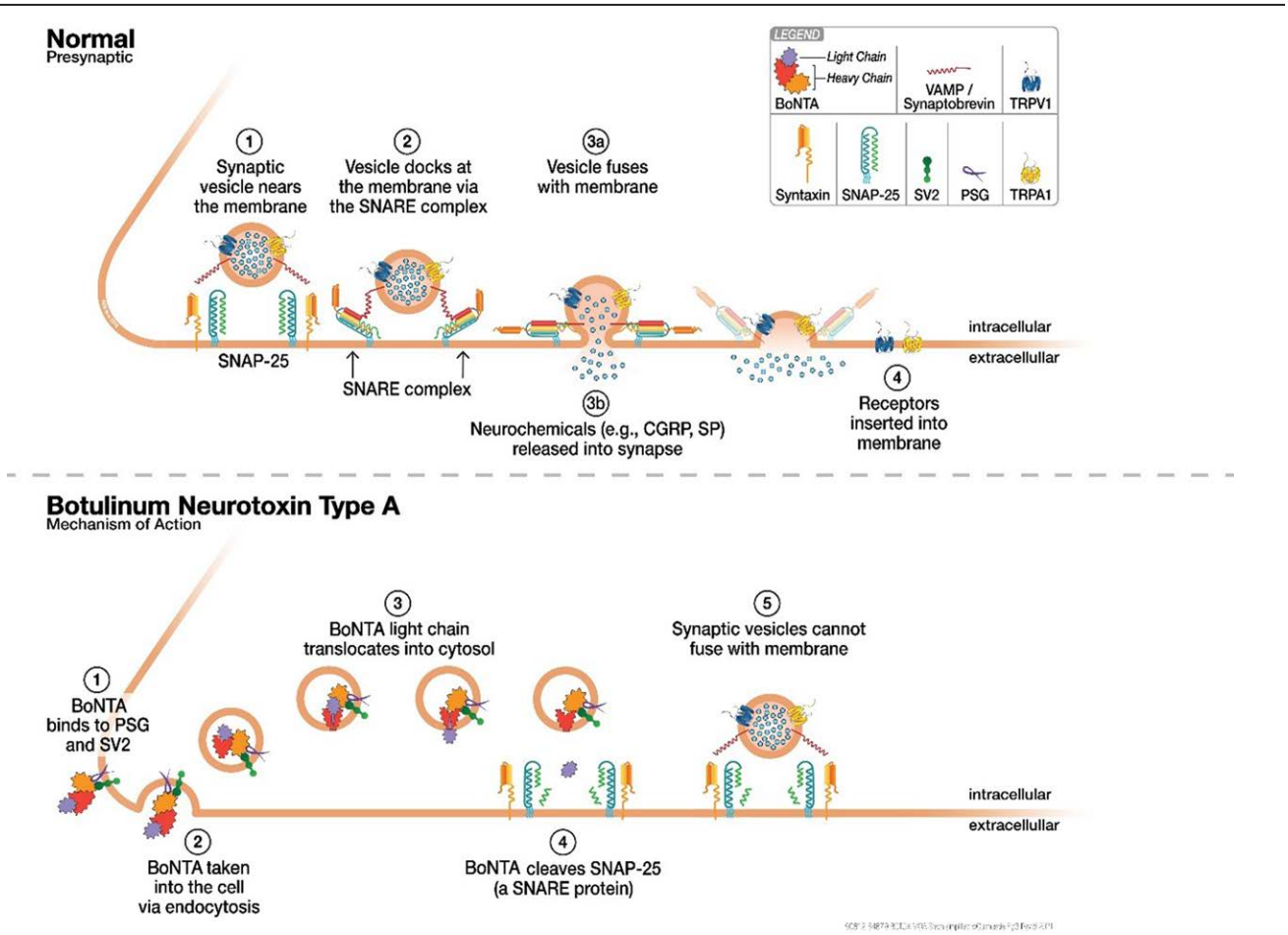

Figure 1. Botulinum neurotoxin type A (BoNT/A) mechanism of action. Events at the synapse are shown in the absence (top panel) and presence (bottom panel) of BoNT/A.

3. Exploration Into Autonomic and Smooth Muscle Effects

Autonomic effects of BoNT were first reported in the 1800s by Justinus Kerner.[1,38] In the 1920s, Dickson and Shevky documented effects of BoNT on autonomic fibers[5] and in 1951 Ambache confirmed that BoNT/A acted on cholinergic postganglionic autonomic fibers regardless of whether they were sympathetic or parasympathetic.[39] This provided the basis for trials of BoNT/A studies in Frey's syndrome in 1995[40] and axillary hyperhidrosis shortly thereafter.[41] Compared with neuromuscular conditions, the action of onabotulinumtoxinA in hyperhidrosis is longer, with a mean duration between treatments of approximately 7 months following axillary injections.[42]

Dr. Brin: *I remember when I examined the topline data for our axillary hyperhidrosis studies and saw that the clinical effects of a single treatment lasted more than a year in 27% of patients. This was remarkable because we were used to the effects lasting 3 to 4 months in other indications. I began asking colleagues why the effects lasted so long in axillary hyperhidrosis and no one really knew. One current hypothesis is that the autonomic nerve endings that innervate sweat glands do not sprout after Botox injection, and this may be a contributing mechanism in the bladder, as later demonstrated by Haferkamp.*[43]

In the late 1980s and throughout the 1990s, clinicians began exploring the injection of BoNTs into smooth muscle to treat disorders of the alimentary tract[44] and detrusor sphincter dyssynergia.[45] As experience with bladder injections accumulated, clinicians noted that the effects of onabotulinumtoxinA persisted for at least 6 months.[46] This finding is paralleled by a lack of axonal sprouting in the bladder of patients treated for detrusor sphincter overactivity,[43] although it is not known whether the lack of sprouting is responsible for the long duration.

More recently, BoNT/A injections have been explored for the prevention of atrial fibrillation after coronary bypass and valvular open chest surgery. The epicardial adipose pads exhibit a complex neurochemical anatomy. They receive extrinsic input from the autonomic nervous system, but contain intrinsic ganglia that regulate this input.[47] Intrinsic neurons in the right atrial ganglionated plexus contain acetylcholine and nitric oxide, with some also containing noradrenergic markers.[48] Most of the intrinsic neurons receive cholinergic innervation, but also peptidergic, nitrergic, and noradrenergic innervation.[48] Initial preclinical studies found that injection of BoNT/A into the epicardial fat pads prevented the effects of vagal stimulation on the sinus node and eliminated the induction of atrial fibrillation.[49,50] These results have been followed by several randomized studies in humans that have shown promising results.[51–53]

4. Exploration Into Sensory Effects

Exploration of onabotulinumtoxinA's sensory effects resulted largely from a reverse translational approach, whereby clinical observations stimulated basic science research into mechanisms. These observations occurred primarily in two areas: migraine and overactive bladder.

4.1. Chronic Migraine

In the 1990s, Dr. William Binder incidentally noted that several of his patients experienced a decrease in migraine headache symptoms following onabotulinumtoxinA injections for hyperfunctional facial lines (see Turkel et al in this supplement for full description).[54] This led to a study by Drs. Binder, Blitzer, and one of the authors (M.F. Brin) that documented improvement in migraines in patients who had received treatment for facial lines.[55,56]

Improvements in pain had been noted for years in patients receiving onabotulinumtoxinA for the treatment of cervical dystonia[57,58] and spasticity,[59] but these effects were assumed to result from decreased muscle contractions.

Dr. Brin: *When we were conducting trials with Botox at Columbia University in the 1980s and 1990s, we were focused on the toxin's neuromuscular effects. We noticed improvement in pain, for example in cervical dystonia patients,*[58] *but attributed it to reduced muscular contractions. This original assumption about pain reduction was called into question when Bill Binder called Andy Blitzer and me about his observation that Botox relieved migraine headache in his aesthetic patients. Since then we've learned more about how Botox can affect pain disorders.*

Given that migraine is primarily a sensory disorder, researchers began seeking a mechanism by which onabotulinumtoxinA could act to relieve pain. Experimental studies showed that onabotulinumtoxinA inhibited substance P release from cultured embryonic dorsal root ganglion neurons[60] and reduced stimulated release of calcitonin gene related peptide (CGRP) from cultured trigeminal ganglia neurons.[61] Sensory effects were also noted in preclinical models of bladder pain[62] and formalin pain.[63]

Still other sensory effects were observed in experiments of the TRPV1 receptor—a cation channel that plays an important role in the perception of peripheral thermal and inflammatory pain.[64] OnabotulinumtoxinA blocked the insertion of TRPV1 into the cell membrane, indicating that this process is SNARE dependent. In peripheral nociceptive terminals, inflammatory mediators increased the expression of TRPV1 in membranes, which is important for the sensation of pain.[65]

As recently reviewed,[66] researchers examining the mechanism of onabotulinumtoxinA in chronic migraine faced the question of how injections into peripheral muscles of the head and neck could improve symptoms of a condition believed to originate intracranially.

Dr. Burstein: *When I first joined the field of migraine/headache, the standard dogma was that headache originates inside as opposed to outside the head. Pain signals from nociceptors innervating intracranial blood vessels and dura were thought to initiate and maintain the headache phase of migraine. On the other hand, Botox was injected outside the head. Its mechanism of action was therefore an enigma. If Botox didn't act systemically, then how could it affect headache that originates in activated pain fibers inside the head? This was the first question I struggled with.*

I took a few years to read the literature and eventually came across an account of migraine written in the 2nd century by the Greek medical scholar Galen who described 2 types of headaches: "Some believe that they are being hit by a hammer, as it were. Others feel that the head is being pounded, or distended. It is not unreasonable that in some the meninges around the brain are affected, in others, the pericranium. Even among those who feel the pain in one half of the head, usually called hemicrania, some get the feeling that the pain is on the outside of the skull, and others, deep within the head."[67] *After reading this, I began asking patients in the clinic to describe whether their headache pain felt like it was coming from the inside or the outside of the head—whether their head felt like it was about to explode from so much pressure inside or whether it felt like a vise tightening around their heads or crushing their skulls. I referred to these as exploding and imploding headache.*[68]

The discovery that headache beginning outside the head can progress to inside the head led to a new hypothesis: an anatomical connection between the outside and inside of the head.

Dr. Burstein: *Searching for this connection dominated my research for the next few years. It resulted in the discovery of a network of pain fibers in the dura that sent axons through sutures of the skull to outside the head,*[69,70] *and pain fibers in the periosteum that send collateral branches to the inside of the head.*[71] *The number of these axons is large and given that even a single electrical signal can give rise to the perception of*

pain, it is likely that these axons play a role in the perception of headache of some migraine attacks that originate intracranially as well as attacks that originate extracranially. It is actually quite easy to do histology on pain fibers in soft tissue but nearly impossible to identify them in bone. You need to de-calcify the bone for about 6 months, which usually destroys pain fibers. We eventually figured out how to preserve the pain fibers during the decalcification process.

It was nearly impossible to publish our findings—people didn't want to believe that pain fibers exited or entered the skull. At this time, there was still skepticism regarding the benefits of Botox in migraine and the results of the randomized controlled trial in episodic migraine weren't significant. When we sent in the paper on imploding and exploding headache,[68] *the reviewers said that it was excellent work, but the editor-in-chief was skeptical and asked for more data.*

Once we found pain fibers exiting[69,70] *and entering*[71] *the skull, a potential anatomical basis for the actions of extracranial injections of Botox had emerged that allowed us to figure out whether extracranial and suture line injections of Botox can alter the responsivity of intracranial (dural) pain fibers. We first applied Botox directly to the dura and found that it inhibited unmyelinated C fibers and their branches but not the myelinated A-delta fibers.*[72] *We also found that it inhibited the small axonal branches that cross the bones of the calvaria from the inside to outside of the head.*[72] *Our next step was to inject Botox along the skull's suture lines and wait long enough for it to affect the sensitivity of neurons inside the head. We found that if you wait 1-4 weeks after injecting Botox outside the head, it significantly reduces the sensitivity of pain fibers inside the head to cortical spreading depression, an event that activates them during migraine.*[73] *That was the final path in creating the connection that started with observations in humans to discovering the anatomical basis to how it can help us understanding Botox mechanism of action in preventing migraine attacks.*

Dr. Brin: *Translational research has also been a big part of understanding the mechanism of onabotulinumtoxinA in migraine. We've observed certain effects in humans and then moved to animals to find out how and why. Migraine is not simply a disease of pain fiber activation—there are other symptoms. Researchers are trying to determine the roles and relationships between pain fiber effects, inflammation, autonomic and vascular pathways, etc.* (Fig. 2)

4.2. Overactive Bladder

Research into sensory effects of onabotulinumtoxinA in migraine were followed closely by those in the bladder. As use of onabotulinumtoxinA expanded to include urinary bladder injections, patients began to report improved urinary urgency[74]–a sensory symptom–and laboratory studies documented inhibition of ATP from sensory afferents in the bladder following spinal cord injury.[75] In a model of bladder pain, intravesical administration of onabotulinumtoxinA blocked pain responses and concurrently inhibited CGRP release from afferent nerve terminals,[62] also supporting direct sensory effects.

Dr. Brin: *An "aha" moment for me was the discovery by Apostolidis and Fowler that Botox decreased P2X3 and TRPV1 ion channel receptors in bladder nerves following intradetrusor injections.*[76] *These data provided a mechanism by which Botox could decrease the urgency in overactive bladder. A key finding was that patients with detrusor overactivity showed higher levels of these ion channel receptors than controls to begin with, but 4 months after Botox injection, the levels of these ion channel receptors stayed low and essentially were normalized. This indicated that Botox was acting like a rheostat modulating the sensitivity of these neurons. Contemporary research suggests that basal levels of vesicular fusion, and resultant receptor insertion, can continue via a constitutive pathway.*[77]

These findings all support an effect of onabotulinumtoxinA on sensory nerves and/or sensation in the treatment of overactive bladder.

Figure 2. Drs. David Dodick, Aubrey Adams, Mitchell Brin, and Rami Burstein (left to right) in Westport, Ireland.

5. Effects in Depression: Central Effects of Peripheral Injections

Injection of onabotulinumtoxinA for facial lines resulted in another interesting discovery: potential benefits in major depression. Treatment of major depression with onabotulinumtoxinA is now supported by four small randomized, controlled studies[78–81] and one larger phase 2 trial.[82] Additionally, several studies have indirectly identified potential antidepressant effects of BoNT using inverse frequency analyses of the Food and Drug Administration Adverse Event Reporting System (FAERS) database. In one such study that screened more than 8 million adverse event reports for all medications, depression was less likely to be reported as an adverse event in patients treated with onabotulinumtoxinA compared with patients who received any medication for the indication of depression.[83] Another such study that screened more than 13 million adverse event reports among patients treated with BoNTs compared with other therapies for the same conditions (eg, cosmetic, chronic migraine, spasticity), found significantly lower rates of depression in those treated with BoNTs for most conditions.[84]

The potential effects of onabotulinumtoxinA in depression have been attributed to central responses induced by changes in muscle and skin movement of the face.[85] The idea that physical expression of emotion influences the emotion itself dates back at least to Charles Darwin who noted, "The free expression, by outward signs, of an emotion intensifies it."[86] This basic explanation is manifest in the more recent facial feedback hypothesis, which postulates that the action of facial muscles influences the perception of emotion.[87] Indeed, a fundamental principle of the central nervous system is its response to input from the external environment, as in the case of learning to avoid painful stimuli. Examples of improvement in central nervous system disorders following changes in peripheral input include stimulation of the vagus nerve as an FDA-approved treatment for epilepsy and depression, and sensory tricks such as facial touching to mitigate head deviation in cervical dystonia.[88]

The idea that peripheral injections of onabotulinumtoxinA can indirectly affect the central nervous system is not new, having been noted since at least the late 1990s by clinicians treating dystonia.[89,90] An early study of patients with writer's cramp found that BoNT/A injection into hand muscles improved writing and increased activation of brain areas not directly involved in motor control.[91] The authors suggested that this represented either a change in movement strategy or cortical reorganization in response to the lack of motor neuron activity. These explanations are similar to the facial feedback hypothesis in that the reduced muscle activity is itself a message to which the brain responds, possibly via sensory receptors in muscles.

Numerous subsequent studies have reported changes in brain activity following peripheral BoNT/A treatment.[91–97] In a 2018 study, patients with cervical dystonia underwent functional magnetic resonance imaging (fMRI) during a skilled hand motor task before receiving any BoNT treatment (BoNT naïve) and 4 weeks after the first onabotulinumtoxinA injection.[98] In addition to significant improvement in dystonia, BoNT treatment was associated with significantly increased activation of various brain areas that were not limited to cortical and subcortical areas representing the treated muscles.

More recently, patients with non-neurogenic urge urinary incontinence were studied using fMRI during an urgency simulation task before and 6-8 weeks after onabotulinumtoxinA treatment.[99] Patients who responded to onabotulinumtoxinA (13 of 18) showed decreased activation in the right inferior frontal and supramarginal gyri, and right inferior and superior parietal lobules; the author noted that a significant reduction in activity observed in the right insula may reflect the reduction of bladder afferent sensation. These findings demonstrate central nervous system changes following peripheral injection of onabotulinumtoxinA for several different conditions, indicating that central changes are not specific to depression.

Dr. Brin: *As people made the discovery of Botox on depression, this led to a broader context in thinking about affective disease. Fifteen years ago, we thought that if Botox helped depression, people were just feeling better because of the positive effects on their appearance. Now we have a better context, which includes evidence that the peripheral effects of Botox can modulate central activity.*

Sensory information from facial muscles and skin travels along the trigeminal nerve, which has reciprocal connections with sensory and limbic structures such as the amygdala.[100–103] The amygdala is particularly relevant to depression because of its role in emotional processing.[104] A brain imaging study found that BoNT/A reduced activation of the left amygdala normally observed during imitation of angry facial expressions, as well as the functional coupling between the amygdala and brain stem regions implicated in autonomic manifestations of emotional states.[105] This is supported by another study that found that BoNT/A inhibited amygdala activity observed in response to angry faces.[106] More recently, a study found that BoNT/A modulated the response of the left amygdala to viewing happy and angry faces, as well as the response of the right fusiform gyrus to viewing happy faces.[107] Another study found that corrugator muscle activity was positively associated with amygdala and negatively associated with ventromedial prefrontal cortex activity during the viewing of pictures designed to elicit negative emotions.[108] Finally, patients who responded to antidepressant medication showed a greater reduction of activity in the amygdala and several other brain areas than those who did not respond, indicating that early changes in emotional processing involving the amygdala and other structures are associated with clinical improvement.[109]

Dr. Brin: *Thirty years ago, I wouldn't have predicted that Botox would be useful for depression. The observation that peripheral injections of Botox may indirectly have central effects via feedback mechanisms had primarily been discussed for conditions with prominent muscle overactivity. The thought that a major emotional disorder like depression could potentially be improved by peripheral muscle alterations wasn't top of mind. The journey to understand how Botox could treat depression may not be unlike that of migraine—neither condition has a major neuromuscular component.*

6. Effects on Skin Quality

In addition to the well-documented effects of onabotulinumtoxinA in the management of facial lines,[110,111] several studies have reported beneficial effects on skin quality following repeated injections. Improvements in the biomechanical properties of skin such as elasticity and pliability have been identified following injections into the glabella, forehead, and lateral orbit.[112] Another study reported improvement in skin roughness, hydration, elasticity, and transepidermal water loss following BoNT/A injections into the dermis.[113]

The mechanism by which BoNT/A improves the skin's biomechanical properties may be related to stimulation and reorganization of collagen. In one study, intradermal injections of BoNT/A slightly increased in procollagen in facial skin biopsies taken 8 weeks later.[114] In another study, BoNT/A injections led to improved organization and orientation of collagen fibers in biopsies taken from the peri-orbital area 3 months after injection, although this study did not find significant changes in the expression of collagen types I, III, or elastin.[115]

BoNT/A has also been found to bind to FGFR3 in motor neurons[30] and to increase FGFR3 dimerization in a novel receptor dimerization assay.[31] FGFR3 is also expressed in the dermis and epidermis,[116] providing a potential site for BoNT/A actions in skin. Studies of dermal fibroblasts in vitro indicate that BoNT/A

increases production of pro-collagen and several collagen protein chains, and decreases expression of several matrix metalloproteases,[117] suggesting that BoNT/A may promote dermal remodeling.[118]

Another potential mechanism by which BoNT/A may improve skin quality is via an effect on sebum production. Following intradermal injection, BoNT/A has been found to reduce sebum production, skin oiliness, and pore size; these effects may be due to inhibition of acetylcholine release from neuronal cells in the vicinity of sebaceous glands, though there may be other mechanisms.[119,120]

Dr. Brin: *There is more to the Botox mechanism of action than originally believed. From early studies that established BoNT's inhibition of acetylcholine release from motor and autonomic nerve terminals, investigation has expanded to include sensory nerves and skin. The search to understand the mechanisms by which Botox acts in chronic migraine, overactive bladder, and depression has led to findings with implications beyond disease, such as the role of SNARE complexes in TRPV1 delivery to membranes and the role of afferent feedback. It will be interesting to learn more about the effects of Botox in non-motor disorders and I look forward to future insights.*

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