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Botulinum Toxins in Clinical Aesthetic Practice

Volume 1: Clinical Adaptations

Third Edition

Edited by Anthony V. Benedetto

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Series in Cosmetic and Laser Therapy

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Third Edition

Volume One: Clinical Adaptations

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To Dianne, my loving wife of forty years, whose encouragement and support permitted me to accomplish that which seemed at times insurmountable and unattainable.

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Preface

Because of the exponential developments in the clinical use of botulinum toxins (BoNTs), the need for a third edition quickly became a foregone conclusion. Maintaining the original mission of an instructional manual, this completely revamped and updated third edition attempts to record the phenomenal progress that has evolved in the use of BoNTs in clinical medicine over the past seven years. Updates of the literature, expanded indications, improved clinical photographs and illustrations, and newer and innovative ways to utilize the different BoNTs that are presently available worldwide are presented in this newly formatted third edition. It also has become strikingly obvious that BoNTs are injected in a variety of novel ways that differ from East to West. Therefore, a concerted effort has been made to include a profile of as many of the different BoNTs currently available around the world, including how they are utilized in a clinical aesthetic setting in both Western and Eastern cultures.

In the United States, glabellar and lateral canthal lines remain the only areas of the face that are approved by the FDA for the cosmetic use of onabotulinumtoxinA (OnaBTX-A) or BOTOX® Cosmetic. The other BoNTs available in the United States, abobotulinumtoxinA (AboBTX-A), incobotulinumtoxinA (IncoBTX-A), and rimabotulinumtoxinB (RimaBTX-B), have their own similar, but very specific, FDA indications. Consequently, except for glabellar and lateral canthal wrinkles, all the cosmetic injection techniques described in this third edition, as in the previous editions, apply to non-approved, off-label indications, which makes this book unlike most other textbooks in medicine.

It is sobering to realize that throughout human existence women and men have always sought ways to improve their appearance. To commence the in-depth and diverse discussions in this third edition on beautification and rejuvenation with BoNTs, Nina Jablonski, PhD, professor of anthropology at The Pennsylvania State University, and a world-renowned biological anthropologist and paleobiologist, provides us in her Prologue with a brief introduction to the evolutionary and anthropological perspectives on the importance of human facial attractiveness and expressivity. She cautions both patients and treating physicians in the over-use of face altering procedures that can effectively inhibit one's ability to express oneself accurately and in a completely natural manner.

Chapter 1 is written by Jean Carruthers, MD, to whom the world is indebted for her prescient identification of the cosmetic uses of the BoNTs. Dr. Jean Carruthers commences our venture through the fascinating evolving world of the BoNTs by presenting a historical account of the chronological events that led to the discovery, identification, isolation, and eventual synthesis of BoNTs for clinical use. Included is her seminal work in the development and advancement of the clinical uses of BoNT-A in ocular therapeutics, and her serendipitous discovery of its cosmetic properties. Jean describes the role she and her dermatologist husband, Dr. Alastair Carruthers, played in their provocatively sensitive introduction and promotion of the cosmetic uses of BoNT-A to the medical community.

Updates on the current advancements in the pharmacology and immunology of the different BoNTs are discussed by world-renowned scientists who are intimately involved in BoNT research and development. These include Chapter 2 by Mitchell F. Brin, MD, neurologist and one of the earliest clinical injectors of OnaBTX-A and now senior vice president of global drug development and chief scientific officer of BOTOX®, at Allergan Inc. (Irvine, CA). He presents an update on the pharmacology, immunology, recent developments, and future predictions on the use of BoNT-A. Chapter 3 by Juergen Frevert, PhD, head of botulinum toxin research at Merz Pharmaceuticals GmbH, (Potsdam, Germany), discusses the innovative pharmacology and immunology of a noncomplexed BoNT-A, and the advantages of its clinical uses.

Chapter 4 by the visionary dermatologist, Richard Glogau, MD, discusses the fascinating emerging science, development, and effective clinical uses of a new topically applied BoNT-A. Chapter 5 by Gary Monheit, MD, a dermatologist and leader in BoNT clinical research, and dermatologist James Highsmith, MD, elaborates on the recent advances of the different FDA approved BoNT-As and BoNT-B with updates on the pertinent literature and details on recent developments in their clinical use. Chapter 6 by Andy Pickett, PhD, Senior Program Leader & Scientific Expert, Neurotoxins for Galderma Aesthetic and Corrective, and Director and Founder of Toxin Science Limited, Wrexham, UK, identifies some of the different BoNTs used in clinical practice currently available in other parts of the world.

Chapter 7 by Alastair and Jean Carruthers, MD, presents updated and advanced clinical information on the adjunctive uses of the BoNTs in conjunction with injections of soft tissue fillers, and light- and energy-based devices for the aesthetic improvement of the face and body.

In Chapter 8, Arthur Swift, MD, an otorhinolaryngologist, Kent Remington, MD, a dermatologist, and Steve Fagien, MD, an ophthalmologist, add a new dimension to the aesthetic interpretation of how to use injectables when rejuvenating the face, change to including their explanation of facial proportions, geometrical Phi measurements, aesthetics, and beauty as they relate to the use of BoNTs.

For Chapter 9, dermatologists David Pariser, MD, and DeeAnna Glaser, MD, Secretary and President, respectively, of the International Hyperhidrosis Society, have comprehensively revised and updated the material on hyperhidrosis, discussing recent developments as well as new and different areas of treatment.

Chapter 10 by dermatologist Kevin C. Smith, MD, the master of novel injection techniques, along with dermatologists Irèn Kossintseva and Benjamin Barankin continues to enlighten us on unique ways to utilize BoNT-A for cosmetic and therapeutic purposes.

Chapter 11 by dermatologist and attorney David Goldberg, MD, JD, concludes the first volume with a revision and update of his chapter on the important medicolegal aspects of the cosmetic uses of BoNT.

Because of the ever-growing selection of the various BoNT products currently commercially available for clinical use in different parts of the world, the new Appendix 1 written by dermatologist Alica Sharova, MD, PhD, of Pirogov Russian National Research Medical University, Moscow, presents thought-provoking results of her metanalysis comparing consensus statements and recommendations for injecting different BoNT products in the United States, Russia, and different countries in Europe. She identifies and compares the fallacious recommendations of dose ratio equivalencies of the different available BoNTs injected, including number of injection points and dosaging for the different areas of the face and neck in males and females.

In the second volume, Sebastian Cotofana, PhD, a quintessential anatomist, has provided essential new material on functional facial anatomy in Chapter 12.

The nuclear Chapters 13, 14, and 15 on the cosmetic treatment of the face, neck, and chest with injections of BoNTs have been reorganized and expanded, assimilating many improved injection techniques by integrating updated information of recently published clinical and anatomical studies. All the anatomical figures and illustrations have been revised and enhanced throughout the text. The organization of these three chapters has remained the same. Each clinical topic is subdivided according to its facial and functional anatomy, and discussed in seven subheadings. The "Introduction" of each topic identifies the different anatomical changes acquired by men and women as they "age" and develop "wrinkles." Normal "Functional Anatomy" discusses the reasons these disconcerting changes and wrinkles occur so that a suitable plan of correction with a BoNT can be initiated. Functional anatomy is stressed and complemented by clinical photographs and detailed illustrations because the only way a physician injector can utilize any type of BoNT properly is to have an in-depth understanding of how to modify the normal and exaggerated movements of facial mimetic muscles and other potentially treatable muscles elsewhere in the body. When injections of a BoNT are appropriately performed, desirable and reproducible results without adverse sequelae are created. In the "Dilution" subheading, suggestions are given on how much diluent can be added to reconstitute a 100-unit vial of OnaBTX-A in order to arrive at various preferred concentrations per fluid volume dilutions when injecting certain muscles at different anatomical sites. The U.S. FDA-approved manufacturer's recommendation for the reconstitution of a 100-unit vial of OnaBTX-A is to add 2.5 mL of nonpreserved normal saline. This approved and recommended dilution is for injecting glabellar and lateral canthal frown lines only, since these areas on the face are the only approved indications for the cosmetic use of OnaBTX-A. However, when treating other areas of the face and body for cosmetic purposes, albeit in an off-label, unapproved manner, higher or lower dilutions of OnaBTX-A have proven to be more suitable and clinically more effective, depending on the muscles being treated. Options for "Dosing" are presented, with an emphasis placed on what to do and what not to do when injecting OnaBTX-A. Precise dosing and accurate injections of OnaBTX-A will diminish muscle movements of the face and body in a safe and reproducible way. Fastidious injection techniques are necessary to correct a particular aesthetic problem reliably, predictably, and for extended periods of time with any BoNT. "Outcomes" and results of different injection techniques are discussed to avoid "Complications" and adverse sequelae. Finally, how to inject a particular anatomical site and its projected results are summarized in the list of "Implications of Treatment".

Controversial and remarkable treatments for non-surgical breast augmentation for women and men are practiced by dermatologists Francisco Atamoros Perez and Olga Marcias Martinez and discussed in detail in Chapter 16. Their accumulated clinical evidence of the efficacy of BoNT-A injections of the pectoral area is clearly presented with an abundance of clinical illustrations.

Chapter 17, by a prominent and internationally well-known Korean dermatologist, Kyle Seo, MD, discusses the Asian perspective of the use of the different BoNTs currently available in his part of the world. Insight into the East and Southeast Asian cultural aesthetic needs and the Asian perception of aesthetics and beauty, is emphasized. He also presents a detailed description of the racial differences in the anatomy between Asians and Caucasians, which call for different indications and variations in appropriate dosing and injection points of BoNT-A treatments, necessary when treating Asian patients. He also provides some practical guidelines for the innovative use of BoNT-A in facial skin redraping and body muscle contouring injection techniques that are currently very popular in the East.

Many appendices supplying material for procedural reference conclude this second volume.

It is extremely fascinating and encouraging to understand that the cosmetic use of OnaBTX-A was initiated by the insight and convictions of two astute and courageous physicians, an ophthalmologist wife and her dermatologist husband. If it were not for the persistence of Jean and Alastair Carruthers in promoting their serendipitous observations, many other perceptive and insightful physicians would not have had the opportunity or the confidence to learn more about BoNT and its use in clinical aesthetic medicine. The challenge now being passed onto the reader is that with knowledge of how to inject a few drops of BoNT appropriately and safely, while treating patients with compassion and professionalism, additional innovative and ingenious uses of BoNT can be discovered, be they for cosmetic or therapeutic purposes.

We are all indebted to those physicians who have treated and continue to care for patients with BoNT for therapeutic and cosmetic purposes. Their commitment to the improvement of their patients' health and well-being through the advancement of sound and effective medical care is commendable and truly appreciated.

Finally, particular recognition and a special expression of gratitude is due to Kelly Heckler for her organizational skills and secretarial expertise that facilitated the completion of this book.

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PROLOGUE

An anthropological perspective on facial attractiveness and expressivity

Nina G. Jablonski

Humans are large-brained, long-lived primates that evolved in small, stable, and tightly knit social groups. In these groups, in the past and today, social cohesion has been essential for survival and communication has been essential for social cohesion. Communication in nonhuman primate and traditional human societies involves important vocal and tactile components, but is dominated by the exchange of visual information. The face is the primary portal from which this information emanates, and the "information content" of the face is vast. Gender is readily perceived by the relative masculinity or femininity of facial features, while color and texture of facial skin connote age and state of health, symmetry of facial features appears to indicate good health during all stages of development, and facial averageness connotes genetic heterozygosity.[1] Across cultures, the same features are also the primary source of judgements about attractiveness, with the universality of these preferences suggesting that, in the course of evolution, humans come to consider certain features attractive because they were displayed by healthy individuals.[2,3] Facial attractiveness is associated with many positive personal, professional and societal outcomes, especially for women.[4] In some cultures perceived Perceived facial attractiveness declines more in older women than in men, suggesting that there is probably greater selective pressure on older women to maintain high facial attractiveness.[5]

The static features of the face are only one aspect of the face's total information content, however. Facial expressions are as, or more important than the static attributes associated with attractiveness because they convey different kinds of information, about inner mood, intention and empathy. Humans and related species that live in complex social groups must be able to interpret the various meanings associated with facial appearance and the facial displays used in different emotional contexts.[6] The nonverbal information conveyed by postures and gestures (body language) is important in humans, but much of our capacity for nonverbal communication—especially in the expression of fear and anger witnessed by raising the hackles—has been lost as a result of loss of visible body hair in the human lineage.[7] Humans have thus become even more face-centric than our highly communicative nonhuman primate relatives.

The antiquity and importance of rich facial expressivity in humans must be considered in the contexts of cosmetic treatment of the face and facial beauty because practitioners and patients are confronted with a paradox when considering modification of the face. The quest for youthful looks and a face showing less visible evidence of age is at odds with the evolved, nuanced and robust communications functions of the human face. Over the life course, the habitual activities of the muscles of facial expression eventually produce lines and wrinkles in the skin, and the goal of much cosmetic intervention is the mitigation of these effects. But the very activities of human expression that lead to wrinkles are some of the most highly evolved of human signals and the most salient parts of the human communications repertoire. There is no easy or single solution to this paradox, but there is ample room for thoughtful exploration and discussion.

The importance of visual signals from the primate face is reflected in the number, size, and complex interconnections of the brain centers in the visual system, limbic system, and prefrontal cortex associated with the reception and interpretation of sensory information from faces.[8–10] The involvement of multiple homologous centers in the brains of macaque monkeys and humans implies the presence of these features in the last common ancestor of the monkey and human lineages, about 30 million years ago.[11] In nonhuman and human primates, the core areas involved in interpretation of static information from the face are the inferior occipital gyrus, fusiform gyrus, and the superior temporal sulcus. These areas in both hemispheres along with the amygdala, hippocampus, inferior frontal gyrus, and orbitofrontal cortex are recruited in the interpretation of facial expressions, and together comprise an extended system for facial processing.[10] The multiplicity and complex interconnectedness of the neural centers involved in the interpretation of both the invariant and changing modalities of facial input denote the preeminent importance of the face in the human social economy. Interpretation of invariant facial features is central to the recognition of identity, while interpretation of changeable aspects of the face is associated with speech and facial expression.

The primacy of the face and facial expression in human communication in humans is witnessed not only by the richness of the sensory systems associated with perception of facial information, but in the impressively complex motor systems that produce facial expressions.

The number and complexity of the intrinsic facial muscles in humans are far greater than in any other primate or mammal[12], a situation that makes for a wide range of facial expressions, from the most extreme and highly visible at a distance to the most subtle and nuanced perceptible only at close quarters. The muscles that produce these movements are described in great detail in the chapters that follow, but it warrants mention here that the muscles of facial expression that are most strongly conserved among mammals are those involved with the closure of the eyes and mouth, including the orbicularis oris and buccinator involved with chewing and swallowing. The muscles that are unique to humans, and highly structurally and functionally distinct, are the superficial perioral muscles, which are arrayed radially around the oral cavity and serve only mimetic function.[13] The most constant of these are the zygomaticus major, the levator labii superioris, the levator labii superioris alaquae nasi, the depressor anguli oris, and the depressor labii inferioris; the risorius and zygomaticus minor are the most individually variable. The wide range of subtle and finely graded facial expressions is made possible not only by the low innervation ratio of all the intrinsic facial muscles, but also by their polyneuronal innervation, that is, the high percentage of single muscle fibers innervated by multiple motor end-plates coming from different neurons.[13]

The fidelity and universality of the basic facial expressions of happiness, sadness, surprise, fear, disgust, and anger was first explored by Charles Darwin in *The Expression of the Emotions in Man and Animals* in 1872[14] and then placed on a sound empirical footing through the studies of Paul Ekman and colleagues.[15,16] It is widely recognized that, in addition to the six basic expressions, many more exist and are used regularly by humans. These compound expressions, as they have been described[17], include some of the most recognizable emotions: happily surprised, sadly surprised, sadly angry, fearfully disgusted, and appalled (Figure P.1).

The different basic and compound expressions use different facial muscles in different combinations, and to different extents. Among the muscles most commonly recruited in these expressions are those

Figure P.1 Sample images illustrating basic and compound emotions, identified by Du and colleagues (2014). The images depict a neutral face (a), faces exhibiting the six basic emotions: (b) happy, (c) sad, (d) fearful, (e) angry, (f) surprised, and (g) disgusted; and 15 faces demonstrating compound emotions: (h) happily surprised, (i) happily disgusted, (j) sadly fearful, (k) sadly angry, (l) sadly surprised, (m) sadly disgusted, (n) fearfully angry, (o) fearfully surprised, (p) fearfully disgusted, (q) angrily surprised, (r) angrily disgusted, (s) disgustedly surprised, (t) appalled, (u) hatred, and (v) awed. (From Du S, Tao Y, and Martinez AM. *Proceedings of the National Academy of Sciences* 2014; 111(15): E1454–E1462, reproduced with permission of the authors and PNAS.)

most of the upper face commonly targeted in cosmetic procedures: the frontalis (especially the upper and middle fibers), the procerus, and the corrugator supercilii. Contraction of these muscles is required for expressions of recognition and concern, as well as in conveying sadness, anger and disgust.

The key questions, then, are what does treatment with botulinum neurotoxin (BoNT) do to human facial expressivity and mood, and does this matter? Facial expressions communicate emotions and mood, and are modified through social learning, primarily through imitation involving the intentional matching of the facial behaviors of others.[18] Because effective imitation of an emotional expression requires that the observer understand the relationship between production of the expression and the underlying emotional state that the expresser wants to convey, facial imitation involves empathy.[18] When an observer watches another person making an expression, covert activation of the facial muscles involved in producing the expressions occurs in the observer due to activation of neurons in the mirror neuron system.[19] Imitation of emotional facial expressions (such as anger, happiness, fear, and the other basic expressions) also involves activation of the insula and amygdala.[20] If an observer is prevented from making an expression (as when they are asked to hold a pencil firmly in their teeth), they become less able to detect the emotional expression of the observed face.[21,22] Failure to recognize emotion in others is also observed in people with Moebius syndrome, which impedes movement of the facial muscles.[23] Activation of the same cortical areas occurs when people are observing and imitating faces expressing emotion.[24] Thus, in emotion recognition, observation and action are linked together by the mirror neuron system.[25] The mental states and intentions of other people, thus, are embodied and not understood only through linguistic and mental processes.[25] In facial feedback, the motor action of forming an expression is sufficient to experience that expression.[26] The deliberate lowering of the eyebrows as in a frown, for instance, makes a person's mood more negative.[26]

It follows from this evidence that when the activity of facial muscles is partially blocked as the result of treatment with BoNT, there is a decrease in the strength of the emotional experience.[27] In the context of facial feedback theory, people treated with BoNT cannot express certain emotions as well, after treatment as before, and the loss of emotional experience is caused by the loss of feedback from making the expression.[26] The observation that emotions—including powerful negative emotions—are attenuated following treatment of specific facial muscles with BoNT has led to the adoption of BoNT injections as part of the armamentarium of techniques for treating clinical depression.[28] This is especially the case when BoNT injections are used in the upper face, to target fibers of the frontalis, procerus, and corrugator. Under these conditions, negative facial expressions are reduced to a greater extent than positive ones, yielded a net change in the valence of facial expressions and a reduction in the experience of negative emotions.[28–30] The role of positive social feedback and positive self-feedback (from looking in the mirror) probably also reduce depression.[28] A full discussion of the use of BoNT in the treatment of depression is beyond the scope of this prologue, but it is sufficient to state that BoNT is increasingly being used because of its psychoactive rather than its cosmetic effects. Regardless of the primary reasons for BoNT use, other impacts of partial facial immobilization have to be considered.

It has become increasingly common for people to choose to restrict the motion of their faces for cosmetic reasons for periods of many years, and for young adults to elect to start BoNT treatment before the appearance of facial lines. The unintended and

long-term consequences of cosmetic BoNT injections have not been fully explored, and initial accounts have focused on the positive outcomes resulting from making people happier through reduction of the capacity to produce negative expressions. But mediation of facial affect with BoNT is a double-edged sword. There are many people today who cannot frown, and many who can't raise their eyebrows. Expressions of recognition, surprise, and concern for others are conveyed through contraction of the muscles of "negative affect," the frontalis and glabellar complex. Thus, BoNT reduces the ability to produce desirable expressions central to the demonstration of empathy as well as classic negative expressions of sadness, anger, and disgust. To what extent does this matter? Few systematic studies have been undertaken to explore the interpersonal and broader social ramifications of this phenomenon, but the preliminary indication is that chronic reduction of facial expressivity significantly impairs the abilities of treated individuals to interpret the emotions of others.[31] To these reports can be added the anecdotal accounts of people feeling uneasy around coworkers treated with BoNT whose expressions they cannot "read," as well the widely publicized on late-night television about a putative, frustrated child who couldn't interpret their parent's expressions: "I wish my teacher knew that I never can tell when Mommy's angry because her forehead doesn't move".[32] The importance of visible expressions of empathy or expressions of displeasure in the socialization of children cannot be overstated. A mother's scowl tells a child that something has gone wrong and that she is unhappy, and the establishment of this highly visible emotional vocabulary is an ancient and central part of human socialization.[33] A frown establishes a "current of connection," indicating that you understand another's distress.[34] As the visible repertoire of emotions develops and diversifies, a child's ability to immediately understand the actions of others develops and diversifies accordingly.[33] One of the cardinal characteristics of human beings is our ability to deal with sophisticated social environments, during which overt bodily behavior occurring in complex social interchanges is interpreted as an indication of our mental activity.[33] Although rarely discussed in the circles of cosmetic medicine, the reduction of the human capacity for empathy resulting from partial facial immobilization needs to be actively considered, discussed, and researched.

The paradox between the quests for lineless facial beauty and facial expressivity has not been resolved, and many important avenues of research about the consequences, especially, of long-term BoNT use require investigation. Thoughtful cosmetic practitioners will deal with this paradox and the related unknowns by being good scientists, and by undertaking attentive discussion of the costs and benefits of BoNT procedures with their patients. This is not an inconvenience, it's important. In connection with the use of BoNT on the face, the costs and risks are not only the medical ones enumerated in consent forms, but the more subtle ones of loss of efficacy of our highly evolved systems of visually based communication. Human beings are incessant communicators and ceaseless innovators. When we recognize that these two areas of human expertise are merged in cosmetic science, we can design new and nuanced interventions that will augment and not erase the best parts of our humanity.

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1 Botulinum toxin and its development in clinical medicine

Jean Carruthers and Alastair Carruthers

INTRODUCTION

In the aftermath of the Napoleonic wars (1799–1815) in Europe in the early nineteenth century, Dr. Justinus Kerner, an astute German physician and poet, noted that there seemed to be a substance in sausages that was causing people to die of a mysterious paralytic disease. Dr. Kerner postulated that this substance could possibly be helpful in treating overactive muscle conditions. Subsequent characterization of this substance and research led San Francisco ophthalmologist Dr. Alan Scott to consider using botulinum toxin type A (BoNT-A) as an alternative to surgery in the treatment of strabismus. In 1982, Ophthalmologist/Dermatologist Dr. Jean Carruthers had the opportunity to undertake a Fellowship with Dr. Scott and subsequently with Dr. Joseph Tsui and other Vancouver neurologists and published the first study of treating patients with dystonias with BoNT-A. Drs. Jean and her husband Alastair Carruthers then treated the first cosmetic patient, thus beginning a new era in the use of biologic substances considered to be deadly poisons as safe clinical modalities in the cosmetic as well as in the medical world.

SAUSAGE POISONING AND *CLOSTRIDIUM BOTULINUM*

At the end of the eighteenth century, the number of cases of fatal food poisoning throughout the southwest German region of Württemberg increased, likely due to widespread poverty after the devastating Napoleonic Wars (1795–1813) and subsequent unsanitary food production in rural areas.[1] In 1793, after 13 people fell ill and after 6 died during an outbreak in the small village of Wildbad in Württemberg, medical officers in the region scrambled to understand and identify the cause. By 1811, the Department of Internal Affairs of the Kingdom of Württemberg had pinpointed prussic acid in undercooked blood sausages as the culprit. In 1820, the district medical officer and poet, Justinus Kerner (1786–1862) published his first monograph on sausage poisoning, with a complete clinical description and summary of 76 case histories.[2] In a quest to extract and isolate the unknown toxic substance he called "fat poison" or "fatty acid," Kerner began to experiment on animals and himself in the pharmacist's laboratory, eventually publishing the first complete monograph containing the clinical evaluation and summary of 155 cases and accurate descriptions of all gastrointestinal, autonomic, and neuromuscular symptoms and signs of botulism.[3] From his experimentation, Kerner deduced that his fat poison acted by an interruption of the peripheral and autonomic nervous signal transmission, leaving the sensory signal transmission intact. In the final paragraph of his monograph, Kerner discussed the potential use of the toxin for the treatment of a variety of disorders characterized by "sympathetic overactivity" (e.g., St. Vitus' dance or Sydenham's chorea, a disorder characterized by jerky, uncontrollable movements, either of the face or of the arms and legs) and hypersecretion of bodily fluid, as well as for treating ulcers, delusions, rabies, plague, tuberculosis, and yellow fever. Sausage poisoning was eventually named botulism, after the Latin word *botulus*, meaning sausage.

In December 1895, 34 people in the small Belgian village of Ellezelles fell ill with symptoms of mydriasis, diplopia, dysphagia, dysarthria, and increasing muscle paralysis after eating pickled and smoked ham.[4] After examining the ham and conducting autopsies on the 3 patients who died, microbiologist Emile Pierre Van Ermengem (1851–1922) of the University of Ghent isolated an anaerobic microorganism that he called *Bacillus botulinus*—later renamed *Clostridium botulinum*.[5]

In 1904, an outbreak of food poisoning in Darmstadt, Germany involving canned white beans, led to the discovery of two serologically distinct strains of *C. botulinum*; these were eventually classified alphabetically as types A and B by Georgina Burke at Stanford University in 1919.[6] Over the next decades, cases of botulism became more frequent with the increased popularity of canned food products, and additional strains—types C, D, E, F, and G—were identified.[7]

CLINICAL DEVELOPMENT OF BOTULINUM TOXIN

With the advent of war, the potential uses of botulinum toxins took on a more sinister edge. In 1928, Herman Sommer and colleagues at the University of California, San Francisco isolated pure botulinum toxin type A (BoNT-A) as a stable acid precipitate.[8] As World War II approached, the United States government—along with multiple countries engaged in biowarfare programs—began intensive research into biological weapons, assembling bacteriologists and physicians in a laboratory at Camp Detrick (later named Fort Detrick) in Maryland to investigate dangerous and infectious bacteria and toxins.[7] In 1946, Carl Lamanna and colleagues developed concentration and crystallization techniques for the toxin that were subsequently used by Edward J. Schantz, a young U.S. army officer stationed at Fort Detrick, to produce the first batch of BoNT-A which was the basis for the later clinical product.[9,10] In 1972, President Richard Nixon signed the Biological and Toxic Weapons Convention, effectively putting an end to all investigations on biological agents for use in war, and Fort Detrick was closed. Schantz took his research to the University of Wisconsin, where he produced a large amount (150 mg) of BoNTA (batch 79–11) that remained in clinical use in the United States until December 1997.[11]

In the late 1960s and early 1970s, Alan Scott (Figure 1.1), an ophthalmologic surgeon at the Smith-Kettlewell Eye Research Foundation in San Francisco, began to experiment with BoNTA, supplied by Schantz, as a potential non-surgical treatment of strabismus.[12] Scott published his first primate studies in 1973,[13] and human studies with BoNT-A (then named Oculinum®) began in 1977. When he injected the toxin using a newly developed practical electromyographic (EMG) device (Figure 1.2)—a Teflon-coated needle used as an electrode that produced an auditory signal when the tip of the needle came close to motor endplates when the muscle was activated, allowing for precise placement of material[14]—strabismus could be treated relatively easily without invasive surgery for the first time. The publication of his landmark paper in 1980 showing that the toxin could correct gaze misalignment in humans[15] revolutionized the treatment of strabismus and subsequently of many other muscular disorders.

In 1989, the Food and Drug Administration (FDA) approved Oculinum®—subsequently acquired and renamed BOTOX® by Allergan Inc. (Irvine, CA)—for the nonsurgical correction of strabismus, blepharospasm, hemifacial spasm, and Meige's syndrome in adults, and clinical use expanded to include the treatment of cervical dystonia and spasmodic torticollis.[16,17]

THE BIRTH OF BOTOX® COSMETIC

By the late 1980s, nearly 10,000 patients had received multiple injections of BoNT-A for the treatment of benign essential blepharospasm with no evidence of antibody formation or systemic complications over 6 years of continued use,[18] and Scott's work planted

Figure 1.1 Alan B. Scott, MD, San Francisco ophthalmologist and strabismologist who was the first to use BoNT-A therapeutically and to recognize its many potential uses.

the seeds for its future cosmetic applications. In Vancouver, British Columbia, Jean Carruthers noticed a remarkable and unexpected effect in the brow of a patient treated for blepharospasm: a noticeable reduction in the appearance of glabellar furrows, giving her a more serene, untroubled expression. Jean discussed the observation with her dermatologist spouse, Alistair, who was attempting to soften the forehead wrinkles of his patients using soft-tissue augmenting agents available in the late 1980s, including collagen, silicone, or autologous fat, none of which worked particularly well—or with minimal risk—in the glabella. The timing for a non-invasive and easy injectable treatment that carried little risk of complication could not have been more perfect. The Baby Boomers—those 80 million babies born between 1946 and 1964—had all grown up and were clamoring to fix the lines, folds, and wrinkles that made them look older than they felt.[19]

After a conversation with Alan Scott, who confirmed he had treated a few patients for cosmetic purposes in 1985, we injected a small amount of BoNT-A between the brows of our then-assistant—now known as "patient zero"—and awaited the results. Seventeen more patients followed, aged 34–51, who would become part of the first published report on the efficacy of BoNT-A for glabellar rhytides (Figure 1.3).[20] The study attracted a flurry of interest and similar

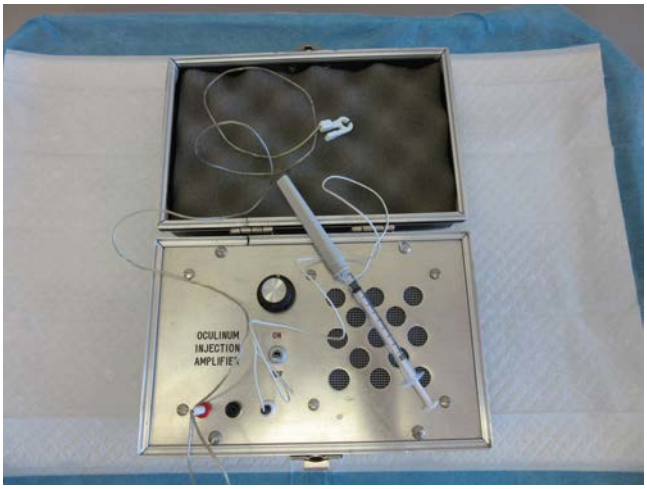

Figure 1.2 Early studies with BoNT-A used with EMG guidance.

trials showing remarkable effects indicating that BoNT-A was indeed a novel and promising treatment for unsightly facial rhytides.[21–23] Between 1992 and 1997, the popularity of cosmetic off-label use grew so rapidly that Allergan's supply temporarily ran out.[24]

By 2002, investigators had established an excellent safety profile for therapeutic doses of the toxin, and numerous open-label studies totaling more than 800 subjects demonstrated the safe and effective use of BoNT-A for improvements in the appearance of hyperfunctional facial rhytides.[25] In the United States, the FDA had approved BoNT-A for strabismus, blepharospasm, hemifacial spasm, and cervical dystonia. Additional approvals had been granted in the United Kingdom for axillary hyperhidrosis, and in Canada for axillary hyperhidrosis, focal muscle spasticity, and for the cosmetic treatment of glabellar wrinkles. In April 2002, on the heels of two large, double-blind, placebo-controlled, randomized, multicenter clinical trials,[26,27] the FDA approved BoNT-A for the non-surgical reduction of glabellar furrows, and the world of facial rejuvenation changed dramatically.

In the 1980s and 1990s, the concept of using botulinum toxin as a therapeutic agent seemed to be at best folly and at worst dangerous. Those of us who had had considerable experience in its use knew that the key to safety, as with any other drug, was the dosage administered. The difficulty was that the units of measurements were in billionths (nanograms) of a gram and the measurement needed to be biologic with "Mouse units."[28] Dr. Ross Kennedy and I performed a prospective randomized clinical trial of patients with misaligned eyes who had no ability to use the eyes together (fusion). We compared BoNT-A to adjustable suture surgery and found the BoNT-A superior in this group of patients. It showed that this modality was safe in this group and yet would not replace traditional surgery for other groups. The periocular safety was also studied in our 1995 paper[29] showing that the production of eyelid ptosis was the specific location of the injecting needle and thus could, with good technique, largely be avoided. In 1995 we used BoNT-A to treat congenital motor nystagmus ("shaking eyes") with a substantial improvement in vision.[30]

The cosmetic uses of BoNT-A spread from its initial use for glabellar frown lines[31] to realizing that we could shape the face in different ways such as being able predictably to elevate the whole eyebrow[32] and to titrate the widening of the eyelid fissure.[33] In 2000 we published on the combined use of BoNT-A with ablative CO_2 laser resurfacing.[34] We started to treat headache pain because our patients were so positive about the effects, even when this was felt not to work with current neurology theories.[35]

By 2003 we had started to use BoNT-A in the mid- and lower face and neck[36] and also were using combination treatments with hyaluronic acid fillers for deep resting glabellar rhytides.[37] With Bob Weiss, Vic Narukar, and Tim Flynn we explored the combination with Intense Pulsed Light (IPL)[38] and in 2004 we showed that injecting BoNT-A with IPL full face caused a 15% improvement in pigment reduction.[39]

By now there was a need to study dose ranging and we looked at men[40] and women[41] and showed that men have much larger dose requirements than women do.

In 2005, we published our first long-term safety review.[42] We started to study Patient Reported Outcomes (PROs) in 2007[43] and we all now realized that this was the hugely important yardstick for the evaluation of cosmetic treatments. The next step was the development of validated rating scales to aid the precision of both patient and investigator ratings.[44–46]

In the early days, fillers were felt to belong only in the lower face and neuromodulators in the upper. With Gary Monheit we did a three-arm prospective randomized study of the separate and combined use of fillers and neuromodulators in the perioral region.[47]

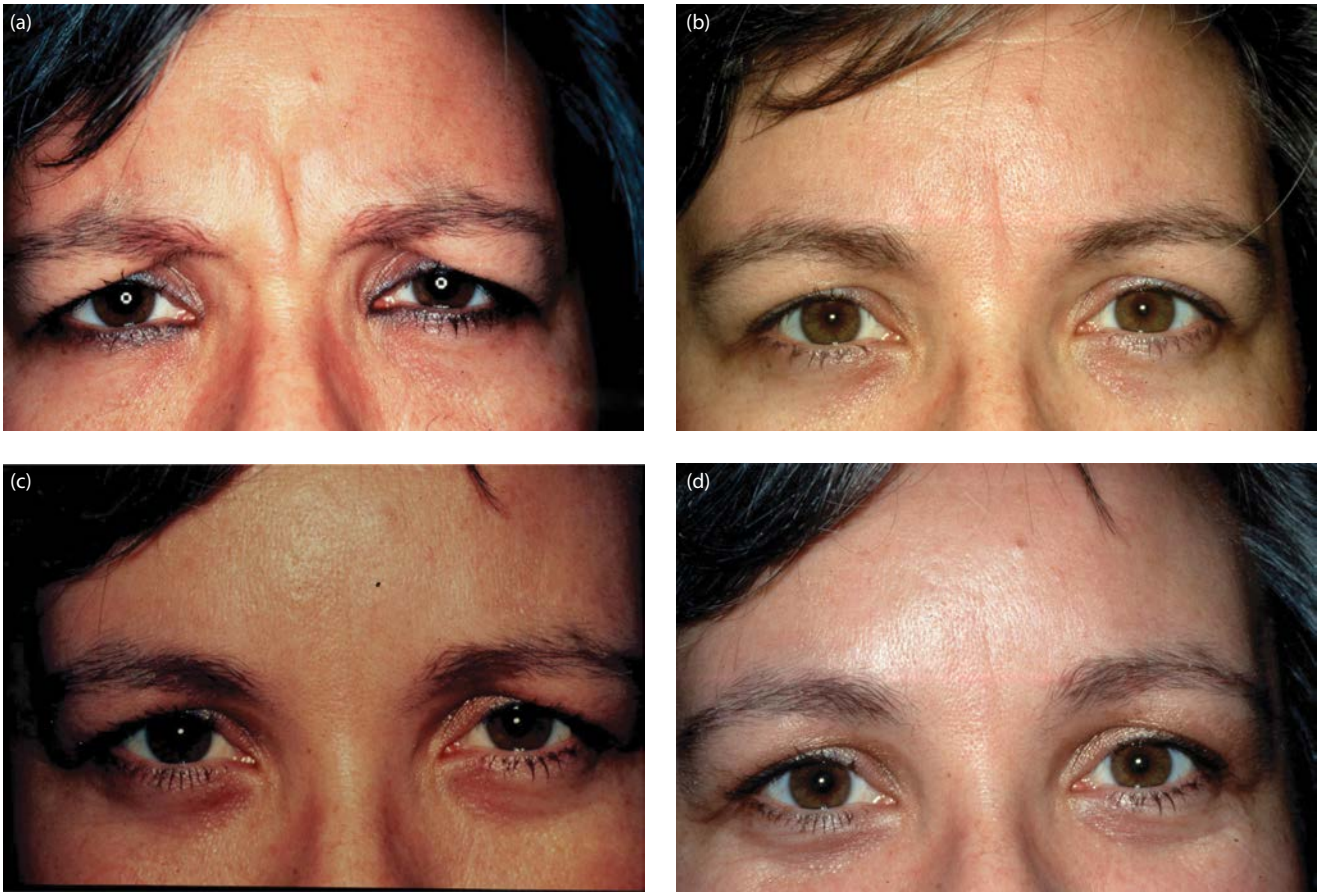

Figure 1.3 Patient zero—BoNT-A for the treatment of glabellar rhytides (a) pre-operative, frowning; (b) pre-operative, resting; (c) post-operative, attempting to frown; (d) post-operative, resting. (From Jean DA et al. *J Dermatol Surg Oncol* 1992; 18: 17, with permission.)

The combination was the clear winner.[47] In October 2012, Jean gave a TEDx talk "How a Feared Poison Became a World Class Multipurpose Drug."

Also in 2012, Jean and Alastair were awarded the prestigious Eugene Van Scott Award from the American Academy of Dermatology. Our presentation was titled "You want to Inject What?"—a phrase some of our many early patients had used when we were discussing treatment options in the early days.[19]

The worldwide popularity of the aesthetic use of BoNT-A has allowed many authors from many countries the opportunity to work together to pool concepts and new ideas for combined uses of botulinum toxins with other treatment modalities.[48,49]

Finally, derivative structures in the molecular structure of BoNT-A as in daxibotulinumtoxinA (DaxiBTX-A) has allowed a second generation of BoNT-A neuromodulators to take their first steps on the cosmetic and therapeutic stage.[50] Also most interesting, a new presentation of a short-acting neuromodulator BoNT-E is currently undergoing clinical trials.

SUMMARY

Thirty years ago, the idea of using a fatal, toxic agent to treat medical disorders and cosmetic rhytides was met with frank disbelief.[19] Today, BoNT-A has become one of the most versatile pharmaceuticals across diverse areas of medicine, with multiple formulations available globally for a broad range of therapeutic and cosmetic applications. Now the treatment of choice for smoothing hyperkinetic lines and shaping the face, alone or in combination with other rejuvenating procedures, and used for a variety of movement, pain, autonomic nervous system, and gastrointestinal and genitourinary disorders, among others, BoNT-A has firmly planted itself in clinical history, thanks to the dedication and sometimes dogged determination of medical innovators.

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2 Botulinum toxins: Pharmacology, immunology, and current developments

Mitchell F. Brin

INTRODUCTION

Like digitalis, atropine, and ziconotide, botulinum toxins (BoNTs) are natural substances that have become useful medicines. As proteins synthesized by living organisms (clostridial bacteria), BoNTs are biological products as opposed to conventional, synthetic drugs. For clinical use, BoNTs are isolated, purified, and formulated into specific products in a complex series of steps strictly regulated by governmental agencies in most countries where the products are approved. The manufacturing method determines not only the purity of the final product, but also the reproducibility of unit activity—the dosage measurement for BoNTs. The final formulations of the products are also critical because they can affect product stability, efficacy, safety, and immunogenicity.

SYNTHESIS AND STRUCTURE

BoNTs are produced as multimeric protein complexes consisting of the ~150 kDa neurotoxin and associated hemagglutinin and non-hemagglutinin proteins. These neurotoxin associated proteins (NAPs) stabilize and protect the ~150 kDa neurotoxin from degradation in the gastrointestinal tract.[1,2] The NAPs also exert biologically relevant *in vivo* activity, as demonstrated by the distinct pharmacodynamic curves in mice following intraperitoneal and intravenous injection of the ~150 kDa versus 900 kDa molecule.[3] Interactions between BoNT proteins and NAPs are influenced by the microenvironment, including pH,[4] but are more difficult to study following therapeutic administration in humans. During the manufacturing of BoNTA for clinical use, proprietary procedures are used to determine which, if any, of the NAPs are retained in the final product.

Different bacterial strains synthesize complexes that vary in size and protein composition, as well as neurotoxin serotype.[5] Seven different BoNT serotypes are recognized: A, B, C1, D, E, F, and G. Serotypes A through F form the 300 kDa complex; serotypes A, B, C1, and D form the 500–700 kDa complex; and only type A forms the 900 kDa complex.[6,7] Type G forms the 500 kDa complex.[8] Some clostridial strains are mosaics, containing genes encoding parts of one serotype and parts of another; the newly identified botulinum toxin may be a new serotype H or may be a mosaic of types A and F.[9,10] Mosaic toxins have previously been described for types C1 and D,[11] and for types F and A.[12] Toxin variants within the serotypes (e.g., A1, A2, etc.) have also been identified, with reported differentiating preclinical *in vivo* profiles.[13,14]

The active BoNT protein in all serotypes is synthesized as a single chain of approximately 150 kDa that must be nicked or cleaved by proteases in order to be active (Figure 2.1).[15] Cleavage results in a di-chain molecule consisting of an approximately 100-kDa heavy chain and an approximately 50-kDa light chain, linked by a disulfide bond.[5] The protein comprises four domains consisting of the ~50 kDa light chain and three domains of the heavy chain: the ~50 kDa H_N membrane translocation domain, the ~25 kDa H_{CN} domain, and the ~25 kDa H_{CC} binding domain.[17]

PHARMACOLOGY

General Mechanism of Action

BoNTs exert their activity through a multistep process: binding to nerve terminals, internalization, translocation of the light chain across endosomal membrane, and inhibition of vesicular neurotransmitter release. This chapter focuses on recent developments in the mechanism of action; several comprehensive reviews are available for additional information.[17,18]

Binding

The binding of BoNTs to nerve cell membranes is characterized by a series of protein-lipid and protein-protein interactions with cellular membrane components that facilitate its internalization. Binding has been explained via a multireceptor model, in which the co-receptor comprises a ganglioside and protein component. BoNTs interact with gangliosides that are highly concentrated on presynaptic terminals.[19–22] Gangliosides are believed to mediate the initial low affinity contact between the BoNT and the neuronal membrane.[22,23] Ganglioside binding increases the local concentration of BoNT at the membrane surface, permitting it to diffuse in the plane of the membrane and bind its high affinity protein receptor (Figures 2.1 and 2.2).[22]

Botulinum neurotoxin A (BoNT-A) binding to gangliosides is mediated not only by the H_{CC} domain,[18] but also by parts of the H_N domain (amino acid residues H_N729-845).[25] A conserved ganglioside binding site motif has been identified in the H_C domain in all serotypes examined thus far except type D,[26] but affinities for various gangliosides differ between and within serotypes (e.g., A1, A2, etc.) produced by different clostridial strains.[27–29] Whether the H_{CN} domain has a function is unknown, but it may be involved in binding phosphatidylinositol phosphate (PIP).[18]

Synaptic vesicle protein 2 (SV2) is a protein receptor for BoNT types A, C1, D, E, and F and is localized to synaptic vesicles.[26,30–32] During exocytosis, portions of SV2 proteins are exposed to the cytoplasm, providing an exposed surface to which BoNTs can bind.[30,31] SV2 has at least three isoforms (SV2A, SV2B, and SV2C) that bind several BoNT serotypes with varying affinities (Table 2.1).

Synaptotagmins I and II are protein receptors for BoNT types B and G.[33,34] Synaptotagmins are localized to synaptic vesicle membranes where they sense calcium and trigger vesicle fusion.[35] Binding of types B and G to these proteins leads to their internalization into neurons.[34,36]

The C terminal domain of BoNTA shows homology with fibroblast growth factors (FGFs) and FGF receptor-3 (FGFR3) has been identified as an additional protein receptor for BoNTA in neuroblastoma cells, although the significance of this binding *in vivo* is not yet known.[37]

Internalization and translocation

After binding to gangliosides and protein co-receptors, BoNTs are internalized via receptor-mediated endocytosis into an endosome/vesicle. The light chain is translocated across the vesicle membrane in a series of steps still under study; recent evidence supports the following mechanism (Figure 2.3).[38,39] ATPase pumps in the vesicle membrane concentrate protons into lumen, decreasing intravesicular pH. The acidic environment of the endosome causes a conformational change in the neurotoxin-receptor complex that promotes insertion of the heavy chain into the endosomal membrane. The H_N domain of the heavy chain forms a channel and the H_C domain is needed for the light chain to unfold so that it can move through the channel into the cytosol.[38] The disulfide bond between the heavy and light chains is necessary for translocation across the synaptic vesicle membrane, but is ultimately reduced for the light chain to separate and interact with SNAP-25 (see the following).

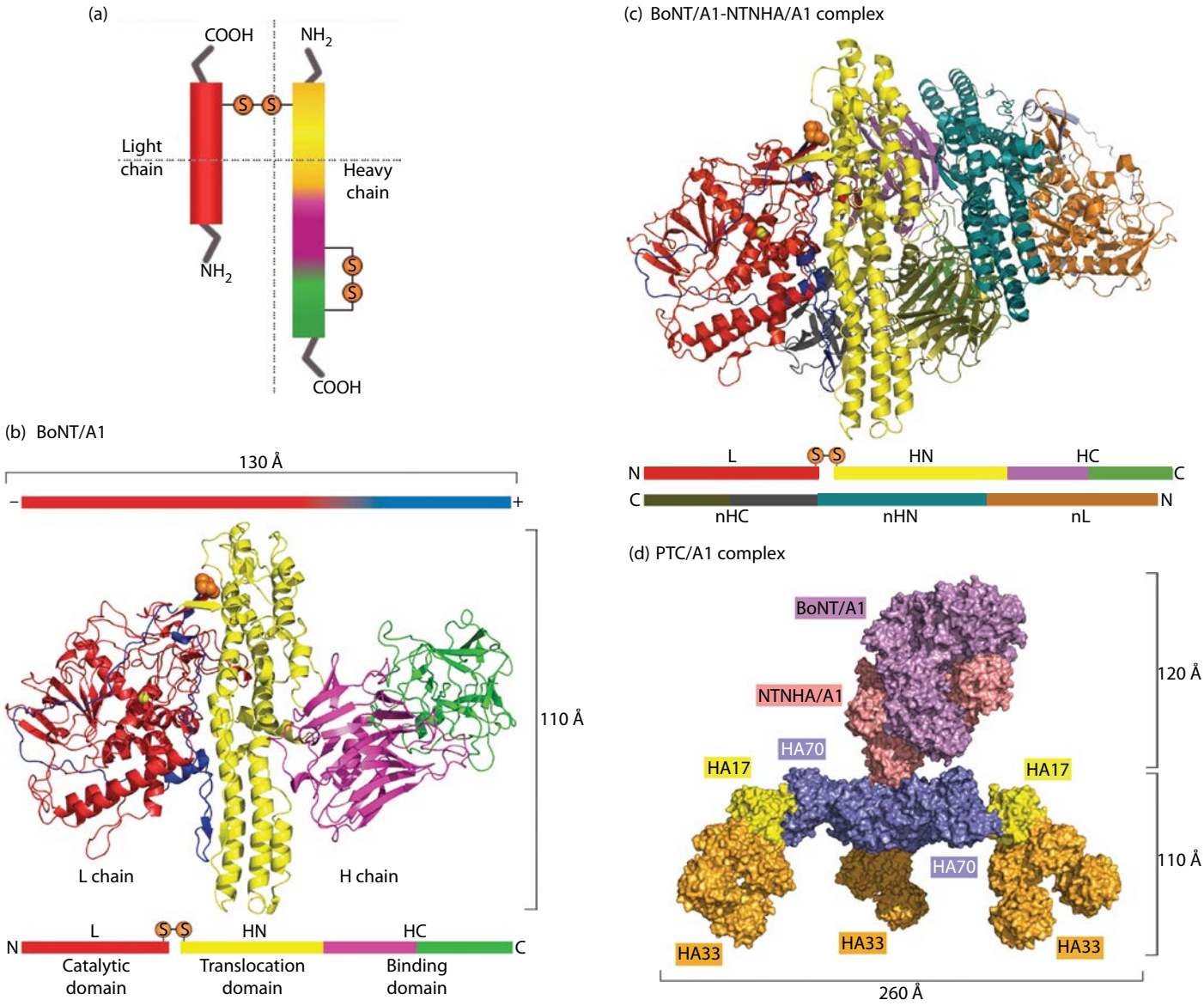

Figure 2.1 Schematic drawing showing structure of BoNT activated di-chain protein ~100-kDaa and ~50-kDaa chains (a) and diagrams of crystal structure of botulinum toxin A1 (BoNT-A1)[16] (b–d). The four individual protein domains interact with cellular membrane components in a series of protein-lipid and protein-protein interactions that facilitate the internalization of BoNT. These include the following: the H_C domain binds specifically to nerve terminals, with the H_{CC}, domain binding gangliosides and the H_{CN} domain possibly binding phosphatidylinositol phosphate (PIP),[18] the H_N domain forms a pore in the endosome that translocates the L chain into the nerve terminal cytosol, and the L chain is a metalloprotease that cleaves one or more SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins that mediate vesicular neurotransmitter release. A peptide belt (dark blue) surrounds the L domain and the inter-chain disulfide bond (orange), links the L chain to the H_N domain. (Figures b–d are reprinted from Rossetto O et al. *Nat Rev Microbiol* 12(8): 535–49. By permission from Macmillan Publishers Ltd., copyright 2014.)

Enzymatic Activity

Inside the cytosol, the light chain cleaves one or more of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins necessary for vesicle docking and fusion (Figure 2.4). Each serotype cleaves a specific peptide bond on one or more of the SNARE proteins in a zinc-dependent process.[43]

BoNT types A and E cleave SNAP-25 at different sites, and the effects of type E are much shorter. Evidence indicates that the type A light chain and its cleavage product ($SNAP\text{-}25_{197}$) localize to the plasma membrane, whereas the type E light chain is distributed throughout the cell cytoplasm.[44] The localization of type A light chain to the plasma membrane is decreased following mutation of the dileucine motif. Mutation of the dileucine motif of type A also leads to rapid recovery of neuromuscular function in rats.[45] More recently, mutation of the two leucines has been found to prevent interactions between the light chain and septins—intracellular structural proteins found clustered with the light chain at the plasma membrane (Figure 2.5).[46] The dileucine mutation also increases degradation of the type A light chain, as does interference with light chain-septin clustering. In contrast, the type E light chain does not interact with septins. These data indicate that the clustering of the type A light chain with septins at the plasma membrane via interactions with the dileucine motif is critical for its stability; these characteristics importantly contribute to the duration of action of BoNTA in clinical use.[44,46] Type A is the only botulinum neurotoxin serotype that contains a dileucine motif at the C terminus of the light chain.[44]

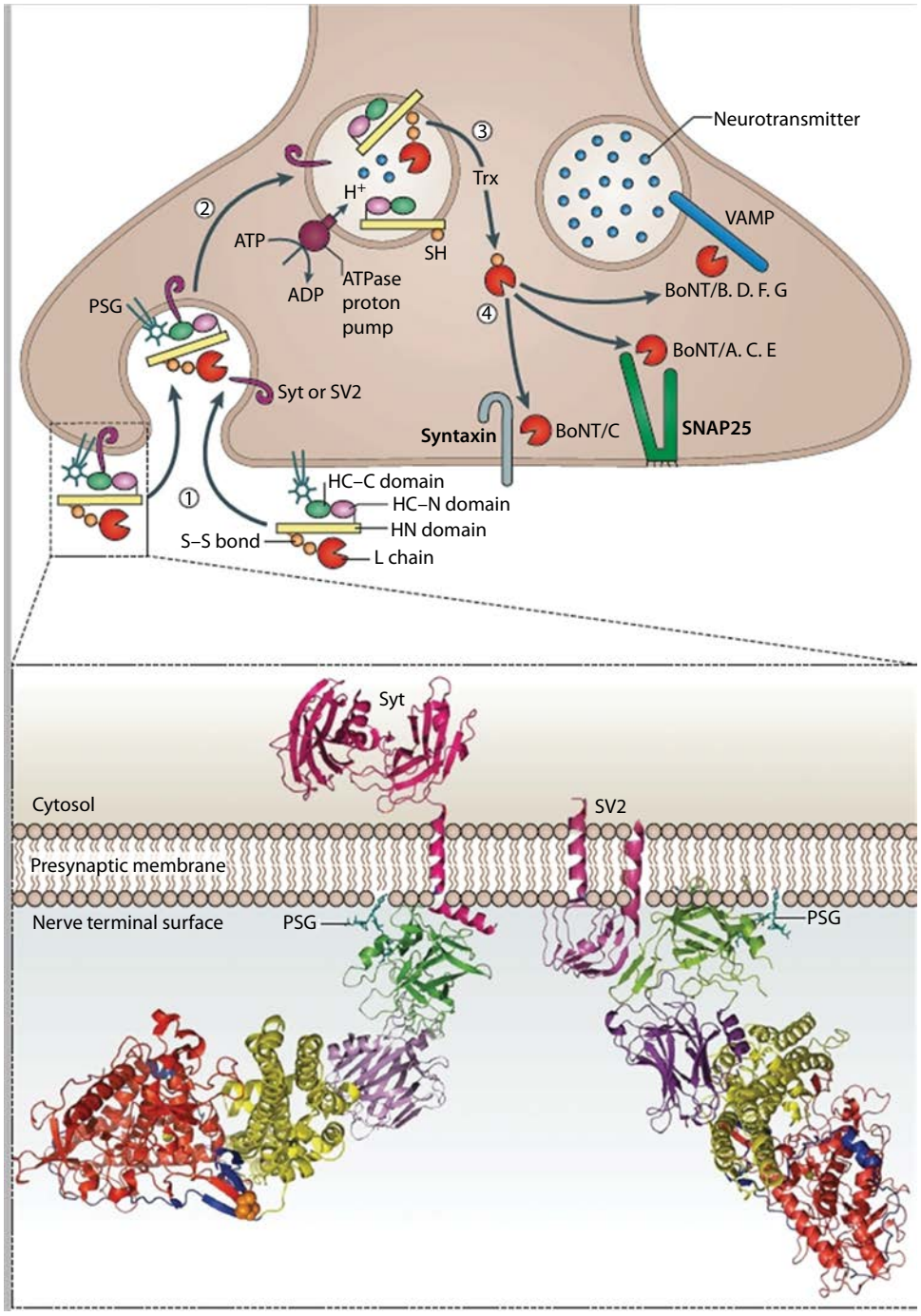

Figure 2.2 Binding and trafficking of BoNTs inside nerve terminals. The carboxy-terminal end of the HC domain (the HC-C domain) binds to a polysialoganglioside (PSG) present on the presynaptic membrane, followed by binding to a protein (either synaptotagmin [Syt] or SV2) located inside the exocytosed synaptic vesicle or on the presynaptic membrane (Step 1). The crystal structure of botulinum toxin B (BoNT-B) bound to Syt and PSG is shown on the lower left-hand side and the crystal structure of BoNT-A bound to PSG and to SV2 is shown on the lower right-hand side. BoNT is then endocytosed inside synaptic vesicles (Step 2), exploiting the vesicular ATPase proton pump that drives neurotransmitter reuptake. As the vesicle is acidified, BoNT becomes protonated, which results in translocation of the L chain across the synaptic vesicle membrane (Step 3) into the cytosol. Translocation can also occur across the endosomal membrane following the fusion of a synaptic vesicle with an endosome (which seems to occur in cultured neurons).[24] The L chain is released from the HN domain following cleavage of the inter-chain disulfide bond (S–S; shown in orange). The L-chain metalloproteases of BoNT-B, BoNT-D, BoNT-F, and BoNT-G cleave VAMP, the L-chain metalloproteases of BoNT-A and BoNT-E cleave SNAP25, and the L-chain metalloprotease of BoNT-C cleaves both SNAP25 and syntaxin (Step 4), all of which inhibit neurotransmitter release. (Reprinted from Rossetto O et al. *Nat Rev Microbiol* 12(8): 535–49. By permission from Macmillan Publishers Ltd., copyright 2014.)

In vitro, under the experimental conditions studied, BoNTA binding and internalization occur within minutes and proteolysis of SNAP-25 can be detected within half an hour.[47] Although traditionally called a neurotoxin because of its potential to cause generalized muscle weakness, BoNTA is not cytotoxic.[48,49]

Clinical Pharmacology

Mechanistically, the universal process of SNARE-mediated synaptic vesicle trafficking is the ultimate pharmacological target for BoNTs in neurons that are capable of binding and internalizing the toxin.[50]

Table 2.1 Receptors for BoNT Serotypes

Serotype	Cell membrane binding	Protein receptor
A	GT1b, GD1a	FGFR3 > SV2C > SV2A > SV2B;
B	GT1b, GD1a	Synaptotagmin II > Synaptotagmin I
C1	GD1b, GT1b	SV2
D	GT1b, GD1b, GD2	SV2B > SV2C > SV2A
E	GD1a	SV2A > SV2B
F	GD1a	SV2
G	GT1b	Synaptotagmin I ~ Synaptotagmin II

Source: Adapted from Lam KH et al. *Prog Biophys Mol Biol* 2015; 117(2–3): 225–31.)
Note: FGFR3 = fibroblast growth factor receptor 3, SV2 = secretory vesicle 2; >indicates comparative *in vitro* affinity.

Pharmacology in Neuromuscular Conditions: Extrafusal and Intrafusal Muscle Fibers

In the extrafusal motor nerve terminal, denervation leads to the increased production of growth factors, such as insulin-like growth factor-1 (IGF-1), and effects on related signaling pathways[51] that stimulate sprout development. Sprouts appear at motor-nerve terminals and nodes of Ranvier within 2 days of BoNTA injection into mammalian soleus muscles that persist and become more complex for at least 50 days.[52] Sprouts may establish functional synaptic contacts,[52] but the role of these sprouts in functional recovery of the neurons is not firmly established. Using a sensitive measure, Rogozhin and colleagues found that quantal neurotransmitter release could be detected in the vicinity of sprouts and the original terminals at about the same time, and the original terminals accounted for more than 80% of total acetylcholine release, suggesting that the spouts are relatively ineffectual.[53,54]

As exocytosis is restored, the original terminals recover and the sprouts regress.[55] After reinnervation is complete, the target tissue is fully functional[52] and there is no clinical indication that post-botulinum reinnervation produces functionally substandard synapses. However, in rats, acetylcholine release recovers more slowly after multiple than single injections.[53]

The SNARE-mediated mechanism inhibiting acetylcholine release occurs not only at alpha motor neurons, which innervate extrafusal muscle fibers, but also at gamma motor neurons, which innervate intrafusal muscle fibers. Intrafusal fibers make up muscle spindles (Figure 2.6)—the proprioceptive organs that are sensitive to stretch and are important in setting the resting tone and reflex sensitivity of muscle. Inhibition of gamma motor neurons decreases activation of muscle spindles, which effectively changes the sensory afferent system by reducing the Ia afferent traffic. However, this mechanism likely does not occur in facial muscles as they are reported to lack muscle spindles.[56,57]

Preclinical and clinical studies indicate that BoNT-A affects afferent pathways via inhibition of neural input to intrafusal fibers.[58–62] Thus, the overall effect of BoNT-A therapy may be a combination of a direct effect on the primary nerve-end organ communication (i.e., the alpha motor neuron innervating muscle) coupled with an indirect effect on the overall system (i.e., via afferent effects associated with toxin-induced chemodenervation of the gamma motor neuron).

The most common BoNT products in clinical use are onabotulinumtoxinA (Allergan), abobotulinumtoxinA (Ipsen), incobotulinumtoxinA (Merz), and rimabotulinumtoxinB (Solstice). BoNTs are most often injected into overactive skeletal muscles that vary depending on the condition to be treated and the patient's individual presentation. The clinical onset of action following intramuscular injection is generally reported to be within 3–7 days, with a peak effect in approximately 2–4 weeks. However, when injected into small muscles for the treatment of glabellar lines, the onset of clinical effects have been reported within 24 hours.[63,64] The duration of beneficial effects of each treatment is approximately 3–5 months following intramuscular injection,[65] although some differences have been noted.[66] The duration of BoNT-B is somewhat shorter than that of type A, and has been reported as 6–12 weeks in the management of facial lines.[67] Most patients respond to BoNT-A for many years without decrements in safety, responsiveness, or quality of life, and without increased doses.[68,69]

Pharmacology in Dermal Conditions

Eccrine sweat glands are widely distributed over the body, with areas around the sweat coil and duct densely vascularized and innervated by sympathetic postganglionic terminals.[70] Unlike most sympathetic neurons, those that innervate eccrine sweat glands are cholinergic; they also co-release neuropeptides such as calcitonin gene related peptide (CGRP) and vasoactive intestinal polypeptide (VIP).[71] Apocrine sweat glands are distributed only in hairy areas such as axillary, mammary, perineal, and genital regions, where they respond to both epinephrine and norepinephrine, although whether they are activated via sympathetic innervation, circulating levels of these neurotransmitters, or local intradermal release is not yet known. Apocrine sweat

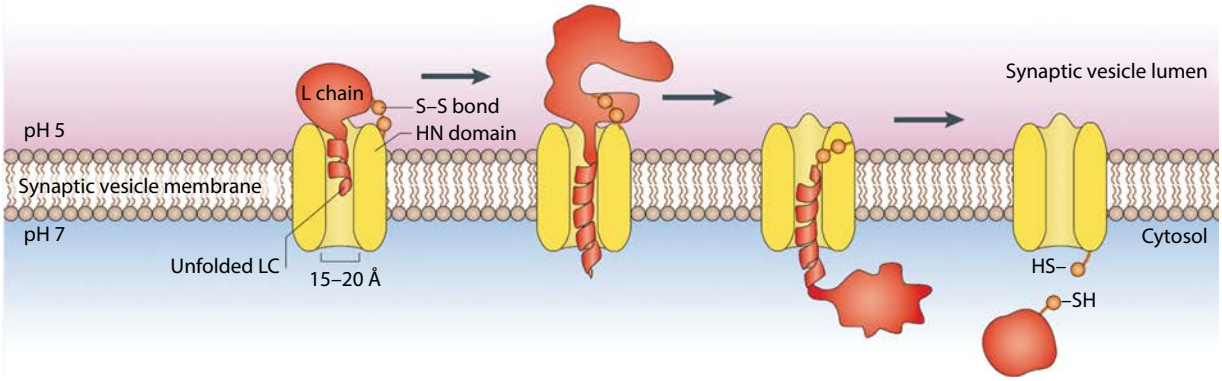

Figure 2.3 Model for the molecular events that occur during L-chain translocation across the synaptic vesicle membrane. Acidification of the synaptic vesicle lumen via action of the ATPase proton pump causes a conformational change in the HN domain, which enables it to penetrate the lipid bilayer. This leads to the formation of a channel that chaperones the partially unfolded L chain across the membrane. The inter-chain disulfide bond (S–S bond) is proposed to cross the membrane at a late stage during translocation, and its reduction on the cytosolic side of the synaptic vesicle membrane releases the L chain into the cytosol. (Reprinted by permission from Rossetto O et al. *Nat Rev Microbiol* 12(8): 535–49, Macmillan Publishers Ltd., copyright 2014.)

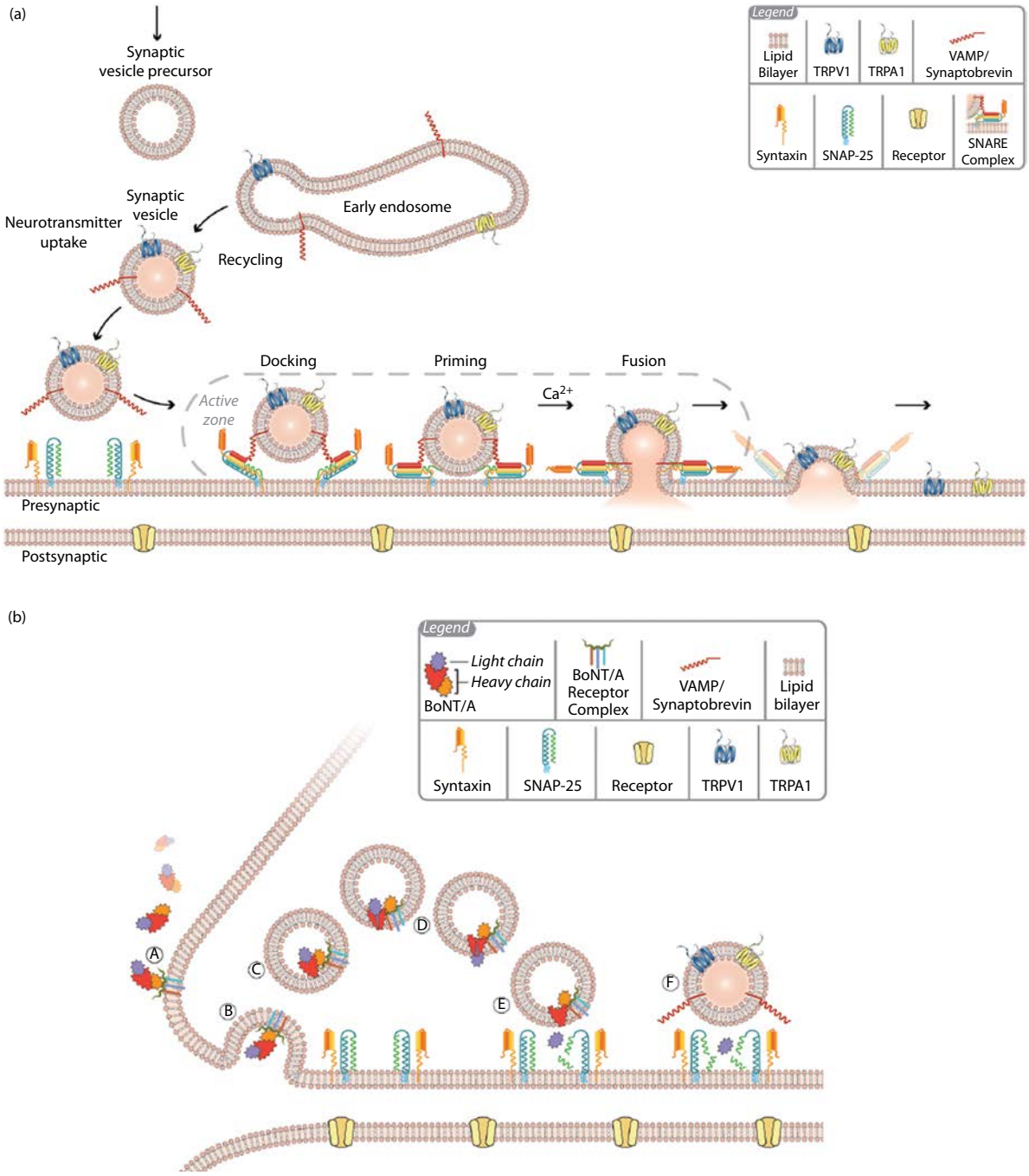

Figure 2.4 BoNT-A mechanism of action: Synaptic vesicle delivery of luminal *content* neurotransmitters and lipid bilayer *cargo* ion channels and receptors. (a) Synaptic vesicle (SC) delivery of luminal contents such as neurotransmitters and lipid bilayer cargo[108] including ion channels and receptors. SVs form a reserve pool at the nerve terminal and may be filled with neurotransmitters. Most SVs are decorated with multiple proteins:[40] membrane-associated protein receptors, transient receptor potential cation channel vanilloid subfamily, member 1 (TRPV1), and transient receptor potential cation channel ankyrin subfamily, member 1 (TRPA1) are depicted. SVs dock adjacent to the nerve terminal and inner membrane active zone and undergo an adenosine triphosphate (ATP)-dependent priming step that enables response to the Ca^{2+} signal that triggers fusion, exocytosis, and consequent delivery of not only SV contents into the extracellular space, but also lipid membrane and associated protein cargo into the cell surface. Successful fusion requires an interaction between the vesicle-associated membrane protein (VAMP)/synaptobrevin with the internal membrane surface proteins synaptosomal-associated protein of molecular weight 25 kDaa (SNAP-25) and syntaxin, which together form the SNARE (soluble NSF [N-ethylmaleimide–sensitive factor] attachment protein receptor) complex; other associated proteins (e.g., Munc18, Rab) are involved but not depicted.[41] The SV membrane may fully fuse into the terminal membrane (full collapse fusion), thus delivering the protein receptors (e.g., TRPV1 or TRPA1) into the cell surface. Excess terminal recycling through one of the endocytosis pathways[42] is not depicted. OnaBTX-A cleaves SNAP-25, impairing SV fusion and the regulated delivery of receptors TRPV1 or TRPA1 to the terminal membrane, thus downregulating receptor activity. An SV with both luminal contents and vesicular lipid bilayer cargo is diagrammed for illustration purposes. (b) OnaBTX-A mechanism of action. (A) OnaBTX-A heavy chain binds to an acceptor complex comprised of three components: ganglioside GT1b, synaptic vesicle glycoprotein 2 (SV2), and fibroblast growth factor receptor 3 (FGFR3); (B) internalization into an endosome that (C) acidifies; (D) conformational change that enables the light chain to traverse the endosomal wall; (E) cytosolic light chain specifically cleaves SNAP-25 (synaptosomal-associated protein of molecular weight 25 kDaa), one of the SNARE attachment protein receptors required for SV membrane docking; (F) SNARE disruption prevents SV fusion with the terminal membrane. This prevents SV content delivery of neurotransmitters to the synaptic cleft in addition to SV cargo delivery and cell surface expression of relevant peripheral nerve receptors and ion channels. (Figures courtesy of Maria Rivero [Allergan, Inc., Irvine, CA]. [a] Modified from Burstein R et al. *Cephalalgia* 2014; 34(11): 853–69; [b] reprinted from Whitcup SM et al. *Ann N Y Acad Sci* 2014; 1329: 67–80 via a Creative Commons License.)

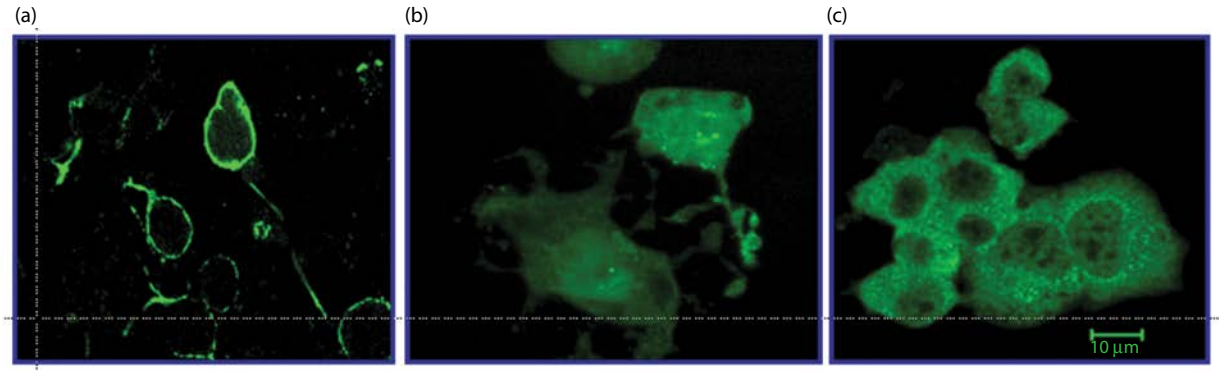

Figure 2.5 Subcellular localization of light chain in differentiated rat pheochromocytoma cells (PC12). Green fluorescent protein-light chain type A (GFP-LCA) localized in a punctate manner in specific areas at the plasma membrane of the cell body and neurites, with no fluorescence in the cytoplasm of cells (a). In contrast, the GFP-LCE (b) localizes in a punctate manner in the cell cytoplasm and the GFP-LCB (c) is dispersed throughout the cell including the nucleus.

glands have also been described in hairy regions where they respond to acetylcholine, norepinephrine, and epinephrine.[70]

Sebaceous glands in the skin are also sensitive to acetylcholine, but they are not directly innervated by autonomic fibers (although nerve fibers are evident in their vicinity).[72] *In vitro*, acetylcholine stimulates sebum production in human sebaceous glands by acting on nicotinic cholinergic receptors, and specifically nicotinic acetylcholine receptors alpha-7 (nAchRα7), which are present *in vitro* and *in vivo*.[73] Notably, acetylcholine is released from non-neuronal sebaceous cells in an autocrine fashion and may not be SNARE mediated; the non-neuronal actions of acetylcholine in skin have been reviewed.[74] Non-neuronal acetylcholine release in human skin is partially mediated via organic cation transporters.[75,76]

Hyperhidrosis

The sympathetic, cholinergic innervation of eccrine sweat glands provides the basis for BoNT-A use in focal hyperhidrosis, in which the medication is injected intradermally. The onset of action of BoNT-A

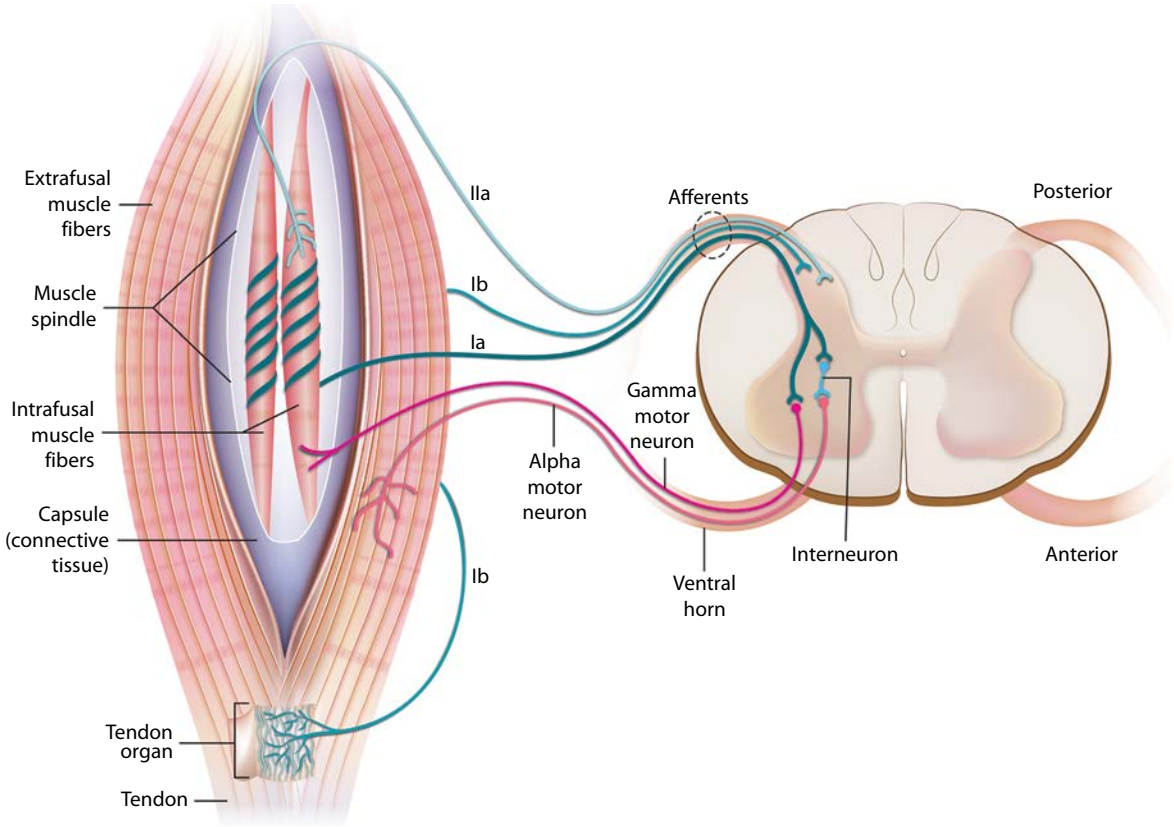

Figure 2.6 Motor and sensory innervation of muscle. Acetylcholine is released from alpha and gamma motor neurons that originate in the spinal cord (right). Alpha motor neurons innervate extrafusal muscle fibers and gamma motor neurons innervate intrafusal fibers of the muscle spindle (left). Activation of gamma motor neurons keeps the muscle spindle taut and sensitive to stretch. Group Ia and Group IIa afferent fibers convey information about muscle length; Group Ia fibers also convey information about the rate of length change. By inhibiting acetylcholine release from gamma motor neurons, BoNTA may affect muscle spindle activity and, consequently, sensory information conveyed back to the spinal cord. Golgi tendon organs sense muscle tension and are innervated by Group Ib afferents. (Figure courtesy of Maria Rivero [Allergan, Inc., Irvine, CA]).

in various forms of focal hyperhidrosis is within 1 week,[77] and benefits last approximately 7 months with OnaBTX-A, although 22%–28% of patients may experience benefits for at least a year.[78,79]

Preliminary studies in other dermal conditions

Serendipitous observations by investigators treating migraine and facial tics suggest that BoNT-A may also have beneficial effects on sebaceous cysts[80] and acne.[81] Several subsequent studies designed to evaluate the effects of OnaBTX-A or BoNT-A (Medytox) on sebum production support this effect.[73,82]

Several case reports and small, open studies have documented beneficial effects of OnaBTX-A and AboBTX-A in rosacea.[83–85] Beneficial effects of OnaBTX-A and AboBTX-A have also been reported in patients with psoriasis and with AboBTX-A in an animal model of psoriasis.[86–88]

BoNT-A has also been studied in cutaneous scarring following speculation that it may reduce the muscle tension that leads to scar production during wound healing.[89] Several small, randomized studies have found that OnaBTX-A injections improve the appearance of scars associated with facial wounds.[90,91] Subsequent case reports have also noted improvement in scarring and pain associated with keloids following BoNT-A.[92,93] A randomized study documented greater improvements in keloid volume and subjective symptoms such as pain following intralesional BoNT-A than corticosteroids.[94]

Studies on fibroblasts isolated from human scar tissue have found that BoNT-A inhibits the growth of fibroblasts and fibroblast differentiation into myofibroblasts, as well as decreases production of the scar-inducing protein, transforming growth factor-beta 1 (TGF-β1).[95,96] In a preclinical scar model, BoNT-A reduced collagen deposition and scarring.[97] In tissue from human keloid scars, BoNT-A has been found to alter expression of multiple scar-related proteins, including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), TGF-β1, and matrix metalloprotease-1 (MMP-1).[98] However, other preclinical work indicates that BoNT-A decreases collagen I production in human dermal fibroblasts.[99] Other researchers have found that BoNT-A significantly antagonizes premature senescence of human dermal fibroblasts *in vitro* induced by ultraviolet radiation, raising the potential of antiphotoaging effects.[100]

Pharmacology in Overactive Bladder/Neurogenic Detrusor Overactivity

Micturition comprises both motor and sensory components. Release of acetylcholine and ATP from parasympathetic nerves mediates the elimination of urine, with acetylcholine dominating under normal conditions and ATP dominating under pathological conditions.[101,102] Sensory mechanisms in the bladder likely mediate the sensation of urgency in overactive bladder. Bladder afferent neurons express numerous receptors, including transient receptor potential vanilloid 1 (TRPV1) that respond to heat, acidic pH, voltage, and endovanilloids,[103] tyrosine kinase receptor A that respond to nerve growth factor, and purinergic receptors (e.g., P2X3) that respond to ATP.[104,105]

The effects of BoNT-A on acetylcholine release from motor terminals are well documented, and growing evidence indicates that BoNT-A has several different sensory actions in the bladder.[105] For example, in preclinical studies, BoNT-A inhibits ATP release from cultured urothelial cells, which may stimulate purinergic receptors on bladder afferents.[106] The effects of BoNT-A have also been studied in a model of spinal cord injury, in which animals show an increase in resting ATP release, an increase in hypoosmotic-evoked ATP release, and a decrease in hypoosmotic-evoked NO release from the urothelium. Although BoNT-A does not affect the increase in resting ATP release, it significantly inhibits the hypoosmotic-evoked urothelial ATP release.[107] BoNT-A also restores the hypoosmotic-evoked inhibition of NO release in these animals. The authors suggest that changes in the ratio of ATP-mediated excitation and NO-mediated inhibition promote hyperactivity in the bladder that can be largely reversed by BoNT-A. Finally, peripheral administration of BoNT-A cleaves SNAP-25 and prevents the SNARE-mediated vesicle-fusion process, which consequently impairs transfer of the vesicular lipid bilayer cargo,[108] TRPV1 and P2X3, to neural membranes.[109,110]

Clinical evidence from patients with neurogenic detrusor overactivity indicates that BoNT-A normalizes disease-associated pathology. Patients with neurogenic detrusor overactivity exhibit increased levels of TRPV1 and P2X3 receptors in the suburothelial bladder.[111,112] The expression of P2X3 and TRPV1 in urinary bladder epithelial cells of these patients decreases significantly (without any loss of fiber density) 4 weeks after BoNT-A treatment, and improvements in patients' sensation of urgency and urodynamic physiology parameters are correlated with the temporal change in P2X3 immunoreactivity.[105] Urinary NGF levels, normalized to creatinine, are significantly higher than controls for untreated patients with either neurogenic or idiopathic detrusor overactivity, and clinical response to OnaBTX-A is associated with the reduction of these levels in both patient populations.[113]

In the treatment of overactive bladder, OnaBTX-A is injected into the smooth detrusor muscle of the urinary bladder and in the Phase 3 program for idiopathic overactive bladder,[114–116] the duration of effect was approximately 7–8 months, with consistent benefits observed following multiple injections up to 3.5 years.[117] Similarly, in the Phase 3 program for neurogenic detrusor overactivity, the duration of effect (time to retreatment) was approximately 8–10 months,[118,119] with consistent benefits observed following multiple injections up to 4 years.[120,121]

Pharmacology in Chronic Migraine

Chronic migraine is characterized by dysfunction in the trigeminovascular pathway, including central and peripheral sensitization involving peripheral release of proinflammatory mediators such as substance P, glutamate, and CGRP.[122,123] Activation of the peripheral pathway via meningeal nociceptors may involve a variety of receptors including TRP channels, P2X3 receptors that are sensitive to ATP, dopaminergic receptors (D1 and D2), and serotonergic 5HT1b/1d receptors.[123]

BoNT-A inhibits the release of substance P from cultured dorsal root ganglion neurons.[124] and the stimulated but not basal release of CGRP from cultured trigeminal ganglia neurons.[125] Moreover, in preclinical studies, BoNT-A reduces mechanical pain in peripheral trigeminovascular neurons in a manner consistent with inhibition or reduction of surface expression of mechano-sensitive ion channels.[123,126] Thus, OnaBTX-A may exert its prophylactic effects in chronic migraine through a dual mechanism that includes inhibition of SNARE-mediated vesicular release of inflammatory neurochemicals and peptides from the peripheral terminals of nociceptive primary afferent neurons, in addition to inhibition/downregulation of relevant peripheral nerve receptors and ion channels in a pathologic state.

For the treatment of chronic migraine, OnaBTX-A is injected into the craniofacial-cervical region as a prophylactic therapy. Beneficial effects are observed by week 4, and injection may be repeated every 12 weeks.[127] The Phase 3 data demonstrated the safety and efficacy of repeated OnaBTX-A injections for up to 56 weeks[128] and medical records of patients receiving OnaBTX-A for up to 9 treatment cycles (~2 years) demonstrated extended efficacy in a real-world setting via reduced headache days.

Overall, at least three lines of evidence indicate that BoNT-A regulates neurotransmitter release and receptor levels in pathological or stimulated states but not in the normal or basal states in conditions with a sensory component: (1) BoNT-A inhibits stimulated but not basal CGRP release from trigeminal cells, of potential relevance to migraine;[125] (2) BoNT-A normalizes the alterations in urothelial ATP and NO release induced by chronic spinal cord injury;[107] and (3) in the pathologic state of neurogenic detrusor overactivity, BoNT-A normalizes the concentration of TRPV1 and P2X3 in the bladder.[105] The latter observation (3) is consistent with the proposed dual regulation of surface expression/insertion of TRPs through the constitutive pathway, in which TRP channels reach the plasma membrane via exocytosis from the trans-Golgi or early endosomes, and the regulated vesicular pathway, in which receptors are transported as cargo in the lipid bilayer of neurotransmitter or neuropeptide vesicles that dock and fuse with the membrane in a SNARE-dependent process.[103] In this model, BoNT-A inhibits the SNARE-regulated mechanism of receptor insertion but not the constitutive expression of these sensory receptors.

Lack of Retrograde Transport at Relevant Preclinical Doses

Historically, a major distinction between tetanus toxin and BoNT has been that the former undergoes retrograde transport and transcytosis across neurons to exert effects in the central nervous system, whereas the latter does not.[129] It is notable that clinical tetanus results in a spastic paralysis and botulinum toxin results in peripheral muscle relaxation. However, over the past decade, several groups have reported the retrograde transport of BoNT-A under experimental conditions that appear to contradict this distinction.[130–132]

Studies reporting retrograde transport and transcytosis used high locally administered doses of BoNT, in marked contrast to the comparatively low doses used clinically. For example, the study by Antonucci and colleagues used a high dose laboratory preparation of BoNT-A injected into a single site of the rat whisker pad (135 pg),[130] which is approximately 450 pg/kg. By way of comparison, patients treated with OnaBTX-A for facial indications typically receive approximately 20 units (or 3 pg/kg) administered into multiple muscles, which, per kilogram, is approximately 150-fold lower than the dose used by the Antonucci and colleagues.[133]

Dose response studies by Dolly and colleagues help clarify the retrograde transport conversation. Using a model of compartmented cultures of rat sympathetic neurons, these investigators applied picomolar (pM) concentrations of BoNT-A to neurites and measured transport to cell bodies as percent total SNAP-25 in the cleaved form.[134] Results showed that BoNT-A acted locally except at high doses; for example, addition of 10 pM BoNT-A to neurites led to approximately one-third of total SNAP-25 cleaved in the neurites but virtually no cleaved SNAP-25 in the cell body compartment. The authors note that this amount of BoNT-A is equivalent to 75 mouse LD_{50} units and exceeds the maximum recommended clinical dose of 50 units per injection site for BoNT-A complex by 50%. At doses of 10^4 pM, which are 1000 times higher than the ~10 pM doses used clinically, BoNT-A applied to distal neurites did induce SNAP-25 cleavage in the central compartment, (indicating some retrograde transmission), but did not block synaptic transmission at cell bodies and therefore had no functional effect. No transcytosis was observed in these studies.

A recent study provided additional insights using a highly selective antibody for the BoNT-A-cleaved substrate ($SNAP25_{197}$) combined with 3-dimensional imaging.[135] In this study, $SNAP25_{197}$ was confined to motor neurons following injection of a low dose into the rat hindlimb; at a higher saturating dose, sporadic staining was observed in distal muscles and associated spinal cord regions, consistent with systemic spread of toxin, but was confined to the motor neuron and there was no evidence for transcytosis.

DIFFERENCES BETWEEN BoNT PRODUCTS

The clinical pharmacology of BoNTs is influenced by the bacterial strain, methods of isolation and purification, serotype, formulation, and procedures used to determine biological activity (see Reference 66 for review). These factors vary for each commercially available BoNT product and can affect its clinical profile.

Units of Biological Activity

Differences in unit potency and the noninterchangeability of units among BoNT products result from differences in the assays used to determine biological activity of bulk drug substance. Each manufacturer uses a unique, product-specific method and reference standard for testing.

Biological assays involving animals are sensitive to variations in animal strain, age, sex, diet, temperature, caging, season, and even the liquid used to dilute the product.[136] Notably, manufacturers of the main BoNT-A products use different diluents for LD_{50} unit testing: Allergan uses saline (the diluent also used for clinical reconstitution),[137] and Ipsen uses gelatin phosphate buffer.[138] Merz adds human serum albumin (HSA) as a stabilizer to their undisclosed diluent,[139] and stabilizers have been shown to enhance the activity of BoNT-A products at low concentrations in preclinical tests.[140]

Difference in LD_{50} assays mean that units are not interchangeable even for products labeled as containing the same number of units per vial. In a comparison of two BoNT-A products, both labeled at 100 units, one of the products (incobotulinumtoxinA) was found to contain substantially fewer units per vial when compared against an Allergan reference standard for OnaBTX-A.[137,141] When these two BoNT-A products were compared in the Merz LD_{50} assay, in which the products are diluted with a solution containing added HSA as a stabilizer and compared against the Merz reference standard, the potency was comparable.[139] These findings confirm that the potencies of the two BoNT-A products are differentially affected by the diluent and stabilizers, indicating that assay conditions markedly influence potency measurements reflecting underlying product differences.

Historically, the mouse-defined LD_{50} has been the global standard for BoNT-A potency testing used by all manufacturers, but the trend is toward less animal use in biological assays. Allergan has implemented a cell-based potency assay optimized for OnaBTX-A that meets the stringent approval requirements of global regulatory agencies for replacement of an animal LD_{50} test.[142] This rigorous, cross-validated assay does not change OnaBTX-A product or potency, and significantly reduces the use of animals for testing.

Current Regulatory Approvals of BoNT-A Products

Most regulatory agencies worldwide require that manufacturers meet strict guidelines governing the manufacture and clinical development of pharmaceutical products. These guidelines promote quality, purity, consistent biological activity, and lack of contamination. An official product approval for a specific disease or condition (i.e., "indication") is granted only after rigorous clinical trials demonstrate efficacy and safety. These studies provide important efficacy, safety, dosing, and injection site information specific to the individual product. The licensed indications for BoNT products differ based on whether the manufacturers have conducted the necessary studies as required by the regulatory agencies (Table 2.2). Approved indications for each product vary by country; practitioners should consult local labeling materials for details.

Table 2.2 Approved Indications[a] for the Main BoNT Products Available in the United States (US) and European Union (EU)[b]

Indication[a]	OnaBTX-A	AboBTX-A	IncoBTX-A
Therapeutic			
Strabismus	US	–	–
Blepharospasm	US, EU	EU	US, EU
Hemifacial spasm	EU	EU	–
Cervical dystonia	US, EU	US, EU	US, EU
Primary axillary hyperhidrosis	US, EU	–	–
Focal upper-limb spasticity	US, EU	US, EU	EU
Focal lower-limb spasticity	US, EU	EU	–
Juvenile cerebral palsy (dynamic equinus foot deformity)	EU	US, EU	–
Chronic migraine	US, EU	–	–
Neurogenic detrusor overactivity	US, EU	–	–
Overactive bladder	US, EU	–	–
Aesthetic			
Glabellar lines	US, EU	US, EU	US, EU
Crow's feet lines	US, EU	–	EU
Forehead lines	US	–	EU

[a]Approved indications, precise indication wording, and associated limitations vary from country to country. Consult local labeling for details.
[b]Majority of EU5 countries (France, Germany, Italy, Spain, United Kingdom).

Unlicensed Products

The noninterchangeability of BoNTs has become even more prominent with the unscrupulous use of counterfeit and unlicensed products. One study evaluated a BoNT-A product CNBTX-A (Nanfeng) that was previously available in China but was not approved there or in any other country.[143] The label on each vial indicated 55 units, however, the product was not accompanied by a package insert or dosing recommendations. Testing against an Allergan reference standard showed that a vial of CNBTX-A contained 243 units of biological activity.[143] Serious consequences could have resulted if clinicians had obtained this nonapproved product and applied it to patients based on doses of an approved product. In another instance, a highly concentrated laboratory preparation of BoNT, labeled for laboratory use only, was illegally administered to four individuals in a Florida clinic for cosmetic purposes.[144] All of the individuals exposed to this laboratory preparation experienced progressive muscle weakness and were hospitalized.[144]

The dangers of using unlicensed BoNT preparations are unambiguous: clinicians risk patient safety and incur professional liability.[145,146] It is critical that clinicians verify the BoNT product they are using and use it at doses recommended by the manufacturer and documented in the published clinical literature.

IMMUNOLOGY

Under certain circumstances (e.g., dose and frequency), BoNTs can elicit immune responses that neutralize the protein's activity. Only antibodies directed against the 150-kDa neurotoxin are neutralizing.[147] Antibodies may occasionally be formed against the nontoxin proteins in the BoNT complex, but these do not appear to affect clinical responsiveness.[147] Others have argued that the NAPs may serve as immune adjuvants,[148] but the low rates of neutralizing antibody formation with OnaBTX-A and AboBTX-A[149–151] suggest that such hypothetical effects are not established.

Within the BoNT-A molecule, antibodies directed against certain peptides within amino acid residues 449–1296 of the heavy chain are neutralizing.[152] Nearly all of the regions overlap or coincide with the regions on the protein that bind to synaptosomes *in vitro*.[152] Similar results have been found for BoNT-B.[153] The pattern of antibody recognition varies among patients with neutralizing antibodies, such that not all patients develop antibodies to the same portion of the BoNT molecule,[152] underscoring the potential role of individual genetic factors in neutralizing antibody development.[154]

In recent clinical studies, the rates of neutralizing antibody formation are low for the main three BoNT-A products:[155] 0% with OnaBTX-A (observed at study conclusion) in glabellar lines and 1.2% in cervical dystonia,[149,150] 0% with AboBTX-A in glabellar lines and less than 3% in cervical dystonia[151] and 1.1% with IncoBTX-A in their overall development program.[156,157] Clinical trials have not directly compared neutralizing antibody rates between different BoNT products, but the aforementioned numbers suggest that studies would not find meaningful differences. Moreover, some patients with neutralizing antibodies continue to respond to BoNT injections.[158]

SUMMARY AND CONCLUSIONS

BoNTs continue to stimulate both basic and clinical research. In the past few years, advances in understanding BoNT binding and internalization mechanisms have been reported, with increasingly detailed information on protein domains and their interactions with protein and lipid components at the plasma membrane. Mechanisms of action beyond the inhibition of acetylcholine release from neurons are also an active area of research, as evidenced by the effects of OnaBTX-A on afferent/sensory mechanisms that are consistent with the treatment of chronic migraine and lower urinary tract disorders.

Clinical studies have evaluated BoNT-A for many dermal conditions beyond hyperhidrosis and hyperfunctional facial lines in aesthetics. Initial reports from these small studies indicate that BoNT-A may reduce dermal sebum production/secretion and scar formation and improve the appearance of keloids. As research and development of BoNTs advance, it seems likely that additional applications will be identified for these important, but noninterchangeable, therapeutic proteins.

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3 Pharmacology and immunology of non-complexed botulinum toxin

Juergen Frevert

INTRODUCTION

Botulinum toxin (BoNT) for therapeutic use was pioneered by Alan Scott. His first experiments to treat strabismus were carried out with the botulinum complex of *Clostridium botulinum* type A,[1] which was prepared by Edward Schantz and Eric Johnson, and at that time was known as crystalline botulinum toxin.[2] The production process starts with the fermentation of *C. botulinum* under anaerobic conditions using a complex medium consisting of several ingredients, including peptides and sugars. This produces a complex of several proteins, one of which is the botulinum toxin, the active substance of all BoNT formulations. Besides serotype A, six further distinct serotypes are known: B, C1, D, E, F, and G.[3] Only serotypes A and B have been developed for human use. Further numerous subtypes exist,[4] for example, for serotype A subtypes A1–A8, and all together, more than 40 subtypes have been described to date. The currently marketed products for aesthetic medicine are all of serotype A1. The subtype of the type B product that is approved only for neurological indications has not been described in the literature.

In 1989, the product developed by Scott was approved for the treatment of strabismus, hemifacial spasm, and blepharospasm. Several other toxins are now licensed in different countries for various indications. A major breakthrough in aesthetic medicine came when Jean and Alastair Carruthers discovered that botulinum toxin could be used for the treatment of wrinkles.[4]

Currently, three products are approved for aesthetic use in Western markets, and all are approved by the FDA: onabotulinumtoxin A (OnaBTX-A; BOTOX®/Vistabel®, Vistabex Allergan Inc., Irvine, CA), abobotulinumtoxin A (AboBTX-A; Dysport®/Azzalure®, Ipsen, Paris, France), and incobotulinumtoxin A (IncoBTX-A; Xeomin®/Bocouture®, Merz Pharmaceuticals GmbH, Frankfurt, Germany).[5–10] There are also several BoNT products originating and approved in Asian countries: in Korea Neuronox® (Medytox Inc.), Nabota® (Daewoong, Inc), and Botulax® (Hugel), and from China BTXA™ or Lantox® (Lanzhou Institute). They are all similar to OnaBTX-A and claim to be based on the 900 kD BoNT complex, but some are formulated with different excipients. They will not be further discussed in this chapter because they contain complexing proteins. In contrast to OnaBTX-A and AboBTX-A and all other botulinum products, IncoBTX-A is the only approved botulinum product free from complexing proteins containing only the pure 150 kD botulinum toxin (here: BoNT), the protein which is responsible for the therapeutic effect. Another non-complexed BoNT known as Purtox® was under development, but has been discontinued for unknown reasons. The company Revance Inc. has stopped developing a purified botulinum toxin product (daxibotulinumtoxinA) as a topical agent but is developing an injectable product (RT002). The botulinum toxin is formulated with peptides that are supposed to bind to the botulinum toxin. It is not known whether this formulation influences the immunogenic potential and pharmacological characteristics of the botulinum toxin.

In the last couple of years, BoNT-A injections have become the most popular cosmetic procedures, especially among dermatologists and plastic surgeons.[11] IncoBTX-A differs in its content of bacterial proteins and in its formulation, which could have an effect on therapy. For optimal use, it is desirable that physicians are aware of the properties of products with complexing proteins and those with pure neurotoxin only. This chapter will describe the similarities and differences between the BoNT products with special regard to IncoBTX-A and examine whether the complexing proteins have any function in BoNT therapy.

MECHANISM OF ACTION OF BoNT

The molecular composition and mechanism of action of BoNTs are described in excellent reviews and are only briefly summarized here.[3,12] The active moiety in all BoNT products is the botulinum toxin, a 1296 amino acid protein with a relatively high molecular weight of 150 kD.[3,12–14] For comparison, insulin, a small protein, has a molecular weight of 5.8 kD.

BoNT is synthesized as a single chain protein, which is nicked into two subunits by a clostridial protease, resulting in two subunits: a heavy chain and a light chain, linked by a disulfide bridge. The C-terminal domain of the heavy chain binds the molecule highly specifically to receptor molecules on the presynaptic membrane of cholinergic neurons. The heavy chain has two binding domains, one for special glycolipids (GT1b) and one for a protein receptor called SV2.[15] The receptor-bound BoNT is taken up into the nerve cell by endocytosis. The second domain of the heavy chain then facilitates the translocation of the light chain into the cytosol, the interior of the neuron. The light chain is a highly specific protease which cleaves a protein, SNAP25, required for the secretion of acetylcholine. Cleaved SNAP25 can no longer function in the secretory process. As a result, the acetylcholine-containing secretory vesicle cannot fuse with the presynaptic membrane, acetylcholine is not secreted, and so the muscle cell is no longer activated and becomes paralyzed.[16] By this mechanism, BoNT blocks cholinergic muscular innervation of striated and smooth muscles as well as the innervation of exocrine glands. The mode of action is identical for all BoNT products (Figure 3.1).

MANUFACTURE OF BoNT PRODUCTS

This is divided into two steps: the manufacture of the drug substance or active pharmaceutical ingredient (API), a highly concentrated solution with the botulinum toxin, and the manufacture of the final drug or drug product, which requires a high dilution, addition of excipients, filling into vials, and a final drying process.

The manufacture of the active pharmaceutical ingredient of all products starts with the fermentation of the anaerobic spore forming *C. botulinum* type A.[2] The details of the fermentation process and of the purification procedure are proprietary and not known in detail. All products use a so-called Hall strain, originally isolated by the microbiologist Ivan Clifford Hall. However, as he kept several *C. botulinum* type A strains, it is not known which is the actual Hall strain. IncoBTX-A is produced with the strain ATCC 3502, which is a defined strain distributed and controlled by the American Type Culture Collection (ATCC). The production strain for OnaBTX-A is called "Hall hyper," which is claimed to produce a higher toxin concentration and not to form spores,[17] although this might be due to fermentation conditions. The identity of the Hall strain used for AboBTX-A has not been published and it is only named "a Hall strain."[18] In any event, the amino acid sequence of the neurotoxin in all products appears to be identical. Of course, one cannot exclude the possibility that the applied strain influences the quality of the product by producing different proteins and therefore a different purity profile, which might also influence

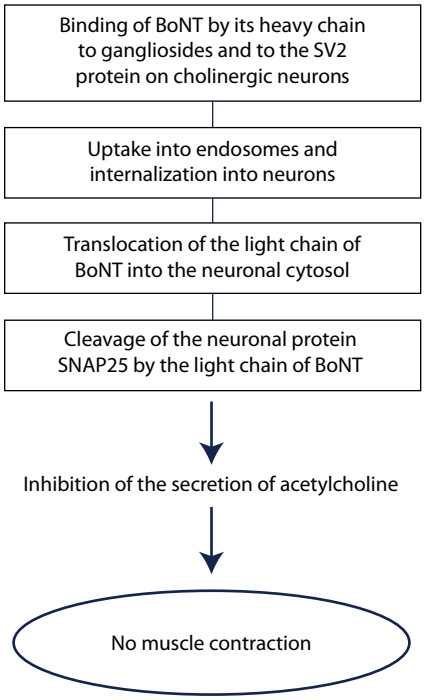

Figure 3.1 Mode of action of BoNT.

the folding of the BoNT molecule and have an effect on its immunological properties (epitope structure).

After fermentation, the biomass is precipitated and the neurotoxin extracted. OnaBTX-A is further purified by precipitation steps (ethanol precipitation) and finally by precipitation with ammonium sulfate, which provides the so-called "crystalline complex" with a molecular weight of about 900 kD.[19,20] Instead of precipitation ("crystallization") steps, the manufacture of AboBTX-A uses chromatography and dialysis,[18] resulting in a drug substance containing complexing proteins accompanied by partly degraded complexing proteins and some impurities, that is, flagellin and a clp protease.[18] The proportion of the different complexing proteins is not consistent with any complex described in the literature. It might be a mixture of complexes (300 and 500 kD), but the complex composition has never been published. For IncoBTX-A, the complexing proteins and other impurities are removed from the neurotoxin in a series of chromatographic steps to end up with the pure neurotoxin.[21] The manufacturing process providing the pure neurotoxin is illustrated in Figure 3.2.

To prepare the final drug product, excipients are added to the diluted drug substance. All products contain human serum albumin (HSA), but in different amounts (Table 3.1). HSA is required to stabilize the tiny amount of drug substance (picogram to nanogram quantities). The molecular effect of HSA is not really understood. It was initially thought that it would block the adsorption of the botulinum toxin to the walls of the vial or other surfaces, but this has never been demonstrated. The addition of sodium chloride during the OnaBTX-A drying process destabilizes the BoNT; it has been shown that sodium chloride causes a loss of activity.[22] If a proportion of the botulinum toxin is inactivated during the drying step, this might be the reason why OnaBTX-A contains a higher amount of botulinum toxin protein,[23] that is, about 50% more or 150 U must be processed to end up with 100 U in the final product. OnaBTX-A is vacuum dried, which means that the solution is not frozen, but only cooled and a low vacuum applied to prepare a thin film on the bottom of the vial. AboBTX-A and IncoBTX-A are produced by freeze drying (lyophilization) providing a loose "cake."

COMPLEXES AND COMPLEXING PROTEINS

OnaBTX-A and AboBTX-A contain the 150 kD BoNT as well as other proteins, known as complexing proteins or neurotoxin-associated proteins (NAPs). It is claimed that these proteins form a complex with the botulinum toxin, which can influence their pharmaceutical properties.[24] The complex agglutinates red blood cells—an activity

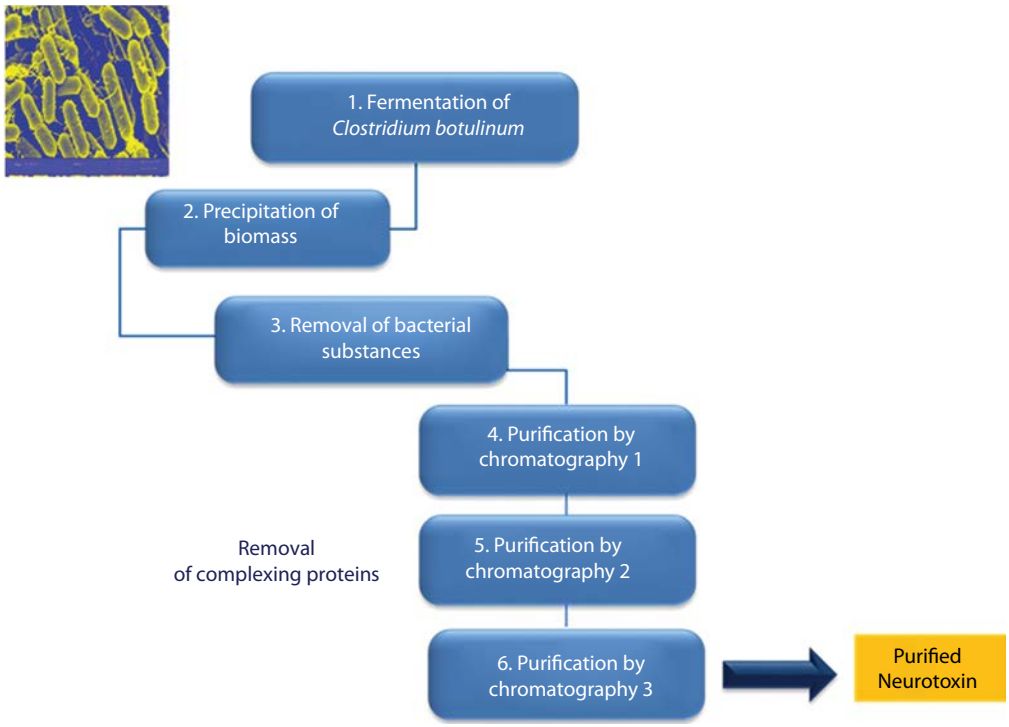

Figure 3.2 Flow diagram of the manufacturing of the purified BoNT.

Table 3.1 Comparison of BoNT-A Formulations

Botulinum toxin type A	AboBTX-A	OnaBTX-A	IncoBTX-A
Brand names	Dysport®, Azzalure®	BOTOX®, Vistabel®	Xeomin®, Bocouture®
Approved aesthetic indication	Moderate to severe glabellar lines	Moderate to severe glabellar lines and crow's feet	Moderate to severe glabellar lines and crow's feet
Presentation	Freeze-dried (lyophilized) powder for reconstitution	Vacuum-dried powder for reconstitution	Freeze-dried (lyophilized) powder for reconstitution
Isolation process	Precipitation and chromatography	Precipitation	Precipitation and chromatography
Composition	*Clostridium botulinum* type A neurotoxin HA and non-HA proteins	*Clostridium botulinum toxin* type A HA and non-HA proteins	*Clostridium botulinum* type A neurotoxin
Excipients	500 U vial[a]: 125 μg human serum albumin 2.5 mg lactose	100 U vial[a]: 0.5 mg human serum albumin 0.9 mg NaCl	100 U vial[a]: 1 mg human serum albumin 4.6 mg sucrose
Molecular weight (neurotoxin), kD	Not published (150)	900 (150)	150
Approximate total clostridial protein content‡	4.35 ng (500 U)	5.0 ng (100 U)	0.44 ng (100 U)
Neurotoxin protein load (neurotoxin per 100 U[a])	0.65 ng	0.73 ng	0.44 ng
Specific neurotoxin potency	154 U/ng	137 U/ng	227 U/ng
Shelf-life	2°C–8°C 2 years	2°C–8°C 2–3 years[b] (or freezer)	Room temperature 3–4 years[b]
Storage (post-reconstitution)	2°C–8°C 4 hours	2°C–8°C 24 hours	2°C–8°C 24 hours

[a] Units of measurement for the three commercially available BoNT-A preparations are proprietary to each manufacturer and are not interchangeable.
[b] Depending on the number of units per vial. HA, hemagglutinin.

certainly not necessary for BoNT therapy—and some of these different molecular weight proteins are therefore called hemagglutinins: HA50, HA34, HA20, and HA17 (slightly different names exist in the literature, e.g., HA34 is also named HA33) In addition, a protein known as non-toxic non-hemagglutinating protein (NTNH) is the direct binding protein for BoNT in the complex.[25] Together, these proteins form a complex under acid conditions (around pH = 5) with the 150 kD neurotoxin.[26] The integration of BoNT into a complex is required for its action as a food poison: the BoNT complex is protected against the hostile conditions of the gastrointestinal tract (low pH, protease attack).[26] The hemagglutinins may also play an important role in the absorption of BoNT from the gastrointestinal tract. They are sugar-binding proteins (lectins), and can bind to E-cadherin and allow BoNT to pass through the mucosa of the intestine and be transported into the blood or lymph.[27,28]

The BoNT progenitor complexes isolated from *C. botulinum* type A cultures adopt three sizes: 900, 500, and 300 kD.[19] It is claimed that the complex size for OnaBTX-A is 900 kD[20] (Table 3.1). The complexes present in AboBTX-A have not been published, but data have shown that complexing proteins are present as both full-length proteins and as a succession of fragments.[18] As most of the NTNH is truncated in AboBTX-A, one can infer that there is little or no 500 kD and no 900 kD complex, and that the 300 kD complex is probably the most abundant.

To determine the identity of the complexes in a vial, the reconstituted products were analyzed by an ultracentrifugation technique, which allows the separation of proteins and complexes of different sizes.[29] According to these data, the botulinum toxin dissociates immediately after reconstitution from the complex in OnaBTX-A, with ≥85% of BoNT present as the 150 kD free form prior to injection into target tissues[29] (Figure 3.3). Data for AboBTX-A show the botulinum toxin completely dissociated from the complexing proteins prior to injection.[29] It can be concluded that molecular weight or protein complex size do not affect biological activity and pharmacological properties, as the BoNT-A botulinum toxin rapidly dissociates from the complexing proteins after reconstitution of the preparation.[29]

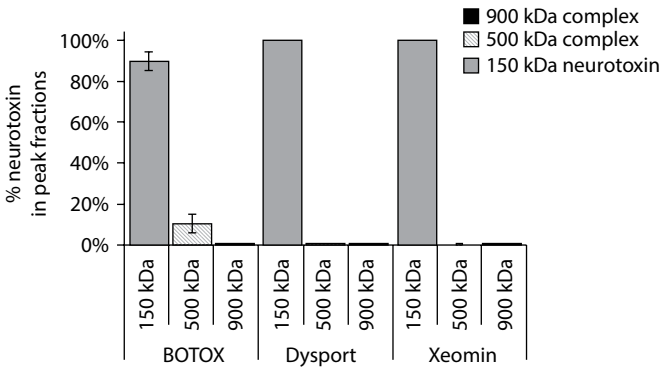

Figure 3.3 Presence of botulinum toxin in complexes after reconstitution of vials.[29] Vials were reconstituted with saline and the complex size determined by sedimentation velocity analysis followed by immunoassay analysis of the botulinum toxin and complexing proteins. (Reproduced from *Toxicon*, 57, Eisele KH et al., Studies on the dissociation of botulinum neurotoxin type A complexes, 555–65, Copyright 2011, with permission from Elsevier.)

BENEFICIAL ROLE OF COMPLEXING PROTEINS?

In the early days of BoNT therapy, it was claimed that it was unlikely that the pure botulinum toxin would ever be used in a clinical setting because pure botulinum toxins "are inactivated on dilution, formulation, and drying."[2] This has certainly been refuted since IncoBTX-A, the botulinum toxin free from complexing proteins, was licensed in Germany. Indeed, IncoBTX-A is the most stable of the BoNT products.

Although complexing proteins do not play a role in the mechanism of action, it was argued that they influence the diffusion or spread of the botulinum toxin out of the injected muscle into other adjacent muscles not intended for treatment.[24] Due to their high specificity for cholinergic neurons (motor neurons and certain neurons that activate glands, e.g., sweat gland, salivary gland), all treatment-related adverse events of BoNT therapy are related to migration of the botulinum toxin in the muscle tissue.

Discussions on botulinum toxin spread and diffusion are hampered by inconsistent use of terminology.[30] Spread occurs when the injected

molecule travels from the original injection site, which is determined by the injection technique, volume of injection, needle size, and by the size of the traveling molecule. In contrast, the physical term diffusion indicates the passive movement of botulinum toxin along a concentration gradient within a fluid.[30] According to Fick's law, the diffusion of molecules is proportional to their molecular mass: a molecule with a higher molecular weight migrates slower than one with a lower molecular weight. This suggests that the complex with the high molecular weight of 900 kD would have a reduced tendency to leave the muscle compared with the markedly smaller non-complexed botulinum toxin, and one would expect a lower rate of off-target effects. However, this has never been demonstrated; the adverse event profile in all head-to-head studies with OnaBTX-A and IncoBTX-A is very similar.[31–33]

Recent studies, which have compared the spread of BoNT-A products by measuring the size of anhidrotic halos following injection of identical volumes and equipotent doses into the forehead of patients, reveal a similar spread, suggesting that there are no differences in migration properties.[34,35] A comparison of OnaBTX-A and AboBTX-A, using dose ratios of 1:2.5, 1:3, and 1:4, showed that the area of anhidrosis was larger with AboBTX-A in 93% of comparisons at all dose ratios and identical injection volumes.[36] A separate study, which used a dose ratio of 1:2.5, observed no significant difference between the mean size of halos produced by the two products.[37] There were no differences in product spread when the same dose was injected with the same technique.[34]

The reason why the complexing proteins do not affect the migration of botulinum toxin in the tissue is very simple: the botulinum toxin is already dissociated from the complexing proteins when it is injected into patients.[29] Even if the complex was still intact, it would immediately dissociate when injected into the muscle because it would not be stable at the tissue pH of 7.3.[26] Similar migration properties have also been demonstrated in a clinical study by intramuscular injection of equivalent doses in the same volume of OnaBTX-A and IncoBTX-A (5 U) or AboBTX-A (12.5 U) into two sites of the forehead of volunteers (split face).[35] After 6 weeks and again after 6 months, the area of anhidrosis was made visible with iodine starch stain (Figure 3.4) and analyzed. The area of anhidrosis was similar for OnaBTX-A and IncoBTX-A, indicating that the complexing proteins do not influence spread of the toxin.[35] The area of anhidrosis for AboBTX-A was larger, but this might have been due to the applied dose ratio. These results were confirmed in a preclinical study in mice, in which spread was visualized by analyzing the expression of a protein (N-CAM). This protein is only detectable in paralyzed muscle and showed no difference between the products.[38] It can be concluded that, in all products, the botulinum toxin migrates unhindered, and that the tendency of the botulinum toxin to leave the injected muscle is the same.

Based on the observation that being part of a complex protects the botulinum toxin against the harsh conditions in the environment, it was hypothesized that the complexing proteins were required to ensure the stability of the BoNT product during storage. This would mean that IncoBTX-A should have a shorter storage stability or more restricted storage conditions than the other products. This has proved not to be the case. Whereas IncoBTX-A has a shelf-life of 3 or 4 years at room temperature, AboBTX-A has a shelf-life of 2 years at 2°C–8°C, and OnaBTX-A can be stored for 2 or 3 years at 2°C–8°C (depending on the number of units) or in the freezer. After reconstitution, IncoBTX-A and OnaBTX-A are stable for 24 hours at 2°C–8°C, and AboBTX-A is stable for 4 hours at 2°C–8°C.[5–10] A recent study, which compared the efficacy of freshly reconstituted IncoBTX-A with IncoBTX-A that had been reconstituted and stored for 1 week at 25°C, has provided further confirmation of the stability of IncoBTX-A.[39] In a split-face design, 10 U of the two formulations were injected into the crow's feet of 21 subjects. Over 4 months of follow-up, there was

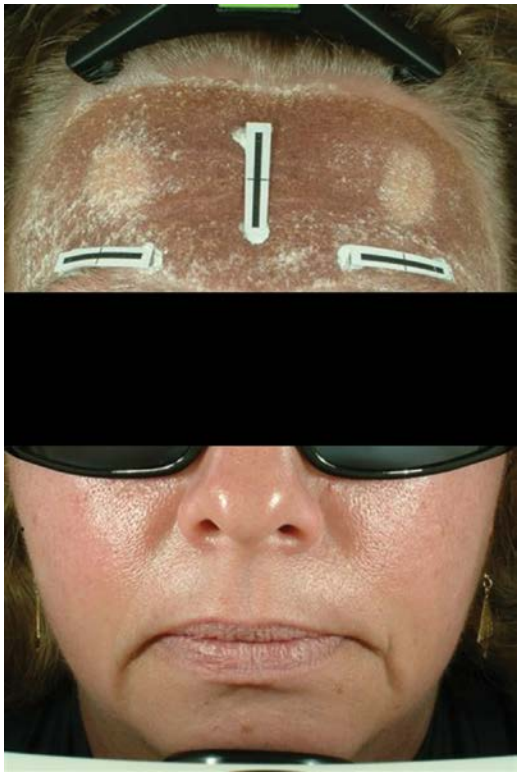

Figure 3.4 Determination of the spread of complexed versus non-complexed BoNT products in a split-face study.[35] 5 U of OnaBTX-A (left side) or 5 U of IncoBTX-A were injected intramuscularly into the forehead of volunteers. After 6 weeks, the anhidrotic halo was made visible with iodine starch stain. (With kind permission from Springer Science+Business Media: *Arch Dermatol Res*, Comparison of the spread of three botulinum toxin type A preparations, 304, 2012, 155–61, Kerscher M et al.)

no statistically significant difference in either efficacy or longevity between the fresh and stored products. The prolonged shelf-life and less stringent temperature restrictions displayed by IncoBTX-A (Table 3.1) and (Figure 3.5) suggest that complexing proteins are not required for BoNT-A stability.[39] It has also been demonstrated that storage of IncoBTX-A at 60°C for 4 weeks does not cause inactivation.[40]

POTENCY AND CLINICAL EFFICACY

The potency of BoNT products is measured in the LD50 assay and given in units. One unit is defined as the dose capable of killing 50% of mice in comparison to a standard preparation of BoNT, which is

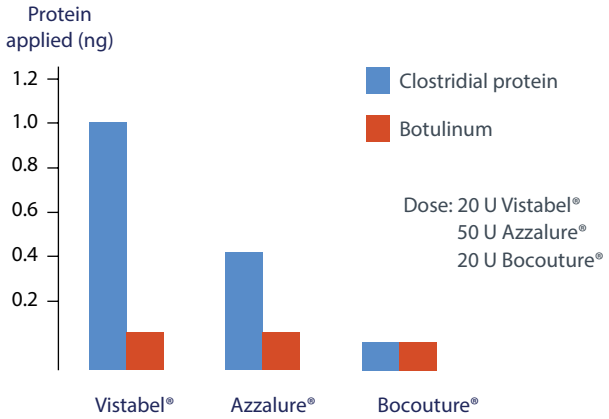

Figure 3.5 Amount of clostridial and botulinum toxin protein (ng) in the treatment of glabellar lines. Dose: 20 U Vistabel®, 20 U Bocouture®, 50 U Azzalure®.

also analyzed in every assay (parallel line assay). The dose for treating patients is related to the LD50 units and therefore an accurate LD50 assay is required. The assays used by the companies differ in various aspects, including dilution procedure, diluents, and stabilizing agents: HSA (IncoBTX-A), gelatin (AboBTX-A), or no stabilizing agent (OnaBTX-A).[41] As the calculation of units depends on the methods that each manufacturer uses in non-standardized assays,[42] a comparison of potency based solely on the units is problematic. This underlines the importance of clinical head-to-head studies to evaluate treatment effects. Interestingly, the potency assay for IncoBTX-A using HSA in the diluent and simulating conditions in the clinic has shown a 1:1 ratio between IncoBTX-A and OnaBTX-A.[43] The LD50 is now being replaced by cell-based assays, which must be cross-validated with the LD50 assay. The manufacturer of OnaBTX-A uses a sensitive neuronal cell line (SiMa cells)[44] approved in different countries, whereas the manufacturer of IncoBTX-A has recently obtained FDA approval for an assay based on differentiated induced pluripotent stem cells.[45] Both procedures quantitate the amount of cleaved SNAP25. The assays are extensively validated before they can replace the animal assay. It would be interesting to analyze the BoNT products with both assays.

The respective amounts of botulinum toxin per 100 U, measured using a high sensitivity ELISA technique, were 0.73 ng for OnaBTX-A, 0.65 ng for AboBTX-A, and 0.44 ng for IncoBTX-A (Table 3.1).[23,46] The specific botulinum toxin potency or biological activity (U) per mass of botulinum toxin protein was calculated based on the overall mean concentration of BoNT-A neurotoxin, giving IncoBTX-A the highest specific biological activity (U/ng botulinum toxin) at 227 U/ng compared with 137 U/ng for OnaBTX-A and 154 U/ng for AboBTX-A.[23] IncoBTX-A contains no other clostridial proteins and, therefore, the specific biologic potency relative to the total clostridial protein is 227 U/ng. As the reported clostridial protein content per 100 U of OnaBTX-A is 5 ng[47] and of AboBTX-A is 4.35 ng, the equivalent specific biologic potency relative to the total clostridial protein load for OnaBTX-A is 20 U/ng and for AboBTX-A is 115 U/ng. The units of AboBTX-A are different from those of OnaBTX-A and IncoBTX-A. However, comparing OnaBTX-A and IncoBTX-A, which have demonstrated similar clinical activity, the findings suggest that 0.44 ng of IncoBTX-A has the same biological activity as 0.73 ng of OnaBTX-A. It is hypothesized that part of the botulinum toxin in OnaBTX-A may be inactived or denatured due to the vacuum drying process used in the manufacture of the final drug in the presence of sodium chloride.[23,48] Figure 3.6 shows the amount of clostridial protein and botulinum toxin protein injected into a patient treated for glabellar lines with 20 U OnaBTX-A or IncoBTX-A, or 50 U of AboBTX-A. Patients treated with products containing complexing proteins are loaded with a markedly higher amount of bacterial protein; for OnaBTX-A the amount is about 10-fold higher.

The complexing proteins do not influence the mode of action of the botulinum toxin. Only the botulinum toxin binds unhindered and independently of any other components to gangliosides (GT1b) and the protein receptor (SV2) of cholinergic neurons and is then taken up by endosomes, followed by translocation of the light chain into the cytosol of the nerve cell. No step in the mode of action requires the presence of other proteins. Although all products contain the botulinum toxin as the active substance, it has been debated whether the biological activity of the products is comparable. Several clinical head-to-head studies in different aesthetic indications (glabellar frown lines, crow's feet) have demonstrated comparable clinical efficacy of IncoBTX-A compared with OnaBTX-A, suggesting a 1:1 conversion ratio between the products (Figure 3.7).[33,49–52] These studies also showed that there was no difference in side effect profile. Comparable efficacy was confirmed in a recent split-face, cross-over study with the same dosage of OnaBTX-A and IncoBTX-A in the treatment of crow's feet (Figure 3.8).[50] Furthermore, the duration of effect was not different in a study comparing OnaBTX-A, IncoBTX-A, and AboBTX-A in the treatment of glabellar frown lines.[53] Several evidence-based consensus reviews on BoNT-A application in aesthetic indications have recapped the evidence confirming a 1:1 conversion ratio between OnaBTX-A and IncoBTX-A.[54–57,58]

A conversion ratio between AboBTX-A and OnaBTX-A or IncoBTX-A is still debated and has not been finally established.[59] A recent consensus review suggests that a conversion ratio of 1:2.5 (IncoBTX-A:AboBTX-A) may be assumed in aesthetic indications.[58] A consensus review from Asia suggests a ratio of 1:2–1:4 (OnaBTX-A:AboBTX-A).[57] RimabotulinumtoxinB (RimaBTX-B), which is based on the botulinum toxin type B complex, is not approved for aesthetic indications and a conversion ratio IncoBTX-A:RimaBTX-B has not been published.

IMMUNOLOGICAL PROPERTIES

BoNT is a bacterial protein and is therefore foreign to the human immune system and an antigen per se. Like any other therapeutic protein product administered repeatedly, BoNT products can elicit the formation of antibodies directed against the botulinum toxin and/or the complexing proteins in the case of OnaBTX-A and AboBTX-A. The immune system might therefore produce antibodies against the foreign

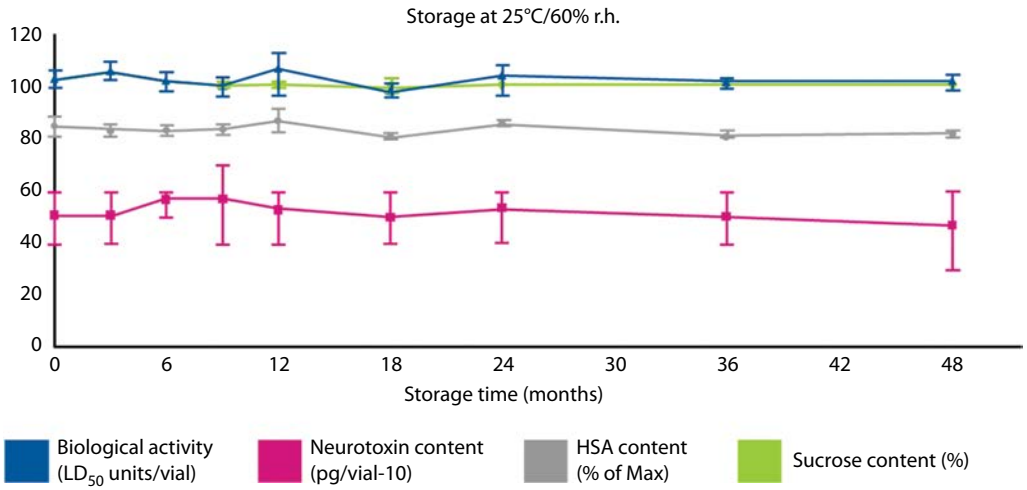

Figure 3.6 Stability of IncoBTX-A at 25°C.[40]

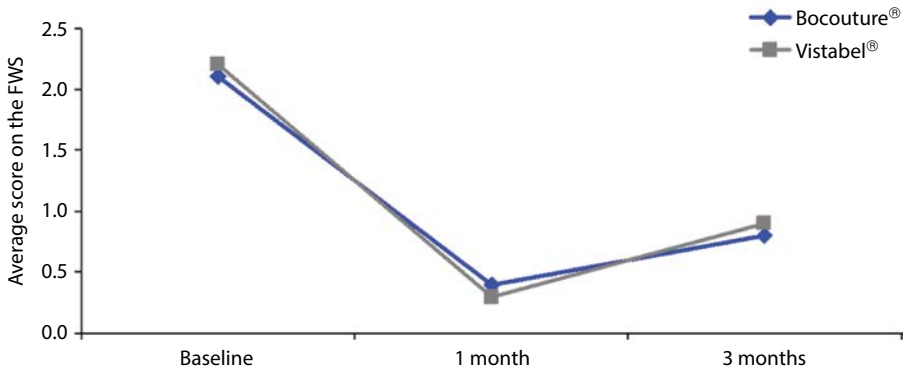

Figure 3.7 Head-to-head noninferiority study of IncoBTX-A (Bocouture®) versus OnaBTX-A (Vistabel®) in the treatment of glabellar frown lines.[33] The outcome of the injection was assessed by an independent rater after 4 and 12 weeks.

protein that will inhibit the therapy when the antibody titer is high enough, leading to a secondary non-response. It is clear that antibodies directed against the binding domain of the botulinum toxin heavy chain will inhibit the binding of the botulinum toxin to the neuron.[60,61] Antibodies directed against the enzymatic domain (light chain) can also neutralize the botulinum toxin's activity because of steric hindrance.[62]

Apart from patient-related factors (sensitivity of the patient's immune system), several product-related factors influence the immunogenicity of biological proteins (see Table 3.2). For BoNT products, these include the manufacturing process, the antigenic protein load, and the presence of complexing proteins as well as treatment-related factors, for example, the interval between injections, booster injections, and prior exposure. The first generation of OnaBTX-A applied in neurological indications contained 10 times more potentially antigenic protein (50 ng of clostridial protein) than the current formulation, which generated a high rate of antibody formation and secondary non-responders.[63] Physicians were advised to keep the dosing interval as long as acceptable for the patient to prevent the formation of antibodies.[63] The amount of botulinum toxin protein in OnaBTX-A has since been markedly reduced to 5 ng clostridial protein (see Table 3.1) and the rate of antibody formation has consequently also decreased.[64] The development of neutralizing antibodies is more common in therapeutic indications because of high doses of the antigen. It had been claimed that antibody production and secondary non-response was negligible in aesthetic indications because of the low doses applied. However, more and more reports about antibody formation in aesthetic indications are appearing in the literature.[65–69] There might also be a high number of unreported cases, as patients treated for aesthetic indications can change physicians or stop treatment when the therapy is not working. Physicians in the aesthetic field are also not as aware of secondary non-response as physicians in the therapeutic field.

Complexing proteins do not play a role in the mechanism of action of BoNT, and so antibodies directed against the complexing proteins cannot block the activity of BoNT. It has been reported that about 50% of patients (treated for a therapeutic indication) develop antibodies against the complexing proteins, but that this has no clinical relevance and is not linked to responsiveness.[70] From this standpoint, complexing proteins would be just inert proteins with no effect on BoNT therapy. However, new data suggest that this might not be the case. A growing body of evidence shows that complexing proteins might interact with the host immune system and therefore be clinically relevant.[71]

In contrast to OnaBTX-A and AboBTX-A, IncoBTX-A does not lead to the formation of antibodies in New Zealand white rabbits after repeated injection of high doses of the product and short treatment intervals.[72] While this study does not reflect the clinical application, it demonstrates that there are clear differences in the antigenic response related to the presence or absence of complexing proteins.

To initiate an immune response, the immune system must be activated. Not only the antigen must be present, but also an activating signal.[73] The first cells to recognize the antigen (i.e., BoNT) are dendritic cells. These present the antigen to T-lymphocytes, which are then activated by the dendritic cells. The activated T-lymphocytes

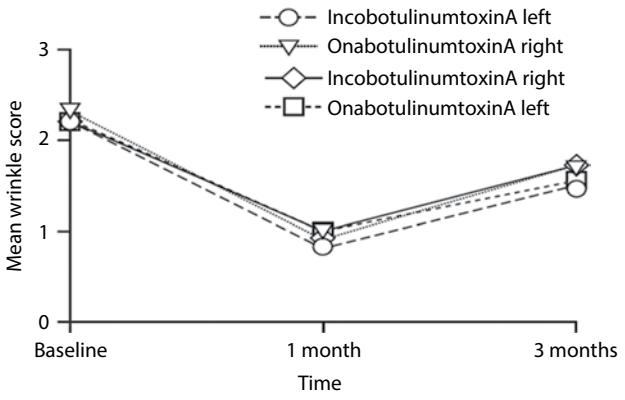

Figure 3.8 Head-to-head clinical trial of IncoBTX-A versus OnaBTX-A in the treatment of crow's feet.[50] A prospective, split face, subject- and rater-blinded, crossover evaluation in two consecutive treatment cycles with 12 U of IncoBTX-A or OnaBTX-A and a 6-month "wash out" between cycles. The graph illustrates pooled results from cycles 1 and 2, showing the mean score for crow's feet severity over time for both IncoBTX-A and OnaBTX-A on both sides of the face at maximum contraction. (Reproduced from Muti G, Harrington L. *Dermatol Surg* 2015; 41(Suppl 1): S39–46, with permission from Wolters Kluwer.)

Table 3.2 Factors Influencing Immunogenic Response According to CHMP Guideline EMEA/CHMP/BMWP/14327/2006 (2008)

Factors that may influence the development of an immune response against a therapeutic protein
Patient and disease related factors
Genetic factors modulating the immune response
Genetic factors related to a gene defect
Age
Disease-related factors
Concomitant treatment
Duration, route of administration, treatment modalities
Previous exposure to similar or related proteins
Product-related factors of immunogenicity
Protein structure
Formulation
Aggregation and adduct formation
Impurities

subsequently activate B-lymphocytes to produce antibodies.[74] Dendritic cells have exposed pattern recognition receptors (Toll-like receptors), which react with different bacterial components, such as bacterial DNA, parts of the bacterial cell wall, and bacterial proteins such as flagellin.[74] Hemagglutinins are known to act as adjuvants, binding and activating dendritic cells.[75,76] It is known that hemagglutinin HA33 is the major immunoreactive protein in the BoNT complex.[69]

The first step of the binding to immune cells has been demonstrated by analyzing the interaction of BoNT, the BoNT complex, and BoNT free from complexing proteins with lymphoblasts, fibroblasts, and a human neuroblastoma cell line (as a control).[71] It was clearly shown that the complexing proteins and the BoNT complex reacted with the lymphoblasts, but not the pure BoNT.[71] Further, the release of inflammatory cytokines was not influenced by pure BoNT, but by BoNT complex and the complexing proteins.[71] It can be concluded that complexing proteins can affect the formation of antibodies against BoNT by stimulating cells of the immune system.[71]

The presence of antibodies in patients does not necessary lead to a secondary non-response, and it is not clear which titer is required to inhibit therapy. The variability in the reported rate of neutralizing antibodies and treatment failure can be attributed to study design, administered doses, indication, assay methodology, timing of serum sample testing, and treatment history.[77,78] Only antibodies that bind BoNT effectively so that its biological activity is sufficiently neutralized, will attenuate its effect on the neuromuscular junction. Thus, the formation of antibodies may have no effect on treatment, or may result in partial or complete clinical unresponsiveness to BoNT-A.[79,80] However, further injections might act as a booster and increase the titer leading to subsequent secondary non-response. This might become relevant given that patients are starting their aesthetic treatments at increasingly younger ages and for several single indications, resulting in an increased overall dose of botulinum toxin per treatment and a high frequency of use over a lifetime. For patients who have developed antibodies following aesthetic therapy, it could be disastrous if the patient later suffers from a stroke and cannot be treated for spasticity with botulinum toxin products.

Clinical studies and case reports in different indications show that a small proportion of patients develops neutralizing antibodies against BoNT after treatment with OnaBTX-A or AboBTX-A, with incidence rates ranging from 0.3% to 6%, which is dependent on the condition being treated and thus treatment dose.[77,81–88] In contrast, there have been no cases of antibody-induced therapy failure with IncoBTX-A in treatment-naïve patients. One case of antibody-induced therapy failure was reported in a patient with progressive hereditary juvenile onset generalized dystonia, whose immune system had already been sensitized by pretreatment with AboBTX-A for 15 years,[89] supporting the hypothesis of reduced immunogenicity with IncoBTX-A.[90] Furthermore, a prospective blinded study in 37 cervical dystonia patients previously treated with OnaBTX-A or AboBTX-A, who developed neutralizing antibodies and partial secondary non-responsiveness, reported that continuous treatment with IncoBTX-A with a high dose of 200 U every 3 months for 48 months, did not result in an increase in neutralizing antibody titer.[91] Despite a transient increase in 10 patients in the first 24 months, neutralizing antibodies in fact declined significantly below the initial titer in 84% of patients ($p < 0.001$), and 62% of patients became seronegative. The decline of the antibody titer was similar to the decline of the titer in a second group of patients who were not treated during that time period.[91] This demonstrates that the immune system did not recognize the neurotoxin molecule in IncoBTX-A as an antigen.

In addition to selecting a product with a low risk of immunogenicity, it is important to establish good practice to minimize the risk of neutralizing antibodies developing. Studies of BoNT-A formulations containing complexing proteins suggest that a higher dosing frequency, short treatment intervals, and greater number of injections may increase the likelihood of their development.[80,92–94]

CONCLUSIONS

BoNT therapies are biological products and their clinical pharmacology depends on many factors, including the bacterial strain used in production, methods of isolation and purification, and the presence or absence of complexing proteins. These factors vary for each commercially available BoNT product, exposing the patient to different proteins and to different quantities of molecules. The active moiety in all BoNT products is the botulinum toxin. The complexing proteins rapidly dissociate from the botulinum toxin on product reconstitution and do not play a role in any of the steps involved in blockade of neurotransmitter release. They are also not required for either the stability of the toxin complex or for limiting the spread of the botulinum toxin. IncoBTX-A is the only pure BoNT commercially available product, free from complexing proteins. It has the lowest amount of foreign protein of all available BoNT preparations, and contains only the purified botulinum toxin as the active substance. BoNT preparations with the lowest amount of proteins provide the best chance for long-term and repeated therapy by minimizing the potential of the patient to form neutralizing antibodies and the possibility of secondary treatment failure. Therapies with a biological product like BoNT are naturally subject to inherent variability. To ensure safe and effective dosing, each batch of BoNT-A must be tested for potency before it can be released onto the market and applied for human use. The potency assays for evaluating the biological activity of currently available BoNT therapies are different, and therefore the products can only be truly compared in clinical head-to-head trials. Results from these have shown that IncoBTX-A and OnaBTX-A are clinically equivalent in terms of efficacy and safety at a 1:1 conversion ratio, confirming findings observed in clinical practice.

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4 Topical botulinum toxin*

Richard G. Glogau

INTRODUCTION

Ten years after the first publication describing the use of botulinum neurotoxin type A (BoNT-A) for the treatment of glabellar lines,[1] BoNT-A was approved in the United States for the "temporary improvement in the appearance of moderate to severe glabellar lines associated with corrugator and/or procerus muscle activity."[2] This was the first cosmetic indication for a botulinum toxin that had previously been approved for therapeutic use only (cervical dystonia, strabismus, and blepharospasm). BoNT-A is now used widely in facial aesthetics not only for glabellar lines but also for many other dynamic facial lines including lateral canthal lines (crow's feet), radial lip lines, horizontal forehead lines, and marionette lines (down-turned corners of the mouth).[3] The target muscles for these areas are the lateral orbicularis oculi, orbicularis oris, frontalis, and depressor anguli oris, respectively.[3] The safety and effectiveness of using BoNT-A in these muscles has now been well established over many years.

At the time of writing, three BoNT-As are available in the United States and Canada (onabotulinumtoxinA [OnaBTX-A], abobotulinumtoxinA [AboBTX-A], and incobotulinumtoxinA [IncoBTX-A]) while injectable daxibotulinumtoxinA [DaxiBTX-A] is in clinical development. All of these agents are administered by injection and therefore have the potential to cause needle anxiety and injection site reactions such as erythema, bruising, discomfort, tenderness, pain, and infection.[4] In an effort to avoid these potential issues, attempts have been made to develop formulations that are suitable for topical delivery.

CURRENT TRANSEPIDERMAL DELIVERY MECHANISMS

Most transepidermal drug delivery systems that have been developed to date are inefficient and can only transport small molecules such as nicotine, progesterone, and scopolamine. As a result, many macromolecules (including insulin, antibodies, and growth hormone) still need to be administered by injection.

In keeping with the skin's primary function—to exclude chemical assaults from the external environment—the stratum corneum and upper layers of the epidermis are lipid-rich barriers that block the entry of most large molecules and so the flux of most proteins across the skin barrier is essentially zero. The stratum corneum and upper layers of the epidermis are essentially a multilayered arrangement of the mature and differentiated horny cells of the epidermis that are interwoven with a lipid matrix that itself has a lamellar structure. Passage through the stratum corneum is less likely to be successful for highly ionic and/or aqueous molecules than lipophilic molecules, and it is also less efficient for larger molecules than smaller molecules. Furthermore, the process is heavily influenced by time and by the concentration of the relevant molecule.

Most attempts to enhance transepidermal delivery by manipulating drug structure have been rudimentary from a biochemical standpoint—because, for example, conjugating a drug to a carrier can compromise its activity and permeation enhancers may disrupt protein linkages and tertiary structures vital to the biological activity of a protein. Iontophoresis has also been explored as an alternative mechanism for drug delivery. Utilizing a direct current of relatively low amplitude, iontophoresis involves placing an active electrode in the drug formulation. The ionic charge imparted to the target molecule allows the drug to be driven into the skin as indifferent ions are pulled from the skin by the indifferent electrode to complete the circuit. However, few molecules are amenable to being delivered by iontophoresis, especially lipophilic molecules. Although it has been reported to be successful with botulinum toxin,[5,6] iontophoretic delivery lacks targeting and delivery specificity, is often painful, and is heavily influenced by time and by drug concentration.

A NOVEL TRANSEPIDERMAL DELIVERY SYSTEM FOR BOTULINUM TOXIN

A novel transepidermal drug delivery system has been developed that may allow BoNT-A to be available commercially as a topical formulation. The investigational product DaxiBTX-A topical gel (RT001, Revance Therapeutics, Inc., Newark, California) consists of a 150-kDa highly purified BoNT-A and a proprietary carrier peptide that binds to BoNT-A electrostatically and then enables it to be delivered transcutaneously. Topical delivery of BoNT-A in this way may be popular with patients because it avoids the need for injections.

The development of the proprietary peptide in DaxiBTX-A topical gel stemmed from the study of a human immunodeficiency virus (HIV) gene called "TAT" (the "transactivator of transcription" gene) that was originally characterized in 1988.[7,8] TAT has within it a protein transduction domain that is capable of penetrating cell membranes and is functionally responsible for the propagation of the viral genome. It causes accelerated production of the HIV double-stranded RNA by binding to cellular factors, controlling their phosphorylation, and resulting in increased transcription of all the HIV genes.

The peptide in DaxiBTX-A topical gel is novel in that it combines a cationic poly-Lysine core with the residues of the TAT gene domain on each end, thus enabling noncovalent binding to the toxin. The peptide backbone (a sequence of consecutive lysines) binds to BoNT-A electrostatically, with the positive charge of the peptide attracted to the relative negative charge of the 150-kDa BoNT-A (Figures 4.1a and b).

The toxin forms a complex with the peptides, with the protein transduction domains directed outward where they are free to attach to cell surfaces. The peptide-covered toxin is absorbed through cell membranes, crosses the cytoplasm to the cell membrane on the other side, and passes out and into the next cell. This is an active energy transport system and is not specific to botulinum toxin—it is a variant of induced macropinocytosis where the cell takes a "drink" of the surrounding media and conveys it out to the other side without harming the cell or the cell membrane.

Once the complex has traversed the cell, it moves through the next cell, and the next, until it exits the epidermis on the dermal side. At this point, the toxin is released from the carrier peptide and is free to exert its usual action on the SNAP-25 protein, producing the cholinergic blockade that is characteristic of BoNT-A. This action appears identical to the action of injected BoNT-A in every way except that the total dose delivered varies depending on the concentration of the toxin, the concentration of the peptide, and how long the complex is in contact with the skin.

* Adapted from Topical botulinum toxin, in *Botulinum Toxins: Cosmetic and Clinical Applications* (ed. Joel Cohen, MD), Wiley-Blackwell, Oxford UK, June 2017.

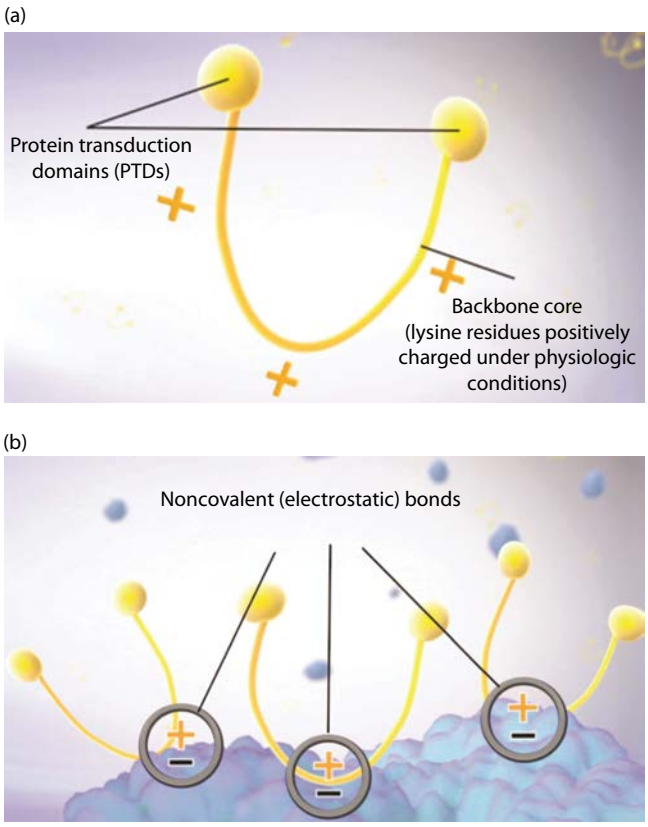

Figure 4.1 (a) Schematic representation of the proprietary peptide with the backbone of lysine residues and TAT domains that will noncovalently bond with the botulinum toxin. (b) The botulinum toxin is negatively charged at physiological pH. The backbone of the peptide then binds noncovalently to the toxin. The protein transduction domains are then projecting outward, available for binding to the cell wall. (With kind permission from Springer Science+Business Media: *Cell-Penetrating Peptides: Methods and Protocols. Methods in Molecular Biology*, Nonclinical and clinical experiences with CPP-based self-assembling peptide systems in topical drug development, 683, 2011, 553–72, Waugh JM et al., Humana Press.)

STUDIES EVALUATING TOPICAL DELIVERY OF BOTULINUM TOXIN

Animal studies first demonstrated the concept that BoNT-A could be transported through the skin and inhibit the contraction of a target muscle if it is applied in the presence of an appropriate peptide carrier[9]. This was evaluated using the digit abduction score assay,[10] which uses a startle reflex of the mouse. When a mouse is lifted up by its tail, its normal startle reflex is to extend its hind limbs and splay its toes apart. However, if the muscle contraction is first inhibited by BoNT-A, such movement is inhibited. Topical application of a peptide-botulinum complex to one leg produced almost complete inhibition of the reflex, compared with no inhibition in the other leg which received topical BoNT-A only without the carrier peptide.

The first reported evidence that topical application of a peptide-botulinum complex is effective in humans came from a randomized, blinded, vehicle-controlled study in patients with primary axillary hyperhidrosis.[11] Four weeks after a single topical application, the peptide-botulinum complex showed a significantly greater inhibition of sweating than vehicle (assessed gravimetrically and by Minor's starch-iodine test).

DaxiBTX-A topical gel was subsequently evaluated in a randomized, double-blind, parallel-group phase 2 study in subjects with moderate to severe primary axillary hyperhidrosis who produced at least 50 mg sweat/5 minutes.[12] The results of this study showed that a single application of DaxiBTX-A topical gel (25 or 50 ng) achieved a clinically meaningful reduction in sweat production—a mean of 214 and 166 mg/5 minutes with 25 and 50 ng, respectively, versus 66 mg/5 minutes with placebo.[13] Although the study was not powered to achieve statistical significance, the reduction in sweat was significantly greater in the higher dose group than the placebo group ($p = 0.003$). An additional important clinical finding was that, even though noninvasive treatments do not generally provide sufficient efficacy to treat severe hyperhidrosis, subjects with profound hyperhidrosis at baseline experienced an excellent reduction in sweating. Adverse events were generally mild, localized, and transient, with the most common treatment-related adverse events being erythema or pain at the application site and folliculitis. Photographic documentation of the effect of topical DaxiBTX-A is shown in Figure 4.2.

DaxiBTX-A topical gel has been studied most extensively in the treatment of lateral canthal lines. Topical delivery of BoNT-A would be highly desirable in this area given the thinness of the skin and the close relationship of the orbicularis oculi (the target muscle) to the skin's surface. Five dose-escalation studies have been performed evaluating the effects of DaxiBTX-A topical gel in the treatment of lateral canthal lines. As the dose of DaxiBTX-A increased, so did the proportion of lateral canthal areas attaining at least a 2-point improvement on the Investigator Global Assessment of Lateral Canthal Line severity scale (IGA-LCL)—8%, 18%, 26%, 34%, and 56% at concentrations of 3.3, 5.5, 11, 22, and 25 ng/mL, respectively.[14] This 5-point scale (of absent, minimal, mild, moderate, and severe) has been shown to be a reliable, appropriate, and clinically meaningful means of assessing lateral canthal line severity.[15] Photographic documentation of the efficacy of DaxiBTX-A topical gel is shown in Figure 4.3.

The escalating doses of DaxiBTX-A did not result in a dose-dependent increase in the severity or frequency of adverse events. Treatment-emergent adverse events were generally mild and transient and none of the studies revealed any safety signals of clinical relevance. Cranial nerve and ECG assessments showed no significant treatment- or dose-related findings and there were no treatment-related increases in antibody titers to the neurotoxin or the carrier peptide relative to predose serum samples.

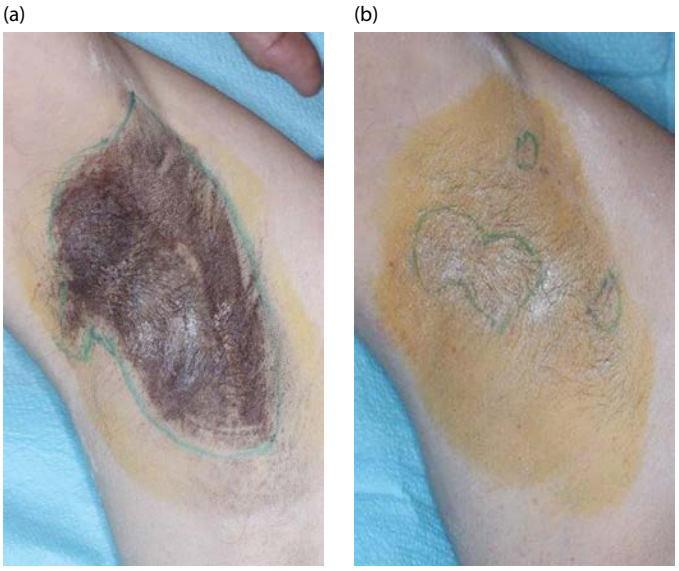

Figure 4.2 Result of Minor's starch-iodine test in a patient with axillary hyperhidrosis. (a) Baseline, (b) 4 weeks after topical application of 50 ng/mL of the peptide-botulinum complex to the axilla. (Reproduced with permission from Revance Therapeutics, Inc., Newark, California.)

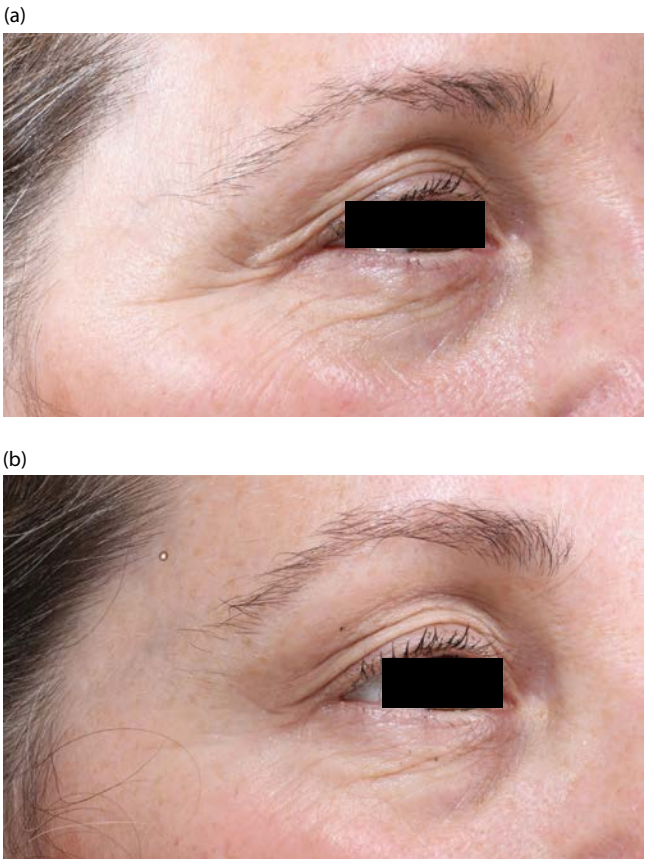

Figure 4.3 Representative appearance of lateral canthal lines treated with a single topical application of the peptide-botulinum complex that was left on the skin for 30 minutes. (a) Baseline, (b) 4 weeks post-treatment. (Reproduced with permission of Revance Therapeutics, Inc., Newark, California.)

A double-blind, placebo-controlled study involving 90 subjects with bilateral moderate or severe lateral canthal lines at rest confirmed the efficacy and tolerability of a single 25 ng dose of DaxiBTX-A (the dose subsequently evaluated in phase 3 studies).[16] A 30-minute topical application of DaxiBTX-A resulted in significantly greater efficacy than placebo for the primary efficacy endpoint (at least a 2-point improvement in both investigator and patient ratings of lateral canthal line severity in both lateral canthal areas at rest)—at week 4, 44% of subjects in the DaxiBTX-A group had achieved this endpoint compared with 0% in the placebo group ($p < 0.001$). DaxiBTX-A topical gel also achieved a significant efficacy advantage for each of five secondary endpoints (a 1- or 2-point improvement in IGA-LCL score in both lateral canthal areas, a 1- or 2-point improvement in patient rating of lateral canthal line severity, and a marked improvement on a patient global impression of change assessment). For example, the proportion of subjects with both lateral canthal areas showing at least a 1-point improvement in IGA-LCL score with DaxiBTX-A topical gel or placebo was 89% versus 28% ($p < 0.001$). For 2-point improvements, the proportions were 58% versus 14% ($p < 0.001$), respectively. A 1-point improvement in severity score was considered clinically relevant and a 2-point improvement was considered a marked improvement. DaxiBTX-A topical gel was found to be well tolerated, with no clinically meaningful or significant differences in safety outcomes observed between DaxiBTX-A topical gel and placebo.

Another double-blind, placebo-controlled study involved a repeat application of DaxiBTX-A topical gel (administered at baseline and again at 4 weeks).[17] At 8 weeks, the proportion of lateral canthal areas showing at least a 1-point improvement from baseline in IGA-LCL severity was 95% versus 15% after treatment with DaxiBTX-A topical gel and placebo, respectively ($p < 0.001$) (Figure 4.4). The corresponding proportions showing at least a 2-point improvement were 50% versus 0% with DaxiBTX-A topical gel and placebo, respectively ($p < 0.001$), (Figure 4.5). No treatment-related adverse events were reported. Photographic documentation illustrates the benefit of repeat dosing, with continued

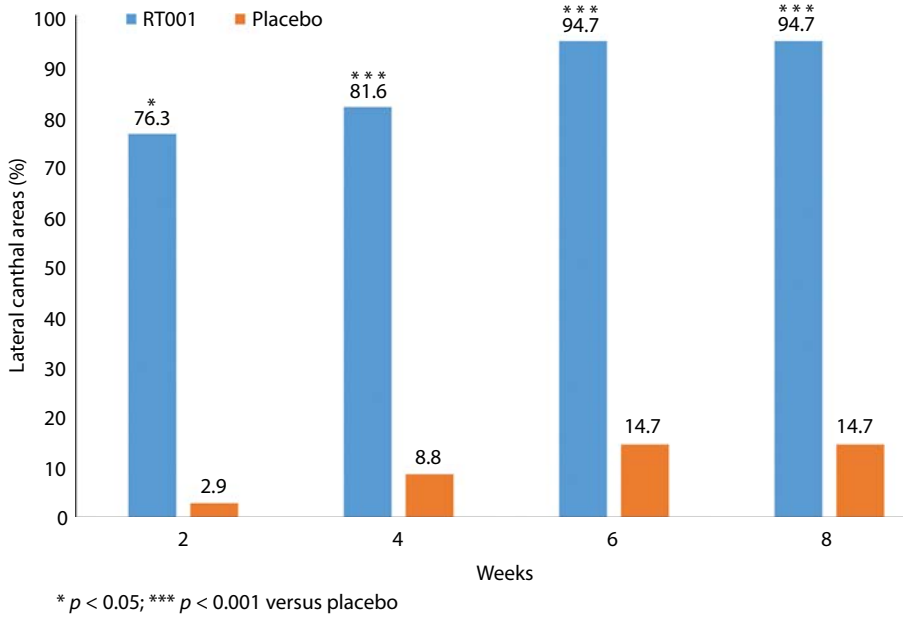

Figure 4.4 Compared with placebo, DaxiBTX-A topical gel resulted in a significantly greater proportion of lateral canthal areas showing at least a 1-point improvement in score on the Investigator's Global Assessment of Lateral Canthal Lines at Rest Severity Scale. Treatment was administered at baseline and repeated at week 4. (From Glogau R et al. *J Drugs Dermatol* 2012; 11: 38–45.)

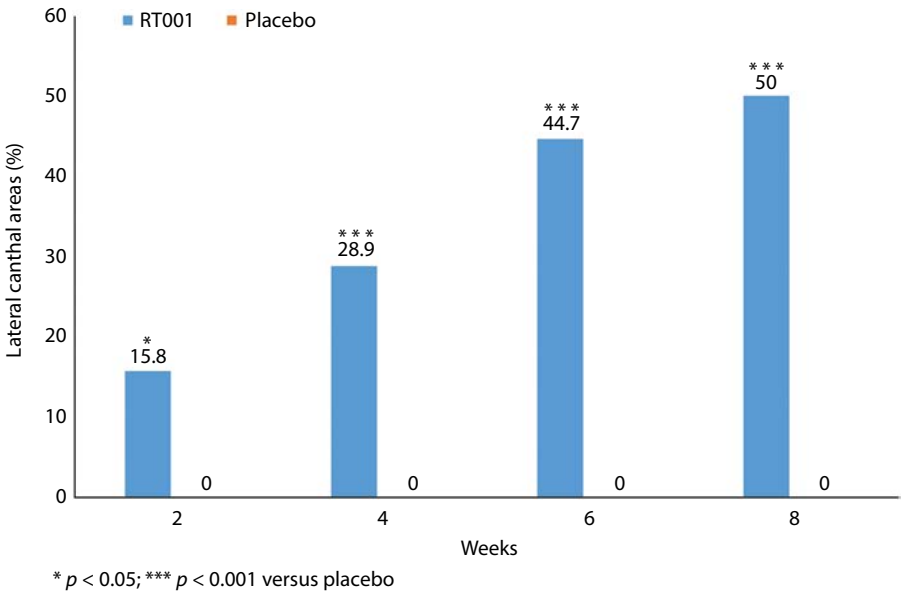

Figure 4.5 Compared with placebo, DaxiBTX-A topical gel resulted in a significantly greater proportion of lateral canthal areas showing at least a 2-point improvement in score on the Investigator's Global Assessment of Lateral Canthal Lines at Rest Severity Scale. Treatment was administered at baseline and repeated at week 4. (From Glogau R et al. *J Drugs Dermatol* 2012; 11: 38–45.)

improvement in lateral canthal lines after the second dose of DaxiBTX-A topical gel (Figure 4.6).

DaxiBTX-A topical gel has also been evaluated in a phase 3 study known as REALISE-1 (clinicaltrials.gov identifier NCT02580370).[18] In this randomized, multicenter, double-blind, placebo-controlled study, 450 subjects with moderate to severe lateral canthal lines received a single treatment with DaxiBTX-A or placebo. The co-primary efficacy endpoints in the trial were composite measurements of at least a 2-point and at least a 1-point improvement in lateral canthal lines between baseline and 28 days after treatment (graded by investigators using the IGA-LCL scale and subjects using the Patient Severity Assessment [PSA]). Results were reported in June 2016. Topical DaxiBTX-A generally appeared to be well tolerated but the co-primary efficacy endpoints were not achieved.[19] As a result, the clinical development of DaxiBTX-A topical gel is not being pursued further at this time for the treatment of lateral canthal lines or axillary hyperhidrosis.

DaxiBTX-A topical gel has also been evaluated for the prevention of chronic migraine headache. In a study conducted in Singapore, patients were considered responders if they had ≥50% improvement versus placebo in at least two of the following parameters (mean scores on the Headache Impact Test [HIT-6™], number of total migraine attacks, and intensity of migraine attacks) plus numerical superiority for the third parameter. At week 4, the proportion of responders was 43.8% with DaxiBTX-A versus 10.5% with placebo ($p < 0.05$).[20] Improvements were evident in the number and severity of headaches and in evaluations of headache-specific quality of life. Adverse events were generally mild. One report of a severe headache was considered serious and possibly related to treatment but resolved without sequelae.

FUTURE DIRECTIONS

Topical delivery of botulinum toxin to the skin could offer opportunities not only to treat areas that are difficult to manage with injectables but also to treat patients who want to avoid injections. Topical BoNT-A could also prove useful as an adjunctive or extender therapy in conjunction with injectable BoNT-A. Some of the most attractive targets for topical delivery may be the upper lip, forehead, and neck for aesthetic improvements, and the hands, scalp, and axillae in patients with hyperhidrosis. However, phase 3 results with DaxiBTX-A topical gel in the treatment of lateral canthal lines have been disappointing and clinical development is not currently being pursued for lateral canthal lines, axillary hyperhidrosis, or migraine.

Figure 4.6 The clinical significance of this study is the demonstration of continued improvement with a second application of the topical peptide-toxin complex gel, observed over the 8-week study period. (a) Baseline, (b) 4 weeks after initial treatment, (c) 8 weeks after initial treatment (4 weeks after repeat treatment). (Reproduced with permission of Wolters Kluwer from: Brandt F et al. *Dermatol Surg* 2010; 36(Suppl 4): 2111–8.)