USING IN-VIVO SNARE PROTEIN ANALYSIS TO ADDRESS PROBLEMS

ASSOCIATED WITH BOTULINUM TOXIN THERAPY

by

DOROTHY DANIEL WHELCHEL

(Under the Direction of Julie A. Coffield)

ABSTRACT

Botulinum neurotoxin (BoNT) causes flaccid muscle paralysis at the neuromuscular junction (NMJ) by cleaving specific SNARE proteins in the presynaptic terminal. BoNT is well known as the agent responsible for causing botulism; however in the past two decades, the toxin has proven to be a valuable therapeutic tool in human medicine. BoNT injections have been used to successfully treat various neuromuscular disorders and currently, formulations of serotypes A and B are approved for specific uses. Although BoNT has proven to be an invaluable therapeutic agent, side effects of treatments include diffusion to undesired muscles, limited duration of treatment, and the development of resistance/tolerance with repeated exposure. In the current study, changes in SNARE protein chemistry following IM injections of BoNT/A (150kDa) were assessed to measure toxin diffusion and determine a temporal correlation with paralysis onset and recovery.

INDEX WORDS: Botulinum neurotoxin, neuromuscular junction, paralysis, SNAP-25, VAMP, sprouting

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DEDICATION

To my parents, for their love and support in my quest to become a professional student. Thanks Mom and Dad!

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

GENERAL BACKGROUND

Botulinum toxin is the agent responsible for botulism, a disease characterized by flaccid muscle paralysis resulting from the inhibition of neurotransmitter release at the neuromuscular junction, (NMJ). The toxin itself is produced under anaerobic conditions by namely, *Clostridium botulinum,* a rod-shaped, spore-forming bacterium (Cato et al., 1986); however, other species such as *Clostridium barati* and *Clostridium butyricum,* are also capable of producing the toxin (Simpson, 2000). Seven immunologically distinct serotypes (A-G) of the toxin exist; serotypes A, B, E, and rarely F are known to cause disease in humans, while the other serotypes (C and D) affect different species (Simpson, 1981; CDC, 1998). Botulism was first described in 1793 during an outbreak from the ingestion of spoiled sausage. In the 1800's Justinius Kerner concluded that the fatal paralysis was caused by an agent in spoiled home fermented sausage, and in 1897, Emile Pierre van Ermengem discovered that botulism was caused by the ingestion of food contaminated with a toxin produced by an anaerobic bacterium (Van Ermengem, 1897; Smith, 1977; CDC, 1998).

Although the natural occurrence of food borne botulism has diminished to approximately 24 cases per year in the United States, botulinum toxin (BoNT) remains a pertinent subject due to its potential use as a biological weapon, and its value to both scientific research and clinical medicine (CDC, 1998). Since the 1940's efforts to develop BoNT as a powerful weapon of mass destruction have been made by numerous countries (Smart, 1997). Presently Iraq and other nations classified as "state sponsors of terrorism" have biological weapons programs in place manufacturing weapons grade BoNT (Cordesman, 1998; US Dept of State, 2000; Bermudez, 2001). Despite the negative connotations associated with BoNT, the toxin has proven to be a powerful tool, enabling scientists to further understand the mechanism of exocytosis and

neurotransmitter release. In addition, BoNT has become a useful therapeutic agent for the treatment for numerous neurological disorders (Jankovic and Brin, 1997).

MECHANISM OF ACTION

Botulinum toxin is an 150kDa metalloprotease. It is composed of two chains, a heavy chain (100kDa) and a light chain (50kDa) joined by a disulfide bond (DasGupta and Sugiyama, 1972). Furthermore, the toxin is divided into three domains: the binding and translocation domains, which constitute the heavy chain, and the catalytic domain which contains the light chain (Lacy et al., 1998). Botulinum neurotoxin is naturally found complexed with hemagglutinins (HA) and other non-hemagglutinin, non-toxic proteins (NTNH). These accessory proteins are thought to protect the toxin from proteolytic enzymes in the gut following ingestion of toxin-contaminated food products. However, the HA and NTNH are not necessary for toxin absorption across the gut (Maksymowych et al., 1999). Once ingested, the toxin binds to a yet unidentified receptor and translocates across the intestinal wall of the small intestine into the general circulation. After entering the general circulation, BoNT travels to peripheral cholinergic nerve terminals, which are the toxin's primary site of action.

At the NMJ, botulinum toxin prevents the presynaptic release of acetylcholine (ACh) through a five-step mechanism, inducing flaccid muscle paralysis (Burgen et al., 1949; Simpson, 1980). First, the toxin binds to receptors located on the motor nerve terminal, stimulating receptor-mediated endocytosis of the toxin. It is currently thought that the toxin's binding domain interacts with two receptors: first in a non-specific, low affinity manner with tri-sialogangliosides located at the nerve terminal and second, in a high affinity and specific nature with a yet unknown protein receptor (Montecucco, 1986; Halpern and Neale, 1995). After endocytosis into the nerve terminal, the disulfide bond joining the light and heavy chains is reduced within the endosome. The acidic

endosomal environment (due to ATP/H^+ pump activity) stimulates a conformational change in the toxin, allowing the catalytic chain to escape into the cytosol. The catalytic chain is a metalloprotease that binds one zinc atom at the active site which is characterized by a HEXXH sequence. Once in the cytosol, BoNT cleaves specific SNARE proteins necessary for exocytosis and neurotransmitter release. Active sites between serotypes differ slightly, giving each serotype both substrate and cleavage site specificity (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000). Toxin serotypes A and E cleave SNAP-25; B, D, F, and G cleave VAMP (synaptobrevin), and C cleaves syntaxin (Schaivo et al., 1992, 1993a,b, 1994; Blasi et al., 1993a,b). Serotype C has also been shown to cleave SNAP-25 in in-vitro preparations (Williamson et al., 1996). Cleavage of these proteins prevents the formation of functional SNARE complexes, which are necessary for vesicular fusion and neurotransmitter release at the nerve terminal (Söllner et al., 1993). Thus, BoNT poisoning prevents ACh release at the NMJ, inducing flaccid muscle paralysis (Raciborska et al., 1998; Kalandakanond et al., 2001).

CLINICAL RELEVANCE

Botulinum neurotoxin has become a widely used therapeutic agent for various neuromuscular disorders. In 1973, Drs. Alan Scott and Edward Shantz collaborated to explore the use of BoNT/A to treat strabismus in non-human primates; these studies moved to human trials in 1981 (Jankovic and Brin, 1997; Schantz and Johnson, 1997). Finally, in 1989, the U.S. FDA approved the use of BoNT/A to treat strabismus, blepharospasm, and focal spasms (including dystonias) in patients older than 12 yrs of age. Currently, there are two formulations of serotype A available for clinical use world-wide: BOTOX™ (Allegran Inc., Irvine, CA) and Dysport™ (Speywood Pharmaceuticals, England). Botulinum toxin treatment has proven to be the most beneficial option for cervical dystonia (CD), having a success rate of over 80%, (Tsui, 1996; Alder et al.,

2000). More recently, serotype B (in the formulation of MYOBLOC™, Elan Pharmaceuticals) was approved by the FDA to treat CD; and in 2002, BOTOX was granted approval for cosmetic use to lessen facial wrinkles.

In addition to the FDA approved uses of botulinum toxin, the toxin is currently used in "off label" treatments of other disorders, and clinical trials investigating new uses are underway. For example, toxin use has been investigated for the treatment of spasticities resulting from post-stroke and post-spinal cord injuries, as well as those occurring in disorders such as multiple sclerosis and cerebral palsy (Tsui, 1996; Jankovic and Brin, 1997). Toxin injections have also shown promise in the treatment of bladder spasticity and gastrointestinal disorders, such as achalasia and anismus (Tsui, 1996; Jankovic and Brin, 1997). Furthermore, recent studies have investigated the use of botulinum toxin for migraine headaches and pain management. Although results in these latter areas are inconclusive, BoNT treatments have been shown to reduce pain in CD patients, reduce the severity of tension headaches, and show antinociceptive characteristics in children with cerebral palsy (Brin et al., 1987; Barwood et al., 2000; Göbel et al., 2001).

Although there are many promising uses for BoNT in clinical medicine, there are also problems associated with its use such as the need for repeat injections, diffusion into unwanted muscle groups, and the development of resistance to further treatment. Since toxin treatments results in a transient paralysis, the desired effects diminish within 3-6 months (BoNT/A), necessitating repeat injections. As a response to the paralysis induced by BoNT and possibly to the toxin itself, neuronal sprouts form in toxin-treated muscles, effectively re-innervating these muscles and promoting functional recovery. Although the mechanism of sprout formation remains largely unknown, it would prove beneficial to design toxin formulations and/or dosing regimes that reduce sprouting and thus prolong the desired paralysis in treated muscles.

Several studies have characterized sprout formation in the mouse levator auris longus muscle after treatment with both serotypes A and D (Angaut-Petit et al., 1990, 1998; Comella et al., 1993; Juzans et al., 1996). Furthermore, de Paiva et al. (1999) documented, in a very elegant manner, the functional role of sprout formation in the recovery of the mouse sternomastoid muscle from BoNT/A induced paralysis. In their study, this group labeled endo/exocytotic activity in nerve living endings with N-(3-triethyl ammonium propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43; Molecular Probes) to monitor sprout formation and measured muscle fiber contraction after BoNT/A treatment to define recovery. Sprouts appeared as early as 4 days post treatment; by day 28 these sprouts appeared to make functional synapses capable of generating muscle contraction. At later periods post BoNT/A treatment, these sprouts appeared to recede and normal function returned to the original endplate regions. In addition, de Paive et al. demonstrated through immunohistochemistry the presence of both SNAP-25 and VAMP in these newly formed sprouts.

Investigators have also proposed potential roles for the SNARE proteins in sprout formation. Shirasu et al. (2000) noted that overexpressing a GFP-SNAP-25 isoform A (found in both differentiated and undifferentiated cells) fusion protein in PC12 cells along with nerve growth factor (NGF) treatment stimulated an increase in neuronal sprouting. Furthermore this study also found that GFP-VAMP 2 over-expression in PC12 cells along with NGF treatment resulted in an increase in neurite length; however, GFP-Syntaxin 1A overexpression induced no significant changes in cell morphology. Similarly, Zhou et al. (2000) studied the effects of SNAP-25 isoform B (found in differentiated cells), VAMP 2, and Syntaxin 1A overexpression in PC12 cells treated with NGF. This group found that Syntaxin 1A inhibited neurite sprouting post NGF treatment, while SNAP-25B overexpression increased neurite elongation. Unlike Shirasu et al.'s findings, Zhou et al. found no significant changes after VAMP 2 over-expression in PC12

cells. Although findings from these two investigators are somewhat contradictory, evidence from both studies suggests a role for SNAP-25 in sprouting.

As sprouts form and treated muscles regain function, larger doses and repeated injections may be needed to achieve the desired clinical effects; however such treatments can further induce other detrimental effects associated with BoNT therapy. Large dosages of the toxin may increase diffusion from the injection site, leading to undesirable weakness in untreated muscle groups. Several studies have documented both local and systemic spread of the toxin in patients undergoing treatment. For instance, Eleopra et al. (1996) used electrophysiological measurements to verify that toxin diffused locally into adjacent, uninjected facial muscles in patients undergoing BoNT/A treatment for blepharspasm and hemispasm. Furthermore, Bhatia et al. (1999) noted three case studies in which patients showed signs of systemic spread of BoNT/A; one patient whom had previously been treated for CD exhibited generalized bilateral upper limb weakness, while another patient undergoing therapy for foot spasms complained of bilateral arm weakness and unilateral drooping of the eyelid. Similarly, leg muscle fiber atrophy was found in 7 out of 11 patients after treatments for CD (Ansved et al., 1997).

Large doses, frequent treatments, and perhaps introduction of the toxin into the systemic circulation can stimulate patients to develop resistance to further treatment, primarily through an immunological response to the toxin (Tsui, 1996). Zuber et al. (1993) noted that 3% of patients treated for focal dystonia exhibited neutralizing antibodies (ABT+) to BoNT/A; of this group, patients with CD had received at least 50ng of toxin and had shorter intervals between treatments, thus increasing their likelihood of developing immunoresistance. In a similar study by Jankovic et al. (1995), 23% of patients undergoing treatments for CD or oromandibular disorder were ABT+ and did not

respond to BoNT/A treatments. These patients received higher mean doses per treatment and higher cumulative doses compared to ABT- patients.

Because the different toxin serotypes are immunologically distinct, clinical uses of other serotypes show promise for patients who have developed resistance to BoNT/A. Recently, the FDA approved the use of BoNT/B (MYOBLOC™) for the treatment of CD, and physicians have successfully treated BoNT/A resistant patients with BoNT/B (Brin et al., 1999; Lew et al., 2000). Furthermore, clinical studies using serotypes C and F in humans are currently underway. In 1995, Jankovic et al. successfully treated 4 out of 5 patients who were ABT+ for BoNT/A with serotype F. Clinical studies by Eleopra et al. (1997) demonstrate that BoNT/C may also be a promising alternative.

Duration of treatment, diffusion, and immunoresistance are problems commonly associated with the therapeutic use of BoNT. The following studies will use an in-vivo mouse model to address the first two problems. Current formulations of BoNT/A used clinically are whole toxin preparations that also include the HA and NTNH accessory proteins; the role of these non-toxin proteins in the development of the problems discussed above is unknown. In the studies herein, a pure formulation of BoNT/A was used which consisted of the 150kDa toxin protein minus these accessory proteins. Small doses of toxin (25 pg) were injected intra-muscularly into the mouse lateral gastrocnemius muscle mimicking the clinical scenario. Mice were then monitored daily for clinical signs of paralysis and recovery, while SNARE protein chemistry was analyzed at select time points post injection to assess toxin diffusion and sprout formation.

REFERENCES

1. Adler CH, Kumar R. Pharmacological and surgical options for the treatment of cervical dystonia. Neurology 2000; 55(12 suppl 5):S9-S14.
2. Angaut-Petit D, Molgó J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 1990; 37:799-807.
3. Angaut-Petit D, Molgó J, Faille L, Juzans P, Takahashi, M. Incorporation of synaptotagmin II to the axolemma of botulinum type-A poisoned mouse motor endings during enhanced quantal acetylcholine release. Brain Res 1998; 797:357-360.
4. Ansved T, Odergren T, Borg K. Muscle fiber atrophy in leg muscles after botulinum toxin type A treatment of cervical dystonia. Neurology 1997; 28:1440-1466.
5. Barwood S, Baillieu C, Boyd R, Brereton K, Low J, Nattrass G, Graham HK. Analgesic effects of botulinum toxin A: A randomized, placebo-controlled clinical trail. Dev Med Child Neurol 2000; 42:116-121.
6. Bermudez JS. The Armed Forces of North Korea. London, England: IB Tauris; 2001.
7. Bhatia KP, Münchau A,Thompson PD, Houser M, Chauhan VS, Hutchinson M, Shapira AHV, Marsden CD. Generalized muscular weakness after botulinum toxin injections for dystonia: a report of three cases. J Neurol Neurosurg Psychiatry 1999; 67:90-93.
8. Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H, Jahn R. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature (Lond) 1993a; 365:160-163.

9. Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J 1993b; 12:4821-4828.
10. Brin MF, Fahn S, Moskowitz C, Friedman A, Shale HM, Greene PE, Blitzer A, List T, Lange D, Lovelace RE, McMahon D. Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm. Mov Disord 1987; 2:237-254.
11. Brin MF, Lew MF, Alder CH, Comella CL, Factor SA, Jankovic J, O'Brien C, Murray JJ, Wallace JD, Willmer-Hulme A, Koller M. Safety and efficacy of NeuroBloc (botulinum toxin type B) in type A-resistant cervical dystonia. Neurology 1999; 53:1431-1438.
12. Burgen ASV, Dickens F, Zatman LJ. The action of botulinum toxin on the neuromuscular junction. J Physiol 1949; 109:10-24.
13. Cato EP, George WL, Finegold SM. Genus *Clostridium*. In: Sneath PHA, Mair NS, Sharpe ME, et al. eds. Bergey's manual of systematic bacteriology. Balitmore: Williams & Wilkins; 1986. Vol. 2, p.1141-1200.
14. Center for Disease Control and Prevention National Center for Infectious Diseases Division of Bacterial and Mycotic Diseases. Botulism in the United States, 1899-1996, Handbook for epidemiologists, clinicians, and laboratory workers. CDC. 1998.
15. Comella JX, Molgó J, Faille L. Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D and the functional recovery of paralysed neuromuscular junctions. Neurosci Lett 1993; 153:61-64.
16. Cordesman AH. Weapons of mass destruction in the gulf and greater Middle East: Force trends, strategy, tactics and damage effects. Washington, DC: Center for Strategic and International Studies; Nov 9, 1998. p.18-52.

17. DasGupta BR, Sugiyama HA. A common subunit structure in Clostridium botulinum type A, B, and E toxins. Biophys Res Comm 1972; 48:108-112.
18. Eleopra R, Tugnoli V, Caniatti L, De Grandis D. Botulinum toxin treatment in the facial muscles of humans: Evidence of an action in untreated near muscles by peripheral local diffusion. Neurology 1996; 46:1158-1160.
19. Eleopra R, Tugnoli V, Rossetto W, Montecucco C, De Grandis D. Botulinum neurotoxin serotype C: A novel effective botulinum toxin therapy in human. Neurosci Lett 1997; 224:91-94.
20. Göbel H, Axel H, Heinze-Kuhn K, Austermann K. Botulinum toxin A in treatment of headache symptoms and pericranial pain syndromes. Pain 2001; 91:195-199.
21. Halpern JL, Neale EA. Neurospecific binding, internalization and retrograde axonal transport. Curr Top Microbiol Immunol 1995; 195:221-241.
22. Jankovic J, Schwartz, K. Response and immunoresistance to botulinum toxin injections. Neurology 1995; 45:1743-1746.
23. Jankovic J, Brin MF. Botulinum toxin: Historical perspectives and potential new indications. Muscle Nerve 1997; 20 (suppl 6):S129-S145.
24. Juzans P, Comella JX, Molgó, J, Failee L, Angaut-Petit D. Nerve terminal sprouting in botulinum type-A treated mouse levator auris longus muscle. Neuromusc Disord 1996; 6 (3):177-185.
25. Kalandakanond S, Coffield JA. Cleavage of intracellular substrates of botulinum toxins A, C, and D in mammalian target tissue. J Pharmacol Exp Ther 2001; 296 (3):749-755.
26. Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nature Struct Biol 1998; 5(10):898-902.

27. Lew MF, Brashear A, Factor S. The safety and efficacy of botulinum toxin type B in the treatment of patients with cervical dystonia: Summary of three controlled clinical trials. Neurology 2000; 55(suppl 5):S29-S35.
28. Maksymowych AB, Reinhard M, Malizio CJ, Goodnough MC, Johnson EA, and Simpson LL. Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces neuromuscular blockade. Infect Immun 1999; 67(9):4708-4712.
29. Montecucco C. How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem Sci 1986; 11:314-317.
30. de Paiva A, Meunier FA, Molgó J, Aoki JR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA 1999; 96:3200-3205.
31. Raciborska DA, Trimble WS, Charlton MP. Presynaptic protein interactions in vivo: Evidence from botulinum A, C, D, and E action at frog neuromuscular junction. Eur J Neurosci 1998; 10:2617-2628.
32. Schantz EJ, Johnson EA. Botulinum toxin: The story of its development for the treatment of human disease. Perspect Biol Med 1997; 40(3):317-327.
33. Schiavo B, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, Montecucco C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature (Lond) 1992; 359:832-835.
34. Schiavo G, Rossetto O, Catsicas S, Delaureto PP, DasGupta BR, Benefenati F, Montecucco C. Identification of the nerve terminal targets of botulinum neurotoxin serotype-A, serotype-D, and serotype-E. J Biol Chem 1993a; 268:23784-23787.

35. Schiavo G, Shone DD, Rossetto O, Alexander FCG, Montecucco CL. Botulinum neurotoxin serotype-F is a zinc endopeptidase specific for VAMP/synaptobrevin. J Biol Chem 1993b; 268:11516-11519.
36. Schiavo G, Malizio C, Trimble WS, Polverino de Laureto P, Milan G, Sugiyama H, Johnson EA, Montecucco C. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J Biol Chem 1994; 269:20213-20216.
37. Shirasu M, Kimura K, Kataoka M, Takahashi M, Okajima S, Kawaguchi S, Hirasawa Y, Ide C, Mizoguchi A. VAMP-2 promotes neurite elongation and SNAP-25A increases neurite sprouting in PC12 cells. Neurosci Res 2000; 37(4):265-75.
38. Simpson LL. Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. J Pharmacol Exp Ther 1980; 212:16-21.
39. Simpson LL. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol Rev 1981; 33:155-188.
40. Simpson LL. Identification of the characteristics that underlie botulinum toxin potency: Implications for designing novel drugs. Biochimie 2000; 82:943-953.
41. Smart JK. History of chemical and biological warfare: an American perspective. In: Sidell FR, Takafuji ET, Franz DR, editors. Medical aspects of chemical and biological warfare. Washington, DC: Office of the Surgeon General; 1997:9-86. Textbook of Military Medicine; part I, vol 3.
42. Smith LDS. Botulism: The organism, its toxins, the disease. Springfield, IL: Charles C. Thomas; 1977.

43. Söllner T, Bennett MK, Whiteheart S, Scheller RH, Rothman JE. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell 1993; 75:409-418.

44. Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic binding sites of *Clostridium botulinum* neurotoxin B. Nat Struct Biol 2000; 7(8):693-699.

45. Tsui JKC. Botulinum toxin as a therapeutic agent. Pharmacol Ther 1996; 72(1):13-24.

46. United States Department of State. Patterns of Global Terrorism in 1999. Washington, DC: US Dept of State; April 2000. Department of State publication 10687. Available at: http://www.state.gov/www/global/terrorism/1999report/1999index.html. Accessed February 1, 2001.

47. Van Ermengem EP. Ueber einen neuen anaeroben Bacillus und seine besiehungen zum botulismus. Z Hyg Infekt 1897; 26:1-56.

48. Williamson LC, Halpern JL, Montecucco C, Brown JE, Neale EA. Clostridial neurotoxins and substrate proteolysis in intact neurons. Botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. J Biol Chem 1996; 271: 7695-7699.

49. Zhou O, Jingnan X, Liu Y. Participation of syntaxin 1A in membrane trafficking involving neurite elongation and membrane expansion. J Neurosci Res 2000; 61:321-328.

50. Zuber M, Shebald M, Bathien N, de Recondo J, Rondot P. Botulinum antibodies in dystonia patients treated with type A botulinum toxin: Frequency and significance. Neurology 1993; 43:1715-1718.

CHAPTER 2

LOCAL AND SYSTEMIC DIFFUSION OF BOTULINUM SEROTYPE A AT VARIOUS TIME POINTS POST INJECTION[1]

[1] Whelchel DD, Brehmer TM, Brooks PM, Darragh N, and Coffield JA. Information from this chapter was submitted to Movement Disorders: official journal of the Movement Disorder Society, 10/28/2002.

INTRODUCTION

Botulinum neurotoxin (BoNT) cleaves specific SNARE proteins at the neuromuscular junction (NMJ), resulting in an inhibition of neurotransmitter release, which in turn produces flaccid muscle paralysis. Seven immunologically distinct serotypes (A-G) of the toxin exist and each serotype cleaves one or more of three SNARE proteins (SNAP-25, Synaptobrevin or VAMP, and Syntaxin). Over the past two decades, the paralysis resulting from BoNT has been exploited clinically to relieve symptoms of various neuromuscular disorders, such as focal spasms (including dystonias), spasticities, tics, and tremors, (Jankovic and Brin, 1997). In addition, clinical studies in humans are continuously investigating novel uses of the toxin in areas such as pain management, (Barwood et al., 2000; Göbel et al., 2001). Currently, the FDA has approved formulations of serotype A (BOTOX™, Allergran Inc., Irvine CA) and B (MYOBLOC™, Elan Pharmaceuticals) for the clinical treatment of specific disorders. Despite the numerous benefits of BoNT therapy, there are problems associated with therapeutic use of the toxin. One such problem is the diffusion of toxin from the injection site into undesired muscle groups. In fact, several human clinical studies have documented undesirable weakness in both nearby and distant muscle groups to those being treated, (Bhatia et al. 1999; Eleopra et al. 1996).

The objective of the study described in this chapter was to evaluate both local and systemic diffusion of a pure formulation of BoNT/A toxin using an in-vivo mouse model. Mice received unilateral injections of BoNT/A in the lateral gastrocnemius of one limb; the contra-lateral limb was injected with saline vehicle. Mice were then monitored daily for clinical signs of paralysis and sacrificed at various time points post injection. Toxin activity and diffusion were identified through the presence of a SNAP-25 cleavage fragment.

MATERIALS AND METHODS

NIH Swiss adult male mice (25-30g; N= 44) were injected in the lateral gastrocnemius with 25 pg of pure BoNT/A (150 kDa) and monitored for clinical changes and signs of paralysis. Mice were sacrificed at selected time points (1 d, 2 d, 4 d, 7 d, 14 d and 28 d) post injection (PI); muscle tissues were then collected and processed. For all studies, SNARE protein chemistry from processed muscle tissues was examined using standard western blot techniques. Cleaved SNAP-25 product was measured at all time points in muscles from the toxin-treated limb, the saline-treated limb and the diaphragm for all mice. Due to the large amount of cholinergic innervation to the muscle itself and the association of respiratory failure with botulism, the diaphragm muscle was selected to further monitor systemic diffusion.

Injection Procedure The mid lateral gastrocnemius muscle of toxin-treated limb was injected with 5 μl of pure BoNT/A (150 kDa; kindly supplied by Dr. Lance Simpson, Jefferson Medical College, Philadelphia, PA; 5pg/μl diluted in sterile saline). Similarly, sham injections (5μl of saline vehicle) were performed in the same muscle of the contra-lateral limb. Injections were directed approximately 12 mm cranial from the hock joint and 3.5 mm proximal to the outer edge of the limb. All injections were performed intramuscularly using a 28-gauge needle attached to a 0.3 cc syringe (BD Micro-Fine IV Needle; BD Co., Franklin Lakes, NJ). Syringes were pre-coated overnight with a 0.01% bovine serum albumin to minimize nonspecific toxin protein binding to the syringe. Throughout the injection procedure, mice were maintained under isofluorane anesthesia.

Clinical Assessment After injections, mice were monitored daily for clinical changes. Paralysis assessments were similar to those preformed in studies by Duchen et al. (1970) and Jurasinksi et al. (2001). Specifically, toe spread and plantar flexion abilities were observed while the mouse was suspended by the tail and while the mouse moved

freely along the top surface of the cage. Movements were assigned a value from 0 to 6, and in all instances, assessments were made by an impartial observer. A score of 0 indicated complete loss of the movement while a score of 6 indicated no impairment of the movement.

Tissue Collection and Preparation At pre-selected time points PI (1 d, 2 d, 4 d, 7 d, 14 d, and 28 d) mice were sacrificed via decapitation, as approved by the University's Institutional Animal Care and Use Committee. Muscle tissues (lateral gastrocnemius, medial gastrocnemius, and soleus) from both the toxin and saline-treated limbs, and the diaphragm were collected. In addition, serum was collected via heart puncture. All tissues were processed on ice as previously described by Kaladakanond and Coffield (2001). Briefly, tissues were homogenized separately using a hand-held electronic homogenizer (Omni 1000). The homogenization buffer contained 255 mM sucrose, 1 mM EDTA, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 20 mM Hepes (pH 7.4). The homogenates were centrifuged at 1,000g for 5 min. The resulting supernatant (S1) was then centrifuged at 10,000g for 10 min. The second supernatant (S2) was then spun at 250,000g for 1 h using an Optima TLX ultracentrifuge (Bechman Coulter, Fullerton, CA) to separate the synaptosomal membrane fraction (P3) and the cytosol fraction (S3). Protein concentrations were determined on the P3 and S3 fractions using a modified Lowry method (Bio-Rad, Hercules, CA).

Western Blot Analysis Proteins from the S3 fractions were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine buffer. Proteins were then transferred electrophoretically to polyvinylidene difluoride membranes (PVDF; Hybond; Amersham Life Science, Inc., Arlington Height, IL). Membranes were blocked overnight at 4 ˚C in 3% nonfat powdered milk diluted in Tris-buffered saline (TBS). After several washes, membranes were incubated with a polyclonal rabbit anti-SNAP-25 antibody for 4 h at 25 ˚C. Following the primary antibody incubations, membranes were

washed and then incubated with a horseradish peroxidase conjugated secondary antibody for 1 h at 25 ˚C, followed by immunodetection with enhanced chemiluminescence (ECL^{+}; Amersham Life Science, Inc., Arlington Height, IL). PVDF membranes were then exposed to hyperfilm-ECL for times adequate to visualize chemiluminescent bands. Full length and cleaved SNAP-25 protein immunoreactivities were analyzed using scanning densitometry (Scion Image; Scion Corporation, Frederick, MD). In addition, the S3 fractions from the corresponding limb muscles of uninjected mice and those solely receiving unilateral saline injections were analyzed and served as controls.

Antibodies A polyclonal rabbit anti-SNAP-25 antibody (1:15,000; Sigma-Aldrich; St Louis, MO) was used for the detection of SNAP-25 (both full length and cleaved product; S3 fractions). A goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Biosource; Camarillo, CA) was used at a 1:5,000 dilution.

Statistics Changes in the percent cleaved product throughout the 28 d period were compared to levels measured at d1; significant differences were determined using a two-tailed Student's t-test.

RESULTS

Clinical Assessments Less than fifty percent of mice treated with BoNT/A appeared to show signs of illness (lethargy, reduced grooming) within 24-48 h PI, these signs resolved by 72 h PI. Impairments in both toe spread and plantar flexion were evident within 24 h PI in the toxin-treated limb of all mice and continued throughout the 28 d period; however, no changes were observed in the saline-treated limb at anytime throughout the 28 d period, (Figure 2.1). By d2-3, severe impairments in both movements were apparent, while slight improvements in both movements were observed between d4-8. However a sharp decline, in both toe spread and plantar

flexion, was again seen between d9-11 PI, but between d12-28, both movements showed a second phase of improvement. Throughout the entire 28 d observation period, neither function recovered completely.

Muscle atrophy was not apparent at early time points post injection, however by d7, decreases in muscle weight became apparent in the toxin-treated limb. On average, weights in the treated limb decreased approximately 16% from those of the saline-injected limb. Atrophy continued to increase through the 28 d period. By d28, muscle weight in the toxin-treated limb was ~50% less than in the saline-treated limb. The time frame for muscle weight loss in the toxin-treated limb suggests that atrophy likely resulted from a lack of use as opposed to denervation.

Local Diffusion Immunoblot analysis of muscles from the toxin-treated leg showed the presence of cleaved SNAP-25 (24kDa) within 24 h PI in both the injected lateral and the adjacent medial gastrocnemius, indicating that local diffusion of the toxin occurred quickly. Cleaved product immunoreactivity was also detected in the soleus muscle of the toxin-treated limb, (not shown). Throughout the 28 d period, cleaved product immunoreactivity continued to increase in the toxin-treated limb, (Figure 2.2).

The ratio of cleaved product (cleaved band immunoreactivity; S3 fraction) to the total SNAP-25 pool (full length SNAP-25 plus cleaved band immunoreactivities; S3 fraction) was used to calculate the percent cleaved product for all processed muscles. Within 24 h PI, the amount of cleaved SNAP-25 product detected in both the toxin-injected lateral gastrocnemius and the adjacent medial gastrocnemius was 48% and 40%, respectively (Figure 2.3a). The percent of cleaved product increased to ~56% by d2 in both the lateral and medial gastrocnemius muscles. By d4, the percent cleaved product in both muscles reached ~60%, and the increases in the medial gastrocnemius were significantly higher than percentages measured at d1, ($p<0.05$). The percent cleaved SNAP-25 in the treated leg peaked between 65-67% by d7, and stabilized at

~60% between d14-28, (Figure 2.3a). Throughout the 28 days PI, cleaved product levels never increased past 67%. However between d7-28, cleaved product percentages were significantly higher than d1 percentages for both the lateral and medial gastrocnemius, (d7 and d14: $p<0.01$ for both Lat G. and Med G.; d28: Lat G: $p<0.01$, Med G: $p<0.05$).

Systemic Diffusion The corresponding muscles of the saline-injected leg, along with the diaphragm muscle, were examined for cleaved SNAP-25 product to determine systemic spread of BoNT/A. Within 24 h PI, cleaved SNAP-25 was detected in the saline-treated limb and the diaphragm, although at much lower percentages (11% and 8% respectively), than those found in the toxin-treated limb, (Figure 2.3b). The presence of cleaved product in non-toxin treated muscles by d1 suggested rapid systemic spread of the toxin. The amount of cleaved product in non-toxin treated tissues remained consistent between d1-2, but increased between d2-4 by 19% in the gastrocnemius muscles and 28% in the diaphragm. Percentages in non-toxin treated muscles peaked between d4-7 (33% in the gastrocnemius muscles; 42% in the diaphragm); and then stabilized between d7-28 at 30% in the gastrocnemius muscles and 21% in the diaphragm, (Figure 2.3b).

Throughout all time points, the amount of cleaved SNAP-25 product present was below 45% and the percentages were usually lower than those found in the toxin-treated limb. Furthermore, cleaved product amounts in the toxin-treated limb were significantly different from levels found in the saline-treated limb at all time points ($p< 0.00011$). Even though low levels of cleaved SNAP-25 were found in the saline-treated limb throughout the 28 d period, suggesting systemic spread of the toxin, signs of clinical paralysis were not observed in these limbs.

Substrate Cleavage and Clinical Signs Ratios expressing the amount of cleaved SNAP-25 product, in muscles from the toxin-treated limb to that measured in the

corresponding muscles of the saline-treated limb, were calculated for each time point. Cleaved product ratios were elevated within 24 h PI for both the lateral and medial gastrocnemius muscles, (Figure 2.4). By d2, these ratios reached their highest levels observed throughout the 28 d period. Furthermore, the high ratios measured on d1 coincided with the onset of paralysis in the toxin-treated limb; while the more severe paralysis evidenced at d2 coincided with peak ratio levels of cleaved product. Following the peak response, ratios decreased between d4-7 concurrent with initial clinical improvements observed from d4-8. However, between d14-28, ratios began to increase once more. Interestingly, clinical observations showed a strong recovery trend spanning this time period (d12-d28), with the highest scores observed between d20-28 (Figure 2.1).

Controls Due to the fact that high levels of cleaved SNAP-25 product were detected 7d after BoTN/A injection, immunoreactivities were analyzed for mice receiving saline-only injections at d7 PI. SNAP-25 cleaved product was not detected in muscles from the non-injected (data not shown) nor saline-only injected mice; furthermore, these mice did not show any signs of paralysis (Figure 2.2).

DISCUSSION

Within 24 h PI, cleaved SNAP-25 product was present, not only in the toxin-injected lateral gastrocnemius and adjacent muscles, but also in muscles distant to the injection site; these findings suggest that both local and systemic spread of the toxin occurred quickly. Although at d1 the percent of cleaved SNAP-25 product was at the lowest point observed throughout the 28 d period, the ratios of cleaved SNAP-25 in the toxin-injected limb to that of the saline injected were high, (Lat G: 7.8; Med G: 5.3). By d2, these ratios peaked at their highest observed levels (7.7-8.3), while the percent cleaved product

continued to increase in toxin-treated muscle. These measurements corresponded with a sharp increase in clinical paralysis between d2-3.

As the percent cleaved SNAP-25 product continued to increase in the toxin-treated limb up to d7, the cleaved SNAP-25 ratio decreased between d4-7, reaching the lowest values at d7 (1.8-2.0). These decreasing ratios coincided with clinical improvements observed between d4-8. From d14-28, cleaved product ratios rose again, while the percent of cleaved SNAP-25 product in the toxin-treated limb remained constant at ~60%. During this same period, clinical observations revealed a strong phase of recovery in the toxin-treated limb. Together, these findings indicate that the toxin-treated limb was able to partially overcome the paralytic effects of BoNT/A by d28 despite the high levels of cleaved product still present at this time.

During the period of strong clinical recovery, cleaved SNAP-25 product immunoreactivity continued to increase and the percent cleaved product remained elevated in the toxin-treated limb (d14-28). Therefore, the presence of cleaved SNAP-25 product indicates ongoing toxin activity; however, sufficient levels of cleaved SNAP-25 product, in relation to the full length protein, must be attained to induce visible signs of impairment. This conclusion is additionally supported by the presence of cleaved product in muscles distant to the injection site, where paralysis was not observed. The percent cleaved product in non-toxin treated muscles remained relatively low (8-18%) during the first two days PI; however, even as cleaved product levels rose, the saline-treated limb did not show signs of paralysis throughout the 28 d period. On the other hand, the amount of cleaved SNAP-25 product in the toxin-treated limb observed by d1 (40 to 48%) was enough to produce initial signs of impaired toe spread and plantar flexion in this limb. Although the amount of cleaved product present in the diaphragm muscle reached 42% by d4, the percent cleaved product in non-toxin treated muscles never consistently maintained the levels observed in the toxin-treated limb throughout

the 28 d period. In fact, percentages remained below 50% at all time points in non-toxin treated muscles, while levels in the toxin-treated limb were above 50% from d2-28. In addition, there were statistically significant differences in cleaved product percentages from muscles in toxin-treated limb compared to those of the saline-treated limb at all time points. While these findings may suggest a threshold level of SNAP-25 cleavage must be reached and maintained to generate observable signs of paralysis, dynamic changes in SNARE protein chemistry that may occur in the in-vivo system preclude drawing such a conclusion. Since previous studies have documented that the formation of axonal sprouts in toxin-treated tissues mediate recovery, it is probable that SNAP-25 protein levels (full length and cleaved) may be altered by this process. Therefore, in order gain further insight into the correlation between the duration of intracellular toxin activity and toxin-induced paralysis, it would prove beneficial to investigate changes in both full length SNAP-25 and other SNARE protein levels.

REFERENCES

1. Barwood S, Baillieu C, Boyd R, Brereton K, Low J, Nattrass G, Graham HK. Analgesic effects of botulinum toxin A: A randomized, placebo-controlled clinical trail. Dev Med Child Neurol 2000; 42:116-121.
2. Bhatia KP, Münchau A,Thompson PD, Houser M, Chauhan VS, Hutchinson M, Shapira AHV, Marsden CD. Generalized muscular weakness after botulinum toxin injections for dystonia: a report of three cases. J Neurol Neurosurg Psychiatry 1999; 67:90-93.
3. Duchen LW. Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: differences between fast and slow muscles. J Neurol Neurosurg Psychiatry 1970; 33:40-54.
4. Göbel H, Axel H, Heinze-Kuhn K, Austermann K. Botulinum toxin A in treatment of headache symptoms and pericranial pain syndromes. Pain 2001; 91:195-199.
5. Eleopra R, Tugnoli V, Caniatti L, De Grandis D. Botulinum toxin treatment in the facial muscles of humans: Evidence of an action in untreated near muscles by peripheral local diffusion. Neurology 1996; 46:1158-1160.
6. Jankovic J, Brin MF. Botulinum toxin: Historical perspectives and potential new indications. Muscle Nerve 1997; 20 (suppl 6):S129-S145.
7. Jurasinski CV, Lieth E, Dang Do AN, Schengrund CL. Correlation of cleavage of SNAP-25 with muscle function in a rat model of Botulinum neurotoxin type A induced paralysis. Toxicon 2001; 39:1309-1315.

FIGURE 2.1 Functional assessment of toe spread (TS) and plantar flexion (PF) movements during the 28 day observation period post BoNT/A injection.

Movements were assessed and scored for both the toxin-treated (Tx, filled symbols) and saline-treated limbs (Ctl, open symbols). Movements were scored on a scale from 0 to 6; a score of 0 represents the complete loss of function/movement while a score of 6 represents no impairment in function/movement. Mean scores (±SEM, n≥3 per day) were recorded throughout the 28 d period PI.

FIGURE 2.1

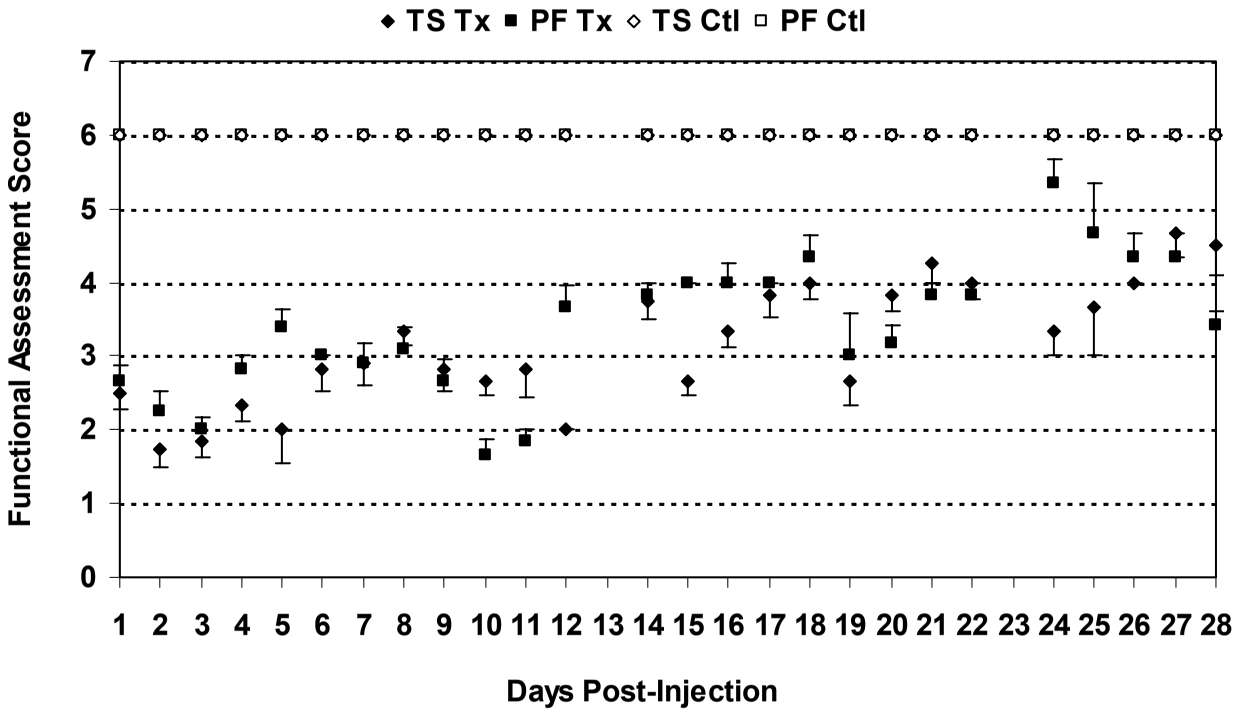

◆ TS Tx ■ PF Tx ◇ TS Ctl □ PF Ctl
Functional Assessment Score
0
1
2
3
4
5
6
7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Days Post-Injection

FIGURE 2.2 Immunoblot detection of full length SNAP-25 and cleaved SNAP-25 product immunoreactivity in a single mouse at various time points post-injection. Full length (25kDa) and cleaved SNAP-25 (24kDa) immunoreactivities were detected in both the injected lateral gastrocnemius (Lat G) and the adjacent medial gastrocnemius (Med G) of both hind limbs as well as the diaphragm (DPH; S3 fractions; 80 µg/lane, Tris-HCL gels). Muscles from the toxin-treated limb (Tx) were compared along side those from the saline-treated limb (Ctl). Immunoreactivities were assessed at 1d, 2d, 4d, 7d, 14d, and 28d post BoNT/A injection. For mice solely receiving unilateral saline injections, the saline-injected limb was designated the treated limb (Tx) and the non-injected limb was defined as the control (Ctl).

FIGURE 2.2

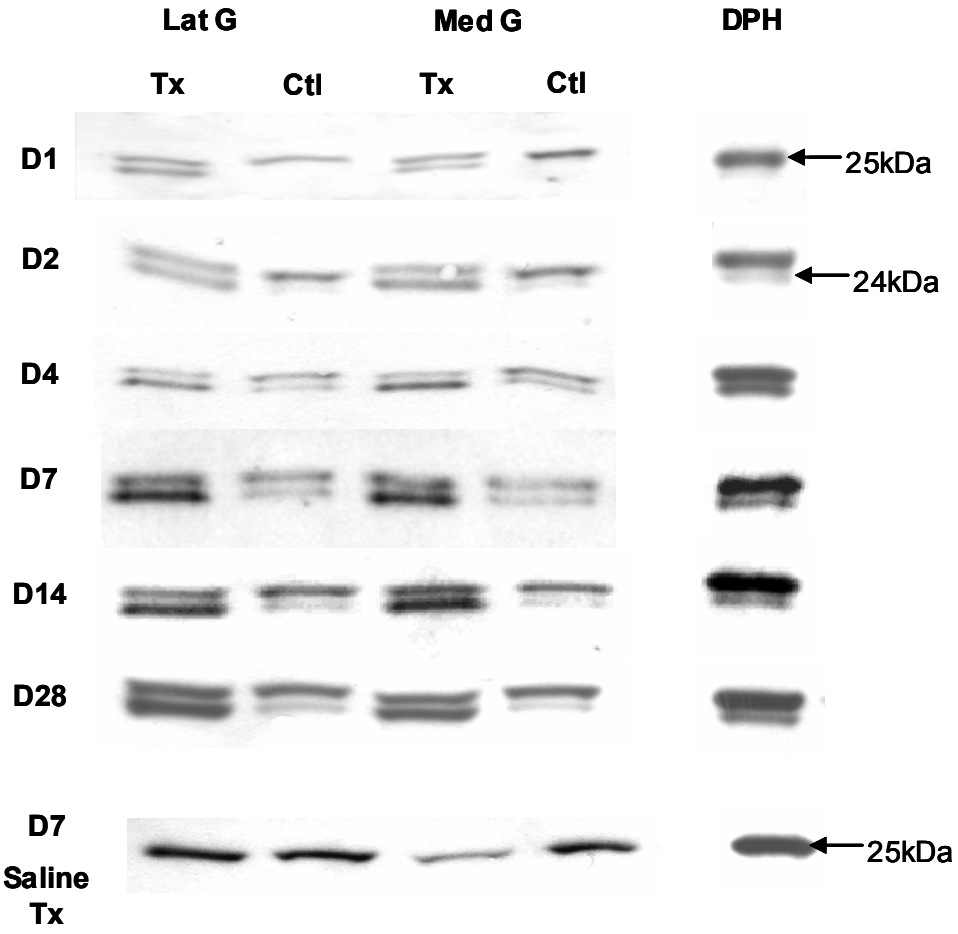

Lat G
Med G
DPH
Tx
Ctl
Tx
Ctl
D1
25kDa
D2
24kDa
D4
D7
D14
D28
D7
Saline
Tx
25kDa

FIGURE 2.3 Changes in the percent cleaved SNAP-25 product found at various time points post BoNT/A injection. The percent cleaved product was calculated as the percentage of cleaved SNAP-25 immunoreactivity (24kDa) out of the total SNAP-25 pool in the S3 fraction [both full length (25kDa) and cleaved]. **a. The percent cleaved product found in muscles from the toxin-treated limb at various time points post-injection.** Cleaved product levels were assessed in the toxin-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) muscles (mean % ± SEM, n≥4). Statistically significant increases in percentages from those measured at d1 were determined using a Student's two-tailed t-test, (**a** (Lat G)**, a'** (Med G) $p<0.01$; **b** (Med G) $p<0.05$.) **b**. **The percent cleaved SNAP-25 product measured in muscles from the saline-treated limb and distant diaphragm at various time point post-injection.** Cleaved product levels were measured in the saline-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) of the contra-lateral limb; cleaved product levels were also assessed in the diaphragm muscle (Diaph; mean % ± SEM, n≥4). Significant increases from d1 percentages were determined as described in 2.3a, (**a** (Lat G)**, a'** (Med G)**, a"** (Diaph): $p<0.01$; **b** (Med G) and **b"**(Diaph): $p<0.05$).

FIGURE 2.3a

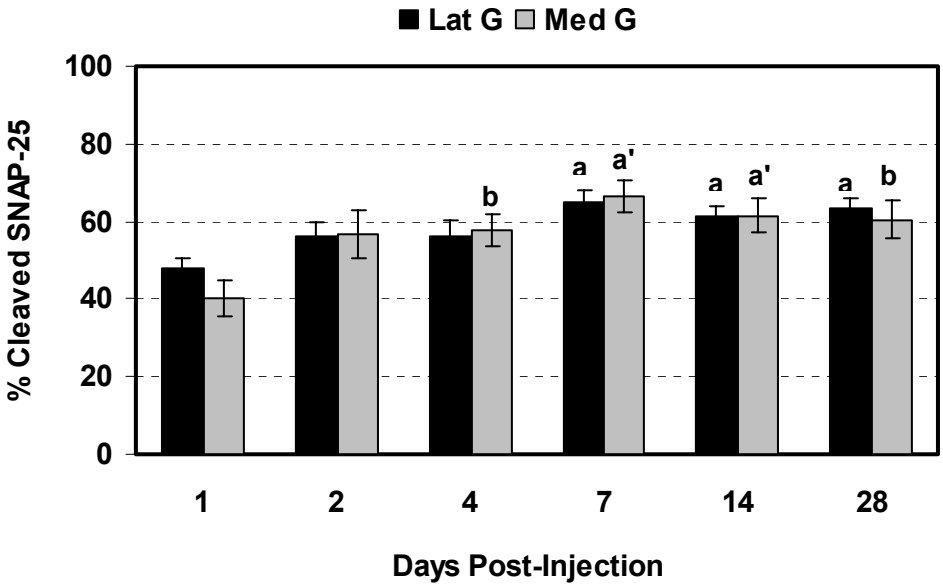


FIGURE 2.3b

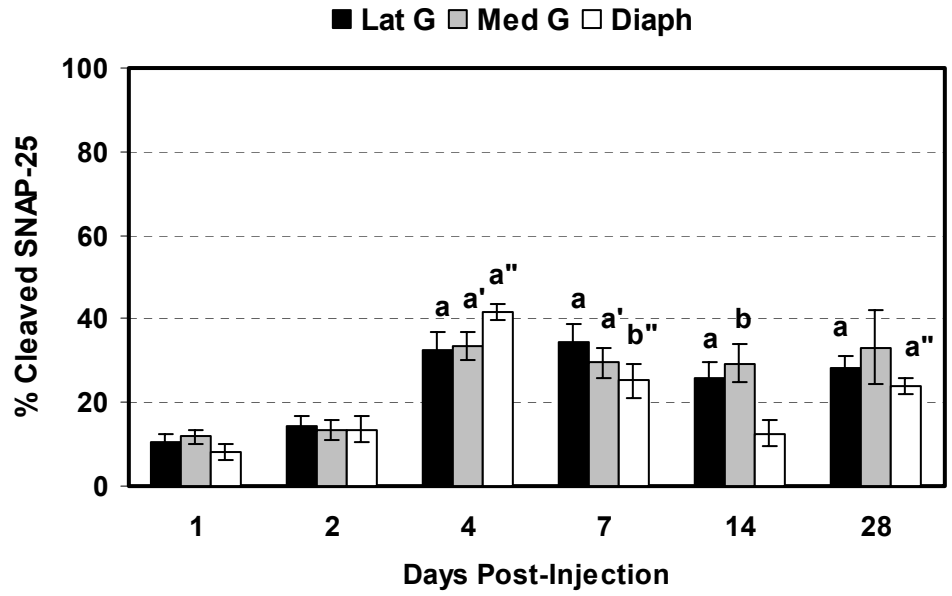
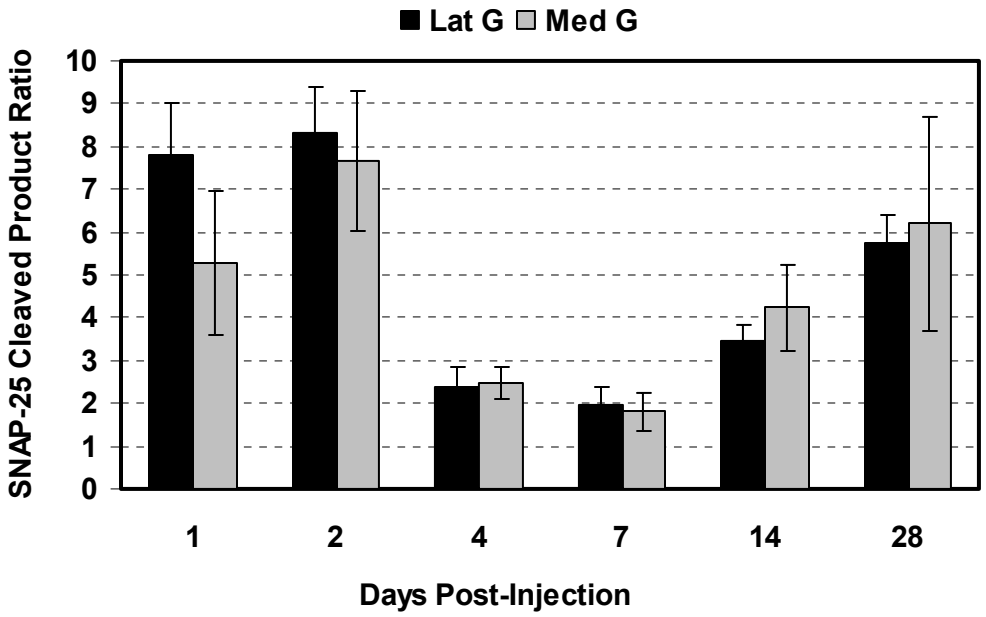


FIGURE 2.4 The ratio of cleaved SNAP-25 immunoreactivity in muscles from the toxin-treated limb to those of the saline-treated limb at various time points post BoNT/A injection. The ratio of cleaved SNAP-25 product was calculated by comparing cleaved product immunoreactivity in muscles from the toxin-injected limb to those of the saline-injected limb at each time point. Ratios of SNAP-25 cleaved product (toxin-treated limb: saline-treated limb) were calculated for both the injected Lat G and adjacent Med G muscles (mean ratio ±SEM, n≥4).

FIGURE 2.4


Lat G
Med G
SNAP-25 Cleaved Product Ratio
10
9
8
7
6
5
4
3
2
1
0
1
2
4
7
14
28
Days Post-Injection

CHAPTER 3

CHANGES IN FULL LENGTH SNAP-25 AND VAMP II IMMUNOREACTIVITY CORRESPOND WITH CLINICAL SIGNS AFTER BOTULINUM SEROTYPE A TREATMENT[1]

[1] Whelchel DD, Brehmer TM, Brooks PM, Darragh N, and Coffield JA. Information from this chapter was submitted to Movement Disorders: official journal of the Movement Disorder Society, 10/28/2002.

INTRODUCTION

Since BoNT poisoning results in a transient paralysis at the NMJ, the desired therapeutic effects of toxin treatment diminish within 3-6 months, necessitating the need for repeat injections. Unfortunately, repeat injections and larger dosages can lead to further complications such as diffusion of the toxin to unwanted muscle groups and the development of resistance to toxin therapy, (Tsui, 1996). Although the continued presence of cleaved SNAP-25 product may indicate ongoing toxin activity in muscle tissues, the findings presented in Chapter 2 demonstrated that the presence of cleaved SNAP-25 fragment did not correspond with the duration of the desired clinical response from toxin treatment.

As a response to the paralysis induced by BoNT/A, transient axonal sprouts form in toxin-treated muscles over time, effectively re-innervating muscles and promoting functional recovery; this, in turn, may decrease the duration of the clinical response. Sprout formation, therefore, is a relevant issue to the therapeutic use of BoNT/A. Numerous studies in mice have shown that nerve terminals in toxin-treated muscles undergo sprouting to form new NMJs capable of eliciting muscle fiber contraction (Angaut-Petit et al., 1990; Comella et al., 1993, Juzans et al., 1996; de Paiva et al., 1999). de Paiva et al. (1999) immunohistochemically identified SNARE proteins (SNAP-25 and VAMP) in these sprouts. Furthermore, other studies have indicated potential roles of SNARE proteins in sprout formation (Shirasu et al., 2000; Zhou et al., 2000).

The aim of this study was to assess changes in SNARE protein chemistry throughout the duration of paralysis and recovery post BoNT/A injection. As previously described, the lateral gastrocnemius was injected with pure BoNT/A, while the corresponding muscle of the contra-lateral leg was injected with saline. At various time points post-injection (PI), mice were sacrificed and changes in full length SNAP-25 and VAMP II immunoreactivity in the toxin-injected and adjacent muscles were compared to

levels in the corresponding muscles of the contra-lateral (saline-injected) limb. In contrast to the results from the analysis of SNAP-25 cleaved product, the results from this study indicate that changes in full-length SNAP-25 and VAMP II immunoreactivities correspond with clinical observations in the toxin-treated limb.

MATERIALS AND METHODS

Tissues from mice, previously injected in the study described in Chapter 2, were used for this study. Furthermore, toxin injection and clinical observations were as described in Chapter 2. Mice were sacrificed at select time points (d1, d2, d4, d7, d14, and d28; N=44) PI; muscle tissues were then collected and processed as previously described, (Ch. 2, Materials and Methods). SNARE protein chemistry from processed muscles was examined using standard western blot techniques. Differences in full-length SNAP-25 and VAMP II immunoreactivities in the toxin-treated limb (lateral gastrocnemius, medial gastrocnemius, and soleus muscles) were compared to those of the corresponding muscles of the saline-treated limb.

Western Blot Analysis The synaptosomal enriched P3 fractions (Ch. 2, Materials and Methods) were resuspended in dH20 with protease inhibitor cocktail, (Sigma-Aldrich; St. Louis, MO). P3 fraction proteins were then separated using SDS-PAGE in a Tris-tricine buffer, and transferred electrophoretically to PVDF membranes, (Hybond; Amersham Life Science, Inc., Arlington Height, IL). Membranes were then blocked for 3 h at 25 ˚C in 3% nonfat powdered milk diluted in TBS. After several washes, the membranes were incubated with a primary antibody cocktail [polyclonal rabbit anti-SNAP-25 and anti-VAMP II antibodies] overnight at 4 ˚C, followed by 4 h at 25 ˚C. After primary antibody incubations, membranes were washed and then incubated with a horseradish peroxidase conjugated secondary antibody for 1 h at 25 ˚C followed by immunodetection with enhanced chemiluminescence as previously described, (Ch. 2, Materials and

Methods; ECL^{+}; Amersham Life Science, Inc., Arlington Height, IL). Full length SNAP-25 and VAMP II immunoreactivity from saline-treated and toxin-treated muscles were determined by scanning densitometry of P3 blots, (Scion Image; Scion Corporation, Frederick, MD). In addition, full length SNAP-25 and VAMPII immunoreactivities were measured in P3 fractions from limb muscles of un-injected and saline-only injected mice; these latter measurements served as additional controls.

Antibodies Polyclonal rabbit anti-SNAP-25 antibody (1:15,000; Sigma-Aldrich, St. Louis, MO) and anti-VAMP II antibodies (1:4,000; Wako Chemicals, Richmond, VA) were used to detect full length SNAP-25 and VAMP II protein (P3 blots). Biosource (Camarillo, CA) goat anti-rabbit horseradish peroxidase conjugated secondary antibody was used at a 1:5,000 dilution.

Statistics To determine significant differences over time, the percent change in both full length SNAP-25 and VAMP II immunoreactivities from each time point were compared to the percentages measured at d1. Statistical significance was determined using a two-tailed Student's t-test.

RESULTS

Clinical Assessments All observations and assessments were as previously described in Chapter 2.

Changes in SNARE Protein Immunoreactivity Within 24 h PI, decreases in full length SNAP-25 and VAMP II immunoreactivities were apparent in the lateral and medial gastrocnemius muscles of the toxin-treated leg compared to levels found in the saline-treated limb, (Figures 3.1 and 3.2). Full length SNAP-25 immunoreactivity decreased approximately 17-24%, while VAMP II decreased approximately 16-21%. No definitive change was apparent in soleus SNAP-25 immunoreactivity, while VAMP II levels decreased slightly in the toxin-treated limb, (3%). By d2, small increases in mean

SNAP-25 and VAMP II immunoreactivity were apparent in the toxin-treated limb, (2-21% and 3-65 %, respectively), however standard error measurements indicate a great deal of variability during these very early time points PI. Notably the onset of clinical paralysis was observed within 24 h PI, and severe impairments in toe spread and plantar flexion were first observed from d2-3.

By d4, immunoblots of the toxin-treated and adjacent muscles showed increased SNAP-25 and VAMP II immunoreactivity levels; these increases became more distinct throughout the 28 d period, (Figures 3.1a and 3.2a). Similarly, d4 mean percent changes in full length SNAP-25 and VAMP II also reflected these increases, (SNAP-25:4-15%, VAMP: 37-49%, Figures 3.1b and 3.2b). The medial gastrocnemius showed statistically significant increases in SNAP-25 (15%) at d4 compared to d1 values, while both the lateral and medial gastrocnemius showed significant increases in VAMP II, (37 and 49%, respectively). Increases in both soleus SNAP-25 and VAMP II immunoreactivities were detected at d4, but not found to be significantly different from those at d1. Concurrent with the first statistically significant increases measured at d4, signs of clinical improvement were apparent in the toxin-treated limb.

Statistically significant increases above d1 levels, in both SNAP-25 and VAMP II immunoreactivities, were also observed at d7. In fact, d7 increases were higher than those observed at earlier time points. SNAP-25 and VAMP II immunoreactivities peaked at 64% and 157%, respectively, in the lateral gastrocnemius and at 36% and 96%, respectively, in the medial gastrocnemius. Increases in both SNARE protein immunoreactivities were also detected in the neighboring soleus, but not found to be significantly different from levels observed at d1. As with d4 observations, the appearance of significant increases in SNARE protein immunoreactivities at d7 coincided with improvements seen in toe spread and plantar flexion from d4-8.

In both the injected and adjacent gastrocnemius muscles, larger increases in SNAP-25 and VAMP II immunoreactivities were seen by d14, (SNAP-25:126 and 83%; VAMP: 174 and 156% respectfully), compared to earlier time points. The increase in full length SNAP-25 in the medial gastrocnemius was significantly higher than levels detected at d1, as were the increases in VAMP II in both the lateral and medial gastrocnemius muscles. However the increases in full length SNAP-25 immunoreactivity in the injected lateral gastrocnemius were not significant due to substantial variability. Further, increases in both SNARE protein immunoreactivities were observed in the neighboring soleus, but these changes were also not significant. These substantial increases further coincided with strong signs of clinical recovery in the toxin-treated limb observed between d12-28.

Similarly, at d28, SNARE protein immunoreactivity was greatly increased in the toxin-treated limb compared to the same time point in the saline-treated limb. In fact, the largest increase in full-length SNAP-25 immunoreactivity was seen at d28 (125-165%), while VAMP II levels were stable. At d28 these increases, observed in both SNARE protein immunoreactivities of the lateral and medial gastrocnemius, were significantly higher than d1 values; however, increases detected in the soleus remained insignificant. Finally, the large increases in SNARE protein immunoreactivities measured at d28 coincided with the highest levels of clinical recovery observed in the toxin-treated limb throughout the observation period.

Controls Due to the fact that increases in full length SNARE protein immunoreactivities were detected 7d after BoNT/A injection, immunoreactivities were analyzed for mice receiving saline-only injections at d7 PI. Non-injected mice and saline-only injected mice did not show substantial changes in full length SNAP-25 or VAMP II levels at d7; furthermore, these mice did not show any signs of paralysis (Figure 3.3).

DISCUSSION

As early as d1 PI, differences in full length SNAP-25 and VAMP II immunoreactivities in the toxin-treated limb, compared to those of the saline-treated limb, were evident. At d1, small decreases in SNAP-25 and VAMP II immunoreactivities in the toxin-treated leg corresponded with the onset of paralysis observed in this limb. Decreases in SNAP-25, particularly in the toxin-injected head, were expected at d1, as BoNT/A cleaved SNAP-25, reducing the amount of full length protein. Similarly, Kalandakanond and Coffield, 2001 showed decreases in full length SNAP-25 immunoreactivity post BoNT/A treatment in the hemidiaphragm in-vitro preparation. However, decreases in VAMP II immunoreactivity were not expected. It is plausible that these initial decreases may have also resulted in part from a post-injection, acute injury or inflammatory response.

Both noticeable and statistically significant increases in SNAP-25 and VAMP II, from d1 values, were apparent in the toxin-treated limb between d4-7, and these increases corresponded to the first signs of clinical improvement observed in this limb, (d4-8). By d7, both the lateral and medial gastrocnemius muscles showed distinct increases in SNAP-25 and in VAMP II immunoreactivities (greater than 30% and 90%, respectively). This marked the first substantial increase in both SNARE protein levels detected in the lateral and medial gastrocnemius muscles of the toxin-treated limb. Furthermore, the largest increases in both SNAP-25 and VAMP II immunoreactivites were observed from d14-28 and corresponded with the strongest improvement observed in both toe spread and plantar flexion.

The temporal increases in full length SNAP-25 and VAMP II immunoreactivities in the toxin-treated limb throughout the post-injection period suggest de novo SNARE protein synthesis. The localization of this de novo synthesis cannot be determined by this study. However three possibilities exist. First, new SNARE protein may be forming

in the intoxicated terminals. Second, the new SNARE proteins may be forming in axonal sprouts. Third, new SNARE protein may be forming in both locations.

The findings of this current study document temporal increases in SNARE protein immunoreactivity and improvements in limb movement before d28; this time period is somewhat earlier than similar studies have previously documented. Such differences may be due to differences in methodology. This study identified increases in SNARE protein immunoreactivity possibly associated with neuronal sprouting using immunoblot techniques. Other studies have identified sprouts through immunohistochemical detection in muscle tissue sections. However, de Paiva et al. identified SNAP-25 and VAMP immunoreactivity in neuronal sprouts formed in the mouse sternomastoid muscle by d9 post BoTN/A injection. Although these sprouts were not characterized as functional endplates at this time, the appearance of SNARE protein immunoreactivity corresponds with the substantial increases in SNARE protein immunoreactivities (d7-28 PI) documented in this study.

Previous studies defined functional recovery from paralysis by conducting electrophysiological measurements in the toxin-poisoned muscles, (Angaut-Petit et al., 1990; Comella et al., 1993; Juzans et al., 1996). In this current study, recovery was defined through visual observations of whole limb function; therefore, improvements were not defined by any one muscle's activity. The assessed movements (toe spread and plantar flexion) are not only controlled by the lateral gastrocnemius muscle but are also influenced by neighboring muscles. Toxin activity may have been lower in these muscles and therefore, neighboring muscles may have "overcome" BoNT/A induced paralysis before the toxin-injected muscle. Recovery in these neighboring muscles may have contributed to the early signs of improvement detected in the toxin-treated limb. It is also possible that the initial improvements observed from d4-7 in this study merely resulted from the cessation of a short-term, post-injection, inflammatory response.

In addition, the early signs of recovery detected in this study may have been due to the increased synthesis of SNAP-25 and VAMP in poisoned terminals. Thus a sufficient amount of exocytosis and neurotransmitter release may have occurred in these terminals, enabling a partial short-term recovery. However, such increases in SNARE protein levels, possibly in the originally poisoned terminals, may have not sufficed to overpower a continuance of toxin enzymatic activity. A more substantial recovery might only be attained through the addition of functional axonal sprouts formed at later time points. This latter period may coincide with when both the strongest recovery and the largest increases in SNARE protein immunoreactivity were observed in the toxin-treated limb.

REFERENCES

1. Angaut-Petit D, Molgó J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 1990; 37:799-807.
2. Comella JX, Molgó J, Faille L. Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D and the functional recovery of parlaysed neuromuscular junctions. Neurosci Lett 1993; 153:61-64.
3. Juzans P, Comella JX, Molgó, J, Failee L, Angaut-Petit D. Nerve terminal sprouting in botulinum type-A treated mouse levator auris longus muscle. Neuromusc Disord 1996; 6 (3):177-185.
4. de Paiva A, Meunier FA, Molgó J, Aoki JR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA 1999; 96:3200-3205.
5. Shirasu M, Kimura K, Kataoka M, Takahashi M, Okajima S, Kawaguchi S, Hirasawa Y, Ide C, Mizoguchi A. VAMP-2 promotes neurite elongation and SNAP-25A increases neurite sprouting in PC12 cells. Neurosci Res 2000; 37(4):265-75.
6. Tsui JKC. Botulinum toxin as a therapeutic agent. Pharmacol Ther 1996; 72(1):13-24.
7. Zhou O, Jingnan X, Liu Y. Participation of syntaxin 1A in membrane trafficking involving neurite elongation and membrane expansion. J Neurosci Res 2000; 61:321-328.

FIGURE 3.1 Changes in full length SNAP-25 (25kDa) immunoreactivity in mouse muscle post BoNT/A injection. **a. Immunodection of changes in full length SNAP-25 in one mouse at various time points post BoNT/A injection.** Using standard western blot analysis, SNAP-25 immunoreactivity was detected in muscles from the toxin-treated (Tx) and saline-treated (Ctl) limb (P3 fractions; 75µg/lane, Tris-Tricine gels). The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb to determine changes in full length SNAP-25 throughout the 28 d period. **b**. **The percent change in full length SNAP-25 immunoreactivity at various time points post BoNT/A injection.** The percent change in full length SNAP-25 is expressed as the mean percent difference (±SEM, n≥4) of SNAP-25 immunoreactivity in muscles from the toxin-treated limb compared to those of the saline-treated limb. For each time point, changes were analyzed in the toxin-injected Lat G and adjacent muscles [Med G and soleus (Sol)]. Baseline values of 100% represent no change from saline-treated muscles, values above 100% represent increases in SNAP-25 immunoreactivity in the toxin-treated limb, and values less than 100% represented decreases. Statistically significant differences from those measured at d1 were determined using a two-tailed Student's t-test, (**a** (Lat G)**, a'** (Med G): $p<0.01$; **b'** (Med G): $p<0.05$).

FIGURE 3.1a

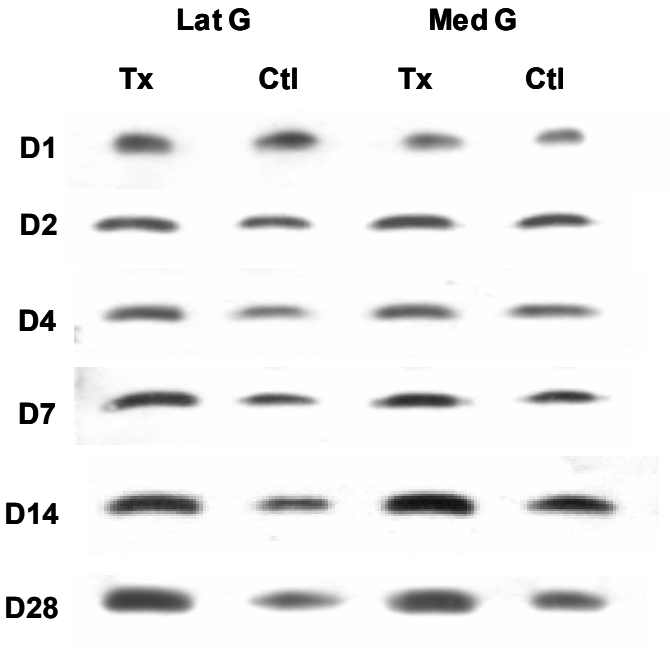


FIGURE 3.1b

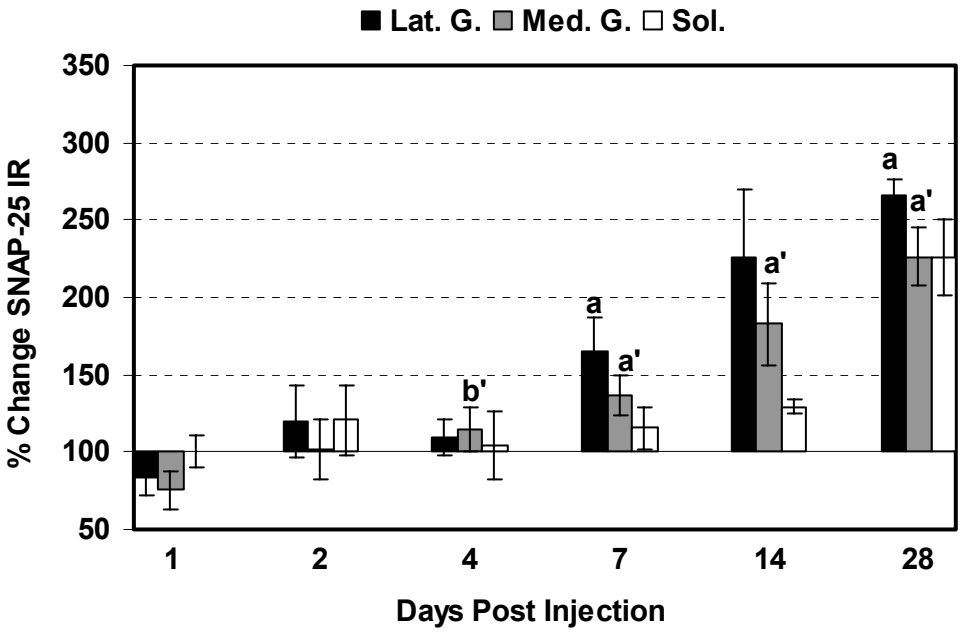


FIGURE 3.2 Changes in full length VAMP II immunoreactivity in mouse muscle post BoNT/A injection. a. Immunodetection of changes in full length VAMP II in one mouse at various time points post BoNT/A injection. Full length VAMP II immunoreactivity was detected in muscles from both the toxin-treated (Tx) and saline-treated (Ctl) limb using western blot analysis (P3 fractions; 75µg/lane, Tris-Tricine gels). The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb to determine changes in VAMP II immunoreactivity throughout the 28 d period. **b. The percent change in full length VAMP II immunoreactivity at various time points post BoNT/A injection.** The percent change in full length VAMP II immunoreactivity in muscles from the toxin-treated limb was determined as previously described for full length SNAP-25 (mean percent change ±SEM, n≥4). The percent change in VAMP II immunoreactivity was analyzed for the Lat G, Med G, and Sol muscles throughout the 28 d period post-injection. Significant increases from d1 percentages were determined as previously described in Figure 3.1b, (**a** (Lat G)**, a'** (Med G): $p<0.01$; **b** (Lat G): $p<0.05$).

FIGURE 3.2a

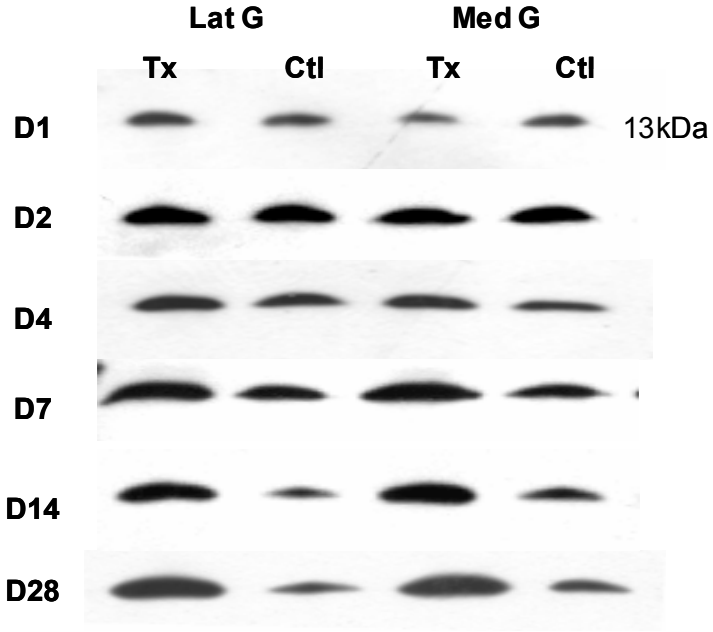


FIGURE 3.2b

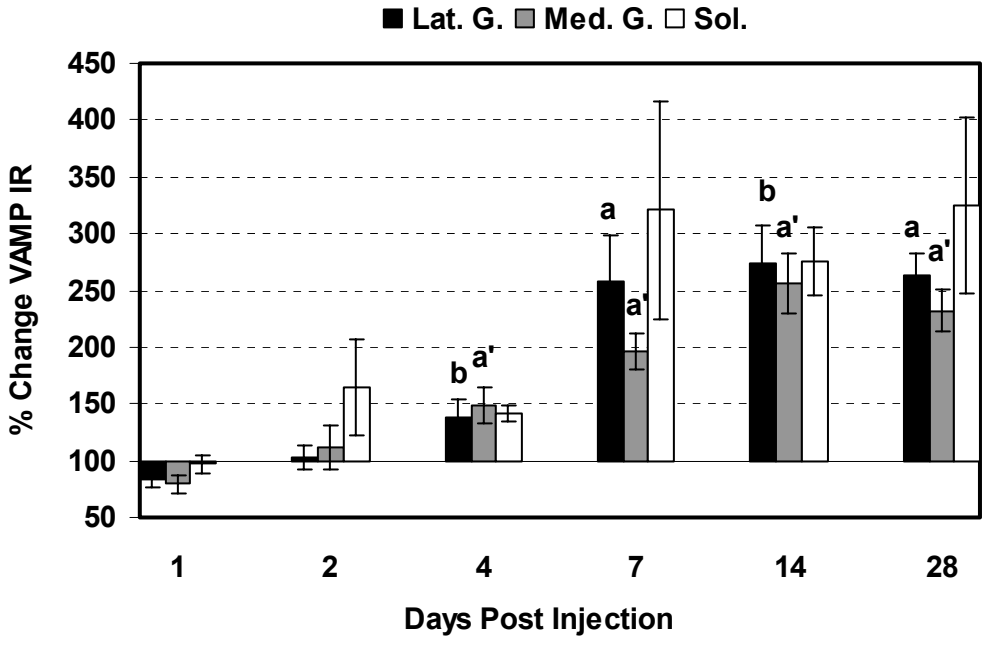
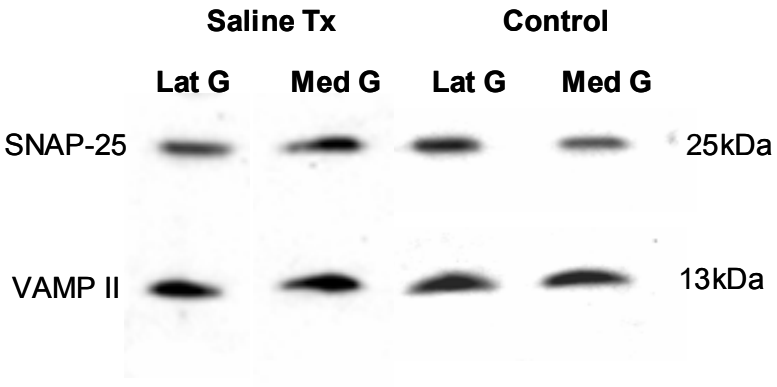


FIGURE 3.3 Immunoblot analysis of full length SNAP-25 and VAMP II immunoreactivity after saline injections. Full length SNAP-25 and VAMP II immunoreactivity was detected in muscles from both a saline-treated mouse and a control (non-treated) mouse using western blot analysis as previously described. Immunoreactivities were assessed 7d after saline injections. The saline-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the non-injected mouse.

FIGURE 3.3


Saline Tx
Control
Lat G
Med G
Lat G
Med G
SNAP-25
25kDa
VAMP II
13kDa

CHAPTER 4

CHANGES IN SNARE PROTEIN IMMUNOREACTIVITY FOLLOWING RE-INJECTION OF BOTULINUM SEROTYPE A

INTRODUCTION

Botulinum neurotoxin has proven to be a valuable tool in the treatment of various neuromuscular disorders. Currently, formulations of serotypes A (BOTOX ™) and B (MYOBLOC™) have received FDA approval for use in human medicine. Serotype A is known to cleave the SNARE protein, SNAP-25 while serotype B cleaves VAMP II. Although there are many promising uses for BoNT/A in clinical medicine, several problems are associated with toxin treatment, one of these being the duration of the desired effect. Depending upon the individual patient and the disorder being treated, the duration of toxin-induced paralysis usually lasts between 3-6 months. Thus, repeat BoNT/A injections are often needed to maintain the desired clinical response.

Several studies, conducted in mice, have documented the formation of neuronal sprouts in muscles previously treated with BoNT/A and D, (Angaut-Petit et al., 1990; Comella et al., 1993; Juzans et al., 1996; de Paiva et al., 1999). de Paiva et al. (1999) correlated sprout formation and maturation with the recovery from BoNT/A induced paralysis in the mouse sternomastiod muscle. These neuronal sprouts formed functional endplates and signs of paralysis resolved within 28-30 days post BoNT/A treatment. At later time points PI, sprouts receded and full function returned to the original endplate region. Although a correlation between sprout formation and recovery has not been documented in human subjects, sprouts likely form in response to BoNT/A-induced paralysis and effectively re-innervate toxin-treated muscles to promote recovery. Therefore, sprout formation may reduce the duration of paralysis, necessitating additional injections to maintain the desired clinical effects achieved from BoNT/A treatment. Thus, in order to maximize the benefits of BoNT therapy, it is necessary to understand the role of sprout formation in recovery and the susceptibility of these new sprouts to toxin action.

The previous studies presented in this thesis have documented increases in full length SNAP-25 and VAMP II immunoreactivities at various time points post BoNT/A injection. These increases may indicate either de novo synthesis of SNARE proteins in the poisoned terminals and/or sprout formation. A question raised by the clinical community is whether these "sprouts" are susceptible to toxin action. Therefore, the experiments conducted in this chapter addressed the susceptibility of these new proteins and/or sprouts to further BoNT/A treatments.

MATERIALS AND METHODS Briefly, six groups of mice received an initial injection of BoNT/A in the lateral gastrocnemius muscle at d0; three of the groups then received a second injection of BoNT/A at a designated time point. Mice were monitored daily for clinical changes and sacrificed at select time points. SNARE protein immunoreactivity was analyzed at select time points post re-injection (PRI) and compared to that found at corresponding times in mice receiving only one injection.

NIH Swiss adult male mice (25-30g) were injected in the lateral gastrocnemius at d0 with pure BoNT/A (150 kDa) and saline vehicle in the corresponding muscle of the contra-lateral limb. Mice were monitored daily for clinical changes and signs of paralysis; toe spread and plantar flexion movements were assessed in both limbs as previously described (CH 2). All dosages and injection procedures were preformed as described (CH 2). Three groups of mice were injected only once at d0 (N=18), one cohort was sacrificed at d9 post-initial injection (PI), the second cohort at d14 PI , and the third at d31 PI (data from d14 PI was collected from previous experiments described in CH 2 and 3). The remaining three groups of mice received a second injection of BoNT/A (N= 21); two groups were re-injected at d7 PI and one at d28 PI. In groups re-injected at d7, one cohort was sacrificed at d2 PRI and the other cohort was sacrificed at d7 PRI. Mice re-injected at d28 were sacrificed 3 d later. Muscle tissues and serum

were collected and processed as previously described (CH 2 and 3). SNARE protein chemistry from processed muscle tissues was analyzed using standard western blot techniques. At designated time points, both cleaved SNAP-25 product and changes in full length SNARE protein immunoreactivity were compared between mice receiving a single injection and those receiving a second injection of BoNT/A.

Clinical Assessment After both initial and repeat injections, mice were monitored daily for clinical changes as previously described (CH 2).

Western Blot Analysis Proteins from soluble, S3 fractions and the synaptosomal enriched P3 fractions were separated using SDS-PAGE and then transferred to PVDF membranes as previously described (CH 2 and 3). Briefly, after blocking, membranes were incubated with primary antibodies. Next, membranes were washed and then incubated with a horseradish peroxidase conjugated secondary antibody for 1 h at 25 ˚C followed by immunodetection with enhanced chemiluminescence (ECL^{+}; Amersham Life Science, Inc., Arlington Height, IL). All immunoreactivities were measured by scanning densitometry (Scion Image; Scion Corporation, Frederick, MD). SNAP-25 cleaved product levels were determined for both toxin and saline-treated limb muscles (lateral and medial gastrocnemius) along with the diaphragm muscle. The percent cleaved product and the cleaved product ratio were calculated as previously described (CH 2). Full length SNAP-25 and VAMP II immunoreactivities were determined in both the lateral and medial gastrocnemius muscles of both limbs. The percent change in full length SNAP-25 and VAMP II was calculated for muscles in the toxin-treated limb as previously described (CH 3). Immunoreactivities were also analyzed in the corresponding muscles of previously un-injected mice; these measurements served as qualitative controls, (not shown).

Antibodies A polyclonal rabbit anti-SNAP-25 antibody (1:15,000; Sigma-Aldrich; St Louis, MO) was used for the detection of full length SNAP-25 (25kDa; S3 and P3

fractions) and cleaved SNAP-25 product (24kDa; S3 fractions). A rabbit polyclonal anti-VAMP II antibody (1:4,000; Wako Chemicals, Richmond, VA) was used to detect full length VAMP II (P3 fractions). A goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Biosource; Camarillo, CA) was used at a 1:5,000 dilution.

Statistics Changes in SNARE protein immunoreactivities were analyzed after re-injection by comparing levels detected in mice receiving two injections to levels measured at the corresponding time point in those receiving a single injection. For instance, SNARE protein immunoreactivities from mice sacrificed 2 d post re-injection (PRI; D7+2) were compared to those of mice sacrificed at d9 post single injection (PI). Likewise, immunoreactivities from mice sacrificed at 7 d PRI (D7+7) were compared to those measured in mice sacrificed at d14 PI. Comparisons were also made between mice sacrificed 3 d after re-injection at d28 (D28+3) and mice sacrificed at d31 PI. All significant differences were determined using a two-tailed Student's t-test.

RESULTS

Clinical Assessment All mice showed a reduction in both toe spread and plantar flexion in the toxin-treated limb within 24 h after the initial injection. In mice re-injected at d7, peak improvements were observed before the second injection, between d6-7 PI. Within 24-48 h after the second injection, further impairments in both movements occurred again in the toxin-treated limb, and paralysis was similar to that recorded shortly after the initial injection. From d5-7 PRI, both assessed movements showed improvement but paralysis remained. During this period, plantar flexion showed the most noticeable improvement (Figures 4.1). In mice receiving only a single injection at d0, nearly complete recovery occurred in the toxin-treated limb by d31 PI, (not shown). However, in mice re-injected at d28, paralysis returned to the toxin-injected limb shortly

after re-injection and no improvement in either movement was observed by d3 PRI (Figure 4.7).

Changes in SNAP-25 Cleaved Product after Re-Injection of BoNT/A Cleaved SNAP-25 immunoreactivity was detected in the toxin-treated limb, the saline-treated limb and diaphragm muscles at all time points PI and PRI.

D7 Re-Injection In mice re-injected at d7, large percentages of cleaved SNAP-25 product were measured in muscles from the toxin-treated limb at both d2 and d7 PRI. Interestingly, the percentages of cleaved product in both groups of re-injected mice were not different from those observed at the same time points after a single injection. However, at d7 PRI, individual immunoblots revealed a noticeable increase in cleaved SNAP-25 product (24kDa) immunoreactivity compared to levels detected at d14 PI (Figure 4.2). Despite this, the mean percent cleaved product maintained levels of ~60% in muscles from the toxin-treated limb at all time points with or without re-injection (Figure 4.3a).

At all observed time points (both PI and PRI), the percent cleaved SNAP-25 product remained below 50% in both the saline-treated limb and diaphragm muscles. Percentages were also below those observed in the muscles from the toxin-treated limb at all observed time points (PI and PRI). Only a small increase in the percent cleaved SNAP-25 product was detected in both the saline-treated limb and diaphragm muscles from d2-7 PRI, (Lat G: 5%, Med G: 8%, Diaph: 2%; Figure 4.3b). Furthermore, little change was observed between percentages at 2d PRI and those found at d9 PI. In fact, only a marginal 2% decrease was detected in the medial gastrocnemius at d2 PRI verses d9 PI. However, percentages at d7 PRI, in both the saline-treated limb and diaphragm muscles, increased significantly from those observed at d14 PI, (Lat G: 14%, Med G: 16%, $p<0.05$; and Diaph: 28%, $p<0.05$).

The ratio of cleaved SNAP-25 product immunoreactivity was calculated as the ratio of cleaved product immunoreactivity in muscles from the toxin-treated limb to that detected in muscles from the saline-treated limb, at designated time points PI and PRI. The ratio of cleaved SNAP-25 remained consistent from d2-7 PRI in all observed muscles. When ratios were compared between re-injected mice and those receiving only one injection, little change occurred at d2 PRI compared to d9 PI, (Figure 4.4). Although the cleaved SNAP-25 product ratio in the medial gastrocnemius increased slightly (0.5%) within 2 d PRI, the ratio in the lateral gastrocnemius muscle maintained levels found at d9 PI, (D7+2: 2.1; D9: 2.3). However at d7 PRI, the ratio cleaved SNAP-25 product decreased in both the lateral and medial gastrocnemius muscles from values observed at d14 PI (1.6 and 2.3, respectively, Figure 4.4). Furthermore, d7 PRI ratios in the lateral gastrocnemius were significantly lower than those found at d14 PI ($p < 0.01$).

D28 Re-Injection Three days post BoNT/A re-injection at d28, similar trends were observed in cleaved product immunoreactivity in all examined muscles. Individual immunoblots revealed strong cleaved SNAP-25 product immunoreactivity in muscles from the toxin-treated limb, (Figure 4.8). Similar to findings post d7 re-injection, the percent cleaved SNAP-25 product maintained levels of ~60% in both the toxin-injected lateral gastrocnemius and neighboring medial gastrocnemius muscles, (Figure 4.9a). Likewise, at d3 PRI, cleaved SNAP-25 product immunoreactivity increased in both the saline-treated limb and diaphragm muscles compared to the levels detected at d31 PI, (Figure 4.9b, Lat G: 10%, Med G: 8%, Diaph: 8%). These increases were significant in the medial gastrocnemius muscle ($p<0.05$). When compared to d31 PI ratios, the ratio cleaved SNAP-25 product at d3 PRI decreased by 1.4 in the lateral gastrocnemius muscle, yet rose by 1.8 in the medial gastrocnemius muscle, (Figure 4.10). Notably, in mice receiving a single injection, the percent cleaved SNAP-25 product decreased from d14-31 PI in muscles of the saline-treated limb (7-10%; Figures 4.3b and 4.10b). During

this period, the ratio cleaved product increased by 2.1 in the lateral gastrocnemius and decreased by 1.2 in the medial gastrocnemius (Figures 4.4 and 4.10).

Changes in Full Length SNARE Proteins after Re-Injection of BoNT/A Mice receiving only a single injection of BoNT/A at d0 showed increases in full length SNAP-25 and VAMP II immunoreactivities in muscles from the toxin-treated limb at d9, d14, and d31 PI, similar to trends previously observed (CH 3). From d9-31 PI, full length SNAP-25 increased 72% in the lateral gastrocnemius and 92% in the medial gastrocnemius muscles of the toxin-treated limb. During the same period, full length VAMP II immunoreactivity decreased 80% in the lateral gastrocnemius, but increased in the medial gastrocnemius muscles of the toxin-treated limb by 116%. The overall increases in full length SNARE protein immunoreactivity coincided with a clinical recovery observed in the toxin-treated limb after a single injection and before re-injection of BoNT/A, (Figures 4.5, 4.6, 4.11, 4.12).

D7 Re-Injection Changes in full length SNARE protein immunoreactivity, in muscles from the toxin-treated limb verses levels in the corresponding muscles of the saline-treated limb, were compared between mice receiving a single toxin injection and those re-injected at designated time points. After re-injection at d7, immunoblots revealed smaller increases in full length SNAP-25 immunoreactivity in muscles from the toxin-treated limb compared to the increases detected at d9 after a single injection of BoNT/A. At d2 PRI, full length SNAP-25 immunoreactivity in the lateral gastrocnemius muscle of the toxin-treated limb was ~69% less than the percentages found at d9 PI (Figure 4.5). However, little change was apparent in the neighboring medial gastrocnemius after re-injection. Similarly, increases in full length VAMP II immunoreactivity, detected in muscles from the toxin-treated limb, at d2 PRI were less than those measured at d9 PI. This difference was significant in the lateral gastrocnemius, (111%, $p<0.01$). Increases in full length VAMP II immunoreactivity in the medial gastrocnemius were 18% less than

d9 PI levels, (Figure 4.6). These smaller increases in both full length SNAP-25 and VAMP II immunoreactivities at 2d PRI coincided with a second onset of impairment observed at this time (24-48 h PRI) in the toxin-treated limb (Figure 4.1).

Both the lateral and medial gastrocnemius muscles of the toxin-treated limb showed substantial increases in both full length SNAP-25 (58% and 33%, respectfully) and VAMP II immunoreactivity (113% and 142%) between d2-7 PRI. However at d7 PRI, increases in full length SNAP-25, in both muscles, were less than those observed at d14 PI (56% less in the Lat G; 28% less in the Med G). Increases in full length VAMP II immunoreactivity at d7 PRI were similar those found at d14 PI; in fact, d7 PRI immunoreactivities increased just slightly from d14 PI values, (Lat G: 17%, Med G: 27%; Figures 4.5 and 4.6).

D28 Re-Injection Unlike the changes in full length SNAP-25 observed after d7 re-injection, less drastic alterations were detected post d28 re-injection. At d3 PRI, full length SNAP-25 immunoreactivity in the lateral gastrocnemius muscle of the toxin-treated limb only increased 7% from d31 PI values. However, in the medial gastrocnemius, increases in full length SNAP-25 at d3 PRI were 38% less than those detected at d9 PI (Figure 4.11). When compared to d31 PI values, full length VAMP II immunoreactivity increased in muscles from the toxin-treated limb after re-injection, (Lat G: 86%, Med G: 11%; Figure 4.12).

DISCUSSION

D7 Re-Injection Although clinical impairment increased in the toxin-treated limb by d2 PRI, both the ratio of cleaved SNAP-25 product and the percent cleaved SNAP-25 product maintained levels observed at d9 PI (Figure 4.3 and 4.4). However, the increases detected in full length SNAP-25 and VAMP II immunoreactivity at d2 PRI were less than those measured at d9 PI; suggesting that full length SNARE protein

immunoreactivity decreased shortly after re-injection (Figure 4.5 and 4.6). Notably, this change was also detected in full length VAMP II immunoreactivity despite the fact that VAMP II is not a substrate of BoNT/A. Further, the decreases observed at 2d PRI mimicked those observed within 48 h post single-injections in CH 3. Therefore, decreases in full length SNARE protein levels observed shortly after both injection and re-injection may be influenced by factors other than toxin enzymatic activity. One possibility may be the influence of a short term, post-injection, acute injury or inflammatory response on SNARE protein chemistry. The lack of clearly definable changes in SNARE protein chemistry, due solely to BoNT/A action at d2 PRI, prevents conclusive determination that de novo SNAP-25 is susceptible to BoNT/A activity shortly after re-injection.

At later time points PRI, slight clinical improvements were apparent, but toe spread and plantar flexion abilities did not recover to the levels observed prior to re-injection. Immunoblots revealed increased cleaved product immunoreactivity from d2-7 PRI as signs of clinical impairment continued in the treated limb (Figures 4.1 and 4.2). Further, cleaved SNAP-25 product immunoreactivity detected in muscles from the toxin-treated limb at d7 PRI increased from the levels observed at d14 PI. However at d7 PRI, the percent cleaved SNAP-25 measured in muscles from the toxin-treated limb did not reveal these increases in cleaved product immunoreactivity. Similar to comparisons between d2 PRI and d9 PI, little change was detected in the percent cleaved product found in muscles from the toxin-treated limb at d7 PRI compared to d14 PI (Figure 4.3a).

Decreases in the ratio of cleaved SNAP-25 product at d7 PRI were likely due to increases in the systemic spread of the toxin, evident through the increase in cleaved SNAP-25 product detected in both the saline-treated limb and the diaphragm muscles at this time (Figures 4.2 and 4.4). When compared to d14 PI, ratios at d7 PRI decreased in both limb muscles, and this change was significant in the lateral gastrocnemius. This

observation, combined with the significant increases in the percent cleaved product at d7 PRI compared to those at d14PI, suggest that toxin activity increased systemically after re-injection. However, at earlier time points PRI, little change was detected in either cleaved product percentages or ratios.

From d2-7 PRI, increases in both full length SNAP-25 and VAMP II immunoreactivity corresponded with signs of slight clinical improvement in the toxin-treated limb. However, when d7 PRI immunoreactivities were compared to those of d14 PI, only differences in full length SNAP-25 levels were found (Figure 4.5). Increases in full length SNAP-25 immunoreactivity detected at d7 PRI were less than those found at d14 PI; this finding suggested that full length SNAP-25 immunoreactivity decreased after re-injection. Full length VAMP II immunoreactivity changed little at d7 PRI when compared to d14 PI levels (Figure 4.6). Thus, the apparent decrease in only full length SNAP-25, combined with the increases in cleaved SNAP-25 product immunoreactivity detected in muscles from the toxin-treated limb were indicative of BoNT/A activity post re-injection. Therefore these findings, combined with the clinical observations, suggest that new SNARE protein, most likely found in sprouts induced by initial injections, were susceptible to repeat toxin treatments.

D28 Re-Injection Despite the strong recovery observed in the toxin-treated limb by d28, severe impairments returned shortly after re-injection at d28. The return of impairment coincided with high levels of cleaved SNAP-25 product detected in muscles from the toxin-treated limb (Figure 4.8). However, similar to findings from d7 re-injections, the percent cleaved product in muscles from the toxin-treated limb at d3 PRI (D28+3) was not different from that found at d31 PI (Figure 4.9a).

Interestingly, percentages detected in the saline-treated limb and diaphragm muscles at d3 PRI were less than those found after d7 re-injection. Further, the percent cleaved SNAP-25 product decreased in the saline-treated limb and diaphragm muscles

from d14-31 PI (Figures 4.3b and 4.9b). Therefore, systemic BoNT/A activity, detected through the presence of cleaved SNAP-25 product in distant muscles, appears to lesson more rapidly than levels found local to the injection site. This may be due to the fact that less BoNT/A reaches distant muscles through systemic diffusion and thus toxin concentration is lower in these terminals. However, systemic BoNT/A activity increased after re-injection. When compared to d31 PI percentages, the percent cleaved SNAP-25 product was greater in both saline-treated limb and diaphragm muscles after d28 re-injection.

Although the increases in full length SNAP-25 immunoreactivity detected in the toxin-treated limb after d7 re-injection were lower than those found at corresponding time points post single-injection, less consistent changes were found after re-injection of BoNT/A at d28. Three days after re-injection (D28+3), full length SNAP-25 levels in the lateral gastrocnemius muscles remained approximately equal to those measured at d31 PI. However, smaller increases in full length SNAP-25 immunoreactivity were detected in the medial gastrocnemius after d28 re-injection. When compared to d31 PI values, full length VAMP II immunoreactivity was greater in both muscles of the toxin-treated limb. Large increases, as those detected in the lateral gastrocnemius, were not observed after d7 re-injections. Unfortunately, a large standard error, calculated for changes in full length SNARE protein at d31 PI hindered meaningful conclusions concerning the susceptibility of de novo SNARE proteins to re-injection at the later time point.

Finally, neither the d2 nor the d3 PRI (D7+2 nor D28+3) time points provided data as useful as d7 PRI (D7+7). This may be partially due to the influence of factors, other than intracellular BoNT/A, on changes in full length SNARE protein immunoreactivities. In fact, trends observed at d7 PRI provided the most valuable

insights concerning the susceptibility of de novo SNARE proteins, possibly formed in sprouts arising from previous BoNT/A injections, to repeated treatments.

REFERENCES

1. Angaut-Petit D, Molgó J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 1990; 37:799-807.
2. Comella JX, Molgó J, Faille L. Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D and the functional recovery of paralysed neuromuscular junctions. Neurosci Lett 1993; 153:61-64.
3. Juzans P, Comella JX, Molgó, J, Failee L, Angaut-Petit D. Nerve terminal sprouting in botulinum type-A treated mouse levator auris longus muscle. Neuromusc Disord 1996; 6 (3):177-185.
4. de Paiva A, Meunier FA, Molgó J, Aoki JR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA 1999; 96:3200-3205.

FIGURE 4.1 Functional assessment of toe spread (TS) and plantar flexion (PF) movements during the observation period before and after d7 re-injection of BoNT/A. Movements were assessed and scored for both the toxin-treated (Tx, filled symbols) and saline-treated limbs (Ctl, open symbols) in mice receiving both an initial injection at d0 and a second injection at d7 of BoNT/A. Movements were scored on a scale from 0 to 6; a score of 0 represents the complete loss of function/movement while a score of 6 represents no impairment in movement/function. Mean scores (±SEM, n≥5 per day) were recorded throughout the 14 d period post initial injection (PI).

FIGURE 4.1

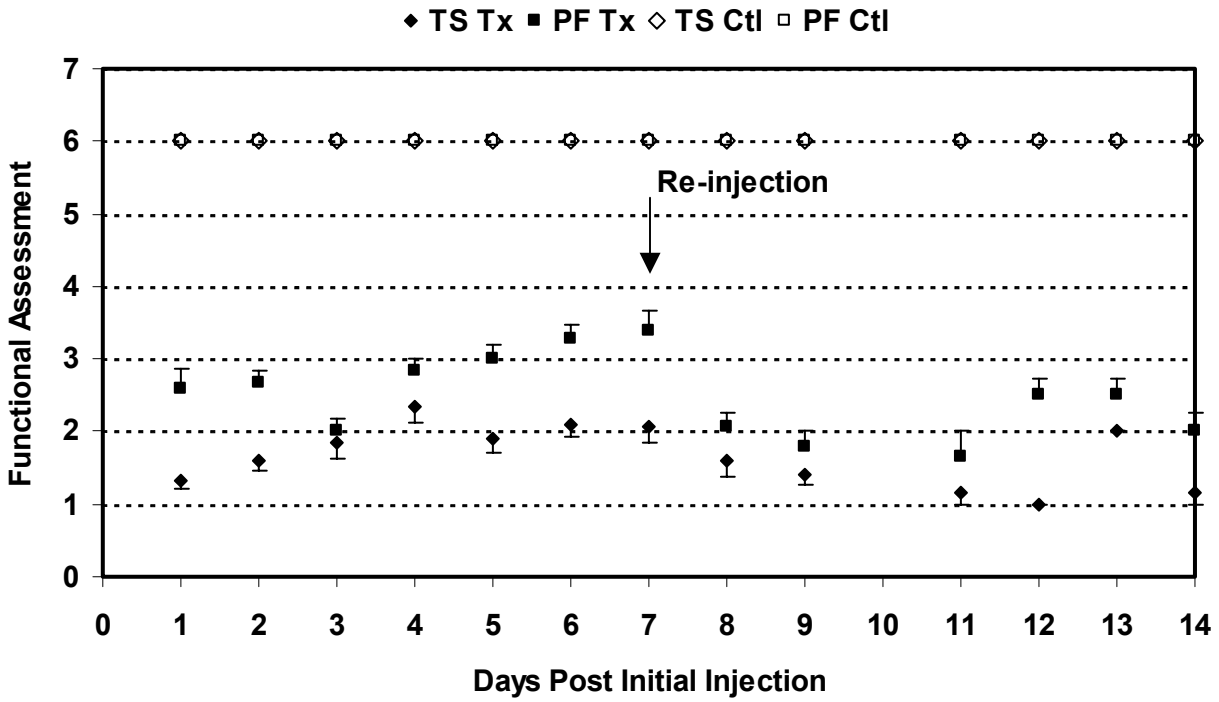

◆ TS Tx ■ PF Tx ◇ TS Ctl □ PF Ctl
Functional Assessment
Re-injection
Days Post Initial Injection
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIGURE 4.2 Immunoblot detection of full length SNAP-25 and cleaved SNAP-25 product immunoreactivity in one mouse at various time points post single injection and d7 re-injection of BoNT/A. Full length (25kDa) and cleaved SNAP-25 (24kDa) immunoreactivities were detected in both the injected lateral gastrocnemius (Lat G) and the adjacent medial gastrocnemius (Med G) of both hind limbs as well as the diaphragm (DPH; S3 fractions; 75 µg/lane, Tris-HCL gels). Muscles from the toxin-treated limb (Tx) were compared along side those from the saline-treated limb (Ctl). Immunoreactivities were assessed after a single injection at d9 and d14 PI, (D9 and D14). Similarly, immunoreactivities were also assessed after d7 re-injection at d2 (D7+2) and d7 (D7+7) post re-injection (PRI).

FIGURE 4.2

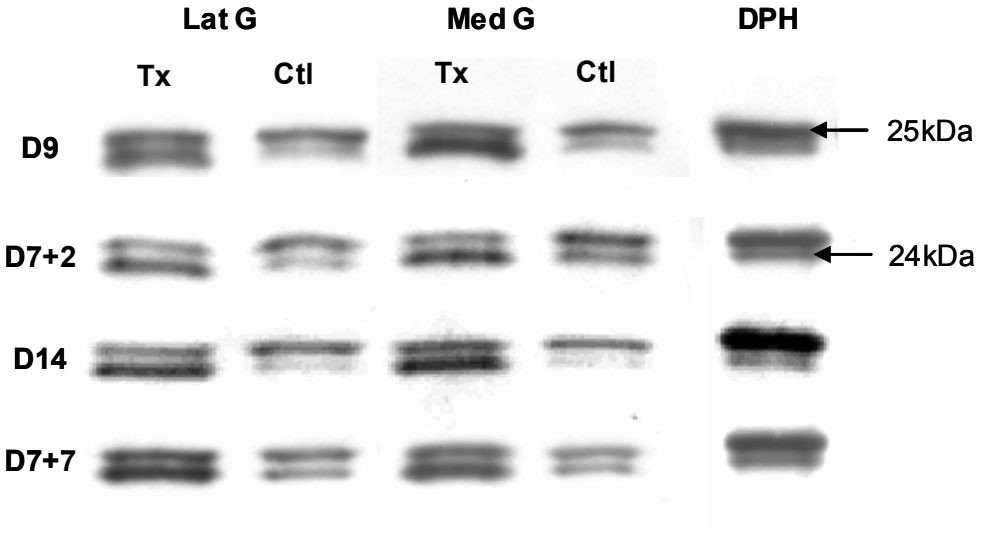


FIGURE 4.3 Changes in the percent cleaved SNAP-25 product found at various times post single injection and d7 re-injection of BoNT/A. The percent cleaved product was calculated as the percentage of cleaved SNAP-25 immunoreactivity (24kDa) out of the total SNAP-25 pool in the S3 fraction [both full length (25kDa) and cleaved]. **a. The percent cleaved product found in muscles of the toxin-treated limb.** Cleaved product levels were assessed in the toxin-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) muscles (mean % ± SEM, n≥5). Percentages were compared between mice receiving a single injection (D9 and D14) and those re-injected with BoNT/A (D7+2 and D7+7). Significant differences between single injection and re-injection data were determined using a two-tailed Student's t-test. **b**. **The percent cleaved SNAP-25 product measured in muscles of the saline-treated limb and distant diaphragm**. Cleaved product levels were measured in the saline-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) of the contra-lateral limb; cleaved product levels were also assessed in the diaphragm muscle (Diaph; mean % ± SEM, n≥4). Significant differences between single injection and re-injection data were determined using a two-tailed Student's t-test. Percentages were compared between mice receiving a single injection (D9 and D14) and those re-injected with BoNT/A (D7+2 and D7+7; **b** (Lat G), **b'** (Med G): $p<0.05$; **a** (Diaph): $p<0.01$).

FIGURE 4.3a

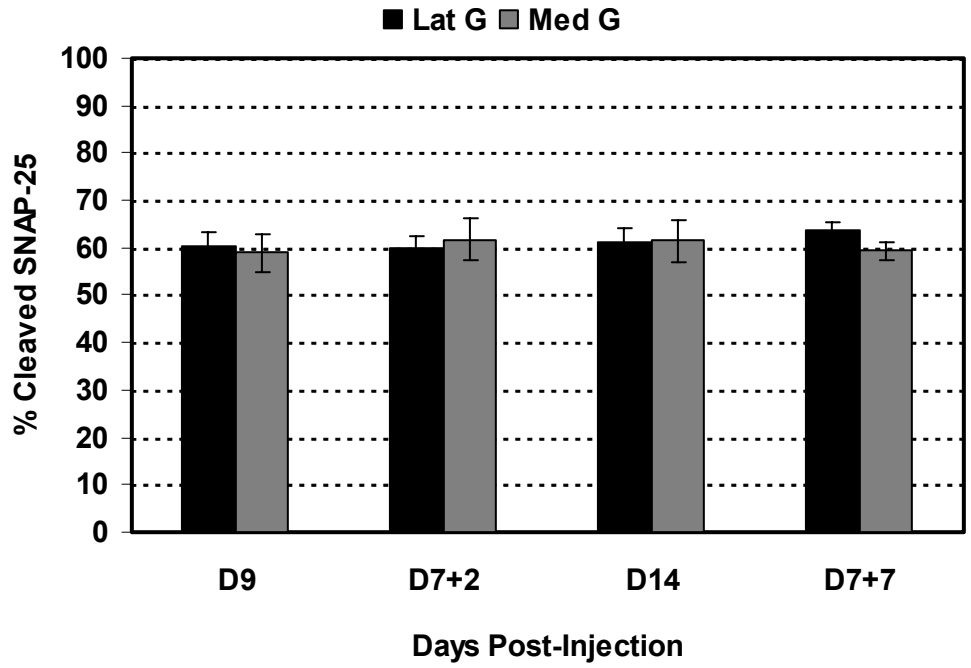


FIGURE 4.3b

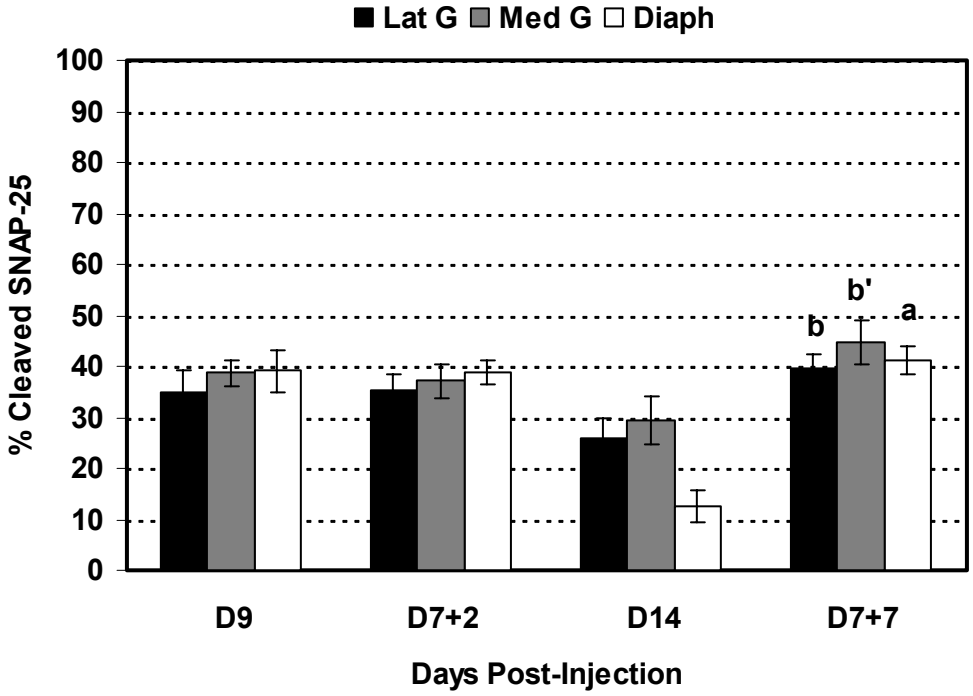


FIGURE 4.4 Comparison of the ratio of cleaved SNAP-25 immunoreactivity in singly-injected and d7 re-injected muscles. The ratio of cleaved SNAP-25 product was calculated by comparing cleaved product immunoreactivity in muscles from the toxin-injected limb to those of the saline-injected limb at each time point PI and PRI. Ratios of SNAP-25 cleaved product (toxin-treated limb: saline-treated limb) were calculated for both the injected Lat G and adjacent Med G, (mean ratio ±SEM, n≥4). Statistically significant changes in ratios after re-injection were determined using a two-tailed Student's t-test; ratios from mice receiving a single injection were determined for D9 and D14 PI and then compared to those from mice re-injected at d7 (D7+2 and D7+7; **a** (Lat G): $p<0.01$).

FIGURE 4.4

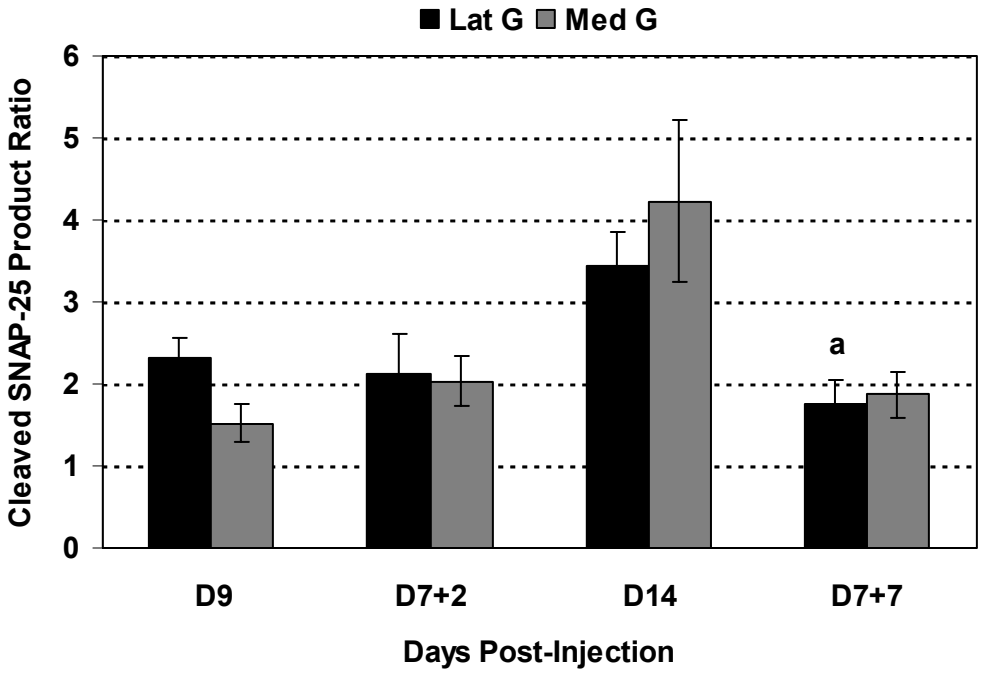

Lat G
Med G
Cleaved SNAP-25 Product Ratio
0
1
2
3
4
5
6
a
D9
D7+2
D14
D7+7
Days Post-Injection

FIGURE 4.5 Comparison of full length SNAP-25 (25kDa) immunoreactivity in singly injected and d7 re-injected muscles. **a. Immunodection of changes in full length SNAP-25 in one mouse.** Using standard western blot analysis, SNAP-25 immunoreactivity was detected in both muscles from the toxin-treated (Tx) and saline-treated (Ctl) limb (P3 fractions; 75µg/lane, Tris-Tricine gels). The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb to determine changes in full length SNAP-25. Immunoreactivities were detected after a single injection of BoTN/A at d9 and d14 PI (D9 and D14). Similarly, immunoreactivities were also assessed at d2 and d7 PRI (D7+2 and D7+7). **b**. **The percent change in full length SNAP-25 immunoreactivity.** The percent change in full length SNAP-25 was calculated as the percent difference of SNAP-25 immunoreactivity in muscles from the toxin-treated limb compared to those of the saline-treated limb. Mean percent changes (±SEM, n≥4) were analyzed in the toxin-injected Lat G and adjacent Med G muscles. Baseline values of 100% represented no change from saline-treated muscles, values above 100% represented increases in SNAP-25 immunoreactivity in the toxin-treated limb, and values less than 100% represented decreases. The percent change was assessed at the designated time points after both single injection and re-injection of BoNT/A as described above. Significant differences between single injection and re-injection data were determined using a two-tailed Student's t-test.

FIGURE 4.5a

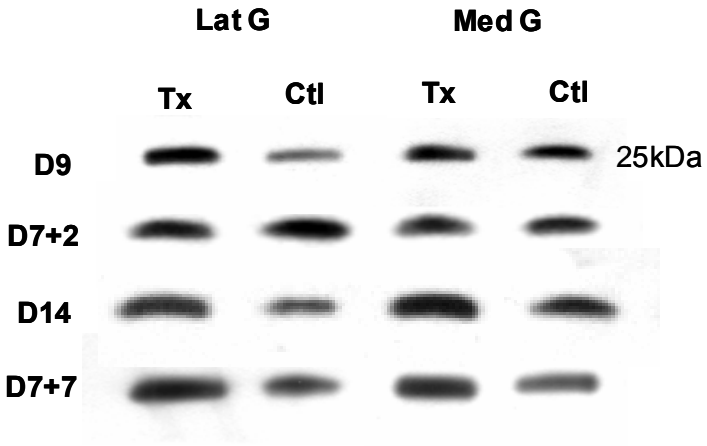


FIGURE 4.5b

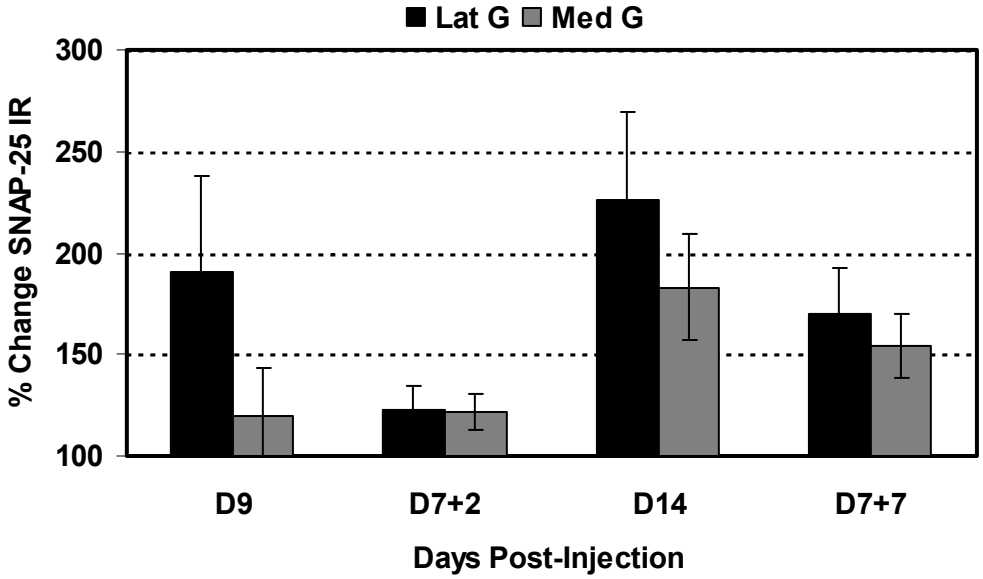


FIGURE 4.6 Comparison of full length VAMP II immunoreactivity in singly injected and d7 re-injected muscles. a. Immunodetection of changes in full length VAMP II in one mouse. Full length VAMP II immunoreactivity was detected in muscles from both the toxin-treated (Tx) and saline-treated (Ctl) limb using western blot analysis (P3 fractions; 75µg/lane, Tris-Tricine gels). The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb to determine changes in VAMP II immunoreactivity. Immunoreactivities were detected at time points after single injection and re-injection as described in Figure 4.5a. **b. The percent change in full length VAMP II immunoreactivity.** The percent change in full length VAMP II immunoreactivity, in muscles from the toxin-treated limb, compared to those from the saline-treated limb, was calculated as previously described for full length SNAP-25 (mean percent change ±SEM, n≥4). Changes in VAMP II immunoreactivity were analyzed for the Lat G and Med G muscles of the toxin-treated limb. The percent change was calculated at designated time points after single injection and re-injection of BoNT/A as described in Figure 4.5b. Significant differences between single injection and re-injection data were determined as described in Figure 4.5b (**a** (Lat G): $p<0.01$).

FIGURE 4.6a

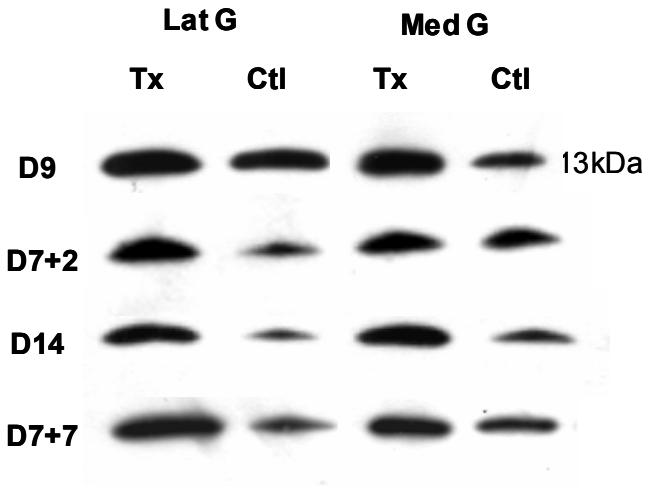


FIGURE 4.6b

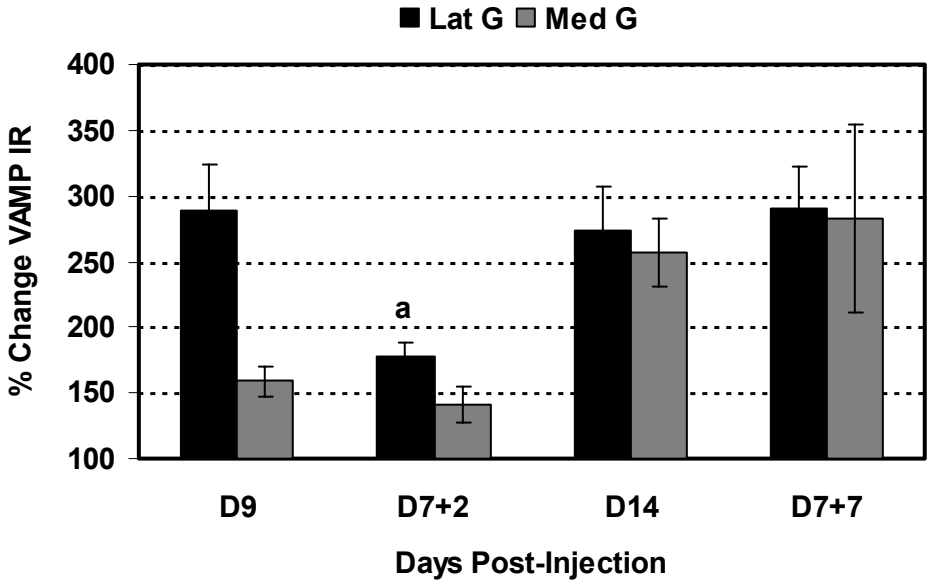


FIGURE 4.7 Functional assessment of toe spread and plantar flexion movements before and after re-injection of BoTN/A at d28. Movements were assessed and scored for both the toxin-treated (Tx, filled symbols) and saline-treated limbs (Ctl, open symbols) in mice receiving both an initial injection at d0 and a second injection at d28 of BoNT/A. Movements were scored as previously described in Figure 4.1. Mean scores (±SEM, n≥3 per day) were recorded throughout the 31 d observation period spanning both initial injection (d0) and re-injection (d28).

FIGURE 4.7

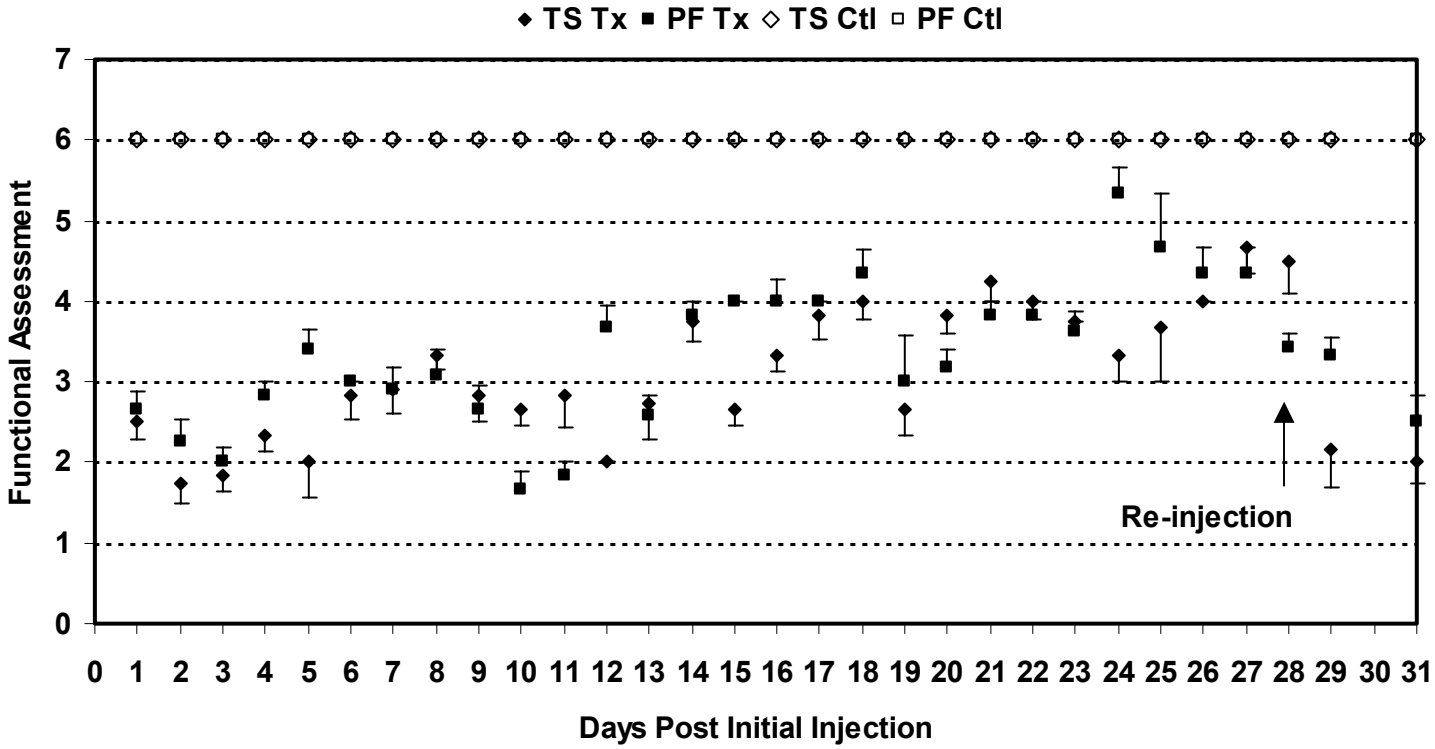

◆ TS Tx ■ PF Tx ◇ TS Ctl □ PF Ctl
Functional Assessment
0
1
2
3
4
5
6
7
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Days Post Initial Injection
Re-injection

FIGURE 4.8 Immunoblot detection of full length SNAP-25 and cleaved SNAP-25 product immunoreactivity after single injection and re-injection of BoNT/A at d28. Full length (25kDa) and cleaved SNAP-25 (24kDa) immunoreactivities were detected in both the injected lateral gastrocnemius (Lat G) and the adjacent medial gastrocnemius (Med G) of both hind limbs as well as the diaphragm (DPH; S3 fractions; 75 µg/lane, Tris-HCL gels). Immunoreactivities were assessed at d31 PI (D31) for a mouse receiving a single injection and compared to those from a mouse re-injected at d28 (D28+3).

FIGURE 4.8

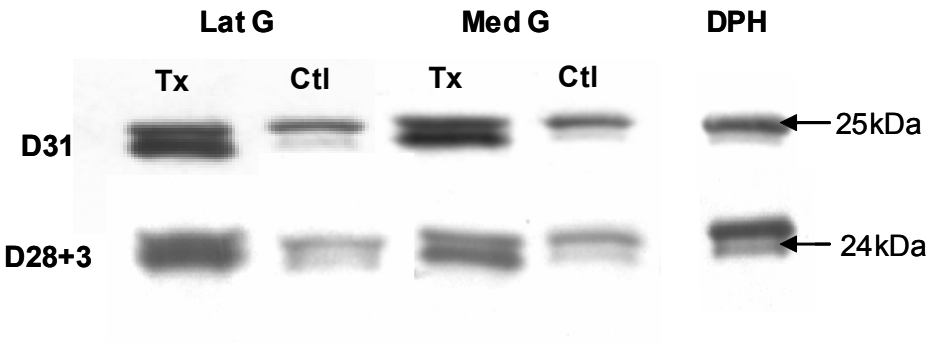


FIGURE 4.9 Comparisons of the percent cleaved SNAP-25 product in singly injected and d28 re-injected muscles. The percent cleaved product was calculated as previously described in Figure 4.3. Percentages were calculated in mice receiving a single injection at d31 PI (D31) and after re-injection at d28 (D28+3) **a. The percent cleaved product found in muscles from the toxin-treated limb**. The percent cleaved product was assessed in the toxin-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) muscles (mean % ± SEM, n≥4). Percentages were compared between mice receiving a single injection (D31) and those re-injected at d28 (D28+3). **b**. **The percent cleaved SNAP-25 product measured in the saline-treated limb and distant diaphragm**. Cleaved product levels were measured in the saline-injected lateral gastrocnemius (Lat G) and adjacent medial gastrocnemius (Med G) of the contra-lateral limb; cleaved product levels were also assessed in the diaphragm muscle (Diaph; mean % ± SEM, n≥4). Percentages were compared as described above. Significant differences between single injection and re-injection data were determined using a two-tailed Student's t-test. (**b** (Med G): $p<0.05$).

FIGURE 4.9a

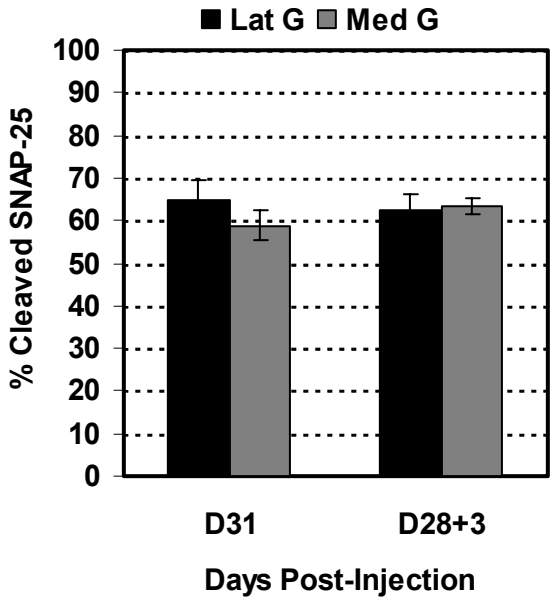


FIGURE 4.9b

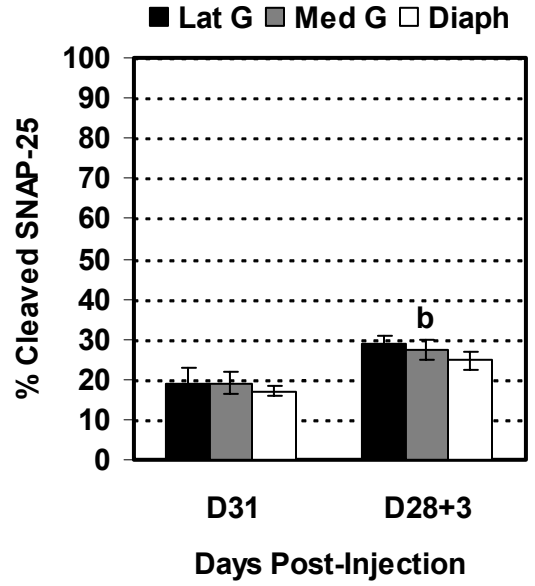


FIGURE 4.10 Comparison of cleaved SNAP-25 product ratios in singly injected and d28 re-injected muscles. The ratio of cleaved SNAP-25 product was calculated as previously described. Ratios were calculated for both the injected Lat G and adjacent Med G, (mean ratio ±SEM, n≥3). Ratios from mice receiving a single injection were determined for d31 PI then compared to those from mice re-injected at d28 (D28+3). Statistically significant changes in ratios after re-injection were determined using a two-tailed Student's t-test.

FIGURE 4.10

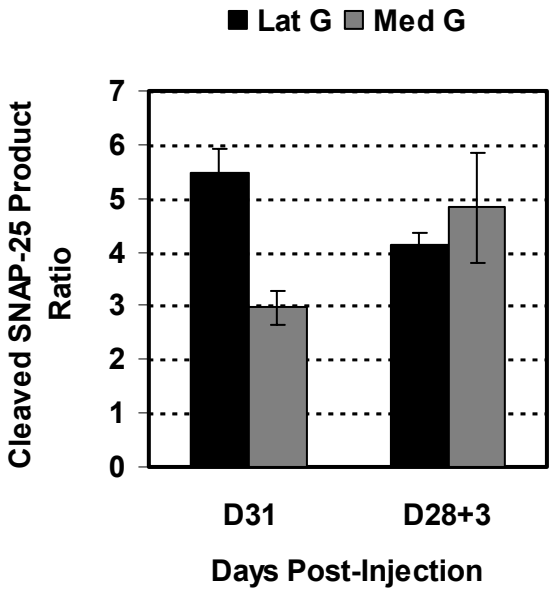

Lat G
Med G
Cleaved SNAP-25 Product Ratio
7
6
5
4
3
2
1
0
D31
D28+3
Days Post-Injection

FIGURE 4.11 Comparison of full length SNAP-25 (25kDa) immunoreactivity in singly injected and d28 re-injected muscles. **a. Immunodection of changes in full length SNAP-25.** SNAP-25 immunoreactivity was detected in both muscles from the toxin-treated (Tx) and saline-treated (Ctl) using western blot analysis as previously described. The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb. Immunoreactivities were detected at d31 PI (D31) from a mouse receiving a single injection and in a mouse re-injected at d28 (D28+3). **b**. **The percent change in full length SNAP-25 immunoreactivity.** The percent change in full length SNAP-25 immunoreactivity detected in muscles from the toxin-treated limb was calculated as described in Figure 4.5b. Mean percent changes (±SEM, n≥5) were analyzed in the toxin-injected Lat G and adjacent Med G muscles after single injection (D31) and re-injection (D28+3). Baseline values of 100% represented no change from saline-treated muscles, values above 100% represented increases in SNAP-25 immunoreactivity in the toxin-treated limb, and values less than 100% represented decreases. Significant differences between single injection and re-injection data were determined as previously described, (Figure 4.5).

FIGURE 4.11a

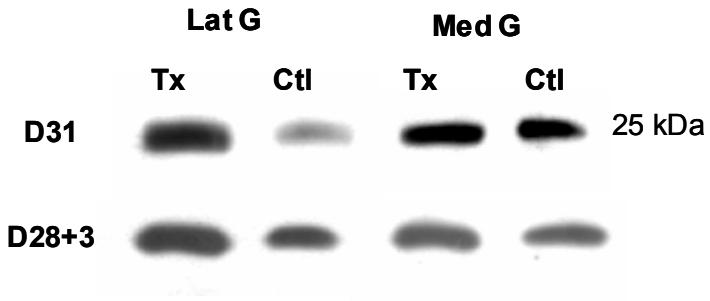


FIGURE 4.11b

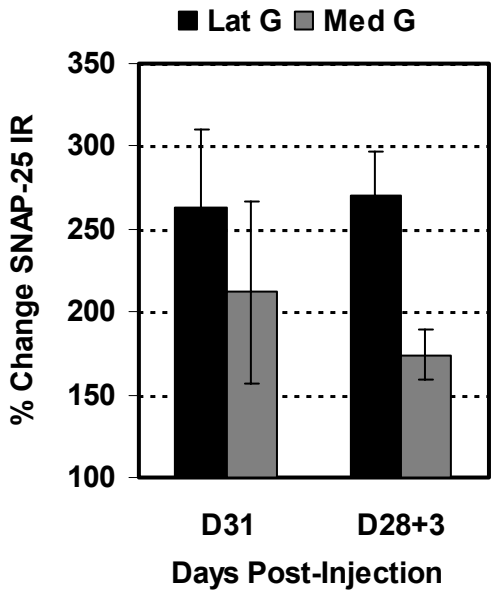


FIGURE 4.12 Comparison of full length VAMP II immunoreactivity in singly injected and d28 re-injected muscles. a. Immunodetection of changes in full length VAMP II. Full length VAMP II immunoreactivity was detected in muscles from both the toxin-treated (Tx) and saline-treated (Ctl) limb using western blot analysis as previously described. The toxin-injected Lat G and adjacent Med G muscles were examined along side the corresponding muscles from the saline-injected, contra-lateral limb. Immunoreactivities were detected after single injection (D31) and re-injection (D28+3). **b. The percent change in full length VAMP II immunoreactivity.** The percent change was calculated for the Lat G and Med G muscles of the toxin-treated limb as previously described (mean percent change ±SEM, n≥3). Percentages were calculated after a single injection at d31 PI (D31) and after d28 re-injection (D28+3). Significant differences between single injection and re-injection data were determined as previously described, (Figure 4.5).

FIGURE 4.12a

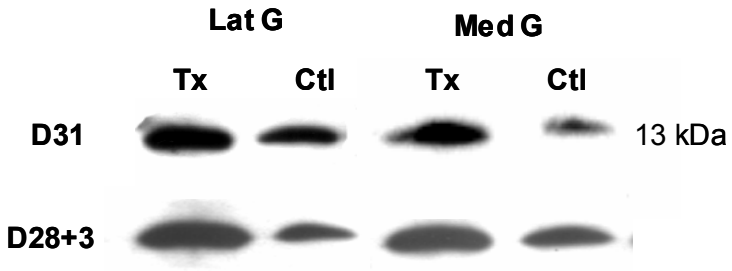
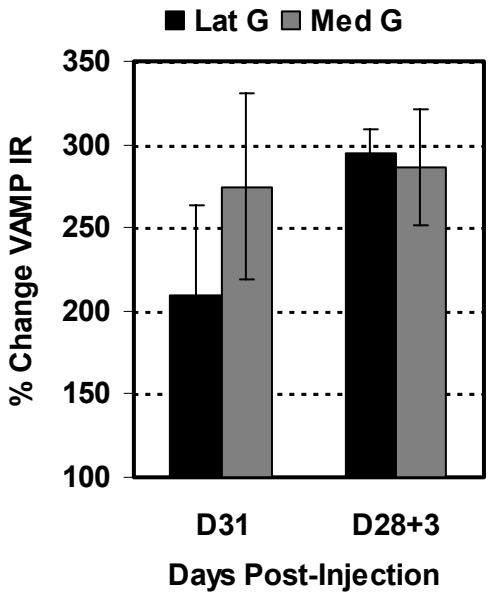


FIGURE 4.12b



CHAPTER 5

CONCLUSIONS

Botulinum toxin is the agent responsible for botulism, a disease characterized by a flaccid muscle paralysis resulting from the inhibition of neurotransmitter release at the neuromuscular junction. However, over past years, botulinum toxin has proven to be a valuable clinical tool in the treatment of various neuromuscular disorders, such as focal spasms (including dystonias), spasticities, tics and tremors, (Jankovic and Brin, 1997). Despite the numerous benefits of BoNT, there are problems associated with the therapeutic use of the toxin; two such problems are the diffusion of toxin into undesired muscle groups and the duration of desired clinical effects. Several studies, in humans, have documented undesirable weakness in both nearby and distant muscle groups to those being treated with BoNT, (Bhatia et al. 1999; Eleopra et al. 1996). Due to the fact that toxin poisoning results in a transient paralysis at the NMJ, the desired effects of toxin treatment diminish within 3-6 months, necessitating repeat injections. Previous studies in mouse muscle have shown that as a response to BoNT induced paralysis, neuronal sprouts form in toxin-treated muscles, temporarily re-innervating these muscles and promoting functional recovery (Angaut-Petit et al., 1990; Comella et al., 1993; Juzans et al., 1996, de Paiva et al. 1999). This, in turn, may decrease the duration of the desired therapeutic effects.

The current study addressed both the problems of toxin diffusion from the injection site and the duration of treatment using an in-vivo mouse model. Briefly, mice received intramuscular injections of BoNT/A (150kDa) in a designated limb and sham (saline vehicle) injections in the corresponding muscle of the contra lateral limb. Changes in SNARE protein chemistries from the injected, neighboring, and distant muscles were then correlated with clinical signs of paralysis and recovery at various time points post-injection. Immunodetection of cleaved SNAP-25 product in both muscles adjacent to and distant from the injection site indicated diffusion of the toxin, while

changes in full length SNAP-25 and VAMP II immunoreactivities indicated increases likely associated with neuronal sprouting.

Cleaved SNAP-25 Product Immunoreactivity Analysis of cleaved SNAP-25 product levels demonstrated that both local and systemic diffusion of the toxin occurred within 24 h PI. High levels of cleaved SNAP-25 product detected in the toxin-treated limb shortly after injection coincided with the onset of impairments observed in this limb. The peak impairments in toe spread observed in this study corresponded with a DAS score of 3 in other in-vivo studies (Aoki et al., 2001). In muscles from the treated limb, the percent cleaved SNAP-25 product increased between d1-7, then stabilized at throughout the rest of the 28 d period. SNAP-25 cleaved product immunoreactivity was detected in both the saline-treated limb and diaphragm muscles throughout the 28 d period; however, impairments were not observed in these muscles and levels were significantly lower than those measured in the toxin-treated limb. Furthermore, cleaved SNAP-25 product increased in these muscles over time at a much slower rate than observed in muscles from the toxin-treated limb. This likely occurred because of the longer time course needed for a sufficient amount of toxin to diffuse systemically and yield detectable cleaved product levels in distant muscles. In addition, a shorter duration of systemic BoNT/A activity was apparent when the percent cleaved SNAP-25 product was compared in muscles from the toxin-treated limb to those of the saline-treated limb from d14-28 PI. This finding is also likely due to the fact that a lower concentration of toxin accumulated in distant terminals. Notably, cleaved SNAP-25 product immunoreactivity was not detected by d7 PI in control mice receiving only unilateral saline injections.

Temporal changes in the ratio of cleaved SNAP-25 product corresponded with clinical observations throughout the 28d period. Despite the high percent cleaved SNAP-25 product detected in the toxin-treated limb, changes in the percent cleaved product over time were likely masked by increases in full length SNAP-25. Although the

highest observed ratios corresponded with the onset of impairment, and the lowest ratios coincided with initial signs of recovery in the toxin-treated limb. Changes in the ratio of cleaved SNAP-25 product appeared to be tri-phasic. Cleaved SNAP-25 product immunoreactivity in the toxin-treated limb had a greater affect on ratios shortly after injection (d1-2), while levels detected in the saline-treated limb had a greater influenced at later times PI (d4-7). However, during the most notable period of clinical improvement in the toxin-treated limb (d14-28), ratios rose once more. Elevated ratios detected at these later time points were likely due to increases in cleaved SNAP-25 product levels in the toxin-treated limb as new full length SNAP-25 protein was conceivably proteolyzed by toxin.

Increases in Full Length SNAP-25 and VAMP II Immunoreactivity Changes in full length SNAP-25 and VAMP II immunoreactivity further corresponded to clinical observations throughout the 28 d period. Initial decreases in full length SNAP-25 and VAMP II protein immunoreactivity were measured at d1 and these changes coincided with the onset of clinical paralysis in the toxin-treated limb. However, the decrease in full length VAMP II levels suggests that these early changes may have resulted from a combination of a direct effect of the toxin on SNAP-25 and an indirect effect from a post-trauma inflammatory response. By d4, full length SNAP-25 and VAMP II immunoreactivities (lateral and medial gastrocnemius muscles) were significantly higher than those of d1 and coincided with the initial signs of clinical improvement in the treated limb. As full length SNAP-25 and VAMP II immunoreactivities continued to increase throughout the 28 d period, the largest increases corresponded with the most distinct signs of recovery in the toxin-treated limb. Moreover, increases in full length SNAP-25 and VAMP II immunoreactivities were not detected in mice receiving saline injections only.

Dynamic Changes in SNARE Proteins and Recovery Post Toxin Treatment

Although the appearance of substantial cleaved SNAP-25 product levels coincided with the onset of impairments in the toxin-treated limb, low levels of cleaved SNAP-25 product immunoreactivity were detected in muscles distant to the injection site. However impairments were not observed in these muscles. Thus, both toxin concentration and/or activity in these distant muscles were too low to produce observable, clinical effects. These findings suggest that a distinct level of toxin activity must be achieved in numerous nerve terminals innervating a muscle to produce visible signs of clinical paralysis. Although it is tempting to propose that a threshold of cleaved SNAP-25 product must be reached to induce muscle paralysis, data from this study suggest that this conclusion does not take into account the dynamic changes occurring in SNARE protein in-vivo.

Previous studies have identified intracellular BoNT/A activity through the loss of full length SNAP-25 (Kalandakanond and Coffield, 2001); however, this current study clearly demonstrated that, due to dynamic changes in SNARE protein levels, the presence of cleaved SNAP-25 immunoreactivity was a better indicator of intracellular toxin activity in-vivo. Although initial decreases in both full length SNAP-25 and VAMP II immunoreactivity occurred within 24 h, immunoreactivities increased by 48h PI and continued to do so drastically throughout the 28 d period as paralysis began to abate in the toxin-treated limb. These finding support the conclusion that de-novo synthesis of new SNARE proteins and/or sprout formation occurred in muscles from the toxin-treated limb, enabling a considerable recovery from BoNT/A induced paralysis by d28 PI.

Although the increases in SNARE protein immunoreactivity detected in this study were likely due to de-novo synthesis and/or neuronal sprout formation, the techniques used in these experiments were incapable of differentiating between these possibilities. Interestingly, immunoblots revealed increases in cleaved SNAP-25 product

immunoreactivity over time, even as improvements were detected in the toxin-treated limb. These increases may have resulted from the cleavage of new SNAP-25 protein, synthesized in the previously poisoned nerve terminals. However, if the increases in full length SNAP-25 solely resulted from the synthesis of new protein in previously poisoned terminals, persistent intracellular BoNT/A activity would likely prevent substantial recovery. Large increases were also detected in full length VAMP II throughout the observation period. Although new SNARE proteins may have been synthesized in the original terminals, the significant increases in both full length SNAP-25 and VAMP II immunoreactivity, coupled with the time course of recovery, supports the conclusion that sprout formation occurred in poisoned muscles. Further, although recovery was defined through visual assessments of whole limb function and a different methodology was used to detect increases in SNARE protein levels, findings from this present study, by and large, agree with the time course for sprout formation and maturation presented in previous works (de Paiva et al. 1999).

Findings from this study demonstrate that the duration of toxin-induced paralysis in-vivo is due to a delicate interplay between the longevity of intracellular BoNT/A activity and changes in SNARE protein chemistry. Interestingly, previous in-vitro studies have shown a prolonged effect of BoNT/A compared to other toxin serotypes. However, a current debate exists over whether this effect is due to a persistence of toxin activity or interactions between the cleaved SNAP-25 product (24 kDa) and other SNARE proteins. Such interactions would potentially form non-functional SNARE complexes, thus shutting down the exocytotic machinery, (Adler et al., 2001; Keller et al., 1999 and 2001). While the current study does not define the lifespan of the 24 kDa product, the increases in cleaved SNAP-25 product detected in the immunoblots from d7-28 PI, suggest a continuance of toxin activity in poisoned terminals.

In order to completely understand the complexities of BoNT action in-vivo, further studies are necessary. Experiments, conducted in a similar manner to those of this work, using different serotypes of BoNT, would provide further insight into temporal changes in SNARE protein chemistry associated with the duration of clinical effects. Although changes in both full length SNAP-25 and VAMP II immunoreactivities were analyzed in this study, changes in Syntaxin 1A immunoreactivity post BoNT/A treatment should also be considered. Furthermore, examination of SNARE protein mRNA levels PI would potentially indicate the upregulation of SNARE proteins associated with de novo protein synthesis and/or neuronal sprouting.

Clearly, findings from the current study offer valuable insight towards the clinical use of BoNT/A. Results documented that both local and systemic diffusion of toxin occur PI. Further, findings suggest that the duration of paralysis is affected, not only by the longevity of intracellular BoNT/A activity, but also by temporal changes in SNARE protein chemistry.

Changes in SNARE Protein Chemistry Post Re-Injection Identify Possible BoNT/A Activity in Neuronal Sprouts The formation of neuronal sprouts in toxin-treated muscles likely influences recovery from BoNT/A induced paralysis and thus the duration of desired therapeutic effects. Therefore, it is necessary to investigate the susceptibility of these newly formed sprouts to additional toxin treatments. In these studies, changes in SNARE protein chemistry were assessed to determine the susceptibility of new SNARE protein, possibly formed in neuronal sprouts, to further BoTN/A treatments.

The return of paralysis in the toxin-treated limb observed after re-injection of BoNT/A suggested that newly formed SNARE proteins, possibly found in sprouts induced from previous treatments, were susceptible to repeated BoNT/A injections. Despite the improvements in toe spread and plantar flexion that had taken place before re-injection, a severe clinical impairment returned to the toxin-treated limb within 24-48 h

PRI. High levels of cleaved SNAP-25 product immunoreactivity detected 2-3 d after re-injection coincided with the return of impairment; however, the percent cleaved SNAP-25 product found in muscles from the toxin-treated limb PRI showed little change from percentages observed at corresponding time points PI.

Changes in the percent cleaved SNAP-25 product did not provide valuable insights concerning the susceptibility of newly formed SNARE protein to repeated BoNT/A treatments. However, percentages detected at d7 PRI (D7+7) and at d3 PRI (D28+3) further emphasized the differences in the time course for the appearance and the duration of cleaved product in distant muscles. Due to the fact that cleaved product levels decreased in distant muscles after a single injection from d14-31 PI, noticeable increases in the percent SNAP-25 cleaved product were detected in both the saline-treated limb and diaphragm muscles at d7 PRI and d3 PRI. These increases were also reflected through decreases in the ratio of cleaved SNAP-25 product found in the lateral gastrocnemius muscle at these time points PRI.

De novo formation of new SNARE proteins, possibly in sprouts resulting from previous BoNT/A injections, may decrease the duration of clinical impairment in toxin-treated muscles. However, the rapid recurrence of clinical impairment PRI implicated a susceptibility of the toxin-treated limb to repeated BoTN/A treatments. Unfortunately, data from days closest to re-injection, (d2 and d3 PRI) yielded inconclusive findings concerning the vulnerability of new SNARE proteins to repeated toxin treatments. Decreases in full length SNARE protein immunoreactivities found at d2 PRI (D7+2) mimicked those found at d1 post single injection, illustrating that changes in SNARE protein chemistry may have been altered by mechanisms other than intracellular toxin activity. However, the trends observed at d7 PRI (D7+7) provided the most convincing evidence that new SNARE proteins, possibly found in sprouts, were susceptible to repeated BoNT/A injections. Decreases solely in full length SNAP-25 combined with the

increases in cleaved SNAP-25 product immunoreactivity detected in muscles from the toxin-treated limb would likely be indicative of BoNT/A activity post re-injection. In order to fully understand the potential susceptibility of newly synthesized SNARE proteins to successive BoNT/A injections, additional studies must be conducted. Findings from this study suggest that examining changes in SNARE protein chemistry at longer intervals PRI may provide the most valuable insights.

Similarly, repeated injections of BoNT/A may further induce additional synthesis of new SNARE proteins and/or neuronal sprouting. Researchers have suggested that in conditions of denervation similar to those induced by the BoNT/A, genes expressed during embryonic neuronal development are once again "turned-on." In such instances, increases in numerous growth factors, adhesion molecules, and peptides appear to play a role in neuronal sprouting (NMJ; Booth et al., 1990; Caroni et al. 1994; Frey et al. 2000, Ishii, DN 1989; Sakuma et al., 2001; Siegel et al, 2000; Yamauchi et al. 2000). Therefore, frequently repeated injections of BoNT/A may induce molecular events atypical to the normal adult NMJ. In order to insure the safety and efficacy of the long-term clinical BoNT/A therapy, it is necessary to fully understand the dynamic changes occurring at the NMJ post injection.

To date, only serotypes A and D have been shown to induce neurite or neuronal sprout formation (Angaut-Petit et al., 1990; Bonner et al., 1994; Comella et al., 1993; Juzans et al., 1996; de Paiva et al., 1999). Therefore, it is plausible that certain serotypes of BoNT may play an additional role in the stimulation of neuronal sprouting. Insights into the molecular events of sprout formation may enable methods to be developed that impede sprout formation and thus prolong the desired clinical effects achieved through BoNT/A treatment.

REFERENCES

1. Adler M, Keller JE, Sheridan RE, Deshpande SS. Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle. Toxicon 2001; 39:233-242.
2. Angaut-Petit D, Molgó J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 1990; 37:799-807.
3. Aoki KR. A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon 2001; 39:1815-1820.
4. Bhatia KP, Münchau A, Thompson PD, Houser M, Chauhan VS, Hutchinson M, Shapira AHV, Marsden CD. Generalized muscular weakness after botulinum toxin injections for dystonia: a report of three cases. J Neurol Neurosurg Psychiatry 1999; 67:90-93.
5. Bonner PH. Botulinum A toxin stimulates neurite branching in nerve-muscle cocultures. Dev Brain Res 1994; 79:39-46.
6. Booth CM, Kemplay SK, Brown MC. An antibody to neural cell adhesion molecule impairs motor nerve terminal sprouting in a mouse muscle locally paralysed with botulinum toxin. Neurosci 1990; 35 (1):85-91.
7. Caroni P, Schneider C, Kiefer MC, Kapf J. Role of muscle insulin-like growth factors in nerve sprouting: suppression of terminal sprouting in paralyzed muscle by IGF-binding protein 4. J Cell Biol 1994; 125 (4):893-902.
8. Comella JX, Molgó J, Faille L. Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D and the functional recovery of parlaysed neuromuscular junctions. Neurosci Lett 1993; 153:61-64.

9. Eleopra R, Tugnoli V, Caniatti L, De Grandis D. Botulinum toxin treatment in the facial muscles of humans: Evidence of an action in untreated near muscles by peripheral local diffusion. Neurology 1996; 46:1158-1160.
10. Frey D, Laux T, Xu L, Schneider C, Caroni P. Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. J Cell Biol 2000; 149 (7):1443-1453.
11. Ishii DN. Relationship of insulin-like growth factor II gene expression in muscle to synaptogenesis. Proc Natl Acad Sci USA 1989; 86 (8):2898-2902.
12. Jankovic J, Brin MF. Botulinum toxin: Historical perspectives and potential new indications. Muscle Nerve 1997; 20 (suppl 6):S129-S145.
13. Juzans P, Comella JX, Molgó, J, Failee L, Angaut-Petit D. Nerve terminal sprouting in botulinum type-A treated mouse levator auris longus muscle. Neuromusc Disord 1996; 6 (3):177-185.
14. Kalandakanond S, Coffield JA. Cleavage of intracellular substrates of botulinum toxins A, C, and D in mammalian target tissue. J Pharmacol Exp Ther 2001; 296 (3):749-755.
15. Keller JE, Neale EA, Olyer G, Adler M. Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett 1999; 456:137-142.
16. Keller JE, Neale EA. The role of synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. J Biol Chem 2001; 276 (16):13476-13482.
17. de Paiva A, Meunier FA, Molgó J, Aoki JR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA 1999; 96:3200-3205.
18. Sakuma K, Watanabe K, Sano M, Uramoto I, Nakano H, Li Y, Kaneda S, Sorimachi Y, Yoshimoto K, Yasuhara M, Totsuka T. A possible role for BDNF,

NT-4 and TrkB in the spinal cord and muscle of rat subjected to mechanical overload, bupivacaine injection, and axotomy. Brain Res 2001; 907:1-19.

19. Siegel SG, Patton B, English AW. Ciliary neurotrophic factor is required for motorneuron sprouting. Exp Neurol 2000; 166:205-212.
20. Yamauchi T, Maeda M, Tamai S, Tamai M, Yajima H, Takakura Y, Haga S, Yamamoto H. Collateral sprouting mechanism after end-to-side nerve repair in the rat. Med Electron Microsc 2000; 33:151-156.