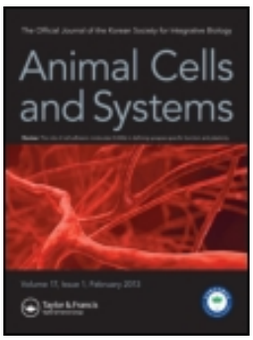

Taylor & Francis
Taylor & Francis Group

Animal Cells and Systems

ISSN: 1976-8354 (Print) 2151-2485 (Online) Journal homepage: https://www.tandfonline.com/loi/tacs20

Phenotypic effect of botulinum toxin A on *Caenorhabditis elegans*

Dong-Wan Kim, Sun-Kyung Lee & Joohong Ahnn

To cite this article: Dong-Wan Kim, Sun-Kyung Lee & Joohong Ahnn (2014) Phenotypic effect of botulinum toxin A on *Caenorhabditis elegans* , Animal Cells and Systems, 18:3, 172-177, DOI: 10.1080/19768354.2014.915883

To link to this article: https://doi.org/10.1080/19768354.2014.915883

Published online: 03 Jun 2014.

Animal Cells and Systems, 2014
Vol. 18, No. 3, 172–177, http://dx.doi.org/10.1080/19768354.2014.915883

Taylor & Francis
Taylor & Francis Group

Phenotypic effect of botulinum toxin A on *Caenorhabditis elegans*

Dong-Wan Kim[a,b†], Sun-Kyung Lee[a,b,c†] and Joohong Ahnn[a,b,c*]

[a]*Department of Life Science, Hanyang University, Seoul 133-791, Republic of Korea;* [b]*BK21 PLUS Life Science for BDR Team, Hanyang University, Seoul 133-791, Republic of Korea;* [c]*The Research Institute for Natural Sciences, Hanyang University, Seoul 133-791, Republic of Korea*

(*Received 20 March 2014; accepted 11 April 2014*)

Botulinum toxins (BoTXs) are potent inhibitors of neurotransmission mediated by exocytosis, cleaving synaptosomal-associated protein of 25 kDa (SNAP-25) to cause paralysis and death in human. Among them, botulinum toxin type A (BoTX/A) is both clinically and cosmetically important because of its wide application in clinical and cosmetic treatment. Therefore, development of feasible and effective model-base assay system might be very helpful to understand the effect of the toxin. In this study, we have examined the physiological effect of BoTX/A on *Caenorhabditis elegans*, a free-living soil nematode model system. Worms treated with BoTX/A showed defective egg-laying, yolk protein consumption, and locomotion while they were normal in developmental process. Worms' susceptibility to BoTX/A suggests potential utility for the assay of BoTX/A.

Keywords: botulinum toxin; SNAP-25; *Caenorhabditis elegans*

Introduction

Botulinum toxin (BoTX) is a deadly poison causing botulism, a paralysis of food-borne intoxication (Davletov et al. 2005). There are seven immunologically distinct BoTX serotypes named A–G according to the order of their discovery. All BoTXs are produced by *Clostridium* bacteria as single polypeptide chains with a molecular mass of 150 kDa having an intramolecular disulfide bond. The polypeptide is cleaved by either bacterial or host protease and produces to the 50 kDa light chain and the 100 kDa heavy chain, which are kept together by the disulfide bond. In the incident of food-poisoning, the two chains traverse the intestinal epithelial cells, enter the bloodstream, and reach peripheral cholinergic nerve endings. They bind to a high-affinity cell surface receptor containing polysialogangliosides. After being internalized, the light chain is dissociated from the heavy chain to become active zinc endopeptidase, which cleaves various soluble NSF-attachment-protein receptors (SNAREs), the neurotransmitter release apparatus. As a result, neurotransmitter vesicle fusion is largely blocked, and synaptic transmission is completely inhibited to cause flaccid paralysis and death.

BoTX/A is the first BoTX identified and the most prevalent cause for human botulism (Davletov et al. 2005). Only one microgram of BoTX/A can kill an adult human, and this extraordinary toxicity also contributes to induce the longest paralysis in human neuromuscular junction among all BoTXs. BoTX/A cleaves the C-terminal nine amino acids of synaptosome-associated protein of 25 kDa (SNAP-25) in human. The truncated SNAP-25 may be retained to interact with plasma membrane syntaxin, thus interfering with the normal vesicle fusion process to result in the highest potency of toxicity.

It was observed that the injection of minute doses of BoTX/A into overstimulated muscles can lead to local relaxation lasting several months in the 1970s (Johnson 1999). This interesting discovery resulted in a clinical use of BoTX/A as a medicine to treat misaligned eyes and hemifacial spasms by ophthalmologists, who often reported a harmless side effect of disappearance of wrinkles around the injection sites. This serendipitous observation led to realization that such injections can be used for cosmetic purposes. BoTX/A can block not only innervation of striated muscles but also of smooth muscles. Furthermore, the cholinergic junctions of the autonomous nervous system that control sweating, salivation, and other types of secretion are sensitive to BoTX/A.

The list of therapeutic targets in human bodies for BoTX/A has been extended, and the use of BoTX/A in clinical medicine includes dystonias, hyperhidrosis, and gastrointestinal disorders (Cherington 2004). Therefore, a variety of assay system has been developed to determine the activity of the toxin, utilizing model systems including chick neurons (Stahl et al. 2007). However, it is unknown whether BoTX/A has a physiological effect on *Caenorhabditis elegans*. We assayed developmental process, locomotion and reproduction of *C. elegans* treated with BoTX/A and found that neuromuscular transmission and endocytosis in embryos might be susceptible to BoTX/A.

*Corresponding author. Email: joohong@hanyang.ac.kr
†Authors contributed equally to this work.

Materials and methods

C. elegans *strains*

N2 and *pwIs23[vit-2::GFP]* worms were obtained from Caenorhabditis Genetics Center (CGC; University of Minnesota, USA). Worms were cultured, according to the established methods (Brenner 1974).

Chemicals

All chemicals were purchased from Sigma-Aldrich (USA). BoTX type A (BoTX/A) was obtained from HUGEL, Inc. (Kangwon, South Korea).

Microscopy

pwIs23[vit-2::GFP] treated with or without BoTX/A were immobilized on 2% agarose pads with 10 mM levamisole. Fluorescent images were collected with an Axio Imager A1 microscope (Carl Zeiss, Germany). Fluorescence intensity was measured using AxioVision image analysis program, AxioVs40 (v.4.6.3.0; Carl Zeiss Imaging Solution GmbH, Germany). Plotting and statistical analysis of data were performed using Prism software (v.5.01; Graph Pad Software Inc., USA). Significance of quantified data was analyzed using unpaired *t*-test with Welch's correction.

Developmental delay, paralysis, and egg-laying

N2 was allowed to lay eggs in Nematode Growth Media (NGM) plates containing BoTX/A for 2 hr, and then the number of worms at each stage was scored at days 3 and 4. Synchronized N2 worms were soaked in M9 buffer or 0.9% NaCl solution containing BoTX/A for 1 hr, transferred to 1 mM aldicarb-containing NGM plates, and then the fraction of paralyzed animals was calculated. In egg-laying assay, serotonin was added after the BoTX/A soaking process, and then the number of laid eggs was counted.

Results

BoTX/A and developmental process in C. elegans

BoTX/A is internalized at the presynaptic ending, activated by acidification in endosomes containing the toxin (Figure 1). It has been unknown whether BoTX/A has a physiological effect on *C. elegans*. The hermaphrodite is able to self-fertilize its own oocytes with sperms produced in its own gonad. Larvae are hatched from laid eggs and develop through four stages or molts, designated as L1–L4. They usually become adults on Day 4, when cultured under standard laboratory condition at 20°C (Brenner 1974). In order to see whether BoTX/A has any effect on general development, we counted the number of animals at different developmental stages at days 3 and 4 after hatching. When

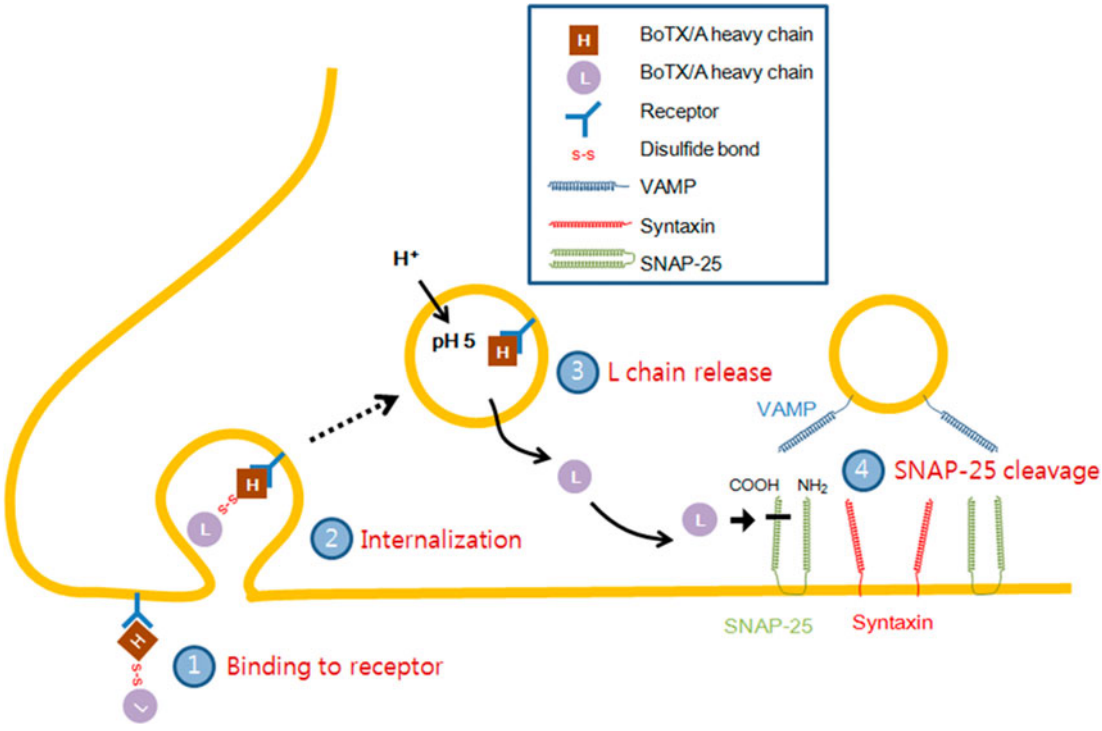

Figure 1. The action mechanism of BoTX/A. (1) BoTX/A heavy chain binds to a high-affinity receptor on the cell surface of the nerve terminal. (2) BoTX/A-Receptor complex is internalized via endocytotic route. (3) Acidification of the endocytotic vesicle containing the BoTX/A reduces disulfide bond to release the light chain into the cytosol. (4) The light chain cleaves SNAP-25, a kind of SNARE.

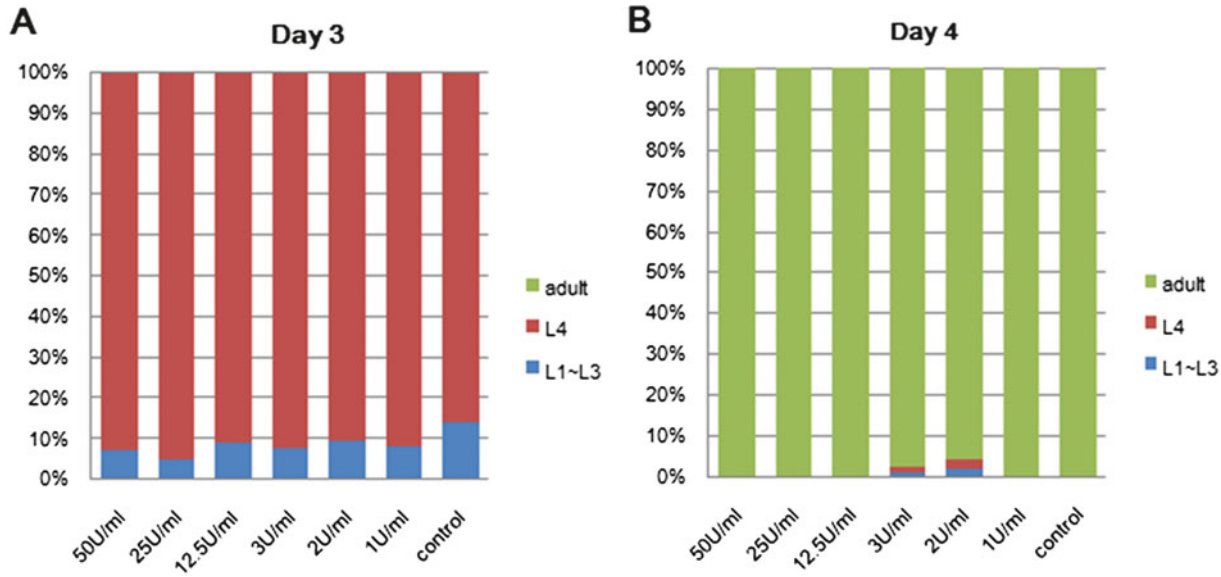

Figure 2. Developmental progress was not delayed by the treatment of BoTX/A. The fractions of animals in each developmental stage from L1 to adults were calculated at day 3 (A) and day 4 (B) after hatch.

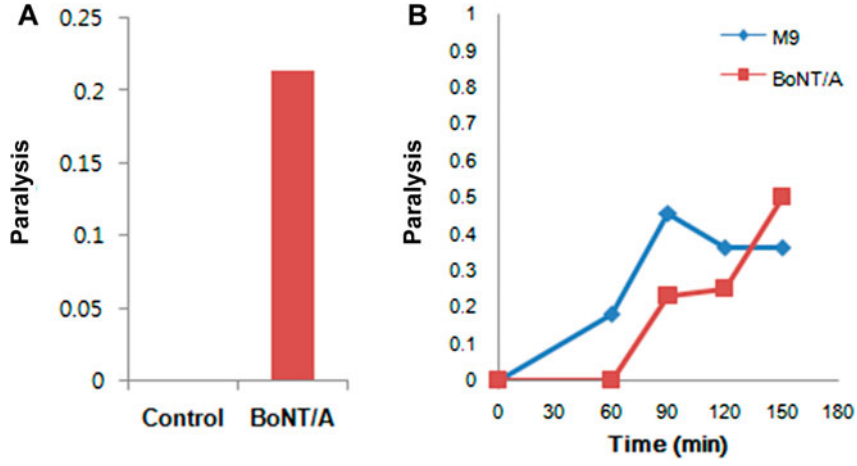

Figure 3. Change in locomotion. Synchronized adult worms were soaked in 400 U/ ml BoTX/A for 1 hr, transferred to NGM plates with (A), or without (B) 1 mM aldicarb, and then paralyzed worms were scored. Fraction of paralyzed worms was plotted.

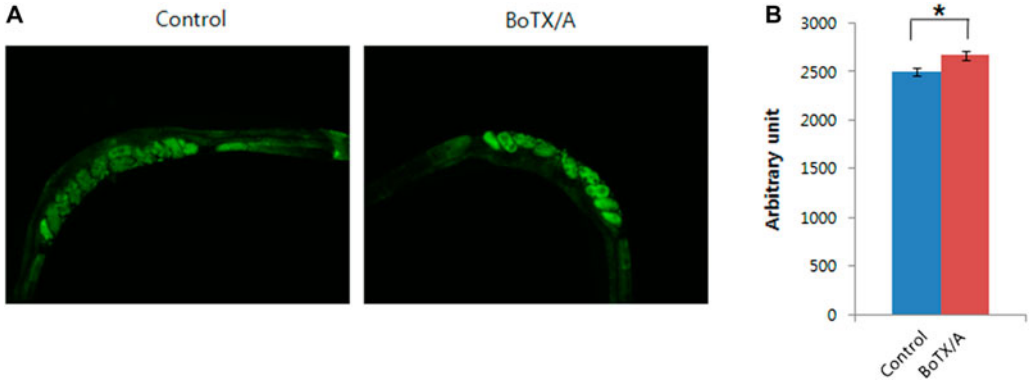

Figure 4. Egg-yolk protein uptake. Fluorescence of YP170::GFP was increased in the embryos of the animals treated with 400 U/ml BoTX/A. Representative figures are shown in (A) and quantified data are plotted (B). *$p < 0.01$.

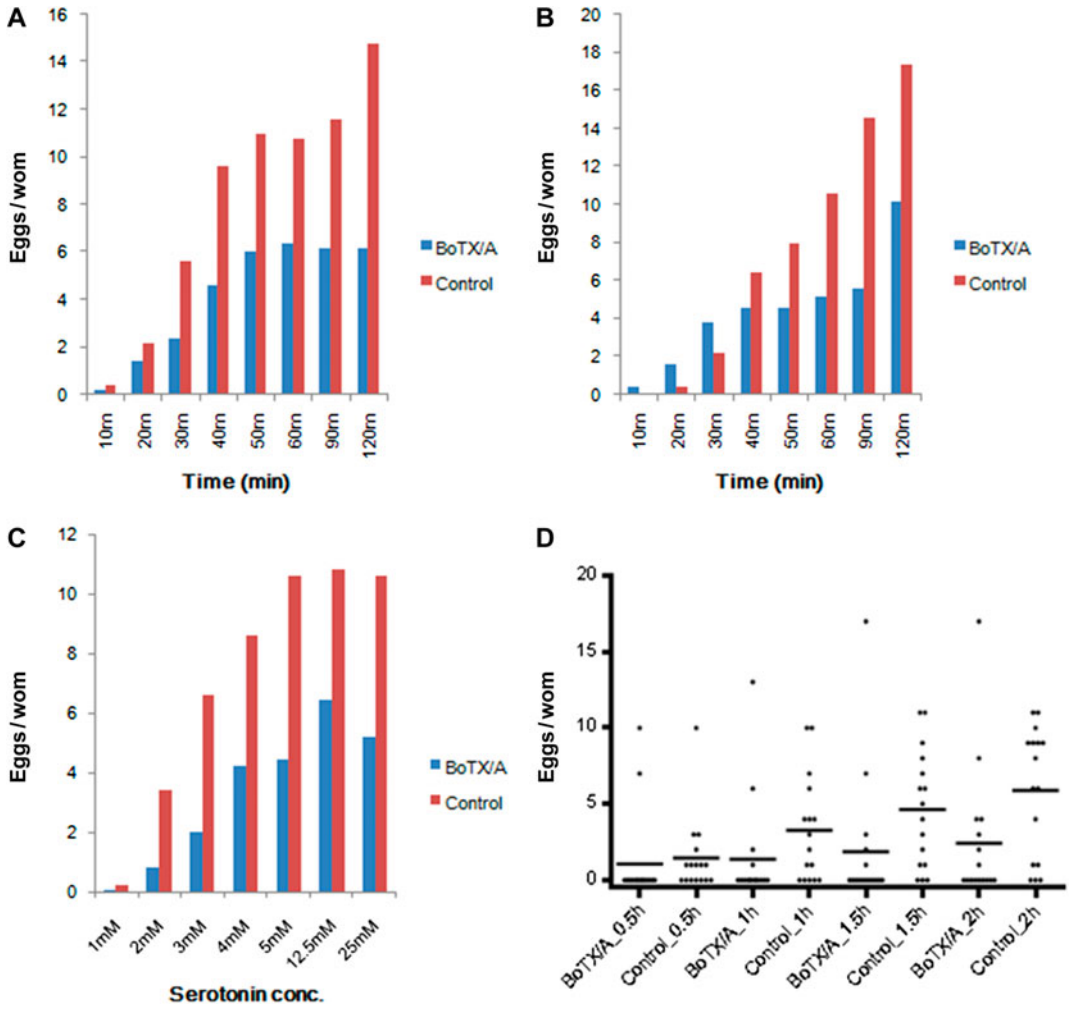

Figure 5. Egg-laying is suppressed by 400 U/ml BoTX/A treatment in *C. elegans*. The numbers of laid eggs of five worms are plotted from the animals treated with (A) 12.5 mM and (B) 25 mM at indicated duration, and for (C) 60 minutes with given concentrations. (D) The number of eggs/worm is shown when animals were treated with 25 mM serotonin for indicated incubation time.

cultured on NGM plates containing 1–50 U/ml BoTX/A, approximately 90% of the animals developed to L4 stage at day 4, and almost all of them became young adults at Day 4 (Figure 2). This result suggests that BoTX/A may not severely affect general developmental process of *C. elegans*.

BoTX/A and locomotion in C. elegans

BoTX/A blocks the release of neurotransmitter at the neuromuscular junction (NMJ) by inhibiting neurotransmitter vesicle fusion (Breidenbach & Brunger 2005). Therefore, we directly tested whether BoTX/A causes paralysis on *C. elegans*. After being soaked in 400 U/ml BoTX/A for 60 minutes, approximately 20% of the worms were not able to move freely, while no control animal showed abnormal locomotion (Figure 3A).

Aldicarb is an inhibitor for acetylcholine esterase and induces hypercontracted paralysis in worms (Wormbook. org). Mutant worms, which are impaired in acetylcholine synthesis, release or reception are resistant to aldicarb. In order to see whether BoTX/A affects locomotion in *C. elegans* in the presence of aldicarb, worms were placed on aldicarb-containing NGM plates after being treated with BoTX/A. Overall, worms soaked in M9 containing 500 U/ml BoTX/A were resistant to aldicasrb at the early response, although there was no difference at the later stage of incubation (Figure 3B). These results suggest that BoTX/A has some effects on locomotion of *C. elegans*.

Egg-laying behavior of C. elegans treated with BoTX/A.

Recently, BoTXs have been reported to be translocated into cells via endocytosis (Restani et al. 2012). In *C. elegans*, YP170, a vitallogenin yolk protein, is produced and secreted by the intestine, and then endocytosed into oocytes which are eventually fertilized to become embryos (Kim et al. 2011). To test whether BoTX/A has any effect on

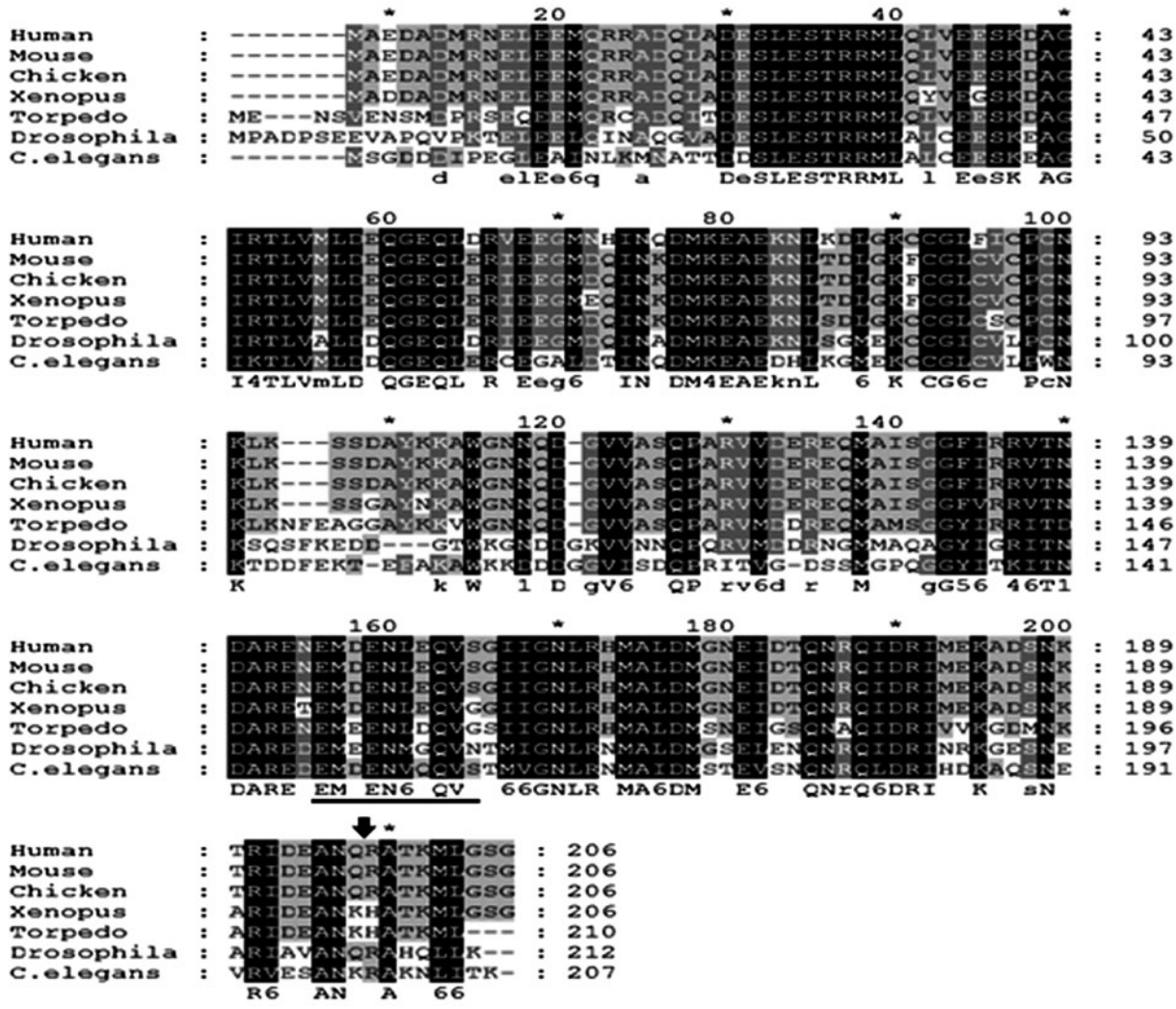

Figure 6. The sequences of SNAP-25 from various species are aligned using CLUSTAL X. An arrow indicates cleavage site of BoTX/A, and SNARE motif required for the cleavage is underlined. Accession numbers for SNAP-25 are AAH10647.1 for human, NP_035558.1 for mouse, NP_990789.1 for chicken, AAI70206.1 for *Xenopus*, AAA49284.1 for *Torpedo*, NP_001036641.1 for *Drosophila*, and CAA16322.2 for *C. elegans*.

endocytosis in *C. elegans*, *pwIs23[vit-2::GFP]*, a YP170: GFP-expressing transgenic line was treated with BoTX/A. Fluorescent intensity in embryos of worms treated with BoTX/A was slightly, but significantly increased (Figure 4A and 4B).

Effect of BoTX/A on vitallogenin endocytosis in worms led us to investigate egg-lyaing behavior of worms treated with the toxin. When 12.5–25 mM serotonin was added, worms treated with 400 U/ml BoTX/A laid about 25–50% less eggs compared to control animals over 2-hr time period (Figure 5A and 5B). Egg-laying was also suppressed, when the worms were treated with various concentrations between 1 and 25 mM serotonin for 1 hr (Figure 5C). Distribution of the number of single-worm laid eggs in case of 25 mM serotonin also indicated that BoTX/A interferes with normal egg-laying (Figure 5D). These results might suggest that BoTX/A prevents normal embryogenesis and egg-laying behavior in *C. elegans*.

Discussion

BoTX/A is a deadly toxin of botulism and also a useful material to treat a variety of clinical and cosmetic conditions. SNAP-25, a SNARE protein functioning in exocytosis at synapse, is known as a sole substrate for BoTX/A. We show here that BoTX/A affects some neurological physiology such as locomotion and egg-laying, but not on developmental process in *C. elegans*.

Worms treated with BoTX/A showed slight paralysis and resistance to aldicarb, though the development proceeded at normal rate. The toxin treatment also resulted in the increase of yolk protein concentration in embryos, indicating that

BoTX/A either facilitates uptake of yolk protein or interrupts its degradation. BoTX/A cleaves SNAP-25 present in neurons of affected animals including mammals and chicken (Stahl et al. 2007). However, exocytosis of *Xenopus* is significantly inhibited by BoTX/A, despite of relatively low sequence preservation in SNAP-25, near the cleavage site for BoTX/A (Yao et al. 1999; Kanno et al. 2009; Figure 6). As BoTX/A mildly affects *C. elegans*, CeSNAP-25 might be a weak substrate of BoTX/A because of less-sequence conservation (Figure 6).

BoTX/A is exceedingly potent in all tested-mammals including human, mice, and rat. Truncated SNAP-25 without C-terminal nine amino acids by BoTX/A is still likely to retain the ability to interact with other SNARE machineries, thus plays a dominant negative role in synaptic transmission in neurons (Restani et al. 2012). Syndet, SNAP-25 isomer present in other tissues than neurons, is not cleaved by BoTX/A, and this explains why BoTX/A attacks selectively on neurons (Washbourne et al. 1997). This implies that the substrate efficiency of the SNAP-25 ortholog for BoTX/A may be a major factor to determine the toxicity of BoTX/A on certain species. The toxicity of BoTX/A to other vertebrates is in contradiction. For example, an electric organ of *Torpedo* is susceptible to BoTX/A, but its SNAP-25 is not the substrate (Marsal 1989; Washbourne et al. 1997). The toxicity of BoTX/A to chicken at organism level is unknown, although chick SNAP-25 in neurons is an effective substrate for BoTX/A (Stahl et al. 2007). Although the sequence conservation in the cleavage site of SNAP-25 seems to be moderate in *Xenopus*, several researches reported that BoTX/A potently interferes with synaptic transmission (Yao et al. 1999; Kanno et al. 2009). It has not been biochemically tested whether *Xenopus* SNAP-25 is cleaved by BoTX/A.

Based on the sequence conservation, *C. elegans* SNAP-25 (CeSNAP-25) has been predicted to be resistant to BoTX/A (Humeau et al. 2000). Overall, CeSNAP-25 shows highly conserved sequences and also contains the SNARE motif which was previously reported to be required for the cleavage by BoTX/A (Figure 6). BoTX/A cleaves between Gln^{197} and Arg^{198} in human SNAP-25, and these amino acids are highly conserved. However, SNAP-25 of *Drosophila* is not a substrate for BoTX/A, although it reserves these amino acids (Washbourne et al. 1997). In addition, *Xenopus* and *Torpedo* neurons are affected by BoTX/A, despite substitution of those amino acids with lysine and histamine, respectively. Above all, human SNAP-25 whose Gln^{197} is substituted with lysine is still an effective substrate for BoTX/A (Vaidyanathan et al. 2002). In CeSNAP-25, lysine replaces glutamine at the corresponding position to Gln^{197} in human SNAP-25 (Figure 6). In overall, *C. elegans* treated with various concentrations of BoTX/A at various duration of incubation time shows that worms are affected by BoTX/A. Further, the investigation to assess the activity of BoTX/A on CeSNAP-25 will reveal whether CeSNAP-25 is a sole target for the toxin in *C. elegans* which shows susceptibility to BoTX/A.

Acknowledgment

This research is supported by Korean Ministry of Education (Basic Science Research Program no. 2013R1A1A2005836) and Women Scientist program [No. 2013R1A1A3A04006010] in the Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by Korean Ministry of Science, ICT & Future Planning.

References

Breidenbach MA, Brunger AT. 2005. New insights into clostridial neurotoxin-SNARE interactions. Trends Mol Med. 11:377–381.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77:71–94.

Cherington M. 2004. Botulism: update and review. Semin Neurol. 24:155–163.

Davletov B, Bajohrs M, Binz T. 2005. Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. Trends Neurosci. 28:446–452.

Humeau Y, Doussau F, Grant NJ, Poulain B. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie. 82:427–446.

Johnson EA. 1999. Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. Annu Rev Microbiol. 53:551–575.

Kanno T, Yaguchi T, Nagata T, Tanaka A, Nishizaki T. 2009. DCP-LA stimulates AMPA receptor exocytosis through CaMKII activation due to PP-1 inhibition. J Cell Physiol. 221:183–188.

Kim D-W, Sung H, Shin D, Shen H, Ahnn J, Lee S-K, Lee S. 2011. Differential physiological roles of ESCRT complexes in *Caenorhabditis elegans*. Mol Cells. 31:585–592.

Marsal J, Egea G, Solsona C, Rabasseda X, Blasi J. 1989. Botulinum toxin type A blocks the morphological changes induced by chemical stimulation on the presynaptic membrane of Torpedo synaptosomes. Proc Natl Acad Sci USA. 86:372.

Restani L, Giribaldi F, Manich M, Bercsenyi K, Menendez G, Rossetto O, Caleo M, Schiavo G. 2012. Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. PLoS Pathog. 8:e1003087.

Stahl AM, Ruthel G, Torres-Melendez E, Kenny TA, Panchal RG, Bavari S. 2007. Primary cultures of embryonic chicken neurons for sensitive cell-based assay of botulinum neurotoxin: implications for therapeutic discovery. J Biomol Screen. 12:370–377.

Vaidyanathan VV, Yoshino K, Jahnz M, Dörries C, Bade S, Nauenburg S, Niemann H, Binz T. 2002. Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E. J Neurochem. 72:327–337.

Washbourne P, Pellizzari R, Baldini G, Wilson MC, Montecucco C. 1997. Botulinum neurotoxin types A and E require the SNARE motif in SNAP-25 for proteolysis. FEBS Lett. 418:1–5.

Yao Y, Ferrer-Montiel AV, Montal M, Tsien RY. 1999. Activation of store-operated Ca^{2+} current in Xenopus oocytes requires SNAP-25 but not a diffusible messenger. Cell. 98:475–485.