Chapter 3

Experimental Comparative Effects of Botulinum Toxin A between Subtypes A1 and A2 in Movement Disorders in Rats

Tomoko Kohda, Yasushi Torii and Hidemitsu Nakajima

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80886

Abstract

In the present review, we describe here experimental comparative and beneficial effects of botulinum neurotoxin A (ANTX) between subtypes A1 and A2 in the pathology of movement disorders, particularly rat Parkinson's disease model. We and other laboratories have shown the beneficial effects, and this novel strategy for intractable brain disorders might confer potent and safety therapy in bedside. First, we show the characteristics of ANTXs in the genetic aspects of these subtypes, and our intriguing findings of immunological profiles in the subtypes between A1NTX and A2NTX. Then, we state the distinct diffusion in the body between A1NTX and A2NTX. Importantly, we describe that the intra-brain treatment of small animals with A2NTX subtype results in improvements of pathologies more effectively and provides greater safety than those of A1NTX in a rat 6-OHDA Parkinson's disease (PD) model. Finally, we represent that the different efficacies between ANTXs are likely due to each localization in the brain; A2NTX is strictly limited in the injected regions, while A1NTX diffused other brain regions. Thus, therapeutic avenue using A2NTX in incurable PD including other movement disorders could be a druggable target in the future.

Keywords: botulinum neurotoxin type A, pharmacokinetics/pharmacodynamics, Parkinson's disease, therapeutics, safety, experimental, rats

1. Introduction

Clostridium botulinum produces highly potent neurotoxin, which causes a persistent paralysis of peripheral nerve terminals. The toxin is classified into seven serotypes (A–G). Type A,

B, E, and F toxins are responsible for human botulism, whereas type C and D toxins cause botulism in other animals. The toxins are large complexes, known as progenitor toxins, which differ in terms of molecular size. The progenitor toxins are containing a neurotoxin (NTX) and several nontoxic components. The nontoxic proteins compose a nontoxic non-hemagglutinin component (NTNHA) and several hemagglutinin (HA) component proteins. In botulinum complex, the proteins are not covalently linked, but their association occurs in culture. The sizes of complex toxins differ, from 900 kDa (LL toxin for type A) to 500 kDa (L toxin for types A, B, C, D, and G), down to 300 kDa (M toxin for types A, B, C, D, E, and F). The LL toxin is a dimer of L toxin, which consists of NTX, NTNHA, and HAs. The M toxin consists of NTX and NTNHA. The complex toxin is also stable at acidic pH but dissociates at alkaline pH (pH ≧ 7).

NTXs are released from *C. botulinum* as single polypeptides with molecular mass about 150 kDa, which are proteolytically activated and composed of light chain (50 kDa) and heavy chain (100 kDa) by disulfide bond. The light chain (LC) acts as a zinc-dependent endopeptidase. The heavy chain is divided into two different functional domains: the amino-terminal (H_N) domain and the carboxyl-terminal (H_C) domain. The H_C acts as the receptor-binding domain, and H_N acts as pH-dependent translocation domain of LC from endosome to cytosol. Neuronal endocytosis is driven by the formation of protein complex between the vesicle N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), VAMP2, and the plasma membrane SNAREs, synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin [1]. Type C toxin cleaves both SNAP25 and syntaxin; type B, D, F, and G toxins only target VAMP; and type A and E toxins cleave SNAP25. NTX inhibits endocytosis by the cleavage of one of the three proteins. Due to high efficiency and longevity, the toxins are the most widely used therapeutic proteins.

Due to their high efficacy, tolerance, longevity, and safety property, botulinum toxins are the most widely used therapeutic proteins. Most of the toxins have several subtypes based on amino acid sequence variability. The type A toxins have been subclassified into 10 subtypes (A1–A10) [2]. Especially, the toxin products used as treatment for neurologic disorders are LL toxin and NTX, produced by botulinum toxin subtype A1. The other subtype of toxins is not used clinically, however has been conducted in researches. We have been studying the biological characteristic and pharmacology of A2 toxin.

Several species of botulinum neurotoxin are known to act on cholinergic terminals of the peripheral neuromuscular junction and the central nervous system (CNS) [3–5]. NTXs cause robust inhibition of the voluntary nervous circuits by blocking the release of acetylcholine (ACh) [6]. The therapeutic application of A1NTX for neurological disorders such as bradykinesia, urinary dysfunction, hemifacial spasm, and cervical dystonia is well established [7]. The type A organisms have been classified into 10 subtypes (A1 to A10) based on the amino acid sequence variability of NTX [2]. All 10 subtypes bind to presynaptic protein SNAP-25 with similar affinity, but A1NTX and A2NTX cleave SNAP-25 more efficiently than that of other subtypes [4, 8, 9].

Recent studies investigated the direct administration of ANTX to the CNS as a therapeutic strategy for the treatment of neurological disorders [3–5]. Parkinson's disease (PD) is characterized by imbalanced cholinergic hyperactivity in the striatum of affected individuals [10, 11]. Interruption of ACh release in the striatum by direct injection of BoNT/A has been reported in the rat unilateral 6-hydroxydopamine (6-OHDA) model of PD [12].

This paper will review the recent advance in the genetic, immunological, diffusion in the body and experimental animal model of PD in botulinum toxin A.

2. Genetic diversity between A1NTX and A2NTX

2.1. Genetic diversity of gene clusters encoding ANTX complexes

The neurotoxin and nontoxic protein genes are defined as the NTX gene cluster. There are two types of nontoxic components of gene organization (HA and Orfx clusters), and *C. botulinum* type A strains were classified according to their harboring of these clusters. The NTX genes are encoded by mobile genetic elements that enable horizontal transfer among different isolates, which is thought to contribute to evolution of the NTX gene loci and thereby to the large number of distinct NTXs that are currently known [13]. Further type A strains have been classified as boNT and HA gene cluster typing to be applied for molecular characterization of type A strain. Genes encoding components of the A1NTX and A2NTX complex are arranged clusters. Type A strains possess HA cluster genes and A1NTX to NTX gene cluster typing 1, Orfx cluster genes and A2NTX to NTX gene cluster typing 2, and HA cluster genes, Orfx cluster genes, and A1NTX with unexpressed or expressed BNTX to NTX gene cluster typing 3 [14]. Umeda et al. have reported that *C. botulinum* type A isolates genotypes by combining the results of NTX subtype (subtype A1 or A2) gene detection with ha33 and/or p47 gene detection by multiplex PCR. Ten isolates associated with infant botulism in Japan were divided into NTX gene cluster typings 2 and 3 by origin (honey feeding or not) and period (1986–1987 and 1999–2007). And, four isolates associated with food-borne botulism in Japan were divided into NTX gene cluster typings 1 and 3. The multiplex PCR method is easily capable of classification of NTX gene cluster typing [15]. Further, genetic characterization was performed in ten botulism cases in Japan between 2006 and 2011. Except two type B isolates, eight type A isolates are NTX gene cluster typings 1 and 3 which are associated with HA cluster genes [16]. NTX gene cluster typing 2 is predominant in Europe, while NTX gene cluster typings 1 and 3 are predominant in the USA [14, 17, 18]. As *C. botulinum* type A is rarely found in Japanese soil, there is a possibility that imported foods are related to botulism cases.

2.2. Immunological differences between A1NTX and A2NTX

The difference in amino acid sequence between subtype A1 and A2 toxins' light chain is 5%, while the difference in heavy chain is 13%. The similarity of heavy chain is lower than light chain. These differences appear to indicate that characteristic antigenicity in the heavy chain is more conserved than that in the light chain [19]. Differences in antigenicity among subtypes were evaluated using monoclonal and polyclonal antibodies [20–23]. Among eight and seven monoclonal antibodies against A1NTX and A2NTX, respectively, each of which recognized different epitopes, each three specifically reacted with A1NTX and A2NTX. Neutralizing single monoclonal antibodies against A1NTX and A2NTX that recognized LC, H_N, or H_C have been reported, respectively (**Tables 1** and **2**). Each neutralizing antibody mostly neutralized only toxins of their own subtypes. It is suggested that the epitopes of neutralizing are present in every domain of both subtypes. The 3B10 and 5G2 that are reacting with LC and H_N, respectively, specifically

recognized and neutralized A2NTX. These monoclonal antibodies recognizing epitopes are considered to function as A2NTX properties. In type B, differences in biological activities among the subtypes B1, B2, and B6NTX appeared to be attributable not only to the function in H_C but also to the function in H_N [24]. For binding of monoclonal antibodies to NTX, KD values of 1F11 for A1NTX were 500 hold higher than that for A2NTX and only neutralized A1NTX. However, the KD values of 5C7 for A2NTX were 16 hold higher than that for A1NTX did not neutralize both NTXs. The neutralization of monoclonal antibody did not correspond to its affinity. And, OD values obtained by ELISA did not necessarily correlate with KD values (**Table 3**).

Type A antitoxin in standard and therapeutic preparation is a polyclonal antibody purified from immunized sera with A1NTX; however, there was no report on the reactivity of the standard type

mAb[1]	ELISA (OD_{450})[2]			Blotting	Neutralization[3]	
	A1NTX	A2NTX	Toxoid/A1NTX		A1NTX	A2NTX
1D4	0.879	0.795	0.787	L	-	-
1E2	0.741	0.820	0.725	L	++	+
1B12	0.748	0.372	0.505	H_N	++	-
1F11	0.953	0.404	0.853	H_N	++	-
10H3	0.725	0.090	0.453	H_N	-	-
4E4	0.967	0.005	0.002	Hc	++	-
6D9	0.565	0.235	0.151	Hc	-	-
9A3	0.643	0.040	0.051	Hc	++	-

[1]: MAbs were raised against A1NTX.

[2]: Values were obtained with each mAb at 1 μg/ml.

[3]: Mix mAb (10 μg/ml) 300 μl with A1NTX (40 LD_{50}/ml) 300 μl in GPB, and incubate at room temperature for 30 min. Inject intraperitoneally 0.5 ml of the mixture to a mouse and monitor the mouse for 4 days. (-): time to death≤24 h; (+): 24 h< time to death≤96 h; (++): time to death>96 h

Table 1. Properties of mAbs raised against A1NTX.

mAb[1]	ELISA (OD_{450})[2]			Blotting	Neutralization[3]	
	A1NTX	A2NTX	Toxoid/A2NTX		A1NTX	A2NTX
3B10	0.063	0.824	0.305	L	-	++
4G12	0.745	0.805	0.432	L	-	+
9B3	0.614	0.894	0.466	L	-	-
2A12	1.185	0.969	0.404	H_N	-	-
5G2	0.025	0.539	0.222	H_N	-	++
5C7	0.601	0.591	0.085	Hc	-	-
6A5	0.015	0.865	0.338	Hc	-	+

[1]: MAbs were raised against A2NTX.

[2]: Values were obtained with each mAb at 1 μg/ml.

[3]: Mix mAb (10 μg/ml) 300 μl with A2NTX (40 LD_{50}/ml) 300 μl in GPB, and incubate at room temperature for 30 min. Inject intraperitoneally 0.5 ml of the mixture to a mouse and monitor the mouse for 4 days. (-): time to death≤24 h; (+): 24 h< time to death≤96 h; (++): time to death>96 h

Table 2. Properties of mAbs raised against A2NTX.

mAb[2]	against NTX	K_D (M)[3] (A1NTX)	K_D (M)[4] (A2NTX)
1D4	A1	3.8×10^{-9}	3.3×10^{-9}
1E2	A1	9.2×10^{-10}	8.5×10^{-10}
1B12	A1	2.5×10^{-9}	2.7×10^{-10}
1F11	A1	6.2×10^{-10}	1.3×10^{-8}
10H3	A1	1.3×10^{-9}	ND[5]
4E4	A1	4.2×10^{-10}	ND
6D9	A1	2.8×10^{-9}	3.2×10^{-9}
9A3	A1	8.1×10^{-11}	ND
3B10	A2	ND	8.9×10^{-10}
4G12	A2	7.7×10^{-9}	3.7×10^{-8}
9B3	A2	7.7×10^{-9}	1.5×10^{-8}
2A12	A2	ND	ND
5G2	A2	ND	8.7×10^{-8}
5C7	A2	1.6×10^{-8}	2.7×10^{-9}
6A5	A2	ND	2.7×10^{-9}

[1]: Association (k_a) and dissociation (k_d) rate constants were determined by surface plasmon resonance in biacore, and K_D was calculated as k_d/k_a.

[2]: MAbs were raised against A1NTX.

[3]: K_D for mAbs binding to A1NTX.

[4]: K_D for mAbs binding to A2NTX.

[5]: Not determinable.

Table 3. Equilibrium dissociation constants (***KD***)[1] of A1NTX and A2NTX with mAbs against A1NTX and A2NTX.

A antitoxin with other subtype toxins. The A1 antitoxin had equivalent potency both the A1NTX and A2NTX; however, neutralization titer of A2 antitoxin was 4–9 hold higher against A2NTX than against A1NTX. It seems that the difference between the antibody titers against the test NTX was due to the standard antitoxin having different reactivities with the NTXs. The binding analysis comparing these antitoxins and NTXs by SPR showed that the A1 antitoxin had a higher binding affinity and slower dissociation speed with the A1NTX than with the A2NTX. The A2 antitoxin showed a higher binding affinity than with the A1NTX [22]. Although these NTXs show a low level of sequence difference, they have marked a difference in antigenicity, and antitoxin preparation should be used for each subtype's diagnosis and therapy of botulism.

3. Diffusion into the body of botulinum toxins A1 and A2

Botulinum toxins type A have been researched and developed for use as important therapeutic agents for neurological disorders such as blepharospasm, hemifacial spasm, various dystonias, and overactive bladder [7, 25]. Botulinum toxin type A products, which are used as treatment for neurologic disorder, are produced from LL toxin or NTX derived from subtype A1 organisms [26]. The toxins show high-level efficacy at very low doses, but their adverse effects are

becoming an issue. In the treatment for torticollis, cervical dystonia, and cosmetic cases, patients showed dysphagia or respiratory compromise [27–29]. In clinical studies of treatment for spasm, patients who received high-dose toxin showed weakness around the site of administration as well as symptoms of botulism [30–32]. The A1 toxins spread to distant regions is considered to be due to transport via the body fluid or nerves [33–35]. In addition, A1 toxin was reported to transport via a retrograde axonal route in visual nerve and facial motoneurons in rats [3].

The first report of the diffusion of A2 toxin in the body was grip strength study in mice to compare with A1 toxin [36]. This study was evaluated by measurement of contralateral grip strength as indicator of toxins' diffusion. The toxins used were A1 L + LL toxin, onabotulinum-toxinA (A1LL toxin), and A2M toxin and were injected into one side of the gastrocnemius muscle, and grip strength of the contralateral hind leg was measured. The study evaluated that the doses causing a 20% reduction in the grip strength before injection were calculated and these values were termed the 20% toxic doses (TD_{20}). The TD_{20} of A1L + LL toxin, A1LL toxin, and A2M toxin were 17.0, 16.2, and 37.3 U/kg, respectively. The grip strength test was conducted for change in toxins' forms, measurement sites, and animal species [37]. The grip strength test using rats' forelegs was conducted using A1 neurotoxin (A1NTX), A1LL toxin, and A2NTX (**Figure 1**). The study evaluated that 50% toxic doses (TD_{50}), which caused a 50%

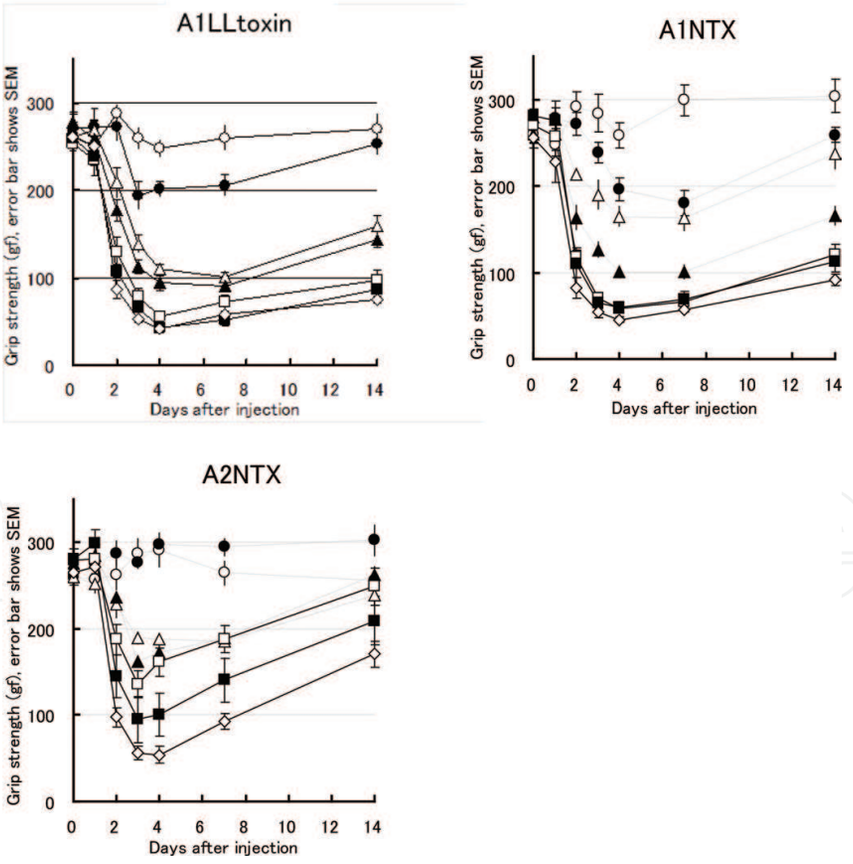

Figure 1. Time-course of the grip strength of the contralateral foreleg after toxin injection. Rats received A1LLtoxin, A1NTX, or A2NTX injection in the left foreleg (each at ○: 1 U, ●: 4 U, △: 8 U, ▲: 12 U, □: 16 U, ■: 20 U, and ◇: 24 U). The grip strength was measured in the right foreleg of each rat at 0 (before administration), 1, 2, 3, 4, 7, and 14 days after injection. Each point is the mean ± S.E.M. (n = 5). These data are cited from Toxicon (Trii, *et al.*, Vol; 57(1), [2011] pp. 97) with the permissions of ELSEVIER.

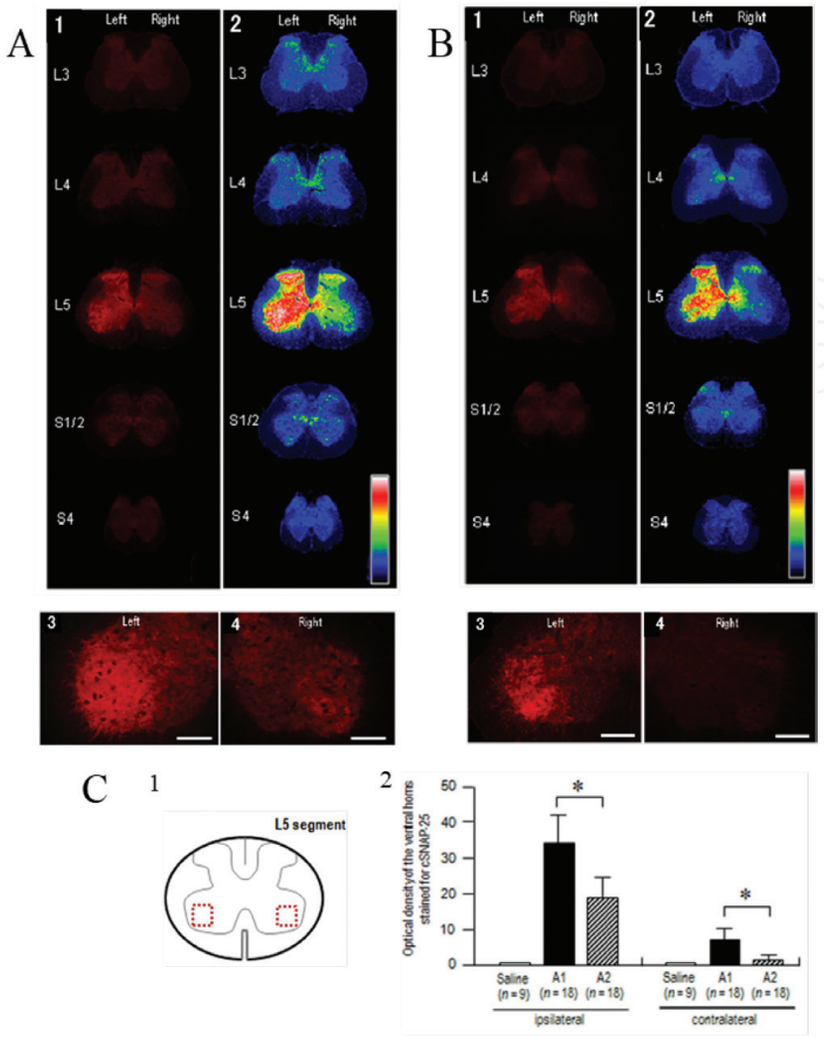

Figure 2. Appearance of botulinum toxin type A-cleaved SNAP-25 (cSNAP-25) in the spinal cord after intramuscular injection of A1- and A2NTX. Densitometric analysis on the spinal ventral horns stained for cSNAP-25. A, B: Immunohistochemical detection of cSNAP-25 was carried out in the spinal cord 4 days after unilateral injection of A1- or A2-NTX (10 U) into the left gastrocnemius muscle. (A: A1NTX, B: A2NTX) 1, 2: Displayed are multiple transverse spinal cord sections stained for cSNAP-25 in the toxin treated rats (1) and their graded color-converted images (2), in which labeling intensity is indicated in a standard pseudocolor scale from blue (lowest level) through green, yellow, red, and white (highest level). 3,4: Photomicrographs of the ventral horns stained for cSNAP-25 ipsilateral (3) and contralateral (4) to peripheral toxin injection. Scale bars = 200 mm. C: Densitometric analysis on the spinal ventral horns stained for cSNAP-25. 1: The scheme shows the transverse spinal cord section at the L5 segment, in which measured areas in the bilateral ventral horn are indicated by dashed open boxes colored in red. 2: Optical densities of the ventral horns stained for cSNAP-25 in rats treated with saline (n = 3), A1NTX (A1) (n = 6), or A2NTX (A2) (n = 6). For each animal, measurements were made in the ventral horns of three spinal cord sections ipsilateral and contralateral to the toxin-injected sites. Values are means ± SD. *P < 0.05, A1 versus A2; Mann–Whitney U-test. These data are cited from frontier in neurology (Torii, *et al.*, [2014] pp. 97. 2014; 5:98) with permissions of frontier media.

reduction in the grip strength before injection, were calculated. The TD_{50} values of A1NTX, A1LL toxin, and A2NTX were 7.54, 6.35, and 15.62 U/head, respectively. These results indicated that A2NTX required higher dosage than A1 toxins to relax on the contralateral muscle and suggested that A1 and A2 toxins have different diffusions in the body.

Why do these toxins make a difference in diffusion in the body? The pathway of A1 and A2 toxins was physiologically investigated in the immunohistological study [38].

Spinal cords (bilateral ventral and dorsal horns), in which A1NTX and A2NTX were injected into the gastrocnemius muscle, were strained using botulinum toxin type A-cleaved SNAP-25 (cSNAP-25). The L5 nerve dominantly innervates the gastrocnemius muscle. The A1NTX was observed to have a strong immunoreactivity for cSNAP-25 in the ventral and dorsal horns of the spinal cord not only at the segmental level of L5 ipsilateral to the peripheral toxin injection site but also to a lesser extent on the contralateral side (**Figure 2A**). The A2NTX was observed to have a strong immunoreactivity at the L5 spinal segment ipsilateral side as A1NTX but to a lesser extent on the contralateral side than A1NTX (**Figure 2B**). In addition, the ventral horns stained for cSNAP-25 at the L5 spinal segment in the toxin-treated rats were compared by optical density measurements. In both the ipsilateral and contralateral ventral horns, cSNAP-25 labeling in rats injected with A1NTX was spread wider than with A2NTX (**Figure 2C**).

The diffusion of A2NTX in the body summarized the previous reports as follows (**Figure 3**). After unilateral intramuscular toxin injection, the catalytically active toxin can be axonally transported to the spinal cord through motor and sensory nerves. Subsequently, the toxin can spread throughout the gray matter of the spinal cord, including the bilateral ventral and dorsal horns, via a transcytosis (cell-to-cell trafficking) mechanism by which a ligand penetrates the neuron at one side, followed by its movement and release at the opposite end, with possible uptake by second-order neurons. Differential delivery routes by which injected A1NTX and A2NTX affect contralateral muscles have also been proposed as A1NTX is transported almost equally to the contralateral muscles via this neural pathway and the blood circulation, while A2NTX is mainly transported to contralateral muscles via the bloodstream only at higher doses.

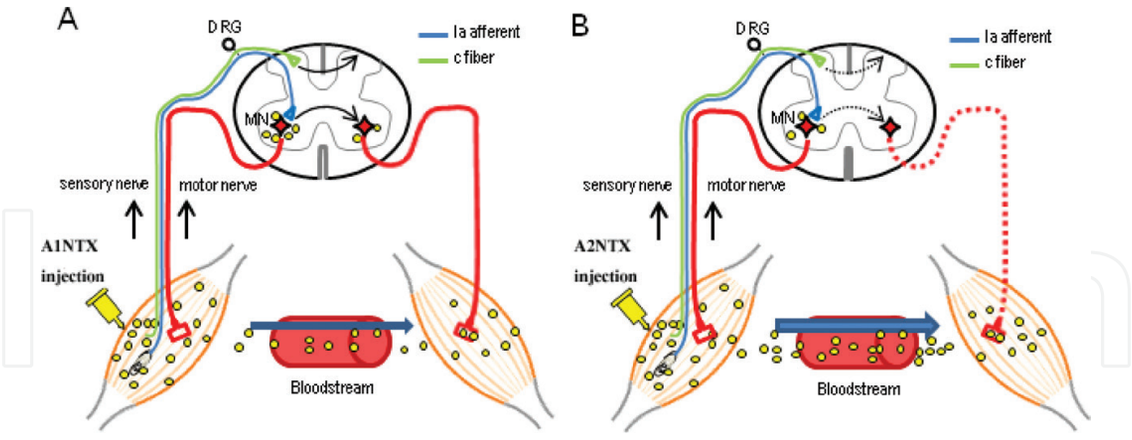

Figure 3. Possible mechanisms for the central actions of intramuscularly injected botulinum toxin type A in the spinal cord. Following unilateral intramuscular A1NTX (A) or A2NTX (B) injection, the catalytically active toxin can be axonally transported to the spinal cord through motor and sensory nerves. Subsequently, the toxin can spread throughout the gray matter of the spinal cord, including the bilateral ventral and dorsal horns, via a transcytosis (cell-to-cell trafficking) mechanism by which a ligand penetrates the neuron at one side, followed by its movement and release at the opposite end, with possible up take by second-order neurons. Differential delivery routes by which injected A1NTX and A2NTX affect contralateral muscles have also been proposed as A1NTX (A) is transported almost equally to the contralateral muscles via this neural pathway and the blood circulation, while A2NTX (B) is mainly transported to contralateral muscles via the bloodstream only at higher doses. These data are cited from frontier in neurology (Torii, *et al.*, [2014] pp. 5:98) with permissions of frontier media.

4. Therapeutic application of botulinum toxins A1 and A2 in Parkinson's disease

Parkinson's disease (PD) is one of the most common movement disorders and is characterized by a progressive degeneration of nigrostriatal dopaminergic signaling, which leads to the unbalanced release of acetylcholine in the striatum [10]. The disturbance of these neuronal circuits elicits parkinsonian motor symptoms with muscular dysfunctions, such as resting tremor, spontaneous dystonia, akinesia, sialorrhea, urinary dysfunction, and pain [10, 39]. While palliative therapies for PD subjects having sialorrler and urinary dysfunction using onabobotulinamtosinA (nealy equal to A1NTX) are going in bedside [12], there is currently a lack of curative therapies using ANTXs.

Several studies demonstrated that the intrastriatal injection of A1NTX reduces pathologic behavior in the rat 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease model (rat 6-OHDA PD model) [11, 40]. These studies demonstrate the feasibility of clinical A1NTX application to treat PD without adverse side effects such as memory dysfunction [11, 40]. However, it is not clear which A1NTX has the greatest efficacy for treatment of PD. Therefore, we first compared the effect of A1NTX with that of A2NTX on pathogenic rotation behavior and in vivo cleavage of striatal SNAP-25 in the 6-OHDA PD rat model.

As a result, intrastriatal treatment of 6-OHDA-lesioned rats with A1NTX or A2NTX significantly reduced the pathogenic rotation behavior in a dose-dependent manner (**Figure 4**). The highest tested dose of A1NTX (1 ng) conferred significant reduction of pathogenic behavior, as did all tested A2NTX doses (0.1, 0.5, and 1 ng). These results suggest that A2NTX has more potent inhibition of ACh release in the striatum than that of A1NTX [40]. Indeed, intrastriatal injection of the 6-OHDA-lesioned rats with A1NTX or A2NTX caused a dose-dependent

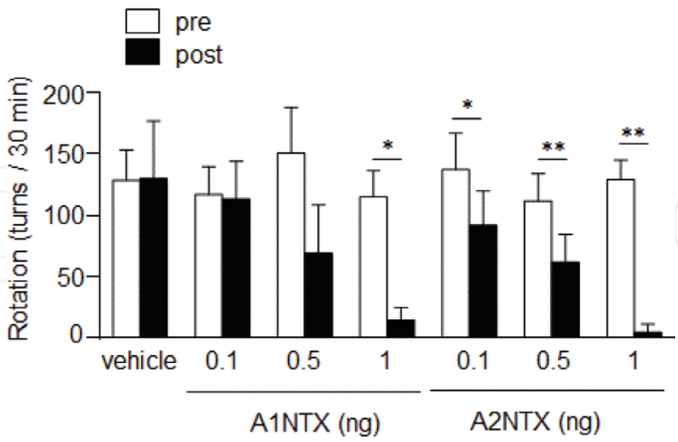

Figure 4. Effects of intrastriatal injection of A1NTX (0.1, 0.5, or 1 ng/rat; n = 6 per dose), A2NTX (0.1, 0.5, or 1 ng/rat; n = 6 per dose), or vehicle (n = 7) on methamphetamine-induced rotation behavior. All rats received ANTX or vehicle injected into the lesioned striatum induced by 6-OHDA injection. For the tests, pre (white columns) represents before injection of ANTX, and post (black columns) represents after injection of ANTX. Data represent means ±S.E.M.; statistical significance is determined as pre versus post in a paired Student's t-test; *$p < 0.05$; **$p < 0.01$. These data are cited from biochemical and biophysical research communications (Itakura et al., Vol;. 447(2), [2014] pp. 312 with the permissions from ELSEVIER).

decrease in the level of full-length SNAP-25 in the striatum [40]. These results support the observed effects of A1NTX and A2NTX on rotation behavior (**Figure 4**). Additionally, we investigated the localization of cleaved SNAP-25 and choline acetyltransferase in the ANTX-treated striatum by performing fluorescent immunocytochemical analysis [40]. These results indicate that A2NTX has greater efficacy for SNAP-25 cleavage in striatal terminals than that of A1NTX. Therefore, their dose-dependent efficacies in the striatum appear to differ, although the therapeutic effects of both toxin species on reducing pathologic rotation behavior in a PD rat model are likely due to their cleavage of SNAP-25 [40].

Several side effects have been reported after therapeutic treatment with ANTXs for cervical dystonia and cosmetic cases, such as dysphagia and respiratory compromise [28, 29]. Our studies also demonstrated that the effects of botulinum toxin could spread from the injection site to other areas of the body causing symptoms similar to those of botulism [41]; A1NTX, but not A2NTX, was transported via axons to the contralateral side after injection into the foreleg muscles as described in Section 3. These results suggest that A2NTX may have a wider safety margin than that of A1NTX for therapeutic applications for PD. Thus, we investigated side effects after intrastriatal injection of either A1NTX or A2NTX in the rat 6-OHDA PD model [42].

To investigate the distribution of A1NTX or A2NTX in the striatum, an immunofluorescent analysis of the cleaved SNAP-25, which is produced by ANTXs, is performed. The area of survey is shown in **Figure 5A**. Compared to the treatment with vehicle control (**Figure 5B**), the treatment with A1NTX increased the cleaved SNAP-25 in both the ipsilateral and contralateral striata (**Figure 5C** and **E**). In contrast, for A2NTX, the cleaved SNAP-25 signals were observed only in the ipsilateral striatum (**Figure 5D** and **F**). These results indicated that A2NTX was retained at the injection site, whereas A1NTX was diffused into the contralateral striatum.

Indeed, the previous study showed that ANTXs were retrogradely transported by central neurons and motoneurons and were then transcytosed to afferent synapses. The SNAP-25 cleaved by ANTXs was observed in the contralateral hemisphere after unilateral ANTX injection to the hippocampus [12, 43]. Moreover, this finding is supported by our findings showing that A1NTX injected into the foreleg muscles was transported via axons to the contralateral side more readily than A2NTX as indicated in Section 3.

Furthermore, we evaluated changes in body weight as an index of the adverse effects of ANTX application. Body weights were measured 1 and 9 days after the 1.0 ng ANTX injection. Treatment with A1NTX resulted in significant loss of body weight compared to both the vehicle and A2NTX groups (**Figure 6**). Together with **Figures 3** and **5**, these results suggest the possibility that A1NTX, but not A2NTX, diffuses into the contralateral hemisphere leading to dysfunction in food/water intake.

Why does the difference between A1NTX and A2NTX arise in a rat PD model? Interestingly, A2NTX enters neuronal cells faster than A1NTX [44]. Additionally, we found that A1NTX and A2NTX have distinctly different distributions in the peripheral neuromuscular system in Section 3. Unfortunately, these findings only are not sufficient to explain the differences of ANTX subtypes in vivo. Thus, further studies are needed to elucidate the variation among ANTXs from the views of genetic, immunological, and neurological aspects.

http://dx.doi.org/10.5772/intechopen.80886

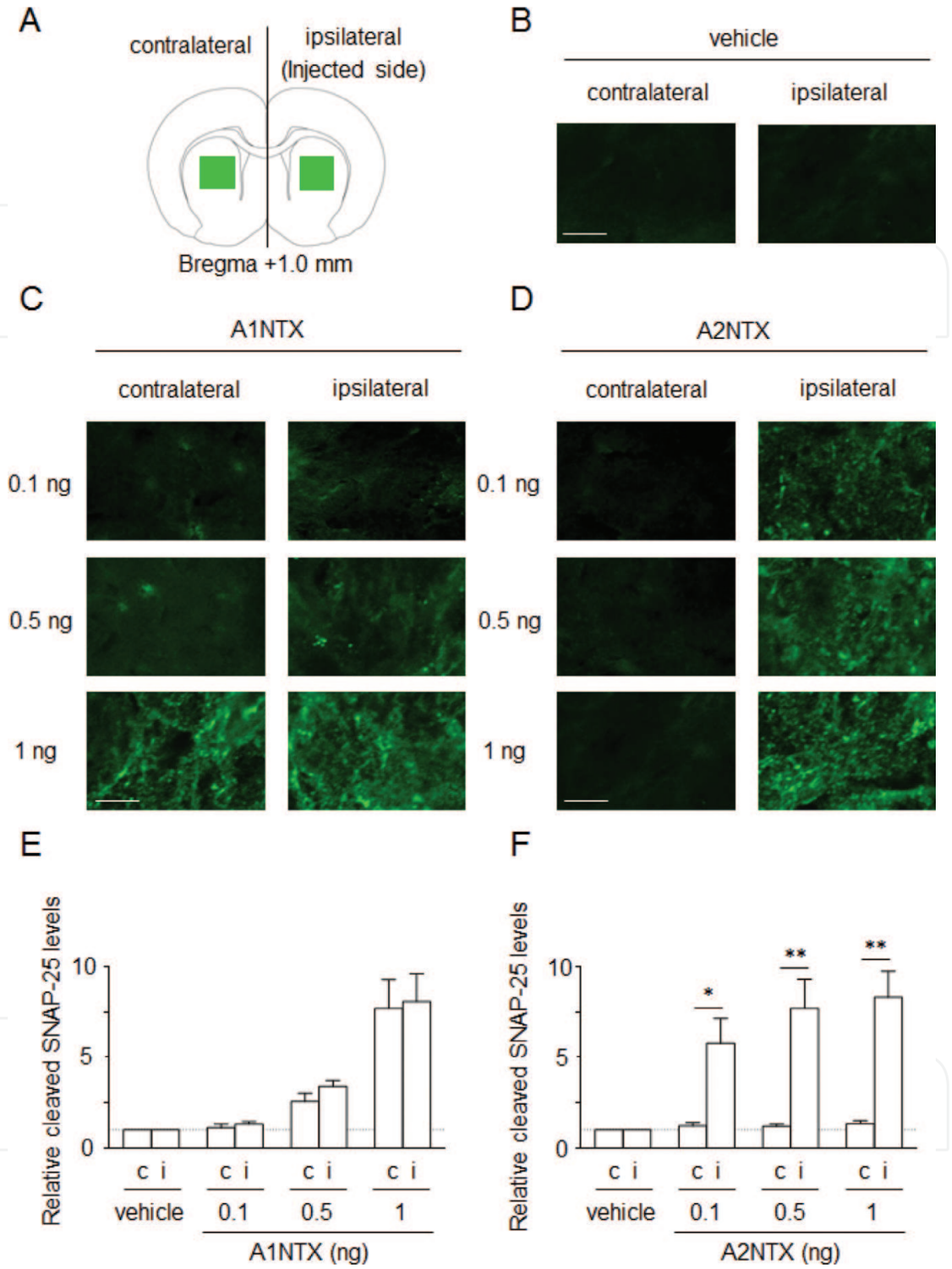

Figure 5. Distribution of A1NTX and A2NTX in the striatum. In (A), green squares represent the brain regions analyzed for the following experiments. Immunofluorescent analysis of cleaved SNAP-25 in the striatum following the intrastriatal injection of vehicle (n = 3) (B), ANTX1 (0.1, 0.5, 1.0 ng/rat; n = 3 per dose) (C) and A2NTX (0.1, 0.5, 1.0 ng/rat; n = 3 per dose) (D) are shown. Semiquantification of the cleaved SNAP-25 signals are shown for the contralateral (indicated as "c") or ipsilateral (indicated as "i," injected side) striatum relative to the vehicle-treated group (E and F). scale bars = 50 μm. Data represent means ± S.E.M.; statistical significance was determined as contralateral versus ipsilateral using a paired Student's t-test; *$p < 0.05$, **$p < 0.01$. These data are cited from Journal of Veterinary Medical Science (Itakura et al., Vol. 76(8), [2014] p. 1191 with the permissions from The Japanese).

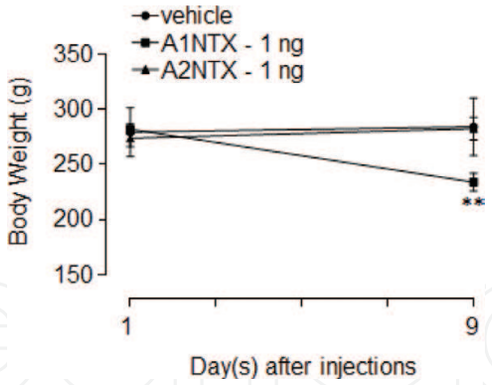

Figure 6. Loss of body weight induced by A1NTX injection. At one and 9 days after vehicle (n = 5), 1.0 ng A1NTX (n = 5) or 1.0 ng A2NTX (n = 4) injection, body weights were measured for all groups. Data represent means ± SE; statistical significance is determined as ANTX-treated groups versus vehicle using a Student's t-test; **p < 0.01. These data are cited from Journal of Veterinary Medical Science (Itakura et al., Vol. 76(8), [2014] p. 1191 with the permissions from The Japanese Society Veterinary Science).

5. Conclusion

Considering the available evidence, it can be concluded that (1) the isolates associated with infant botulism were epidemiologically divided into NTXA gene cluster types. And, A1NTX and A2NTX have marked a difference in antigenicity. (2) A2NTX caused less muscle flaccidity of nontoxin-treated muscle than A1 toxins. The variation in the amino acid sequence between A1NTX and A2NTX causes the difference in the spreading pathways. (3) A2NTX provides anti-PD effectiveness more effectively and confers greater safety than those of A1NTX. These findings might open a new therapeutic avenue for not only PD subjects but be useful also for application to other parkinonisms.

Acknowledgements

We entirely thank Drs. Shunji Kozaki and Ryoji Kaji (Tokushima University) for both their excellent discussion and encouragement to undergo this review. These works were supported in part by a grant-in-aid (C; 22580339, C; 25450428, and B; 16H05029, to H.N.) and (B; 21380188, S.K.) from the Japan Society for the Promotion of Science.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

6-OHDA	6-hydroxydopamineACh acetylcholine
ANTX	*Clostridium botulinum* neurotoxin subtype A

CNS	central nervous system
A1NTX	ANTX subtype A1
A2NTX	ANTX subtype A2
GPS	gelatin phosphate buffer (pH 6.2)
HA	hemagglutinin component
KD values	the affinity constant calculated as dissociation (kd) rate constant/association (ka) rate constant
LD_{50}	50% lethal dose
mAb	monoclonal antibody
OD	optical densities
Orfx	unknown function open reading frame gene
PD	Parkinson's disease
SNAP-25	synaptosomal-associated protein of 25 kDa

Author details

Tomoko Kohda[1], Yasushi Torii[2] and Hidemitsu Nakajima[3]*

*Address all correspondence to: hnakajima@vet.osakafu-u.ac.jp

1 Department of Veterinary Epidemiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisaso-shi, Osaka, Japan

2 Department of Animal Science, Tokyo University of Agriculture, Atsugi, Kanagawa, Japan

3 Department of Veterinary Pharmacology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisaso-shi, Osaka, Japan

References

[1] Brunger AT. Structure and function of SNARE and SNARE-interacting proteins. Quarterly Reviews of Biophysics. 2005;**38**:1-47. DOI: 10.1017/S0033583505004051

[2] Rossetto O, Pirazzini M, Montecucco C. Botulinum neurotoxins: Genetic, structural and mechanistic insights. Nature Reviews Microbiology. 2014;**12**:535-549. DOI: 10.1038/nrmicro3295

[3] Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. Long-distance retrograde effects of botulinum neurotoxin A. The Journal of Neuroscience. 2008;**28**:3689-3696. DOI: 10.1523/JNEUROSCI.0375-08.2008

[4] Hawlitschka A, Antipova V, Schmitt O, Witt M, Benecke R, Mix E, et al. Intracerebrally applied botulinum neurotoxin in experimental neuroscience. Current Pharmaceutical Biotechnology. 2013;**14**:124-130. DOI: 10.2174/1389201011314010015

[5] Knuepfer S, Juenemann KP. Experience with botulinum toxin type A in the treatment of neurogenic detrusor overactivity in clinical practice. Therapeutic Advances in Urology. 2014;**6**:34-42. DOI: 10.1177/1756287213510962

[6] Rossetto O, Megighian A, Scorzeto M, Montecucco C. Botulinum neurotoxins. Toxicon. 2013;**67**:31-36. DOI: 10.1016/j.toxicon.2013.01.017

[7] Jankovic J. Botulinum toxin in clinical practice. Journal of Neurology, Neurosurgery, and Psychiatry. 2004;**75**:951-957. DOI: 10.1136/jnnp.2003.034702

[8] Arndt JW, Jacobson MJ, Abola EE, Forsyth CM, Tepp WH, Marks JD, et al. A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1-A4. Journal of Molecular Biology. 2006;**362**:733-742. DOI: 10.1016/j.jmb.2006.07.040

[9] Henkel JS, Jacobson M, Tepp W, Pier C, Johnson EA, Barbieri JT. Catalytic properties of botulinum neurotoxin subtypes A3 and A4. Biochemistry. 2009;**48**:2522-2528. DOI: 10.1021/bi801686b

[10] Obeso JA, Rodriguez-Oroz MC, Benitez-Temino B, Blesa FJ, Guridi J, Marin C, et al. Functional organization of the basal ganglia: Therapeutic implications for Parkinson's disease. Movement Disorders. 2008;**23**(Suppl 3):S548-S559. DOI: 10.1002/mds.22062

[11] Wree A, Mix E, Hawlitschka A, Antipova V, Witt M, Schmitt O, et al. Intrastriatal botulinum toxin abolishes pathologic rotational behaviour and induces axonal varicosities in the 6-OHDA rat model of Parkinson's disease. Neurobiology of Disease. 2011;**41**:291-298. DOI: 10.1016/j.nbd.2010.09.017

[12] Antipova V, Hawlitschka A, Mix E, Schmitt O, Drager D, Benecke R, et al. Behavioral and structural effects of unilateral intrastriatal injections of botulinum neurotoxin a in the rat model of Parkinson's. Journal of Neuroscience Research. 2013;**91**:838-847

[13] Popoff MR, Bouvet P. Genetic characteristics of toxigenic Clostridia and toxin gene evolution. Toxicon: Official Journal of the International Society on Toxinology. 2013;**75**:63-89. DOI: 10.1016/j.toxicon.2013.05.003

[14] Franciosa G, Floridi F, Maugliani A, Aureli P. Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in Clostridium botulinum type A, Ab, and A(B) strains. Applied and Environmental Microbiology. 2004;**70**:7192-7199. DOI: 10.1128/AEM.70.12.7192-7199.2004

[15] Umeda K, Seto Y, Kohda T, Mukamoto M, Kozaki S. A novel multiplex PCR method for Clostridium botulinum neurotoxin type A gene cluster typing. Microbiology and Immunology. 2010;**54**:308-312. DOI: 10.1111/j.1348-0421.2010.00213.x

[16] Kenri T, Sekizuka T, Yamamoto A, Iwaki M, Komiya T, Hatakeyama T, et al. Genetic characterization and comparison of Clostridium botulinum isolates from botulism cases in Japan between 2006 and 2011. Applied and Environmental Microbiology. 2014;**80**:6954-6964. DOI: 10.1128/AEM.02134-14

[17] Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, et al. Genetic diversity among botulinum neurotoxin-producing clostridial strains. Journal of Bacteriology. 2007;**189**:818-832. DOI: 10.1128/JB.01180-06

[18] Johnson EA, Tepp WH, Bradshaw M, Gilbert RJ, Cook PE, McIntosh ED. Characterization of Clostridium botulinum strains associated with an infant botulism case in the United Kingdom. Journal of Clinical Microbiology. 2005;**43**:2602-2607. DOI: 10.1128/JCM.43.6.2602-2607.2005

[19] Kozaki S, Nakaue S, Kamata Y. Immunological characterization of the neurotoxin produced by Clostridium botulinum type A associated with infant botulism in Japan. Microbiology and Immunology. 1995;**39**:767-774. DOI: 10.1111/j.1348-0421.1995.tb03269.x

[20] Garcia-Rodriguez C, Levy R, Arndt JW, Forsyth CM, Razai A, Lou J, et al. Molecular evolution of antibody cross-reactivity for two subtypes of type A botulinum neurotoxin. Nature Biotechnology. 2007;**25**:107-116. DOI: 10.1038/nbt1269

[21] Kalb SR, Lou J, Garcia-Rodriguez C, Geren IN, Smith TJ, Moura H, et al. Extraction and inhibition of enzymatic activity of botulinum neurotoxins/A1, /A2, and /A3 by a panel of monoclonal anti-BoNT/A antibodies. PLoS One. 2009;**4**:e5355. DOI: 10.1371/journal.pone.0005355

[22] Torii Y, Shinmura M, Kohda T, Kozaki S, Takahashi M, Ginnaga A. Differences in immunological responses of polyclonal botulinum A1 and A2 antitoxin against A1 and A2 toxin. Toxicon: Official Journal of the International Society on Toxinology. 2013;**73**:9-16. DOI: 10.1016/j.toxicon.2013.06.020

[23] Zhao H, Nakamura K, Kohda T, Mukamoto M, Kozaki S. Characterization of the monoclonal antibody response to botulinum neurotoxin type A in the complexed and uncomplexed forms. Japanese Journal of Infectious Diseases. 2012;**65**:138-145. DOI: 10.1371/journal.pone.0005355

[24] Kohda T, Nakamura K, Hosomi K, Torii Y, Kozaki S, Mukamoto M. Characterization of the functional activity of botulinum neurotoxin subtype B6. Microbiology and Immunology. 2017;**61**:482-489. DOI: 10.1111/1348-0421.12540

[25] Sadick NS. Botulinum toxin type B. Dermatologic Surgery. 2003;**29**:348-350. discussion 350-341. DOI: 10.1046/j.1524-4725.2002.29083.x

[26] Dressler D, Benecke R. Pharmacology of therapeutic botulinum toxin preparations. Disability and Rehabilitation. 2007;**29**:1761-1768. DOI: 10.1080/09638280701568296

[27] Borodic GE, Joseph M, Fay L, Cozzolino D, Ferrante RJ. Botulinum A toxin for the treatment of spasmodic torticollis: Dysphagia and regional toxin spread. Head & Neck. 1990;**12**:392-399. DOI: 10.1002/hed.2880120504

[28] Camargo CH, Teive HA, Becker N, Baran MH, Scola RH, Werneck LC. Cervical dystonia: Clinical and therapeutic features in 85 patients. Arquivos de Neuro-Psiquiatria. 2008;**66**:15-21. DOI: 10.1590/S0004-282X2008000100005

[29] Cote TR, Mohan AK, Polder JA, Walton MK, Braun MM. Botulinum toxin type A injections: Adverse events reported to the US Food and Drug Administration in therapeutic

and cosmetic cases. Journal of the American Academy of Dermatology. 2005;**53**:407-415. DOI: 10.1016/j.jaad.2005.06.011

[30] Mancini F, Sandrini G, Moglia A, Nappi G, Pacchetti C. A randomised, double-blind, dose-ranging study to evaluate efficacy and safety of three doses of botulinum toxin type A (Botox) for the treatment of spastic foot. Neurological Sciences. 2005;**26**:26-31. DOI: 10.1007/s10072-005-0378-9

[31] Willis AW, Crowner B, Brunstrom JE, Kissel A, Racette BA. High dose botulinum toxin A for the treatment of lower extremity hypertonicity in children with cerebral palsy. Developmental Medicine and Child Neurology. 2007;**49**:818-822. DOI: 10.1111/j.1469-8749.2007.00818.x

[32] Goldstein EM. Safety of high-dose botulinum toxin type A therapy for the treatment of pediatric spasticity. Journal of Child Neurology. 2006;**21**:189-192. DOI: 10.2310/7010.2006.00041

[33] Tang-Liu DD, Aoki KR, Dolly JO, de Paiva A, Houchen TL, Chasseaud LF, et al. Intramuscular injection of 125I-botulinum neurotoxin-complex versus 125I-botulinum-free neurotoxin: Time course of tissue distribution. Toxicon. 2003;**42**:461-469

[34] Wiegand H, Erdmann G, Wellhoner HH. 125I-labelled botulinum A neurotoxin: Pharmacokinetics in cats after intramuscular injection. Naunyn-Schmiedeberg's Archives of Pharmacology. 1976;**292**:161-165

[35] Dressler D, Saberi FA, Barbosa ER. Botulinum toxin: Mechanisms of action. Arquivos de Neuro-Psiquiatria. 2005;**63**:180-185. DOI: S0004-282X2005000100035

[36] Yoneda S, Shimazawa M, Kato M, Nonoyama A, Torii Y, Nishino H, et al. Comparison of the therapeutic indexes of different molecular forms of botulinum toxin type A. European Journal of Pharmacology. 2005;**508**:223-229. DOI: 10.1016/j.ejphar.2004.12.007

[37] Torii Y, Kiyota N, Sugimoto N, Mori Y, Goto Y, Harakawa T, et al. Comparison of effects of botulinum toxin subtype A1 and A2 using twitch tension assay and rat grip strength test. Toxicon. 2011;**57**:93-99. DOI: 10.1016/j.toxicon.2010.10.009

[38] Koizumi H, Goto S, Okita S, Morigaki R, Akaike N, Torii Y, et al. Spinal central effects of peripherally applied botulinum neurotoxin a in comparison between its subtypes A1 and A2. Frontiers in Neurology. 2014;**5**:98. DOI: 10.3389/fneur.2014.00098

[39] Lester DB, Rogers TD, Blaha CD. Acetylcholine-dopamine interactions in the pathophysiology and treatment of CNS disorders. CNS Neuroscience & Therapeutics. 2010;**16**:137-162. DOI: 10.1111/j.1755-5949.2010.00142.x

[40] Itakura M, Kohda T, Kubo T, Semi Y, Azuma YT, Nakajima H, et al. Botulinum neurotoxin A subtype 2 reduces pathological behaviors more effectively than subtype 1 in a rat Parkinson's disease model. Biochemical and Biophysical Research Communications. 2014;**447**:311-314. DOI: 1016/j.bbrc.2014.03.146

[41] Torii Y, Akaike N, Harakawa T, Kato K, Sugimoto N, Goto Y, et al. Type A1 but not type A2 botulinum toxin decreases the grip strength of the contralateral foreleg through

axonal transport from the toxin-treated foreleg of rats. Journal of Pharmacological Sciences. 2011;**117**:275-285. DOI: 10.1254/jphs.11121FP

[42] Itakura M, Kohda T, Kubo T, Semi Y, Nishiyama K, Azuma YT, et al. Botulinum neurotoxin type A subtype 2 confers greater safety than subtype 1 in a rat Parkinson's disease model. The Journal of Veterinary Medical Science. 2014;**76**:1189-1193. DOI: 10.1292/jvms.14-0184

[43] Holzmann C, Drager D, Mix E, Hawlitschka A, Antipova V, Benecke R, et al. Effects of intrastriatal botulinum neurotoxin A on the behavior of Wistar rats. Behavioural Brain Research. 2012;**234**:107-116. DOI: 10.1016/j.bbr.2012.06.008

[44] Pier CL, Chen C, Tepp WH, Lin G, Janda KD, Barbieri JT, et al. Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1. FEBS Letters. 2011;**585**:199-206. DOI: 10.1016/j.febslet.2010.11.045

IntechOpen