

Botulinum Neurotoxin Type A Subtype 2 Confers Greater Safety than Subtype 1 in a Rat Parkinson's Disease Model

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ABSTRACT. Botulinum neurotoxin type A (BoNT/A) cleaves SNAP-25 and interrupts the release of acetylcholine. We previously reported that BoNT/A subtype 2 (BoNT/A2) ameliorates pathologic behavior more effectively than subtype 1 (BoNT/A1) in a rat Parkinson's disease model. Here, we further show BoNT/A2 has fewer adverse effects than BoNT/A1. We first confirmed that intrastriatal treatments of both BoNT/As had no-effect on dopaminergic terminals in the striatum. SNAP-25 cleaved by BoNT/A2 was strictly localized to the striatum on the injected side; however, SNAP-25 cleaved by BoNT/A1 diffused contralaterally. Furthermore, treatment with BoNT/A1 caused a significant reduction in body weight, while BoNT/A2 treatment did not. These results suggest that BoNT/A2 is more beneficial for clinical application against Parkinson's disease than BoNT/A1.

KEY WORDS: adverse effect, botulinum neurotoxin, Parkinson's disease, rat, subtype

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Botulinum neurotoxin type A (BoNT/A) acts on cholinergic neurons, cleaves presynaptic protein SNAP-25 (a 25 kDa synaptosomal-associated protein) and interrupts the release of acetylcholine [4, 19, 20]. Recently, BoNT/A has been applied clinically for several diseases characterized by hyperactive cholinergic nerve terminals [7, 9]. BoNT/A exists as subtype 1 (BoNT/A1) and subtype 2 (BoNT/A2), and studies have demonstrated that these subtypes differ both immunologically and biologically [10, 15, 18, 22, 23].

Parkinson's disease 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 [16]. The disturbance of these neuronal circuits elicits parkinsonian motor symptoms, such as resting tremor, rigidity and akinesia [13, 16]. Several studies demonstrated that the intrastriatal injection of BoNT/A reduces pathologic behavior in the rat 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease model (rat 6-OHDA PD model) [1, 14, 24]. In addition, our recent report indicated that intrastriatal injection of BoNT/A2 reduces pathologic behavior, *i.e.* methamphetamine-induced rotational behavior, more effectively than BoNT/A1 in the rat 6-OHDA PD model [8].

Several adverse effects have been reported after therapeutic treatment with BoNT/A for cervical dystonia and cosmetic cases, such as dysphagia and respiratory compromise [5, 6]. Other 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 [12]. Interestingly, one study showed that BoNT/A1, but not BoNT/A2, was transported via axons to the contralateral side after injection into the foreleg muscles [23]. These results suggest that BoNT/A2 may have a wider safety margin than that of BoNT/A1 for therapeutic applications. In the present study, we investigated adverse effects after intrastriatal injection of either BoNT/A1 or BoNT/A2 in the rat 6-OHDA PD model.

All animal experimental procedures were approved by the Animal Ethical Committee of Osaka Prefecture University and were performed according to the animal ethical guidelines of the Osaka Prefecture University. Male Wistar rats (SLC Japan, Shizuoka, Japan), weighing about 250–300 g, were maintained under controlled 12 hr light/12 hr dark cycles and had free access to food and water. BoNT/A1 and BoNT/A2 were purified according to the method reported previously [11, 21]. In brief, *Clostridium botulinum* type A strains 62A (A1 subtype) and Chiba-H (A2 subtype) were used for the purification of the complex toxins. BoNT/A was isolated from M toxin by anion-exchange chromatography. BoNT/A was stored in 50 mM phosphate buffer (pH 7.5) at –80°C until use.

The experimental design was based on our previous report [8]. Rats were anesthetized by the intraperitoneal (*i.p.*) injection of sodium pentobarbital (50 mg/kg, Abbott Japan, Tokyo, Japan) and fixed to a stereotaxic apparatus (Narishige, Tokyo, Japan). A middle skin incision was made

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with subsequent drilling of the skull. 6-OHDA (20 μ g/rat, 4 μ l of 5 mg/ml dissolved in 0.9% saline containing 0.01% ascorbic acid; Sigma-Aldrich, St. Louis, MO, U.S.A.) was injected into the right striatum at a rate of 1 μ l/min via a 28-gauge Hamilton syringe (coordinates: 1.0 mm anterior to bregma, 3.0 mm lateral to midline and 5.0 mm ventral to the surface of the skull) [17]. After the injection was complete, the syringe was left in place for 4 min and then removed at a rate of 1 mm/min. Successful lesion of right striatum was confirmed by the methamphetamine-induced rotation test. Rats were treated with methamphetamine (2 mg/kg, i.p., Dainippon Sumitomo Pharm, Osaka, Japan) seven days after the injection of 6-OHDA, and the rotational behavior was assessed as described previously [3, 8]. The rats that rotated 50–200 turns within 30 min after methamphetamine treatment (a complete turn is defined as a 360° turn to the right) were used in the following studies. Two weeks after the injection of 6-OHDA, rats were anesthetized and injected with BoNT/A1 or BoNT/A2 solution (0.1, 0.5 or 1.0 ng/rat; 2 μ l of 0.05, 0.25 or 0.5 μ l/ml in phosphate-buffered saline [PBS] containing 0.1% bovine serum albumin) or a control vehicle. All injection coordinates and infusion rates were the same as those for the injection of 6-OHDA.

Western blotting analysis was accomplished with anti-tyrosine hydroxylase (TH) antibodies as an indicator of the levels of dopaminergic terminals. Anti- β -actin antibodies were used as the internal standard. The nine day after BoNT/A injection, rats were overdosed with sodium pentobarbital (200 mg/kg, i.p.), brains were quickly removed after decapitation and the striatum was dissected. Striatum was homogenized on ice with a sonicator (model Q-125, Qsonica LLC, Newton, CT, U.S.A.) in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF and a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The samples were centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatants (5–10 μ g) were subjected to electrophoresis using 5–20% sodium dodecyl sulfate-polyacrylamide gel (DRC, Tokyo, Japan). Proteins were transferred to polyvinylidene difluoride membranes (Millipore Japan, Tokyo, Japan). The membranes were incubated for 1 hr with 5% nonfat milk (BD Difco, Franklin Lakes, NJ, U.S.A.) in PBS containing 0.05% Tween-20 and 0.02% NaN_3 (PBST) to block nonspecific binding. The membranes were then incubated for 1 hr with mouse monoclonal anti-TH antibodies (1:200, #1017-381; Roche Diagnostics) or mouse monoclonal anti- β -actin antibodies (1:4,000, #AC-15; Sigma-Aldrich) in 0.5% nonfat milk/PBST. The membranes were washed three times with PBST for 5 min and then incubated for 1 hr with anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (1:5,000, Invitrogen, Carlsbad, CA, U.S.A.). Signal development was achieved using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific Japan, Yokohama, Japan). Band intensities were quantified with the LAS-3000 image analyzer (Fujifilm, Kanagawa, Japan).

For immunofluorescent analysis, rats were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) at nine

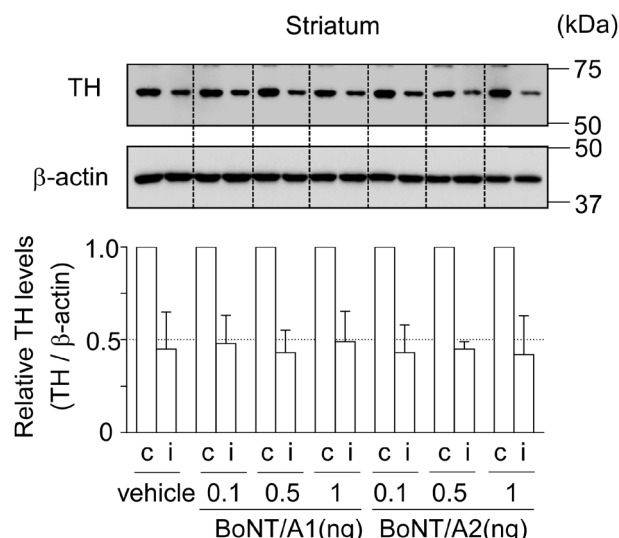


Fig. 1. Effects of the intrastriatal injection of BoNT/A1 (0.1, 0.5 or 1.0 ng/rat; $n=3$ per dose), BoNT/A2 (0.1, 0.5 or 1.0 ng/rat; $n=3$ per dose) or vehicle ($n=3$) on the relative levels of tyrosine hydroxylase (TH) in the contralateral (indicated as "c") or ipsilateral (indicated as "i", injected side) striatum. Values were calculated as the ratio of the TH band intensity relative to the β -actin band intensity. Data represent means \pm SE.

days after BoNT/A injection and perfused with ice-cold 4% paraformaldehyde in PBS. Brains were removed, post-fixed in ice-cold 4% paraformaldehyde in PBS at 4°C overnight and stored in 30% sucrose in PBS for 2–3 days. The OTC-compound-embedded frozen sections (10 μ m) were then prepared. The sections were incubated with 10% donkey serum in PBS and then incubated at 4°C overnight with rabbit anti-cleaved SNAP-25 antibodies (1:50, prepared in our laboratory according to [8]) in PBST containing 1% bovine serum albumin. The signals were visualized by staining with an anti-rabbit Alexa488-conjugated secondary antibody (1:1,000, Invitrogen) using a confocal scanning microscope (model C1si-TE2000-E, Nikon, Tokyo, Japan). The raw images were first grayed and then made binary using the Scion image software package (version 4.0.3., Scion cooperation, Fredrick, MD, U.S.A.). The numbers of binary pixels in the square images were measured automatically.

We first examined the influence of BoNT/A1 and BoNT/A2 treatment on the levels of dopaminergic terminals in the striatum, which were decreased by 6-OHDA injection. As shown in Fig. 1, the ipsilateral reduction of TH levels elicited by 6-OHDA was confirmed (roughly ~50%). There were no differences in TH levels among the vehicle, BoNT/A1 and BoNT/A2 groups, suggesting that both BoNT/A1 and BoNT/A2 exerted no-effect on the dopaminergic terminals in the striatum.

To investigate the distribution of BoNT/A1 or BoNT/A2 in the striatum, an immunofluorescent analysis of the cleaved SNAP-25, which is produced by BoNT/As, was performed. The area of survey is shown in Fig. 2A. Compared to the treatment with vehicle control (Fig. 2B), the treatment

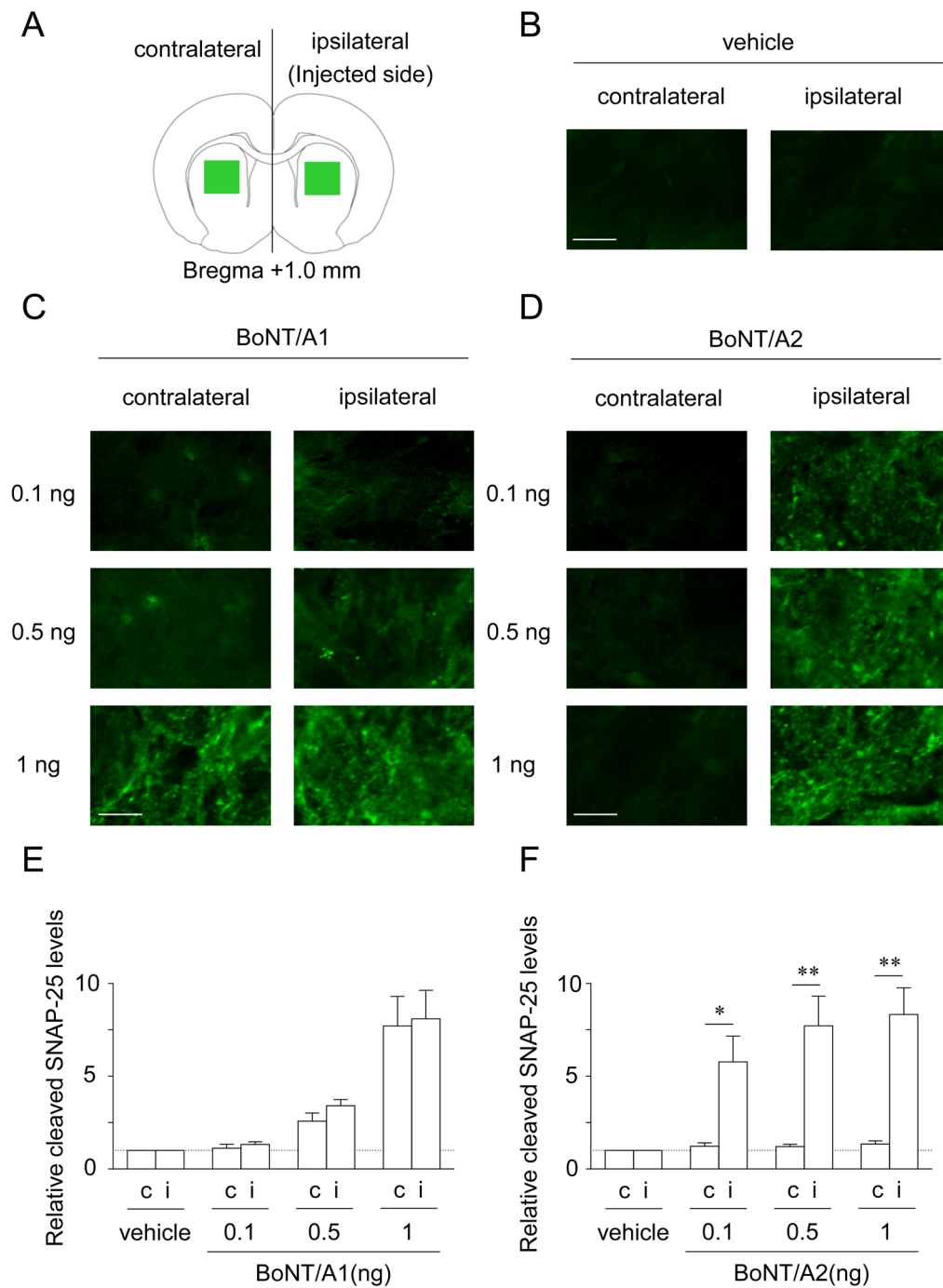


Fig. 2. Distribution of BoNT/A1 and BoNT/A2 in the striatum. In (A), green squares represent the brain regions analyzed for the following experiments. Immunofluorescent analysis of cleaved SNAP-25 in the striatum following the intrastriatal injection of vehicle ($n=3$) (B), BoNT/A1 (0.1, 0.5 or 1.0 ng/rat; $n=3$ per dose) (C) and BoNT/A2 (0.1, 0.5 or 1.0 ng/rat; $n=3$ per dose) (D) are shown. Semi-quantification of the cleaved SNAP-25 signals is 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 μ m. Data represent means \pm SE; statistical significance was determined as contralateral versus ipsilateral using a paired Student's t -test; * $P<0.05$, ** $P<0.01$.

with BoNT/A1 increased the cleaved SNAP-25 in both the ipsilateral and contralateral striata (Fig. 2C and 2E). In contrast, for BoNT/A2, the cleaved SNAP-25 signals were observed only in the ipsilateral striata (Fig. 2D and 2F). These results suggest the possibility that BoNT/A2 is retained at the injection site, whereas BoNT/A1 might diffuse into the contralateral striatum. Indeed, a previous study showed that BoNT/A was retrogradely transported by central neurons and motoneurons and was then transcytosed to afferent synapses. The SNAP-25 cleaved by BoNT/A was observed in the contralateral hemisphere after unilateral BoNT/A injection to the hippocampus [2]. Moreover, this finding is supported by the previous report showing that BoNT/A1 injected into the foreleg muscles was transported via axons to the contralateral side more readily than BoNT/A2 [23].

Finally, we evaluated changes in body weight as an index of the adverse effects of BoNT/A application. Body weights were measured one and nine days after the 1.0 ng BoNT/A injection. Treatment with BoNT/A1 resulted in significant loss of body weight compared to both the vehicle and BoNT/A2 groups (Fig. 3). Together with Fig. 2, these results suggest the possibility that BoNT/A1, but not BoNT/A2, diffuses into the contralateral hemisphere.

In our previous report, we showed that the treatment of 6-OHDA-lesioned rats with BoNT/A2 reduced pathologic rotational behavior in a dose-dependent manner (0.1–1.0 ng), whereas BoNT/A1 treatment caused a reduction in rotational behavior in the high-dose treatment group only (1.0 ng) [8]. Based on the findings in the present study (Figs. 1–3), it appears possible that, like botulism, the injection of BoNT/A1 reduced the rotation behavior as a result of the motor dysfunctions.

In conclusion, our study demonstrated that the levels of SNAP-25 cleaved by BoNT/A2 were localized more strictly to the injection site compared to that of BoNT/A1. Furthermore, BoNT/A2 did not cause the same loss of body weight that was induced by BoNT/A1. These findings suggest that BoNT/A2 has a wider safety margin and, therefore, greater potential for clinical application against neurological disorders, such as Parkinson's disease.

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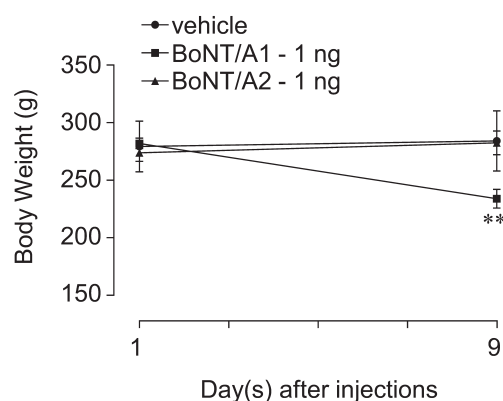


Fig. 3. Loss of body weight induced by BoNT/A1 injection. At one and nine days after vehicle (n=5), 1.0 ng BoNT/A1 (n=5) or 1.0 ng BoNT/A2 (n=4) injection, body weights were measured for all groups. Data represent means \pm SE; statistical significance is determined as BoNT/As-treated groups versus vehicle using a Student's *t*-test; ***P*<0.01.

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