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Botulinum neurotoxin A subtype 2 reduces pathological behaviors more effectively than subtype 1 in a rat Parkinson's disease model

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ABSTRACT

Recent reports indicate that interruption of acetylcholine release by intrastriatal injection of botulinum neurotoxin type A (BoNT/A) in a rat Parkinson's disease model reduces pathogenic behavior without adverse side effects such as memory dysfunction. Current knowledge suggests that BoNT/A subtype 1 (BoNT/A1) and BoNT/A subtype 2 (BoNT/A2) exert different effects. In the present study, we compared the effects of BoNT/A1 and BoNT/A2 on rotation behavior and *in vivo* cleavage of presynaptic protein SNAP-25 in a rat unilateral 6-hydroxydopamine-induced Parkinson's disease model. BoNT/A2 more effectively reduced pathogenic behavior by efficiently cleaving SNAP-25 in the striatum compared with that of BoNT/A1. Our results suggest that BoNT/A2 has greater clinical therapeutic value for treating subjects with Parkinson's disease compared to that of BoNT/A1.

1. Introduction

Several species of botulinum neurotoxin (BoNT) are known to act on cholinergic terminals of the peripheral neuromuscular junction and the central nervous system (CNS) [1–3]. BoNT causes robust inhibition of the voluntary nervous circuits by blocking release of acetylcholine (ACh) [4]. The therapeutic application of botulinum neurotoxin type A (BoNT/A) for neurological disorders such as blepharospasm, hemifacial spasm, and dystonia is well established [5]. The type A organisms have been classified into five subtypes (A1–A5) based on the amino acid sequence variability of BoNT [6]. All five subtypes bind presynaptic protein SNAP-25 (synaptosomal-associated protein of 25 kDa) with similar affinity, but BoNT/A1 and BoNT/A2 cleave SNAP-25 more efficiently than that of other subtypes [6–8]. The cleaved SNAP-25 by BoNT/A blocks transmission of pathological neural signal [1,3].

Recent studies investigated the direct administration of BoNT/A used clinically, which is essentially identical to BoNT/A1, to CNS as a therapeutic strategy for the treatment of neurological disorders [1–3]. Parkinson's disease (PD) is characterized by imbalanced cholinergic hyperactivity in the striatum of affected individuals [9,10]. 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 [11]. That study demonstrates the feasibility of clinical BoNT/A application to treat PD without adverse side effects such as memory dysfunction [12,13]. However, it is not clear which BoNT/A subtypes have the greatest efficacy for treatment of PD. Also, we previously purified BoNT/A2 produced by *Clostridium botulinum* type A strains Chiba-H [14,15]. Therefore, we compared the effect of BoNT/A1 with that of BoNT/A2 on pathogenic rotation behavior and *in vivo* cleavage of striatal SNAP-25 in the 6-OHDA PD rat model.

2. Materials and methods

2.1. Preparation of BoNT/A1 and BoNT/A2

Clostridium botulinum type A strains 62A (A1 subtype) and Chiba-H (A2 subtype) were used for the purification of toxins. The complex toxins were purified according to a method reported previously [16]. BoNT/A was isolated from M toxin by anion-exchange chromatography as described previously [15]. BoNT/A was stored in 50 mM phosphate buffer (pH 7.5) at −80 °C until use.

Abbreviations: 6-OHDA, 6-hydroxydopamine; ACh, acetylcholine; BoNT/A, botulinum neurotoxin subtype A; CNS, central nervous system; ChAT, choline acetyltransferase; PD, Parkinson's disease; PBS, phosphate-buffered saline.

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2.2. 6-Hydroxydopamine model of Parkinson's disease in rats

The 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 250–300 g were maintained on ordinary laboratory chow and tap water *ad libitum* under constant 12-h light/12-h dark cycle. The induction of Parkinson's model was performed by unilateral injection of 6-OHDA into the right striatum [17]. Rats were anesthetized by intraperitoneal treatment with sodium pentobarbital (50 mg/kg, i.p., Abbott Japan, Tokyo, Japan), and fixed to a stereotaxic apparatus (Narishige, Tokyo, Japan). Injection of 6-OHDA (20 μg/rat; 4 μl of 5 μg/μl in 0.9% saline containing 0.01% ascorbic acid; Sigma–Aldrich, St. Louis, MO) or control vehicle into the right striatum was performed at a rate of 1 μl/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 skull surface) [18]. After injection was complete, the syringe was left in place for 4 min, and then removed at a rate of 1 mm/min.

2.3. Animal experimental procedures

The study design is shown in Fig. 1. Successful lesion of right striatum was confirmed in each animal by recording the number of rotation behaviors evoked by treatment with methamphetamine (2 mg/kg, i.p., Dainippon Sumitomo Pharm., Osaka, Japan) at 7 days after injection of 6-OHDA [first rotation test (designated as pre); Fig. 1]. The rotation test ($n = 43$) was performed as described previously [17]. Rats were equally divided to seven groups based on the rotation criterion of 50–200 turns within 30 min after methamphetamine treatment (a complete turn is defined as a 360° turn to the right). Two weeks after the injection of 6-OHDA, rats were given an injection of BoNT/A1 or BoNT/A2 (0.1, 0.5, or 1 ng/rat; 2 μl of 0.05, 0.25, or 0.5 ng/μl in phosphate-buffered saline containing 0.1% bovine serum albumin) or control vehicle into the right striatum under anesthesia. All injection coordinates and infusion rates were the same as those for injection of 6-OHDA. To validate dose-dependent effects of BoNT/A1 or BoNT/A2 on the pathogenic rotation behavior induced by 6-OHDA lesions, the methamphetamine treatment was conducted at 15 days after BoNT/A injection using the same protocol as that used for the first methamphetamine test [second rotation test (designated as post); Fig. 1]. Subsequently, rats received an overdose of pentobarbital (200 mg/kg, i.p.) at 23 days after the 6-OHDA injection (Fig. 1) and were decapitated.

2.4. Protein extraction and Western blotting

Rat brains were quickly removed after decapitation and striatum were dissected. Striatum were homogenized on ice for 10 s with a sonicator (model Q-125, Qsonica LLC, Newton, CT) 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 clear supernatants were retained. The supernatant protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Supernatants (5–10 μg) were subjected to SDS–PAGE using 5–20% sodium dodecyl sulfate–polyacrylamide gels (DRC, Tokyo, Japan). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Japan, Tokyo, Japan). The membranes were incubated for 1 h with 5% nonfat milk (BD Difco, Flanklin Lakes, NJ) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 0.02% NaN_3 (PBST) to block nonspecific binding. The membranes were incubated for 1 h with rabbit polyclonal anti-SNAP-25 antibody (1:500, #Ab5666; Abcam, Cambridge, UK) or mouse monoclonal anti-β-actin antibody (1:4000, #AC-15; Sigma–Aldrich) in 0.5% nonfat milk/PBST. Membranes were washed three times with PBST for 5 min, then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies [1:5000, anti-rabbit (for anti-SNAP-25) or anti-mouse (for anti-β-actin) IgG; Invitrogen, Carlsbad, CA]. Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific Japan, Yokohama, Japan). Band intensities were quantified with the LAS-3000 image analyzer (Fujifilm, Kanagawa, Japan).

2.5. Immunofluorescent analysis

Rats were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) and perfused transcardially with ice-cold 4% paraformaldehyde in PBS at 23 days after the 6-OHDA injection (Fig. 1). The brains were removed and postfixed in 4% paraformaldehyde in PBS at 4 °C overnight, stored in 30% sucrose in PBS for 2–3 days, and frozen at −80 °C until sectioning. The frozen tissue was cut on a cryostat into 10 μm coronal sections. Sections were taken through the following brain region, relative to bregma; +2.16 to 0.0. The brain sections were incubated for 1 h with 10% donkey serum in PBS, and then incubated overnight at 4 °C with both rabbit anti-cleaved SNAP-25 polyclonal antibody (1:50, prepared in our laboratory according to [1]) and goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:100, #1978747; Millipore) in 1% bovine serum albumin/PBST. Antibody detection was visualized by staining with an Alexa488- or an Alexa568-conjugated secondary antibody (1:1000, Invitrogen) using a confocal scanning microscope (model C1si-TE2000-E, Nikon, Tokyo, Japan).

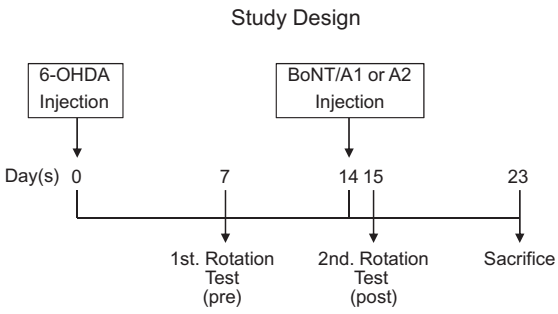

Fig. 1. An overview of the study design and schedule of BoNT/A1 and BoNT/A2 injection into the striatum of 6-OHDA-lesioned rats.

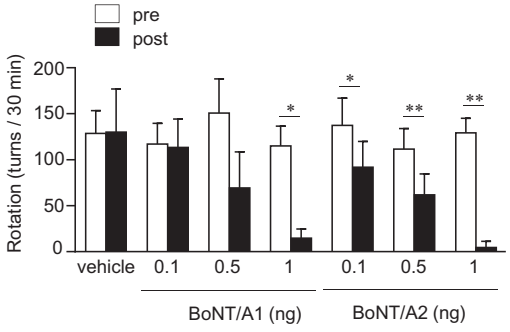

Fig. 2. Effects of intrastriatal injection of BoNT/A1 (0.1, 0.5, or 1 ng/rat; $n = 6$ per dose), BoNT/A2 (0.1, 0.5, or 1 ng/rat; $n = 6$ per dose), or vehicle ($n = 7$) on methamphetamine-induced rotation behavior. All rats received BoNT/A or vehicle injected into the lesioned striatum induced by 6-OHDA injection. For the tests, pre (white columns) represents before injection of BoNT/A, and post (black columns) represents after injection of BoNT/A. Data represent means ± SE; statistical significance is determined as pre versus post in a paired Student's *t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$.

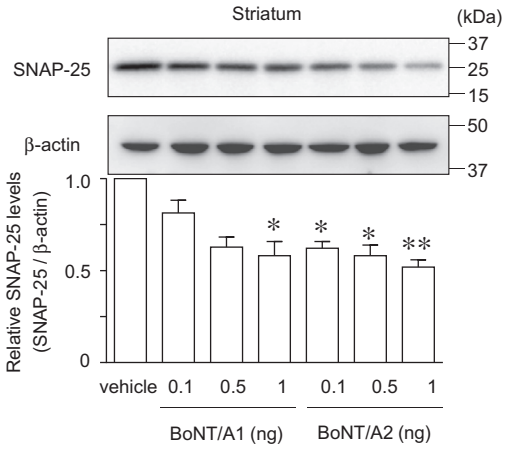

Fig. 3. Effects of intrastriatal injection of BoNT/A1 (0.1, 0.5, or 1 ng/rat; $n = 6$ per dose), BoNT/A2 (0.1, 0.5, or 1 ng/rat; $n = 6$ per dose), or vehicle ($n = 7$) on the relative levels of full-length SNAP-25 in the striatum. Values were calculated as the ratio of the SNAP-25 band intensity (quantified in arbitrary units) relative to the β-actin band intensity. Data represent means ± SE; statistical significance is determined as test versus vehicle using ANOVA followed by Dunnet's multiple test; $^{*}p < 0.05$; $^{**}p < 0.01$.

3. Results and discussion

Intrastriatal treatment of 6-OHDA-lesioned rats with BoNT/A1 or BoNT/A2 significantly reduced the pathogenic rotation behavior in a dose-dependent manner (Fig. 2). The highest tested dose of BoNT/A1 (1 ng) conferred significant reduction of pathogenic behavior, as did all tested BoNT/A2 doses (0.1, 0.5, and 1 ng). These results suggest that BoNT/A2 has more potent inhibition of ACh release in the striatum compared with that of BoNT/A1.

To elucidate the mechanism of BoNT/A reduction of rotation behavior, we examined the effects of BoNT/A1 and BoNT/A2 on the level of full-length SNAP-25 in the BoNT/A-injected side of the striatum (Fig. 3). Treatment of the 6-OHDA-lesioned striatum with BoNT/A1 or BoNT/A2 caused a dose-dependent decrease in the level of full-length SNAP-25 (i.e., both toxin subtypes cleaved SNAP-25 in the striatum). These results support the observed effects of BoNT/A1 and BoNT/A2 on rotation behavior (Fig. 2). 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, their dose-dependent efficacies in the striatum appear to differ.

We investigated the localization of cleaved SNAP-25 and choline acetyltransferase (ChAT) in the BoNT/A-treated striatum by performing fluorescent immunocytochemical analysis (Fig. 4). Treatment with 0.1 ng BoNT/A1 did not change the level of cleaved SNAP-25 in the striatum compared with that of the vehicle-treated group. By contrast, treatment with 0.1 ng BoNT/A2 significantly increased the level of cleaved SNAP-25, which was visualized as prominent green dots observed in the images [1]. In the BoNT/A2-treated rats, these green fluorescent dots were located closely to ChAT-positive cells (red). These results indicate that BoNT/A2 has greater efficacy for SNAP-25 cleavage in striatal terminals than that of BoNT/A1.

In the present study, we determined a minimum effective dose of 0.1 ng/rat for therapeutic treatment of pathological behaviors

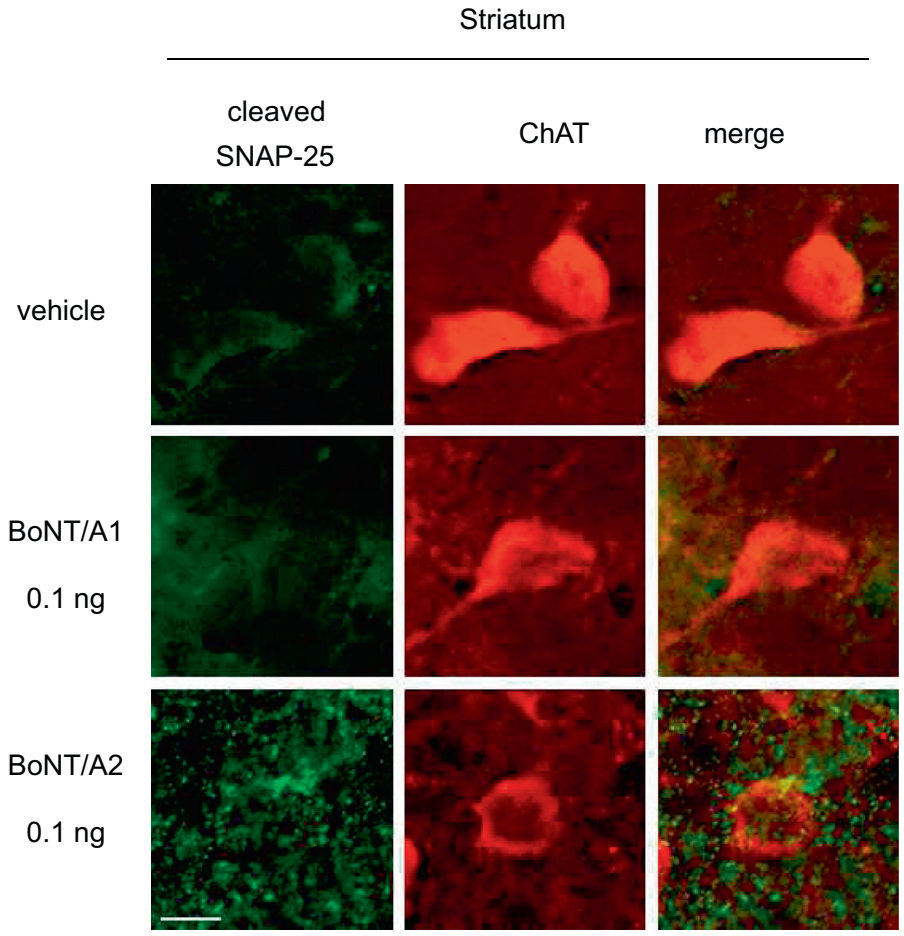

Fig. 4. Immunofluorescent analysis of cleaved SNAP-25 (green) and choline acetyltransferase (ChAT, red) expression in the striatum following intrastriatal injection of vehicle, BoNT/A1, or BoNT/A2. Scale bar = 5 μm.

with BoNT/A2 in a PD model (Figs. 2–4). This result is consistent with those of previous studies, which use commercially available BoNT/A and report an effective dosage range of 1–2 ng [11–13]. The current results also support previous observations that BoNT/A2 has greater potency compared with that of BoNT/A1 [19,20]. The observed difference between BoNT/A1 and BoNT/A2 in the rat PD model may be due to differences in the rate of toxin entry into neuronal cells; BoNT/A2 enters neuronal cells faster than BoNT/A1 [21]. BoNT/A1 and BoNT/A2 also have distinctly different distributions in the peripheral neuromuscular system [22]. We showed that BoNT/A2 provided greater reduction of pathological rotation behavior resulting from 6-OHDA-induced lesions and methamphetamine treatment compared to that of BoNT/A1. This effect was likely mediated by higher SNAP-25 cleavage activity in the striatum by BoNT/A2 compared with that of BoNT/A1. These results support the therapeutic application of BoNT/A2 to treat CNS neurological disorders including Parkinson's disease.

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