

nal region of the heavy chain) and cell-binding domain (C-terminal region of the heavy chain). The light chain contains the endopeptidase domain, which cleaves proteins associated with intracellular vesicular transport, such as SNAP-25 (synaptosomal-associated protein of 25 kDa) for type A toxin, and consequently inhibits acetylcholine release from neurons, leading to paralysis. Type A organisms have been classified into five subtypes (A1 – A5) based on the amino acid sequence variability of the produced NTX (6, 7). Botulinum toxin type A products, which are used as a treatment for neurologic disorder, are produced from LL toxin or NTX derived from subtype A1 organisms (8). 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 (9 – 11). 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 (12 – 14). The U.S. Food and Drug Administration announced 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 (15). The major problem with the toxin products was the relaxation of non-target muscles due to spread. It was reported that this spread might be the result of transport of the toxin via nerves (16):

To search for a toxin product with fewer adverse effects, we produced NTX from subtype A2 (A2NTX), with an amino acid sequence different from that of NTX subtype A1. In this study, to compare the spread dose of LL toxin from subtype A1 (A1LL), NTX from subtype A1 (A1NTX), and A2NTX, we conducted grip strength tests involving the side contralateral to the toxin-treated foreleg in rats as an indication of spread. To investigate the route of toxin spread, we carried out grip strength tests of the contralateral side of rat models treated with neurotomy or colchicine before the administration of toxins and in the rats treated with antitoxins after toxin administration. In addition, we used immunoblotting to determine if toxins were present in the contralateral foreleg after toxin administration.

Materials and Methods

Purification of toxins

Botulinum type A neurotoxins (150 kDa, NTX) were prepared as described in the previously reported method, with modifications (17). *Clostridium botulinum* type A strains 62A and Chiba-H, which belong to subtype A1 and A2, respectively, were cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glu-

cose, and 0.025% sodium thioglycolate by allowing them to stand at 30°C for 3 days. M toxin was purified from the culture fluid by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration. Each M toxin subtype was adsorbed onto a DEAE Sepharose column equilibrated with 10 mM phosphate buffer and eluted with a 0 – 0.3 M NaCl gradient buffer for NTX and non-toxic component separation. The different types of NTX were stored at –70°C until use.

For the test control, commercial progenitor LL toxin (BOTOX; Allergan Inc., Irvine, CA, USA) was used.

Experimental animals

ICR/CD-1 mice (4 weeks of age, female, about 20 g; Charles River Laboratories Japan, Yokohama) and S/D rats (8 weeks of age, female, about 200 g; Charles River Laboratories Japan) were used for the toxic activity assay and grip strength test, respectively. Animals were maintained under controlled light/dark conditions and had free access to food and water. This study was performed in accordance with the guidelines concerning experimental animals established by The Japanese Pharmacological Society and was approved by the Animal Ethics Committee of our institute.

Toxic activity measurements

The toxic activities of A1LL, A1NTX, and A2NTX were determined employing the mouse intraperitoneal (i.p.) LD₅₀ method (18). The mouse i.p. LD₅₀ was determined using a 7-dose assay with a dilution interval of 1.25 and 20 mice per dose. The evaluation period was the first 96 h after administration, and the LD₅₀ was calculated by the probit method. One mouse i.p. LD₅₀ was defined as 1 unit (U).

Investigation of toxin spread

We compared flaccid paralysis of the contralateral side muscles to that of those injected with A1LL, A1NTX, and A2NTX as an indicator of toxin spread (adverse effect). The toxins were serially diluted to 1.00 – 1.90 log U/mL with physiological saline containing 0.5% human serum albumin (diluent). Rats were anesthetized by the i.p. injection of 40 mg/kg of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo). After the disappearance of the eyelid reflex, the foreleg of the rat was shaved and 0.1 mL of each toxin concentration was injected into the foreleg muscles using an insulin syringe (Becton Dickinson, Tokyo).

The grip strength of the left and right forelegs of each rat was measured using a Grip Strength Meter (Muromachi Kikai, Tokyo) and the modification of a previously reported method (19). Two Grip Strength Meters were placed side by side, and a T-bar was attached to each. A

rat gripped the T-bar with each foreleg and was fixed horizontally, and then the rat was pulled steadily by the root of its tail away from the T-bar until its grip was broken. The peak grip strength was measured. The grip strength of each rat was measured five times, and the average was used. The grip strength was measured at 0 (before administration), 2, 3, 4, and 7 days after injection. The rat grip strength was expressed in units of gram-force (gf).

Investigation of route of toxin spread using a neurotomy model

To investigate whether toxins are transported via nerves to the contralateral muscles, denervation of the left brachial plexus of toxin-treated muscle was carried out as follows (Fig. 1): rats underwent general anesthesia by i.p. injection of pentobarbital sodium at 40 mg/kg, and regional anesthesia around the surgical site by the intramuscular injection of 5 mg/kg xylazine (Nippon Zenyaku Kogyo, Koriyama). A surgical incision was made in the left thoracic region, exposing the left brachial plexus. The division of this plexus was ligated at two sites and cut between those sites. After the surgical operation, the muscle and skin were sutured, and 75 mg of kanamycin (Meiji Seika, Tokyo) was injected subcutaneously (s.c.) to prevent infection. The non-neurotomy group received a sham operation.

A1LL and A1NTX were serially diluted with the diluent to 1.60–1.90 log U/mL and A2NTX, to 1.80 log U/mL. A volume of 0.1 mL with each toxin at various concentrations was injected into the left foreleg muscles in the neurotomy and non-neurotomy groups. The vehicle group was injected only with the diluent.

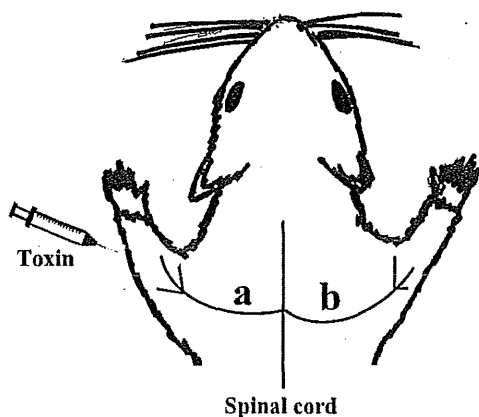


Fig. 1. Treatment of the neurotomy and colchicine models. a: left brachial plexus was cut (neurotomy model) or was treated with colchicine (left colchicine model), b: right brachial plexus was treated with colchicine (right colchicine model). The toxins were injected into the left foreleg in these models.

The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Investigation of route of toxin spread using a colchicine model

To investigate whether the toxins are transported axonally to the contralateral foreleg, the left and right brachial plexus of rats was chemically treated with colchicine (Wako, Osaka) (left and right colchicine model, respectively) (Fig. 1). Colchicine selectively inhibits axonal transport by acting on neuronal microtubules (20). The left or right brachial plexus was surgically exposed in the same way as in the neurotomy model and then covered with agars containing colchicine (10 μ g). The agars were themselves covered with Vaseline (Wako) to avoid the leakage of colchicine into non-target tissues. The non-colchicine-treated rat group received a sham operation.

A1LL and A1NTX were diluted to the dose of 0.60 log U and injected into the left foreleg muscles in these colchicine models and non-colchicine-treated groups. A2NTX was diluted to the dose of 0.78 log U and injected in the left colchicine- and non-colchicine-treated groups. The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Investigation of route of toxin spread employing treatment with antitoxin

To investigate whether toxins are transported via the body fluid, we intravenously administered antitoxin following toxin injection. Antitoxin against type A1 toxin (type A1 antitoxin) was equine-derived Japanese standard botulinum antitoxin type A (National Institute of Infectious Diseases, Tokyo). Antitoxin against type A2 antitoxin (type A2 antitoxin) was the equine-derived F(ab')₂ fragment, obtained as follows: Horses were immunized with toxoid of type A2 toxin, and sera were collected. The F(ab')₂ fragment was purified by peptic digestion from immunoglobulin which was extracted from the obtained sera.

The dose of toxins used was higher than the previous one because the toxins may possibly be transported through pathways other than axonal transport. A1LL and A2NTX were diluted to the dose (A1LL: 0.84 log U, A2NTX: 1.20 log U) that caused a 50% reduction in the grip strength of the contralateral foreleg, as reported in a previous experiment (21), and injected into the left foreleg muscles. In the antitoxin-treated groups, A1LL- or A2NTX-treated rats were intravenously given 1 unit of type A1 or A2 antitoxin (A1-antitoxin- and A2-antitoxin-treated groups, respectively) at 1 h after toxin administration. One unit of these antitoxins can neutralize 4.00 log U of type A1 or A2 toxin, respectively (22). In addition, in the prepared neurotomy model, A1LL and anti-

toxin type A1 were administered in the same way. These antitoxin doses were set so that the antitoxin dose did not affect the grip strength of the toxin-injected foreleg. The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Immunoblotting

To show the presence of the toxins in the contralateral foreleg, we investigated whether SNAP-25 was cleaved by the toxins in the contralateral nerve ends. The presence of cleaved SNAP-25 was demonstrated by immunoblotting, according to the previously described method (16). The neurotomy and left colchicine-treated models were used. Control rats received a sham operation. A1LL and A2NTX were each diluted to the appropriate dose (A1LL: 0.60 log U, A2NTX: 0.78 log U) and injected into the left foreleg muscles. The right foreleg muscles and nerve were isolated at 4 days after injection. The muscles and nerve were homogenized, and proteins were extracted with lysis buffer of the following composition: 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 10% glycerol, 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na₂MoO₄, 0.5 mM Na₃VO₄, 5 mM Na₄P₂O₇, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 0.01 mM PMSF. Protein extracts were separated by electrophoresis and electroblotted to a PVDF membrane (GE Healthcare Japan, Hino), which was then incubated with primary antibody (anti-botulinum toxin type A-cleaved SNAP-25) for 1 h at room temperature. The anti-botulinum toxin type A-cleaved SNAP-25 was produced as described previously method (23). Blots were then reacted with HRP-conjugated anti rabbit IgG (GE Healthcare Japan) and developed by ECL (GE Healthcare Japan).

Statistical analysis

The grip strength is presented as the mean ± S.E.M., and the time course is shown graphically. The change in the grip strength of each toxin-treated group, neurotomy model, colchicine model, antitoxin-treated group, and vehicle group over the 4 days after administration was analyzed by one-way ANOVA, Tukey's test, and two-way ANOVA. SAS (ver. 9.1; SAS Institute Japan, Tokyo) was employed for statistical analysis. The significance level was set to two-sided 5%. The *P*-value was rounded to 4 decimal places.

Results

Comparison of the adverse effect of toxins on contralateral muscles

The adverse effect of each toxin was evaluated by measuring the grip strength of both forelegs after intra-

muscular injection into the left foreleg. The grip strength decreased with increasing toxin concentration in the contralateral foreleg (Fig. 2). The grip strength of the toxin-treated (ipsilateral) foreleg was about 0 gf at all toxin concentrations used (data not shown).

The decrease in the grip strength of the contralateral

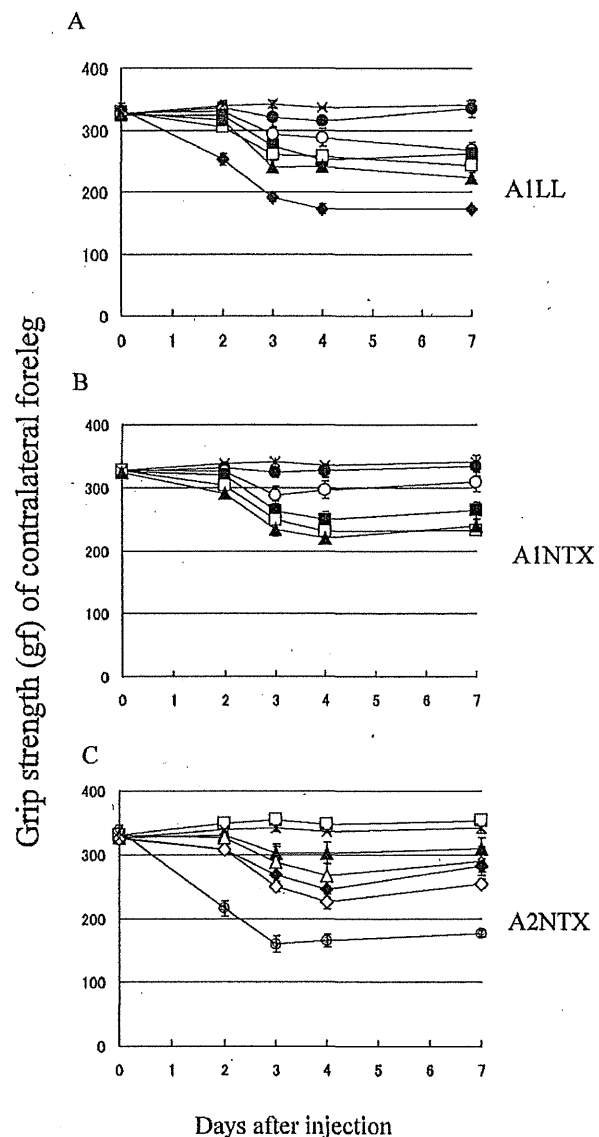


Fig. 2. Time-course changes in the grip strength of the contralateral forelegs after toxin injection in rats. Rats were injected with A1LL (A), A1NTX (B), or A2NTX (C) at various concentrations (0–1.20 log U) into left foreleg muscles. The grip strength was measured in the contralateral foreleg of each rat before (0) and at 2, 3, 4, and 7 days after injection. Each point is the mean ± S.E.M. (*n* = 5). 0 (closed circle), 0.30 (open circle), 0.48 (closed square), 0.60 (open square), 0.70 (closed triangle), 0.78 (open triangle), 0.84 (closed diamond), 0.90 (open diamond), 1.20 (double circle) log U, and Vehicle (cross mark).

foreleg reached a maximum on day 4 after administration. Thus, the change during the 4-day period was calculated by subtracting the value before administration from that on day 4. The change over the 4 days after injection between toxin-treated and vehicle groups was analyzed by one-way ANOVA. A1LL and A1NTX showed a significant difference from the vehicle at 0.30 log U or more, respectively. In contrast, A2NTX showed a significant difference at a dose higher than 0.78 log U (Fig. 3).

Effects of A1LL and A1NTX on contralateral muscles in the neurotomy model

To investigate the route of toxin spread to the contralateral foreleg, a rat neurotomy model was used. The change over the 4 days after injection among neurotomy, non-neurotomy, and vehicle groups was analyzed using two-way ANOVA and Tukey's test. Two-way analysis of variance (toxins dose by neurotomy treated) was performed among neurotomy, non-neurotomy, and vehicle groups. All groups of A1LL and A1NTX showed a significant difference from the vehicle group (main effect of toxin dose: $P < 0.0001$, main effect of neurotomy treated: $P < 0.0001$, toxins dose by neurotomy treated: $P = 0.0008$

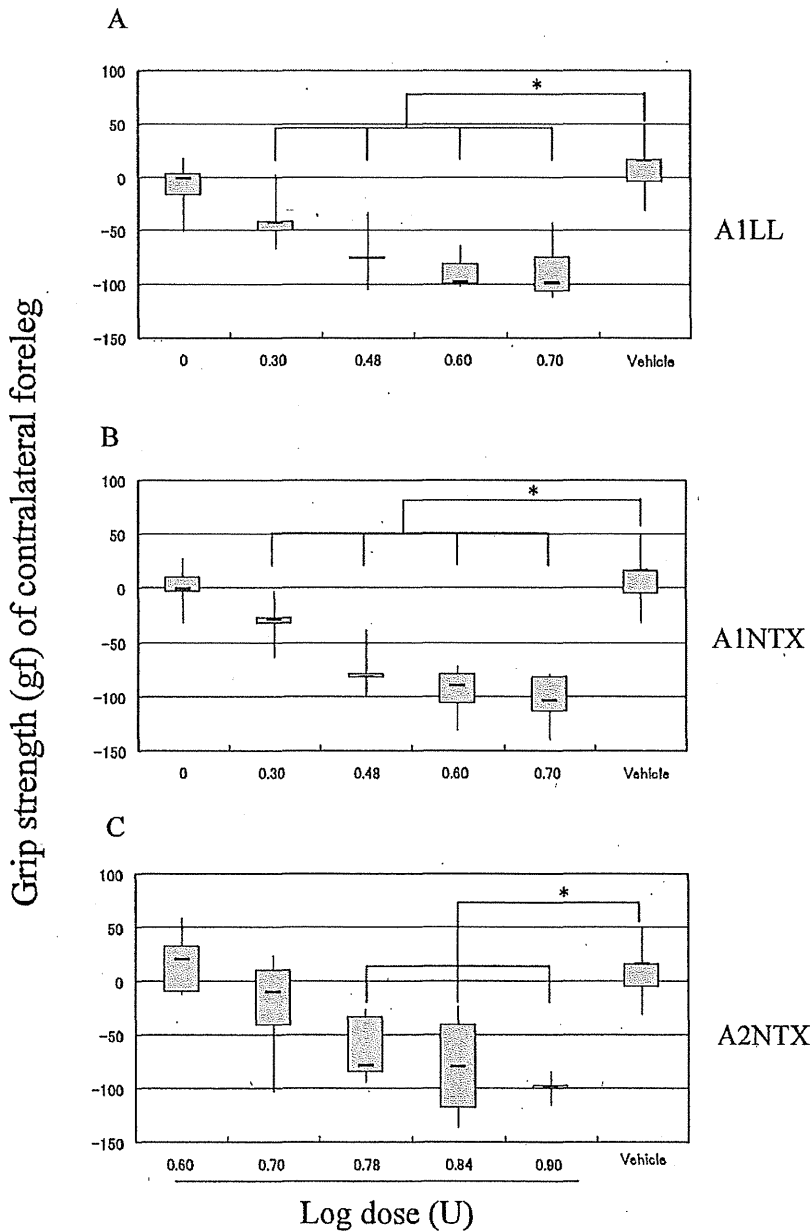


Fig. 3. Change in the grip strength of the contralateral foreleg during the 4 days after toxin injection [A1LL (A), A1NTX (B), and A2NTX (C)] ($n = 5$; $*P < 0.05$, one-way ANOVA). A1LL, A1NTX, and A2NTX showed a significant difference from the vehicle at more than 0.30, 0.30, and 0.78 log U, respectively.

in A1LL; main effect of toxin dose: $P < 0.0001$, main effect of neurotomy treated: $P < 0.0001$, toxins dose by neurotomy treated: $P = 0.0028$ in A1NTX). Tukey's test was performed among the groups. In the neurotomy group, the grip strength of the contralateral foreleg after the injection of 0.78 log U or less of A1LL or A1NTX did not decrease, and there was no significant difference between the neurotomy and vehicle groups ($P = 0.1859$, $P = 0.8453$ in A1LL and A1NTX at 0.78 log U, respectively). In contrast, the grip strength decreased in the non-neurotomy group, and there was a significant difference between the neurotomy and non-neurotomy groups ($P < 0.0001$ in A1LL and A1NTX at 0.78 log U). Moreover, a significant difference was observed between the neurotomy and vehicle groups at 0.90 log U of each toxin ($P = 0.0346$, $P = 0.0140$ in A1LL and A1NTX, respectively) (Fig. 4). In the neurotomy and non-neurotomy groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Effects of A1LL and A1NTX on the contralateral muscles in the colchicine model

The results using the neurotomy model suggested that both A1LL and A1NTX were transported to the contralateral muscles via a nerve pathway. In order to clarify this toxin pathway, left and right colchicine model rats were produced by treating the left and right brachial plexus with colchicine, respectively. The left and right colchicine models were blocked regarding axonal transport from the ipsilateral muscle to the spinal cord and from the spinal cord to the contralateral muscle, respec-

tively. The change over the 4 days after injection among colchicine-treated, non-colchicine-treated, and vehicle groups was analyzed using one-way ANOVA and Tukey's test.

The left colchicine- and non-colchicine-treated groups were injected with 0.60 log U of A1LL or A1NTX. The grip strength of the contralateral foreleg did not decrease in the left colchicine-treated rats, similar to the results seen in the neurotomy model. One way ANOVA was performed, and there was a significant difference among the left colchicine-treated, non-colchicine-treated, and vehicle groups ($P < 0.0001$ in A1LL and A1NTX). Tukey's test was performed, and there was no significant difference between left colchicine-treated and vehicle groups ($P = 0.8862$ and $P = 0.6132$ in A1LL and A1NTX, respectively). In contrast, the grip strength of the contralateral foreleg decreased in the non-colchicine-treated group, resulting in a significant difference between the left colchicine- and non-colchicine-treated groups ($P < 0.0001$ in A1LL and A1NTX) (Fig. 5A).

The right colchicine- and non-colchicine-treated groups were injected with 0.60 log U of A1LL or A1NTX. The grip strength of the contralateral foreleg did not decrease in the right colchicine-treated rats, similar to the results seen in the left one. One way ANOVA was performed, and there was a significant difference among the right colchicine-treated, non-colchicine-treated, and vehicle groups ($P < 0.0001$ and $P = 0.0002$ in A1LL and A1NTX, respectively). Tukey's test was performed, and there was no significant difference between the right colchicine-treated and vehicle groups

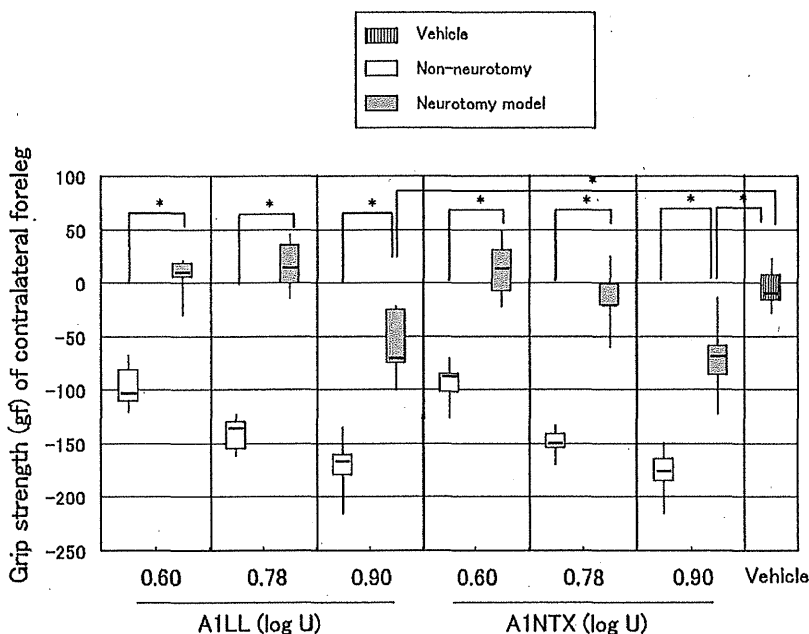


Fig. 4. Change in the grip strength of the contralateral foreleg in the neurotomy and non-neurotomy models during the 4 days after A1LL or A1NTX injection ($n = 5$; $*P < 0.05$, Tukey's test). Denervation was performed at the left brachial plexus, and then denervated rats received an injection of A1LL or A1NTX at 0.60, 0.78, and 0.90 log U into the left foreleg. There was no significant difference between neurotomy and vehicle groups at 0.78 log U or less of each toxin, but a significant difference appeared at 0.90 log U. At all doses used, the grip strength of the non-neurotomy group significantly differed from that of the neurotomy group.

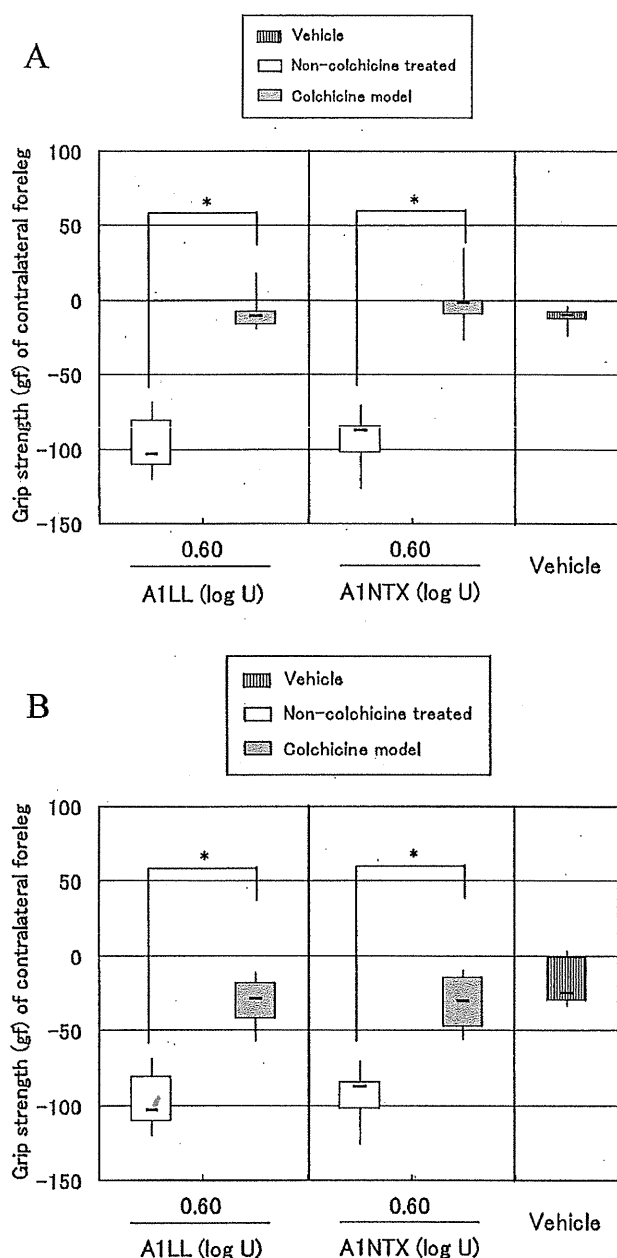


Fig. 5. Change in the grip strength of the contralateral foreleg in colchicine model rats during the 4 days after toxin injection ($n = 4 - 5$; $*P < 0.05$, Tukey's test). **A:** The left brachial plexus of rats was treated with colchicine, and then the rats received 0.60 log U of A1LL or A1NTX in the left foreleg. The grip strength of the contralateral foreleg did not decrease in the left colchicine model rats. There was no significant difference between the left colchicine model and vehicle rat groups, but there was a significant difference between the left colchicine model and non-colchicine-treated rat groups. **B:** The right brachial plexus of rats was treated with colchicine, and then the rats received 0.60 log U of A1LL or A1NTX in the left foreleg. The grip strength of the contralateral foreleg did not decrease in the right colchicine model rats. There was no significant difference between the right colchicine model and vehicle rat groups, but there was a significant difference between the right colchicine model and non-colchicine-treated rat groups.

($P = 0.5896$ and $P = 0.5560$ in A1LL and A1NTX, respectively). In contrast, the grip strength of the contralateral foreleg decreased in the non-colchicine-treated group, resulting in a significant difference between the right colchicine- and non-colchicine-treated groups ($P < 0.0001$ and $P = 0.0018$ in A1LL and A1NTX, respectively) (Fig. 5B).

In the left colchicine-, right colchicine-, and non-colchicine-treated groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown). To verify whether the colchicine-treated nerve was intact, the left brachial plexus of toxin-nonadministered colchicine model rats was stimulated 7 days after surgery. Muscular contraction of the left foreleg was normal on electrical stimulation.

Effect of A2NTX on the contralateral muscles in the neurotomy and colchicine model

We determined the grip strength in the neurotomy and non-neurotomy groups treated with 0.78 log U of A2NTX. One way ANOVA was performed, and there was a significant difference among the neurotomy, non-neurotomy, and vehicle groups ($P = 0.0017$). Tukey's test was performed, and neurotomy and non-neurotomy groups were significantly different from the vehicle group ($P = 0.0093$ and $P = 0.0020$ in the neurotomy and non-neurotomy groups, respectively). Unexpectedly, there was no significant difference in the effect of A2NTX between the neurotomy and non-neurotomy groups ($P = 0.6609$) (Fig. 6A). The grip strength in the colchicine- and non-colchicine-treated groups treated with 0.78 log U of A2NTX significantly differed from that of the vehicle group ($P < 0.0001$, in colchicine- and non-colchicine-treated groups). There was no significant difference in the effect of A2NTX between the colchicine- and non-colchicine-treated groups ($P = 0.8297$) (Fig. 6B). In all groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Effect of antitoxin treatment on the contralateral foreleg

To investigate the route of toxin spread to the contralateral muscles excluding axonal transport, we injected antitoxin following toxin administration. As a result, the grip strength of the contralateral foreleg did not decrease in the A2-antitoxin-treated group, and there was no significant difference between A2-antitoxin-treated and vehicle groups (Fig. 7A).

In contrast, the grip strength of the contralateral foreleg markedly decreased in the A1-antitoxin-treated group. This group significantly differed from A1LL-alone ($P = 0.0032$) and vehicle groups ($P = 0.0017$) (Fig. 7A). The neurotomy rats injected with A1-antitoxin were not

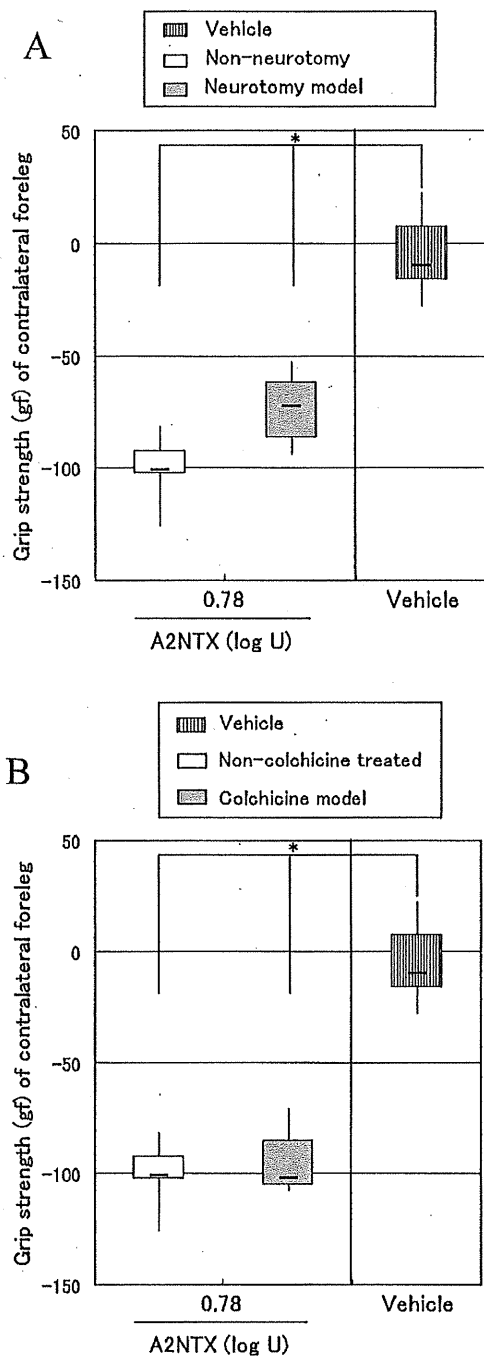


Fig. 6. Change in the grip strength of the contralateral foreleg in neurotomy and colchicine-treated models during the 4 days after A2NTX injection ($n = 5$; $*P < 0.05$, Tukey's test). **A:** Rats were denervated at the left brachial plexus and then injected with 0.78 log U of A2NTX into the left foreleg muscles. There was a significant difference between the neurotomy and vehicle groups, but no significant difference between the neurotomy and non-neurotomy groups. **B:** The left brachial plexus of rats was treated with colchicine, and then the rats received 0.78 log U of A2NTX in the left foreleg. There was a significant difference between the colchicine-treated and vehicle groups, but no significant difference between the colchicine- and non-colchicine-treated groups.

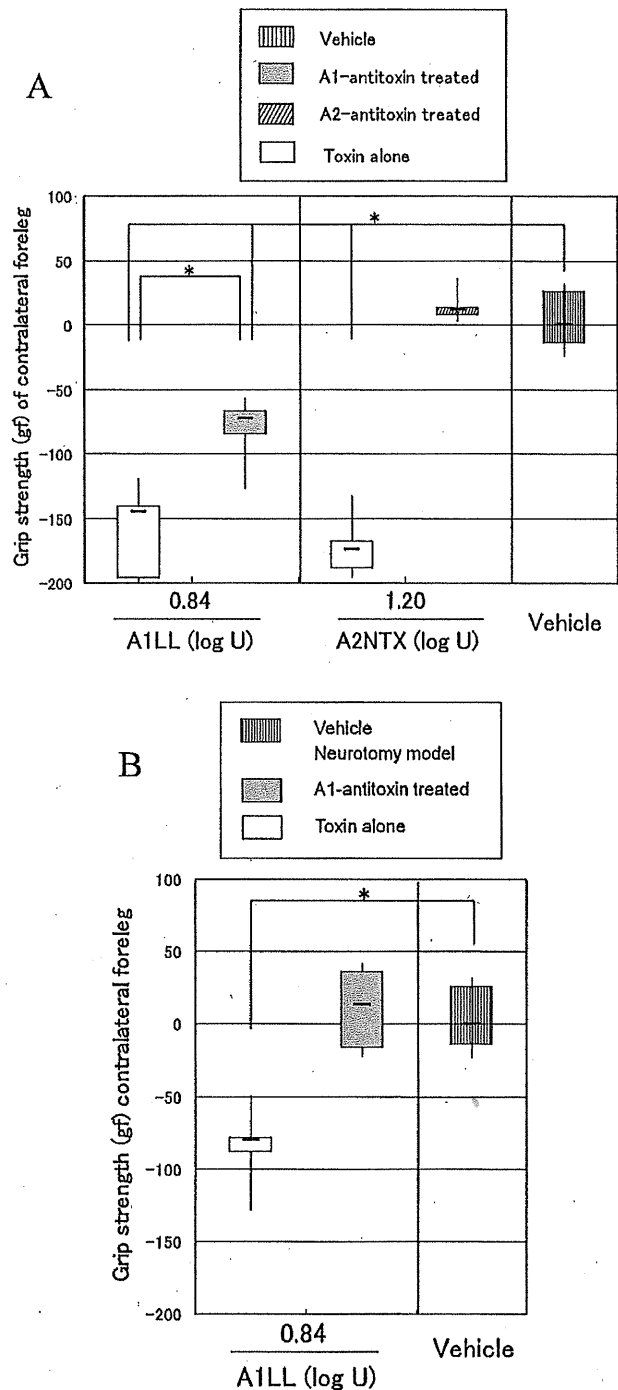


Fig. 7. Change in the grip strength of the contralateral foreleg in the antitoxin-treated rats during the 4 days after toxin injection ($n = 5$; $*P < 0.05$, Tukey's test). **A:** Injection of A1LL (0.84 log U) or A2NTX (1.20 log U). In the antitoxin-treated groups, where A1LL- or A2NTX-treated rats were intravenously injected with type A1 or A2 antitoxin, 1 h after toxin administration. The A1-antitoxin-treated group significantly differed from the A1LL-alone and vehicle groups. The grip strength in the A2-antitoxin-treated group did not decrease. **B:** Injection of A1LL (0.84 log U) in the neurotomy model. The grip strength of the neurotomy model rats that received antitoxin did not decrease.

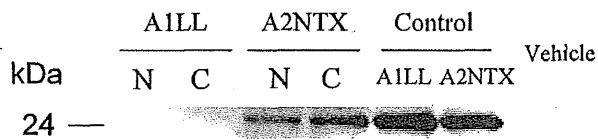


Fig. 8. Immunoblotting for A1LL- or A2NTX-cleaved SNAP-25 in protein extracts from the contralateral foreleg of the neurotomy (N) and colchicine-treated (C) model.

significantly different from the vehicle group ($P = 0.9263$) (Fig. 7B). In the toxin-treated groups (including antitoxin-treated groups), the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Immunoblotting

To show the presence of the toxins in the contralateral foreleg, we used immunoblotting to detect cleaved SNAP-25 in the muscle and nerve of the contralateral foreleg as an assay of toxins trafficking. No cleavage of SNAP-25 was observed in the A1LL-administered neurotomy and colchicine-treated models, but SNAP-25 cleavage was observed in all control rats treated with this toxin. All groups given A2NTX showed cleavage of SNAP-25 (Fig. 8). These results indicate that A1LL was not present in the contralateral muscle of these models, but in contrast, A2NTX did diffuse into these muscles.

Discussion

We investigated the route of botulinum toxin spread. The present results clearly indicate that both A1LL and A1NTX decrease the grip strength of the contralateral foreleg via both axonal transport and body fluid, while A2NTX is not transported via nerve pathways but body fluid.

The maximum doses of A1LL and A1NTX that did not reduce the grip strength of the contralateral foreleg were 0 log U (5 U/kg). The dose was 0.70 log U (25 U/kg) for A2NTX. This indicates that A2NTX requires a greater dosage for diffusion to the contralateral muscles than A1LL and A1NTX. A2NTX was 2.2- and 1.5-times more effective in decreasing the rat grip strength than A1LL and A1NTX in the toxin-injected foreleg, respectively, as previously reported (21). Therefore, A2NTX possibly causes a lower incidence of adverse effects than commercial products of botulinum toxin such as A1LL and A1NTX.

Several adverse effects of botulinum toxin preparations have been reported, and an adverse effect involving the relaxation of non-target muscles is a concern. In previous reports, the adverse effects were thought to be caused by the erroneous injection of toxin into non-target

muscle or spread due to a high dose of toxin (24, 25). Toxin spread to distant regions is considered to be due to transport via the body fluid or nerves (26–28). An RI study showed when labeled toxins were injected into gastrocnemius muscle of rats, the toxins were detected in the sciatic nerve, plasma and contralateral muscle. The toxins were not detected 48 h after injection in the contralateral muscle. More than 70% of the toxins were passed out of the body within 48 h of administration. Another RI study showed the retrograde axonal transport of toxin to the 6th lumbar spinal cord vertebra (L6) within 48 h after injection into the cat gastrocnemius muscle. Because of the elapsed time, it was assumed that the toxin was inactivated during transport (27). However, it has recently been reported that toxin that has entered neurons retains its activity for a prolonged period (16).

In this study, the results indicated that the grip strength of the contralateral foreleg reduced when the toxins were injected at a high dose. We considered it most likely that the toxins moved in the nerves to affect the grip strength of the contralateral foreleg, so we investigated whether the toxins moved to the contralateral foreleg through nerves or another pathway. When we treated the rats with A1LL or A1NTX, the grip strength of the contralateral foreleg decreased in the non-neurotomy and non-colchicine groups, but not in the neurotomy nor left colchicine model rats, indicating that A1LL and A1NTX were transported axonally from the ipsilateral muscle to the spinal cord and acted on the innervated contralateral foreleg muscles. To investigate the route of A1LL and A1NTX to the contralateral side, right colchicine model rats were treated with these toxins. The grip strength of the contralateral foreleg decreased in the non-colchicine groups, but not in the model rats. These results suggested that A1LL and A1NTX were retrogradely transported from the ipsilateral foreleg muscle to the spinal cord, and then the toxin was anterogradely carried to the nerve endings. To show that the toxin moves to the contralateral side, we used detection of cleaved SNAP-25 in contralateral nerve ends as an assay of the toxins trafficking. Botulinum toxin acts at the picogram level in rats. In order to detect the toxins directly in vivo, ^{125}I -labeled toxins would be required since otherwise a high dose exceeding the lethal dose would have to be used (26). However, we used a non-isotope-requiring method in this study that had been reported to be the most sensitive test to monitor the presence of active toxins in vivo, because a single toxin molecule can proteolyse a large number of SNAP-25 target molecules, providing a dramatic amplifying effect (16). In A1LL, we found cleavage SNAP-25 in the control of the contralateral nerve endings, but no SNAP-25 fragments were detected in the neurotomy and colchicine-treated models. This result

indicated that type A1 toxin transported to the contralateral foreleg via a nerve pathway. For A1LL and A1NTX to act on the innervated contralateral foreleg muscles, these toxins must be transported from the injected muscle to the spinal cord and between the ipsilateral and contralateral nerves in the spinal cord. Botulinum toxin binds to receptors on membranes of nerve endings and is introduced into neurons by endocytosis. The light chain of the toxin is translocated into the cytoplasm after acidification of endocytic vesicle. This vesicle was acidized at nerve endings. Then, the light chain cleaves SNARE protein. On the other hand, tetanus toxin, which has a similar structure to botulinum toxin, is also introduced into vesicle by endocytosis. However, the vesicle is not acidized at nerve ending. The tetanus toxin is retrogradely transported in these non-acidifiable vesicles in nerves. Then, the tetanus toxin is moved to other neurons in the spinal cord by transcytosis and acts on central nerves (29). In this study, the results suggest that A1LL and A1NTX were transported from injected muscle to spinal cord and between nerves as a tetanus toxin. When A1 toxin was injected into the hippocampus, the toxin was reported to cleave SNAP-25 in the contralateral side 3 days after injection (16). The two hippocampi are interconnected by commissural connections. The spinal cord also has nerve fibers that connect the two sides as a pathway that crossed extensor reflex follows. A1LL and A1NTX may be transported to the contralateral side via the nerve fibers. The mechanism of A1LL and A1NTX action in the spinal cord is a subject of future investigation.

In the neurotomy model, the grip strength of the contralateral foreleg after the injection of doses in excess of 0.78 log U of A1LL or A1NTX decreased, indicating that these toxins were transported to the contralateral foreleg not only via nerves but also via other pathways when these toxins were injected at high doses. To clarify the other pathways, antitoxin for botulinum toxin was injected at 1 h after the administration of 0.84 log U A1LL to control and neurotomy model rats. The grip strength of the contralateral foreleg in the A1LL-treated rat group without antitoxin decreased to about 150 gf over the 4 days after administration and that of the A1 antitoxin-treated rat group was about 75 gf. The neurotomy model rats injected with antitoxin 1 h after toxin administration did not show any decrease in the grip strength of the contralateral foreleg. These results indicate that the additional A1LL pathway excluding axonal transport is via the body fluid. The grip strength of the A1 antitoxin-treated rat group was reduced to one half of that in the A1LL-treated rat group without antitoxin. The antitoxin neutralizes toxins diffused from toxin-treated muscle to the body fluid; however, it does not neutralize

toxins inside nerve cells (axonal transport of toxin). These results clearly indicate that A1LL was transported not only via nerves but also via the body fluid to the contralateral foreleg when a high dose beyond the axonal transport capacity (about 0.80 log U or more) was used.

When A2NTX was injected at more than 0.78 log U, the grip strength of the contralateral foreleg in the neurotomy and colchicine-treated groups decreased to almost the same level as that of the non-neurotomy and non-colchicine-treated groups. In addition, we used immunoblotting to determine if A2NTX was present in the contralateral foreleg. A2NTX cleaved SNAP-25 in the neurotomy and colchicine-treated models. These results indicated that A2NTX was not transported via a nerve pathway to the contralateral foreleg. To clarify the transport mechanism of A2NTX, antitoxin was injected 1 h after administration of the toxin at 1.20 log U. The grip strength of the contralateral foreleg did not show any decrease. This indicates that A2NTX was transported via the body fluid. A1LL diffused to the contralateral muscles via axons at a dosage from 0.30 to 0.84 log U and via both axons and the body fluid at high dosages of more than about 0.80 log U. The dose via the body fluid was almost the same as that of A2NTX (about 0.80 log U or more). Therefore, the doses of A1LL and A2NTX transported via the body fluid might be almost the same (about 0.80 log U or more), and the difference between toxins in the dose diffusing to the contralateral foreleg may have been caused by the presence or absence of the axonal transport of toxins.

A2NTX shares 89% amino acid sequence homology with A1NTX, 95% in light chains, and 87% in heavy chains (6). A1 toxins but not A2NTX underwent axonal transport, and the reason is considered to be a difference in the amino acid sequences between them. Very different amino acid sequences in toxins can be seen in the heavy chains, which are involved in the binding of receptors and translocation of the intraneuronal light chains. We considered the following possible mechanisms for the contralateral transport of these toxins: one hypothesis is that A1 toxin binds to receptors not only of acidifiable endocytic vesicles but also non-acidifiable ones as tetanus toxin-containing vesicles. In contrast, A2NTX may bind to receptors of only acidifiable vesicles. In another hypothesis, both toxins are retrogradely transported to the spinal cord. A1 toxin is moved into the contralateral nerve by transcytosis. In contrast, A2NTX is moved in another way.

In this study, A2NTX caused less muscle flaccidity of non-toxin-treated muscle than A1 toxins (A1LL or A1NTX) as commercial botulinum toxin products. It was suggested that the variation in the amino acid sequence between A1NTX and A2NTX causes the difference in

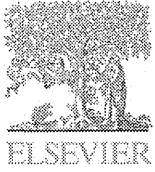
the spreading pathways. The required dose for spread (adverse effect) to the contralateral side is different according to the pathway.

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Editorial

Introduction

Keywords:

Botulinum toxin
Asian
Movement disorder

This supplement is a collection of articles arising from discussions of the Asian Botulinum Toxin Consortium, which consists of Asian movement disorder neurologists (from India, Korea, Japan, the Philippines, Taiwan, Thailand and Singapore) with a research interest in botulinum toxin (BTX). Although Asians account for approximately 30% of the world's population, the clinical data on Asian patients with movement disorders is not as well represented in the literature, except for conditions known to have an Asian preponderance, such as Lubag (sex-linked recessive dystonia parkinsonism of Panay) [1–3]. Intuitively, such information would be interesting. Although the incidence and prevalence of Parkinson's disease in Asia appear to be similar to, or lower than those in Western patients [4,5], Asians are over-represented among patients with hemifacial spasm [6], whereas they appear to be under-represented among patients with dystonia [7]. Asian patients with stroke behave differently from those of Caucasian or African ethnicity [8,9]; yet the presence, or absence, of an 'Asian phenotype' in movement disorders has yet to be established. Interestingly, some authors have reported that Asian patients require lower doses of BTX for the treatment of hemifacial spasm [10] and cerebral palsy [11] than has been reported in Caucasian patients. Clearly, formal clinical trials, comparing patients of different ethnicities, matched for age, gender and weight and using established clinical scales, are required.

BTX, which is one of the most lethal toxins known to man, has emerged in the past few decades as a potent therapeutic agent, used in the treatment of overactive smooth and skeletal muscles, glandular overactivity, as well as in the treatment of painful conditions. Ryuji Kaji has summarised the clinical indications of BTX to date and highlighted new and emerging indications of BTX, such as epilepsy and urgency in the overactive bladder. Roger Aoki examines how BTX ameliorates painful conditions, summarising clinical and non-clinical results to support the hypothesis that BTX reduces the symptoms associated with chronic pain through a two-step process, that is, reduction of local-pain nerve sensitisation through the local inhibition of neuropeptide release, resulting in an indirect reduction of central sensitisation. The benefits derived from the injection of BTX may be negated by spread of the toxin after injection, leading to unintended weakness. This may be reduced by accurate guidance techniques using

electromyography (EMG), ultrasonography, endoscopy or imaging. Erle Lim et al. summarise these techniques and discuss why different techniques are more suited to specific muscle groups or conditions. An important point, previously raised by Simpson et al. [12] in an evidence-based review of BTX in the treatment of spasticity, is reiterated by Lim et al. [13] that is, that though intuitively attractive, localisation techniques, such as EMG or ultrasound-guidance techniques, have not been proven to be more effective than surface localisation techniques. This is partly attributable to the different injection techniques (comprising different formulations, dilution volumes and localisation techniques), lack of uniform rating scales, lack of well-designed multi-centre studies and small patient numbers that are a feature of current studies. Raymond Rosales et al. review the use of BTX in the treatment of upper-limb post-stroke spasticity, commencing with the view of spasticity as a continuum of changes, spanning early to late neural alterations, as well as biomechanical modifications to muscle. They describe how BTX may be used to improve the functional outcomes.

Three articles in this supplement focus on dystonia. Roongroj Bhidayasiri examines the clinical features of complex cervical dystonia and discusses the prevalence of dystonia in Thailand. Finally, Petr Kaňovský and Raymond Rosales discuss how BTX, when used to treat dystonia, can modulate brain plasticity, thence resulting in long-term alleviation of the symptoms of dystonia.

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CASE REPORT

Gait and posture assessments of a patient treated with deep brain stimulation in dystonia using three-dimensional motion analysis systems

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Ryuji Kaji⁴, Satoshi Gotoh⁴, Shinji Nagahiro⁵, Shinjiro Takata¹, and Natsuo Yasui¹

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Abstract : Kinesiologic analysis of gait disorders, postural instabilities and abnormal movements is quite difficult to assess objectively by clinical observation, such as by specific scale and video recordings. In this study, we reported one of the aspects of the usefulness of three-dimensional motion analysis (Vicon Systems, Oxford, United Kingdom), which can measure inclusive data of movement disorders and substitute for conventional assessments. A 49-year-old man who had various dystonic symptoms, mainly on his left side of the body, responded well to deep brain stimulation (DBS). The examination quantified how the involuntary movements or other symptoms with dystonia changed before and after treatments. *J. Med. Invest.* 58 : 264-272, August, 2011

Keywords : motion analysis, dystonia, DBS

INTRODUCTION

Involuntary movements of dystonia interfere with normal posture and gait. Abnormal posture and gait disturbance are associated with frequent falls and restrict activities of daily living (ADL), which can reduce the quality of life (QOL). Deep brain stimulation (DBS) is a safe and successful therapeutic option for patients with gait disturbance in Parkinson's disease and dystonia. Globus Pallidus internus (GPI) DBS is effective for the treatment of generalized

dystonia (1). GPI DBS for X-linked dystonia improves 67.9-80.6% of symptoms (2-5).

Functional neurological impairment has been measured by specific observational analysis, such as a rating scale, video monitoring, conventional three-dimensional kinematic recordings using multiple videos, electromyography and electro-goniometry for the analysis of gait, a force plate for the analysis of postural stability, and multiaxial accelerometers and gyroscopes for the analysis of movement disorders. Conventional three-dimensional kinematic recordings are complicated and time consuming, and it is difficult to extract data (6). The Vicon system is a simplified three-dimensional motion analysis system that integrates conventional approaches. Vicon motion measurements have been used in clinical and research laboratories combined with

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an MX camera, which captures three-dimensional optical marker-based technology, to provide inclusive motion data in detail. We used the Vicon system to assess a patient with generalized dystonia before and after the implantation of GPi DBS electrodes. In this paper, we present the outcome of a case of dystonia treated with DBS to show its therapeutic efficacy to improve postural alignment and gait disturbance.

CASE REPORT

Patient presentation

A 49-year-old Filipino male, native to the island of Panay, was admitted to our hospital to treat dystonia. He had been previously diagnosed with X-linked dystonia-parkinsonism (DYT3 ; "lubag" disease) (7, 8). He first presented with involuntary movements of his bilateral halluxes at the age of 41. He later developed involuntary movements of his upper and lower limbs, cervical dystonia and spinal torsion causing gait disturbance and abnormal posture at the age of 43.

His ambulation progressively worsened. The baseline United Dystonia Rating Scale (UDRS) score performed at our institution was 64/5/112, and his Burk-Fahn-Mardsden Scale was 57/120 (9). He was found to have dystonic symptoms mainly on the left side of his body with a retracted head, twisted trunk and involuntary movements of his upper limbs. He also had difficulties with vocalization, swallowing and eye opening. His dystonia was very disabling, greatly affecting his quality of life (QOL).

Experimental design

Motion analysis using the Vicon system was conducted to record kinematic and kinetic data during static posture and dynamic walking. The patient was evaluated in three-dimensional static posture and consecutive gait using the Vicon MX system (Vicon Motion Systems, Oxford, United Kingdom). Kinematic data were collected at 120 Hz using a passive eight-camera system (Vicon MX T20 ; Vicon Motion Systems). Kinetic parameters were recorded at 120 Hz using a four-embedded ground force platform (AMTI, model OR-06 ; Advanced Mechanical Technology, Watertown, MA). Kinematic and kinetic systems were synchronized for simultaneous collection. Nexus 1.4 software (Vicon Motion Systems) derived the kinematic and kinetic parameters of standing for thirty seconds and five times walking on a ten-meter

walkway.

Markers (14-mm diameter) which reflected infrared rays were placed on landmarks of the whole body following the Plug-in-Gait model (Fig. 1, Vicon Motion Systems). Eight MX cameras captured the motion of markers and Nexus 1.4 processed the motion data as stick images from marker positions in three dimensions. Nexus 1.4 extracted c3d data (three-dimensional coordinated data of model) extracted from the plug-in-gait model parameters. Polygon 3.1 software (Vicon Motion Systems) simulated the human motion (skeletal model) expressed by the rigid body of the plug-in-gait model from c3d data. In addition, Medicaptures (Winpod, Balma, France) were used to record the distribution of foot pressure and the tracks of length (LNG) by postural sway of the center of mass (COM) in static posture, which showed the stability of static balance. He was evaluated before and after DBS (16 days after DBS) using Vicon. He was examined using various parameters such as neck angle (the angles between the head relative to the thorax) for cervical dystonia and spine angle (the angles between the thorax relative to the pelvis) for spinal torsion, COM changes for the stability of dynamic balance, patterns of ground reaction force (the force exchanged between the foot and the ground while walking) for symmetrical motion of limbs, and gait parameters (cadence, speed, step

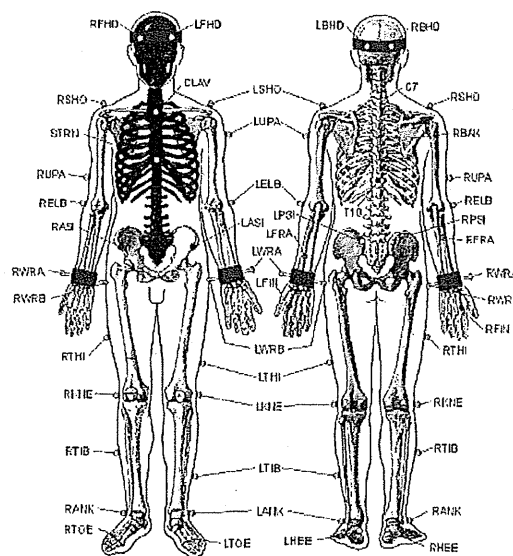


Figure 1. Plug-in gait marker placement
Thirty-five reflective markers were placed on landmark of the whole body. The distribution of markers consisted of head (four), trunk (nine), upper limbs (fourteen) and lower limbs (ten).

length, step width, single support time) for gait disturbance or symmetrical gait. He maintained a static posture for 30 seconds and gait five times and the mean values were calculated. All gait data were normalized by the gait cycle.

RESULTS

1. Posture

1-1) Foot pressure and COM

Skeleton models confirmed the external differences in static posture (Fig. 2). The patient's model before DBS inclined to the left side with poor position of his neck and trunk. His right foot pressure was distributed to his toe and heel (forefoot 51% and hindfoot 49%, average 415 g/cm²) and the left was deviated to his heel (forefoot 19% and hindfoot

81%, average 543 g/cm²). The LNG, which showed postural instability, was 923.2 mm. After DBS, distribution of foot pressure was on the right side (forefoot 65% and hindfoot 35%, average 361 g/cm²) and on the left (forefoot 18% and hindfoot 82%, average 510 g/cm²), and the LNG decreased to 502.9 mm. The balance of weight bearing and right-left ratio of foot pressure showed no difference between before and after DBS. LNG revealed clear shortening of 54.5% and shifted to the middle of his feet after DBS.

1-2) Neck and spine angle

His spine had extended to the left side, bent and rotated to the left before, and was slightly bent and rotated to the left after DBS (Fig. 3). His neck, however, had extended to the right side, bent and rotated to the right before, and was slightly bent and rotated to the left after DBS. His postural alignment

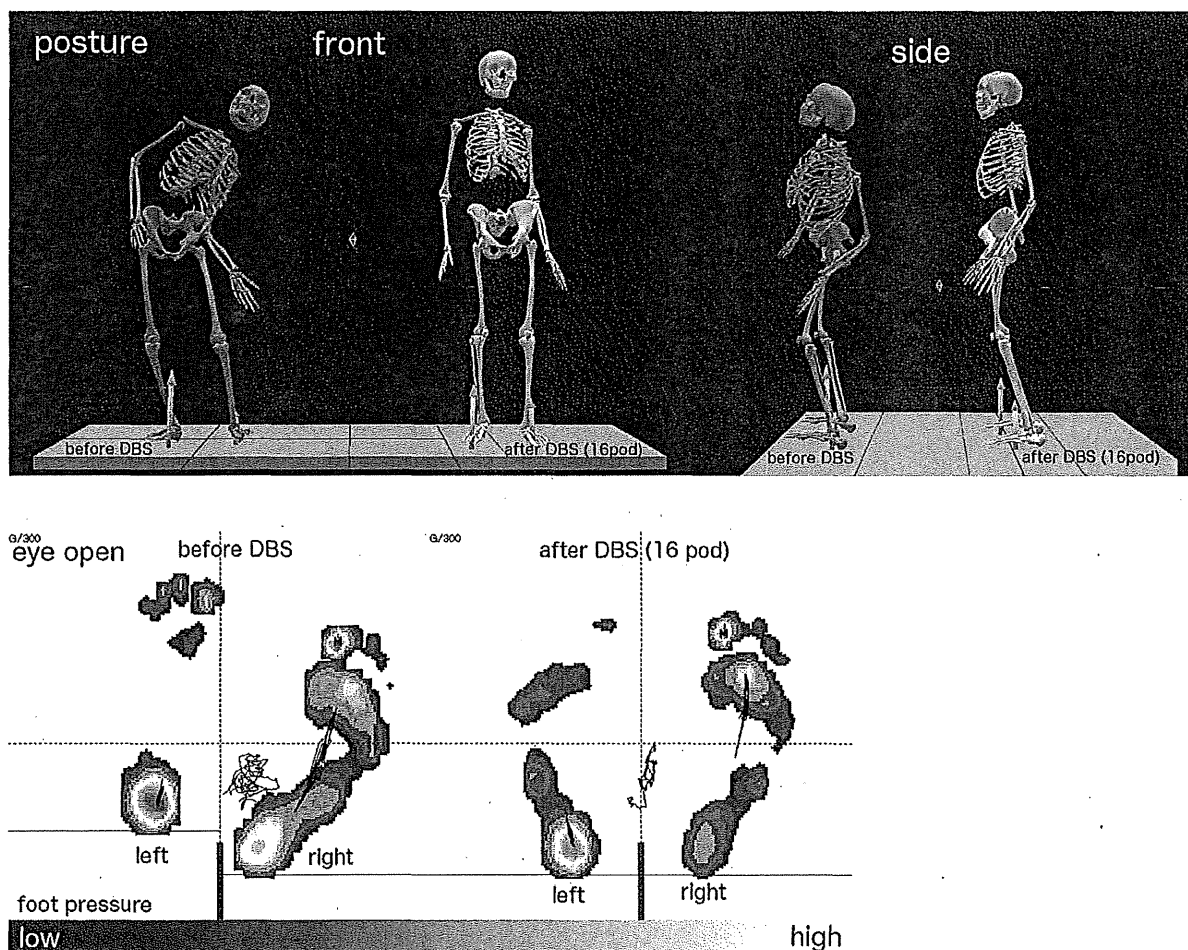


Figure 2. Static posture and foot pressure

Skeleton models from Polygon 3.1 simulated and visualized the postural alignments in frontal and sagittal planes. The patient stood on force plates with the eyes open and relaxed. Abnormality before DBS (left) especially showed spinal torsion with left postural deviation. The lines of the center of pressure showing postural instability improved on middle of the foot images after DBS.

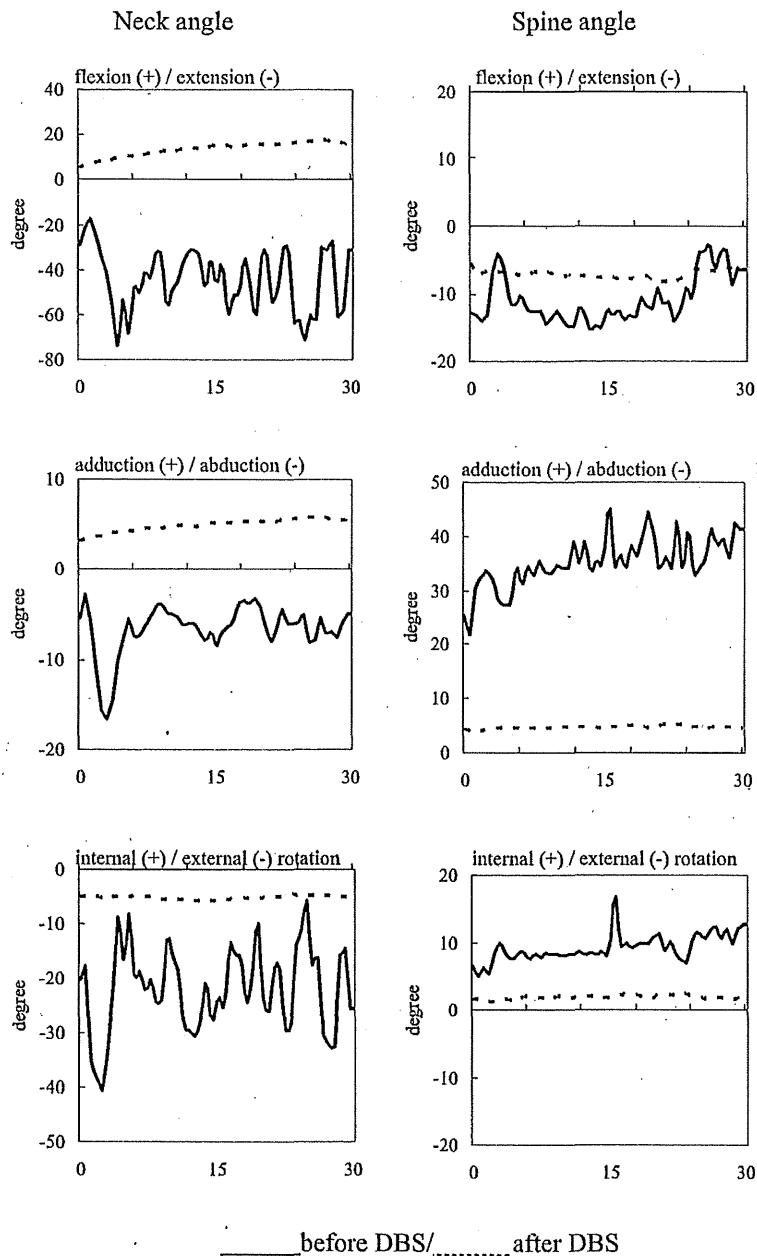


Figure 3. Neck and spine angle in static posture

The degree of neck and spine angles with static standing for thirty seconds improved to maintain a stable position after DBS in three dimensions. Neck movements before DBS were antagonists of those of the spine in lateral bend and vertical directions; these symptoms decreased after DBS.

remained almost straight (offset) from the center of his body (0 degrees) after DBS (neck offset : flexion 13.5 ± 3.4 degrees, left bending 4.9 ± 0.8 degrees, right rotation 5.1 ± 0.7 degrees, spine offset : flexion 13.3 ± 2.8 degrees, left bending 4.7 ± 0.3 degrees, left rotation 1.8 ± 0.8 degrees).

2. Gait

2-1) Gait parameters

The changes in gait parameters are shown in

Table 1. The following parameters were also compared with normal data (normal values) (10-12). All values after DBS were better than before but could not reach normal values for increasing cadence (110-120 steps/min), faster walking speed (1.36 m/sec), longer step length (0.65 m), shorter step width and single support time.

2-2) Neck and spine angle

The alignments of his spine before, which extended, bent and rotated to the left, changed to a

Table 1. Gait parameters

| | Before DBS | | After DBS | |
|-----------------------|------------|-----------|------------|-----------|
| | Left | Right | Left | Right |
| Cadence (steps/min) | 93.8±13.0 | 91.3±18.3 | 100.0±8.79 | 92.7±5.83 |
| Walking speed (m/sec) | 0.65±0.09 | 0.62±0.20 | 0.86±0.15 | 0.86±0.05 |
| Step lengths (m) | 0.26±0.10 | 0.54±0.14 | 0.45±0.18 | 0.58±0.05 |
| Step width (m) | 0.30±0.04 | 0.28±0.02 | 0.20±0.05 | 0.21±0.03 |
| Single support (sec) | 0.56±0.11 | 0.45±0.05 | 0.48±0.07 | 0.49±0.08 |

Values are expressed gait parameters (mean±SD) for five times walking. The parameters became more symmetry after DBS.

lower degree of spinal deviation close to a straight position on gait after DBS (Fig. 4). His neck angles after DBS were still abnormal (flexion, left bending

and right rotation) even after DBS. The standard deviations of each angle stayed in the lower ranges, showing stability during a gait cycle.

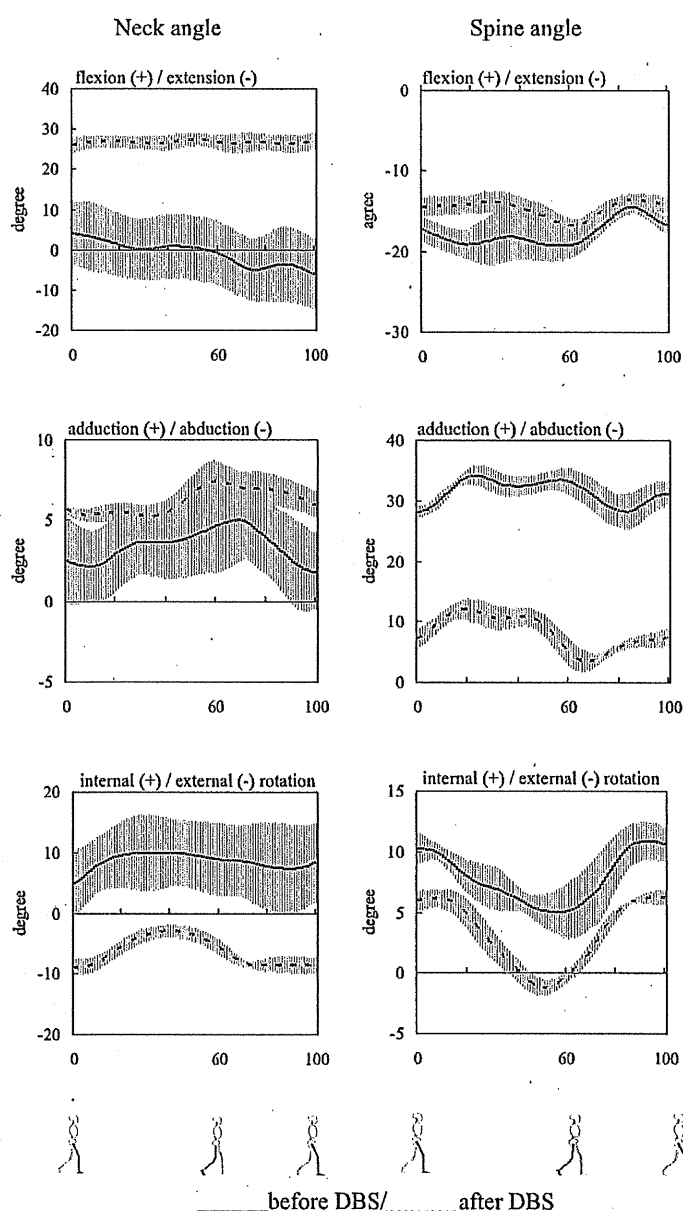


Figure 4. Neck and spine angles during a gait cycle

Angles (mean±SD, degree) of the patient with five times gait. After DBS, the spine angle became close to the median line of the body with moderate standard deviation in all planes (sagittal, frontal and coronal planes). Neck position remained inclined to the left.

2-3) Ground reaction force (GRF)

The asymmetry of GRF patterns (Fig. 5) before DBS became close to symmetrical after DBS (lateral

shear and vertical forces), except for a progressive pattern. Each lower limb had a respective role in braking force for the left and driving force for the

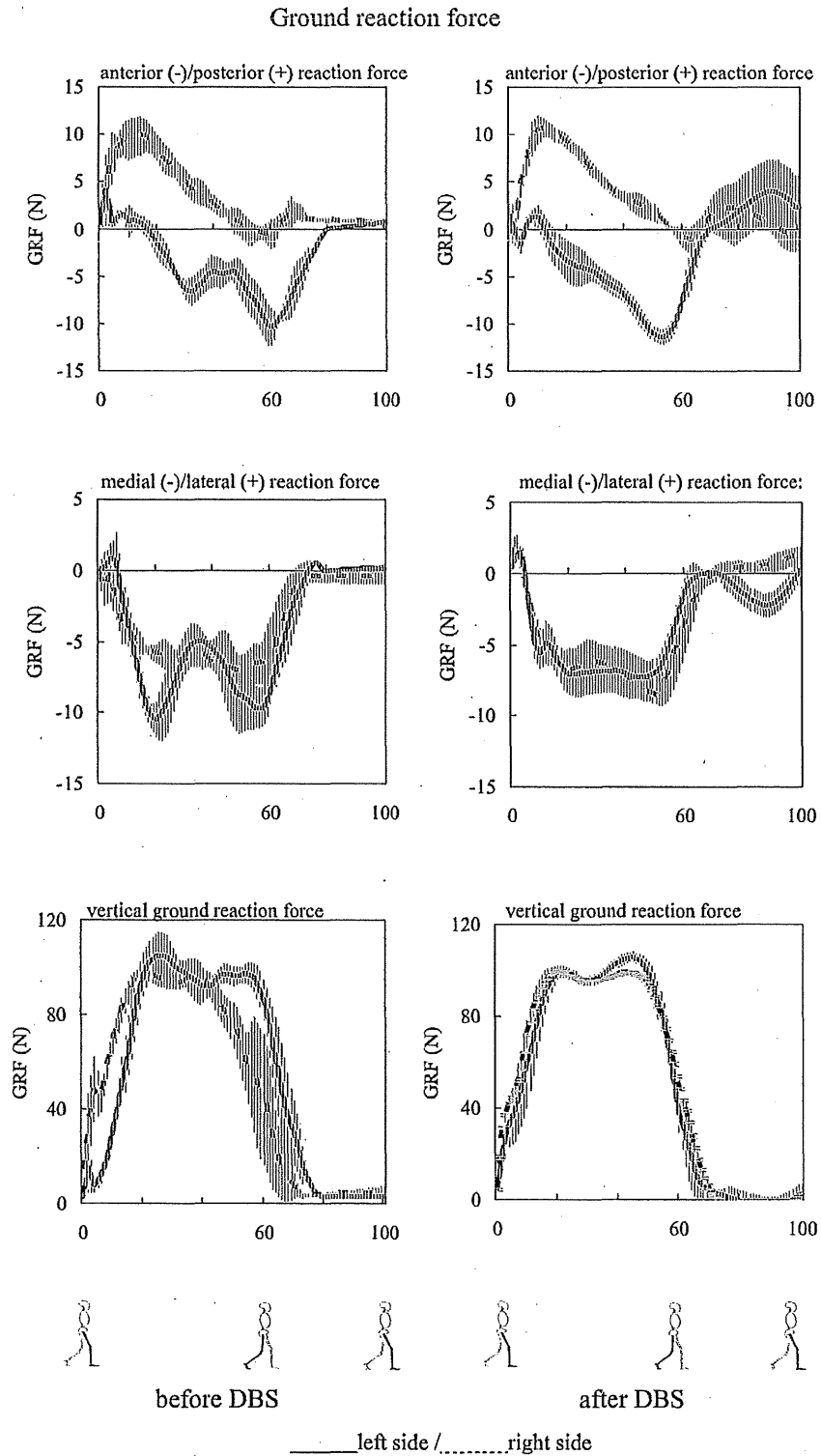


Figure 5. Ground reaction force during a gait cycle GRF (mean±SD, Newton) from force plates with five times gait showed close to symmetrical bilateral gait patterns after DBS, except progressive shear forces. Dystonia side (left) reversed normal side (right).

right in progressive shear force.

2-4) Center of mass (COM)

The ranges (distance) of COM were, before DBS : 173.4±26.7 mm (lateral : L), 66.5±22.4 mm (vertical : V) and after : 70.5±17.2 mm (L), 22.5±4.8 mm (V). The normal values were 58.0±20.0 mm (L) and 48.0±11.0mm (V) (10). The deviation of COM revealed stability in both lateral and vertical directions during a gait cycle (Fig. 6).

DISCUSSION

The mechanism of the DBS effect was associated with the disruption of pathological network activity in the cortico-basal ganglia-thalamic circuits by affecting the firing rates and bursting patterns of neurons and synchronized oscillatory activity of neuronal networks (13). There is a consensus that idiopathic generalized, cervical and segmental dystonia

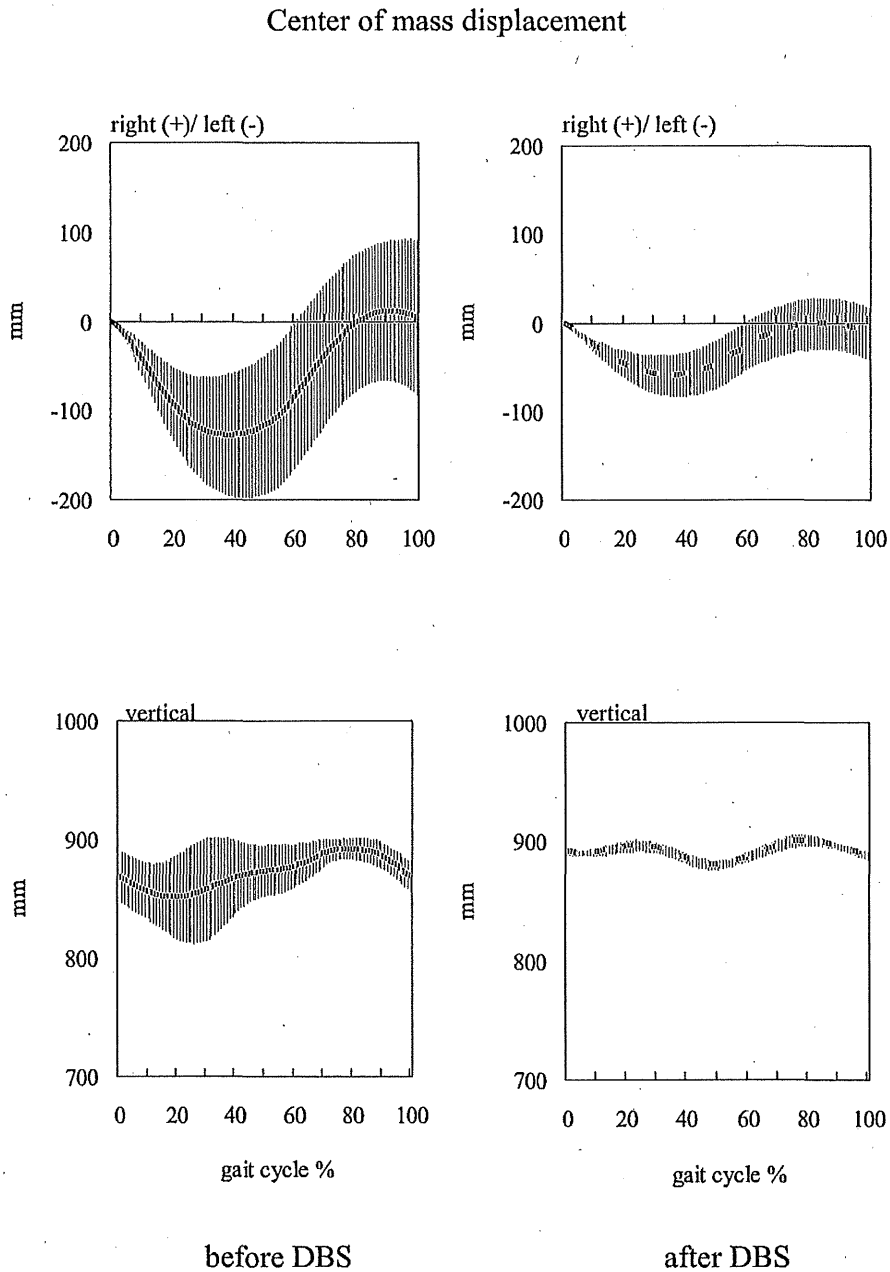


Figure 6. COM patterns during a gait cycle

Displacement of center of mass (mean±SD, mm) with five times gait in lateral and vertical directions for gait instability; wide ranges of standard deviations before DBS revealed less stability than after DBS during the gait cycle.

are good indications of DBS and efficacy is maintained long term. Although pallidal DBS has been shown to be cognitively safe, non-dystonic extremities have not received much attention (14). We clarified which involuntary movements were related to postural instability and gait disturbance by DBS in a dystonic patient using three-dimensional motion analysis in this study. His posture and gait were asymmetrical and unstable before DBS; therefore, he quickly became exhausted easily and fell down frequently (15), but they improved close to symmetrical after DBS. Functional body balance was controlled by changes of symptoms (with partial corrections of neck and spinal alignments in a static posture) and maintained the stability of COM and COP. His neck angles remained abnormal with specific motions during gait compared to the spine, which was not disturbed in walking. Functional improvements of gait, such as gait parameters including increasing of cadence (step rate) and walking speed, increased step length, reduction of a wide base, extension of single support time and symmetrical GRF patterns in lateral and vertical shear force close to normal patterns in consecutive gait showed dynamic stability simultaneously. Gait needs the neuromuscular function of the whole body and involves involuntary as well as voluntary motor elements (11). DBS facilitated the possible relations of gait asymmetry to postural instability in dystonia as well as Parkinson's disease (16). These improvements of gait parameters also explained that of neuromuscular function, which well responded to DBS. Not all symptoms could be treated with DBS (2-4). Our patient improved 80% by BFMS with slightly abnormal movement in his posture and gait. Some dependence on the right side remained for weight bearing to substitute the symptomatically dominant side during gait even after DBS. Other symptoms, including slightly better vocalization, swallowing and keeping his eyes open could help the patient to be independent and active during hospitalization. These remaining symptoms will necessitate a rehabilitation program, using training to establish new movement patterns, to preserve an appropriate activity level, and to treat the specific disability, which resulted from secondary changes of the musculoskeletal system during pathological muscle tension in dystonia (17) after DBS.

The results of this study revealed that three-dimensional motion analysis could inclusively assess the level of improvement in a patient with movement disorders and could assist in diagnosis or effect

measurement. We also need to collect more data from patients with movement disorders and clarify their specific characteristics in future studies.

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