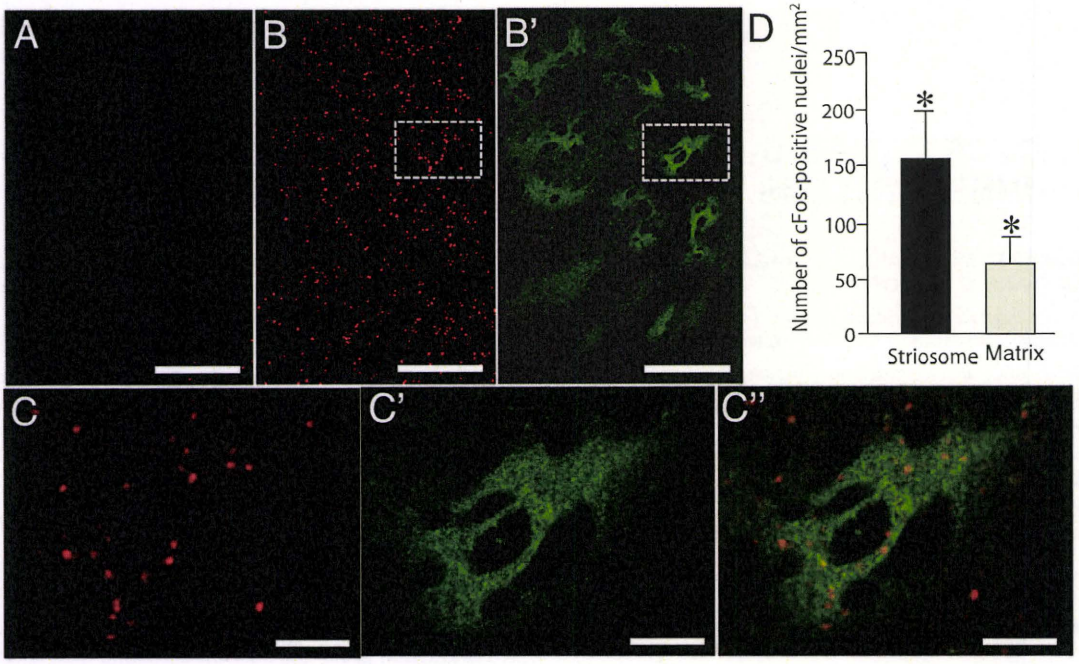

Fig. 1. Striosome-predominant expression of c-Fos protein with apomorphine treatment in adult mice. (A) Immunofluorescence staining for c-Fos in the striatal section from a mouse with no treatment of apomorphine. (B, B′) Dual antigen immunofluorescence staining for c-Fos (B) and MOR (B′) in the striatal section from a mouse with apomorphine treatment. Region shown in dashed open boxes in (B, B′) is illustrated at higher magnification in (C, c-Fos), (C′, MOR) and (C′′, merged). Apomorphine induces patchy striosome-predominant expression of c-Fos in the caudoputamen. (D) Density measurements of c-Fos-labeled nuclei in the striosome and matrix compartments. Data are represented as the mean (SEM) (bars) values (n=25). * indicates P=0.01 striosome vs. matrix. MOR, μ-opioid receptor. Scale bars: (A, B) 500 μm; (C) 100 μm.

Louis, MO, USA), rabbit polyclonal antibody to μ-opiate receptor (MOR; Millipore, Billerica, MA, USA; 1:10,000), rabbit polyclonal antibody to the dopamine and cAMP-regulated phosphoprotein of 32 kDa (the dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32); Cell Signaling, Danver, MA, USA; 1:1000), rabbit polyclonal antibody to phospho-[Thr34]-DARPP-32 (Cell Signaling; 1:1000), rabbit polyclonal antibody to phospho-[Thr75]-DARPP-32 (Cell Signaling; 1:1000), rabbit polyclonal antibody to c-Fos (Oncogene Science, Cambridge, MA, USA; 1:2000), and rabbit polyclonal antibody to Gαolf (Santa Cruz Biotechnology; 1:500) were used as primary antibodies. For detection of the bound antibodies, we used the Histofine Simple Stain kit (Nichirei Company, Tokyo, Japan) with diaminobenzidine (DAB) as a chromogen. Dual antigen immunofluorescence staining for c-Fos and MOR was performed as previously described (Sato et al., 2008). The digital microscopic images from the immunostained sections were acquired with Meta-Morph software (Molecular Devices, Tokyo, Japan), imported into Adobe Photoshop CS4, and processed digitally.

Densitometry and statistics

To estimate the density of Gαolf labeling, we immunostained the striatal sections in parallel at the same time with the same protocols. The optical densities of DAB products were measured as gray levels. For each animal, measurements were made in five striatal fields from five sections. Measurements of the density of c-Fos-labeled nuclei in the striatal compartments were made on the sections doubly-stained for c-Fos and MOR. We counted the number of c-Fos-positive nuclei within the striosomes (n=25) and in the matrix areas (n=25) from five striatal fields of each rat (n=5), and calculated the density of c-Fos-positive nuclei/mm^2 in each compartment. For statistical analysis we used Student's two tailed t-test and P-values less than 0.05 were considered as statistically significant.

RESULTS

To test whether the striosome and matrix compartments differentially respond to dopaminergic stimulation in the adult mice used in the present study, we performed an assay for c-Fos expression after treatment with apomorphine (Fig. 1). Because the induction of immediate early gene reflects acute elevation of cAMP-dependent signaling caused by the activation of postsynaptic D1Rs within striatal neurons, c-Fos induction can be considered as an indicator of D1R-mediated signal transduction in the striatum (Moratalla et al., 1996; Kim et al., 2002). With the dual-antigen detection for c-Fos and MOR, a marker for striosomes (Canales and Graybiel, 2000; Sato et al., 2008), we confirmed preferential localization of the nuclei labeled for c-Fos in the striosomes relative to the matrix compartment in adult mice that were administered a high dose (10 mg/kg) of apomorphine (Fig. 1).

Next, to identify a molecular candidate responsible for the differential responses of the two striatal compartments to the D1R signaling, we reappraised the distributional profile of TH and D1R (Fig. 2) in the striatum of adult mice. Immunohistochemistry for TH, a marker for striatal dopaminergic afferent fibers, showed a nearly homogeneous staining (Fig. 2A). No obvious compartmentalization of D1R-labeling (Fig. 2B) was also found. On the other hand,

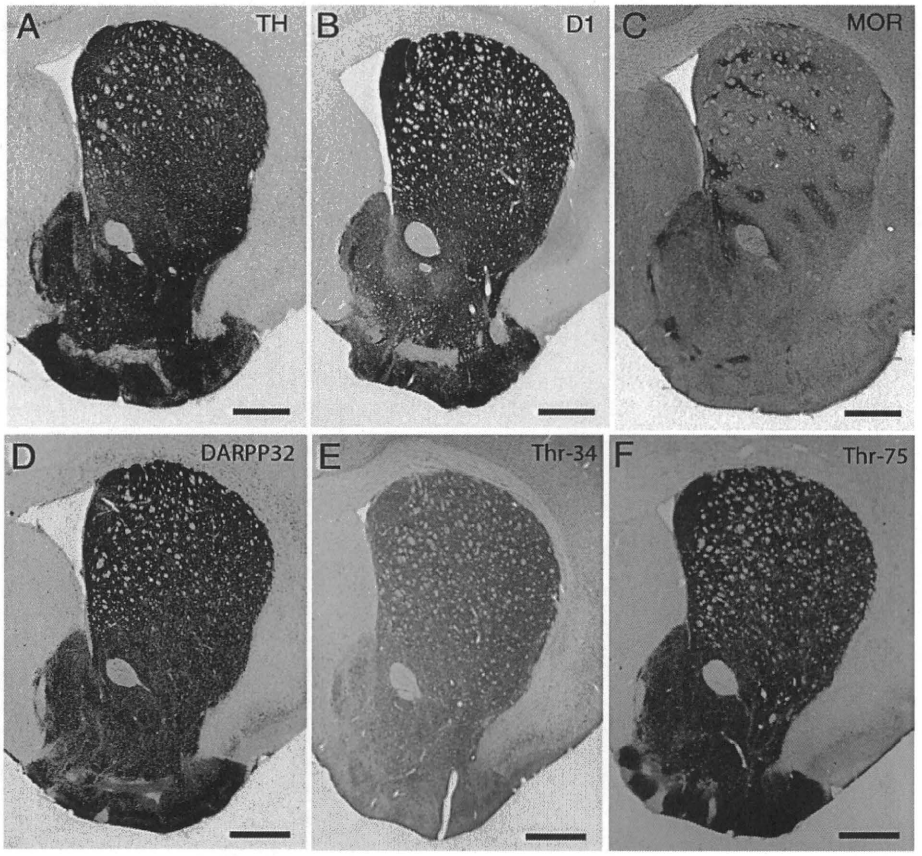

Fig. 2. Striatal localization of D1 dopamine signal-related molecules in adult mice. Striatal sections stained for TH (A), D1R (B), MOR (C), DARPP-32 (D), phospho-[Thr34]-DARPP-32 (E), and phospho-[Thr75]-DARPP-32 (F). TH, tyrosine hydroxylase; D1R, dopamine D1 receptor; MOR, μ-opioid receptor, DARPP-32, the dopamine and cAMP-regulated phosphoprotein of 32 kDa. Scale bars=500 μm.

the striosome and matrix compartments were clearly identified on staining for MOR (Fig. 2C). Hence, it is likely that dopaminergic afferents and D1Rs are almost homogeneously distributed in the striosome-matrix system. In addition, DARPP-32, a major component of the D1R-signaling cascade (Greengard, 2001), also showed no obvious compartmentalized distribution in the striatum (Fig. 2D), as did its site-specific phosphorylated forms such as phospho-[Thr34]-DARPP-32 (Fig. 2E) and phospho-[Thr75]-DARPP-32 (Fig. 2F).

Lastly, we examined the localization pattern of Gαolf in the striosome-matrix systems of adult mice (Fig. 3). For this purpose, we used a rabbit polyclonal antibody to the Gαolf protein. On immunoblots of rat brain extracts, a protein band with an approximate molecular mass of 42 kDa, corresponding to the predicted size of the native Gαolf protein, was selectively detected with the antibody (Fig. 3A). From the anterior to posterior levels of the striatum (Fig. 3B–D), immunoreactivity for Gαolf was highly concentrated in the striatum that consists of the caudoputamen, nucleus accumbens, and olfactory tubercle. Under high-power magnifications, Gαolf labeling was strongly expressed in numerous neuronal fibers but less intensely in neuronal perikarya in the striatum (Fig. 3E). Of particular interest was the finding that within the caudoputamen, there were focal zones of particularly high Gαolf immunostaining. These zones of heightened Gαolf labeling corresponded to the striosomes identified in the adjacent sections stained for MOR (Fig. 3F, G). Serial section analysis on negative images (Fig. 3H, I) also illustrates this higher expression of Gαolf in the striosomes relative to the nearby matrix area. Densitometric measurements confirmed this visual impression (Fig. 3J). A quantitative study revealed a significant difference in the Gαolf staining density between the striosome and matrix compartments (P=0.001). Hence, we inferred that the striosome compartment is enriched with the Gαolf protein in adult mice.

DISCUSSION

Given that c-Fos induction by apomorphine is significantly high in the striosomes relative to the matrix compartment, our results indicate that there exists a predominant responsiveness of the striosome compartment to D1R signaling in the adult mouse striatum. However, according to our data, no apparent compartmentalization was found in adult mice with respect to the distribution of D1R or TH. This finding is consistent with the known expression pattern of D1R and

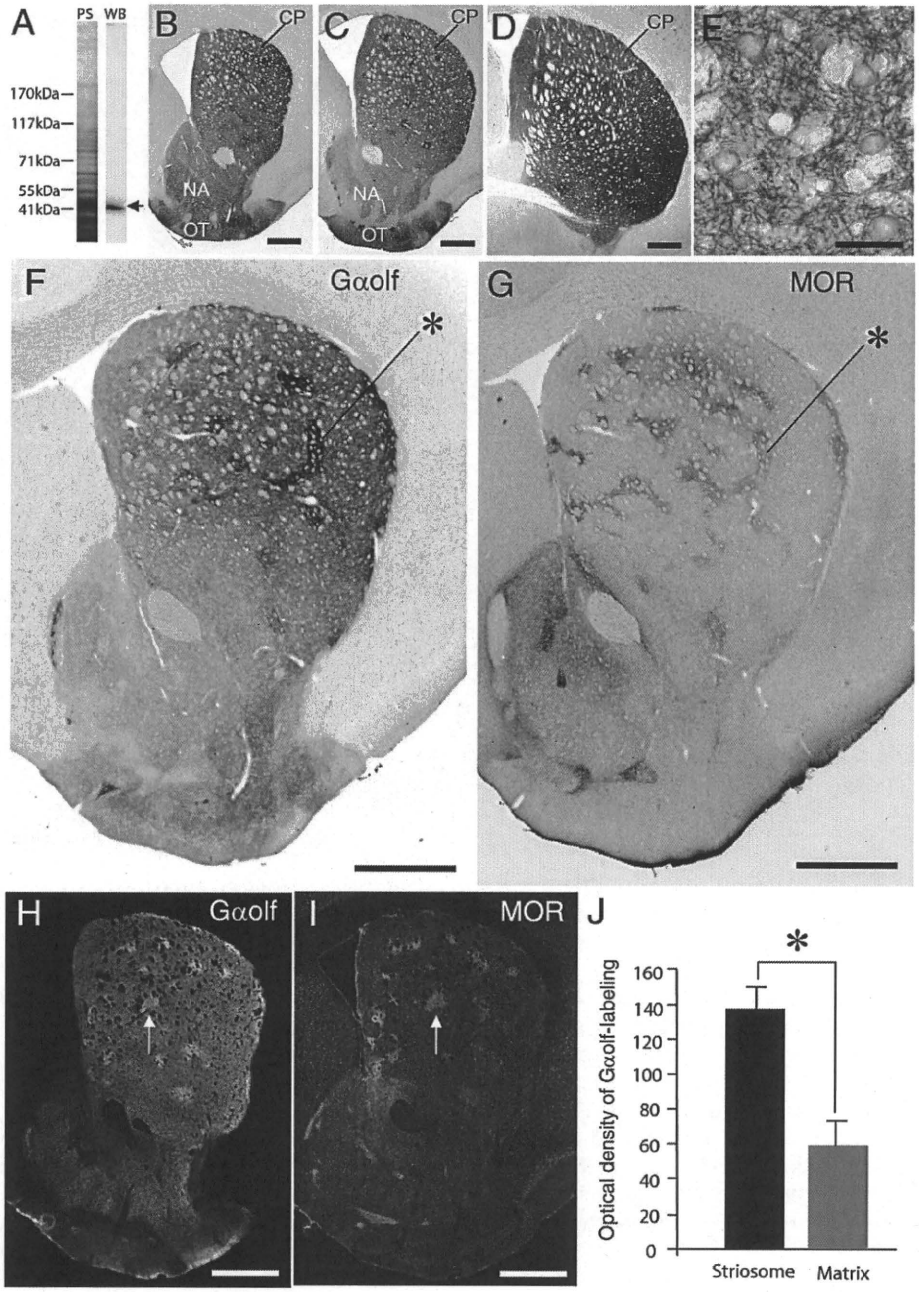

Fig. 3. Striatal localization of Gαolf in adult mice. (A) Western-blot assay. Rat brain extracts (50 μg of protein) were loaded onto 10% SDS-PAGE and then processed for the transimmunoblot technique using anti-Gαolf antibody. Note that a protein band (arrowhead) with an approximate molecular mass of 42 kDa, corresponding to the predicted size of the native Gαolf protein was selectively detected. PS, protein staining; IB, immunoblot. (B–D) Gαolf-immunostaining at the anterior (B), middle (C), and posterior (D) levels of the striatum. (E) High-power photomicrograph of the striatal area stained for Gαolf. (F, G) Serial sections stained for Gαolf (F) and MOR (G). Asterisks indicate a corresponding striosome. MOR, μ-opioid receptor. (H, I) Negative prints of serial sections stained for Gαolf (H) and MOR (I). Arrows indicate a corresponding striosome. (J) Density measurements of Gαolf-labeling in the striosome and matrix compartments. Data are represented as the mean (SEM) (bars) values (n=25). * indicates P=0.01 striosome vs. matrix. Scale bars: (B–D), (F–I), 500 μm; (E) 50 μm.

TH in the mouse striatum (Kim et al., 2002; Granado et al., 2008; Sato et al., 2008). Immunostaining of D1R and TH first appears in discrete "dopamine islands" that correspond to developing striosomes (Graybiel, 1984) at the early postnatal period. With development, the level of D1R- and TH-staining intensity in the matrix compartment is increased to the level found in striosomes, and hence their compartmentalization becomes obscure during adulthood (Kim et al., 2002). Therefore, it is likely that in adult mice, the striosome and matrix compartments differentially re-

spond to D1R signaling, despite no obvious difference in the distribution of D1Rs and dopaminergic inputs as determined by TH-staining between the two striatal compartments. This notion could raise the possibility that certain molecules that play a role in the intracellular dopamine D1 signaling cascade are differentially expressed in the striosome-matrix systems in adult mice. Although a previous study showed a homogeneous pattern of Golf mRNA expression throughout striatal development in rats (Sakagami et al., 1995), in the present study, we have shown that the Gαolf protein is differentially expressed in the striatal compartments of adult mice. The present study first showed compartmentalized distribution of the Gαolf protein in the adult mouse striatum, with a heightened density of Gαolf-staining in the striosomes relative to the matrix compartment. Because activation of D1R increases cAMP production via Gαolf-mediated stimulation of adenylyl cyclase in rodents (Zhuang et al., 2000; Corvol et al., 2001), we suggest that Gαolf could be one of the key molecules for controlling differential response of striosome-matrix systems to D1R signaling in adult mice.

Accumulating evidence has shown that differential involvement of the striosome-matrix dopamine systems could be critical to identifying mechanisms underlying the genesis of movement disorders such as Parkinson's disease (Wilson et al., 1987; Moratalla et al., 1992; Graybiel et al., 2000; Iravani et al., 2005; Crittenden et al., 2009) and dystonias (Sato et al., 2008). It has also been suggested that a significant change in striatal Gαolf expression is strongly linked with the induction of the hypokinetic or hyperkinetic state in models of striatal dopamine deficiency in both animals (Herve et al., 1993, 2001; Marcotte et al., 2001; Cai et al., 2002) and humans (Corvol et al., 2004). Moreover, an important finding indicates that the striatal level of Gαolf serves as a key regulator for acute responses to psychomotor stimulants (Corvol et al., 2007). Taken together, we suggest that striosomal enrichment of the Gαolf protein may give new insights in the compartment pathology of striatal dopamine systems in movement and behavioral disorders.

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Quantitative determination of biological activity of botulinum toxins utilizing compound muscle action potentials (CMAP), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins

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ABSTRACT

The biological activity of various types of botulinum toxin has been evaluated using the mouse intraperitoneal LD_{50} test (ip LD_{50}). This method requires a large number of mice to precisely determine toxin activity, and so has posed a problem with regard to animal welfare. We have used a direct measure of neuromuscular transmission, the compound muscle action potential (CMAP), to evaluate the effect of different types of botulinum neurotoxin (NTX), and we compared the effects of these toxins to evaluate muscle relaxation by employing the digit abduction scoring (DAS) assay.

This method can be used to measure a broad range of toxin activities the day after administration. Types A, C, C/D, and E NTX reduced the CMAP amplitude one day after administration at below 1 ip LD_{50}, an effect that cannot be detected using the mouse ip LD_{50} assay. The method is useful not only for measuring toxin activity, but also for evaluating the characteristics of different types of NTX. The rat CMAP test is straightforward, highly reproducible, and can directly determine the efficacy of toxin preparations through their inhibition of neuromuscular transmission. Thus, this method may be suitable for pharmacology studies and the quality control of toxin preparations.

1. Introduction

Clostridium botulinum produces toxins that have been classified into 7 serotypes, A–G, based on their immunological characteristics (Sakaguchi, 1983). The toxins act on neuromuscular junctions and induce muscle relaxation by inhibiting acetylcholine release (Sakaguchi, 1983; Jahn and Niemann, 1994). The toxins cause muscle flaccidity, and have recently been utilized to treat spasm in myotonus and dystonia (Jankovic, 2004). Type A and B toxins were approved as drugs for treatment, and are clinically applied. Type C, E, and F toxins have been used in clinical studies (Mezaki et al., 1995; Eleopra et al., 1997, 1998, 2004). The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by

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inhibiting the release of acetylcholine from synaptic vesicles. The different types of toxin are selective for specific SNARE proteins. Type A and E toxins cleave SNAP-25, type B toxin cleaves synaptobrevin II, type C toxin cleaves syntaxin and SNAP-25, and type D, F, and G toxins cleave synaptobrevin I and II. The cleavage sites for each toxin differ, even when toxins cleave the same protein (Montecucco and Schiavo, 1994; Schiavo et al., 2000).

The biological activity of botulinum toxins has been evaluated using the mouse intraperitoneal (ip) LD_{50} test (Pearce et al., 1994). This method is not an assessment of toxin efficacy, which is the inhibition of neuromuscular transmission, but of lethality due to respiratory muscle paralysis caused by the toxin. In this method, the results vary due to the individual sensitivity of mice against toxins, and many mice are required to ensure sufficient accuracy levels for the quality control of preparations. Therefore, the method has posed a problem with regard to animal welfare. International meetings on alternative methods for animal testing have been held, and replacements for the mouse ip LD_{50} test have been discussed (Straughan, 2006). The 3 Rs (refinement, reduction, and replacement) have been proposed for alternative methods, and alternative *in-vitro*, *ex-vivo*, and *in-vivo* test systems have been investigated. The *in-vitro* test system, ELISA, determining the endopeptidase activity, does not use animals, but the sensitivity is lower than the mouse bioassay (Hallis et al., 1996; Wictome et al., 1999). This method could determine only light-chain activity in many cases, and the inaccurate determination of toxin function has been reported. The *ex-vivo* test system using the mouse phrenic nerve-hemidiaphragm is sensitive, but it requires a skilled technique, and has a low reproducibility (Bigalke et al., 2001; Yoneda et al., 2005). The *in-vivo* test systems, such as the digit abduction scoring (DAS) and local flaccid paralysis assays, use scores for evaluation (Aoki, 2001; Takahashi et al., 1990; Sesardic et al., 1996). The methods of evaluation involved scoring, and so they have a disadvantage in that the obtained data are discrete quantities. As each test systems has disadvantages; no alternative method has been established.

We attached a greater importance to the following point in devising an alternative method to determine the activity of botulinum toxin: The potency of botulinum toxin should be evaluated based on their pharmacological effect of inhibiting neuromuscular transmission, and not based on their lethal activity, as in the mouse ip LD_{50} assay. There are several test systems to evaluate the inhibition of neuromuscular transmission, and we focused on the measurement of the compound muscle action potential (CMAP) used for the diagnosis of various nervous disorders. The CMAP is generated by the contraction of muscle fibers; the microcurrent generated by muscle contraction is amplified and recorded. Botulinum toxin affects nerve endings to suppress neurotransmission. Therefore, by determining the CMAP amplitude, the action of the toxin suppressing the transmission of electric stimulation to the muscle can be shown numerically. CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which checks the response to the toxin before treatment in patients who might have antibodies to the botulinum toxin (Kessler and Benecke, 1997). It was reported that the CMAP amplitude was measured in the rat gastrocnemius muscle which was injected several times with botulinum toxin (Cichon et al., 1995). The CMAP amplitude decreased as the toxin activity increased, but quantitative determination of the toxin was not carried out.

In this study, based on this previous CMAP study, we investigated the quantification of biological activity (effect of neuromuscular transmission blockage) by different types of botulinum toxin. We also compared toxins with an inhibitory effect on neuromuscular transmission. In addition, we investigated the muscle flaccidity-inducing effect of the toxins, compared CMAP data, and the overall effect of the toxins.

2. Materials and methods

2.1. Preparation of toxin

Botulinum neurotoxin type A, B, C, C/D, D, E, and F (150 kDa, NTX) were prepared using modified culture and purification methods, as previously reported (Sakaguchi et al., 1981). *C. botulinum* type A A2, type B Okra, type C CB-19, type C/D 003-9, type D 1873, type E 35396, and type F Langeland were used. For type A, B, E, and F organisms, PYG medium containing 2% peptone, 0.05% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate was used. For type C, C/D, and D organisms, a basic medium containing 0.8% glucose, 0.5% starch, 1.0% yeast extract, 1.0% ammonium sulfate, and 0.1% cysteine hydrochloride salt was used, and cooked meat (6 g) and 0.5% calcium carbonate were added to 100 mL of the basic medium. The organisms were cultured by allowing them to stand at 30 or 37 °C for 2–3 days. Culture fluid was purified by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration of M toxin. M toxin was adsorbed to 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 protein separation. Each NTX was stored at −70 °C until use.

2.2. Experimental animals

Female ICR/CD-1 mice (4 weeks of age, about 20 g, Charles River Laboratories Japan, Yokohama, Japan) and female S/D rats (8 weeks of age, about 200 g, Charles River Laboratories Japan, Yokohama, Japan) were used for the LD_{50} and CMAP tests, 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.

2.3. Toxin activity (mouse ip LD_{50}) measurements

The LD_{50} of each NTX was determined following ip injection into mice (Pearce et al., 1994). Seven doses with a dilution factor of 1.25 were used to determine the LD_{50}, and 20 animals were used per dose. Mice were evaluated for the first 96 h after administration, and the LD_{50} was calculated by the probit method.

2.4. CMAP measurements

The different types of NTX were serially diluted with physiological saline containing 0.5% human serum albumin. The following dilutions were prepared: 0.1–300 ip LD_{50}/mL type A, 100–1 × 10^5 ip LD_{50}/mL type B, 1–10,000 ip LD_{50}/mL type C, 0.3–100 ip LD_{50}/mL type C/D, 300–1 × 10^5 ip LD_{50}/mL type D, 1–3000 ip LD_{50}/mL type E, and 10–10,000 ip LD_{50}/mL type F. Rats were anesthetized with an ip injection of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). After the eyelid reflex disappeared, the left hind leg was shaved, and 0.1 mL of an NTX dilution was injected into the gastrocnemius muscle of five animals.

The CMAP was measured using a Nicolet Viking Quest (Viasys Healthcare, Tokyo, Japan). Rats were anesthetized and fixed in the prone position. The electrode employed was an alligator clip lead wire (Viasys Healthcare, Tokyo, Japan). The stimulating electrode was placed on the root of the spinal cord, the recording electrode was positioned on the belly muscle of the left hind gastrocnemius muscle, the reference recording electrode was placed on the left hind gastrocnemius tendon, and the earth electrode was positioned on the tail root. Electric stimulation was given at 25 mA for 0.2 ms. The CMAP of anesthetized rats was measured before (0) and 1, 2, 4, 7, and 14 days after administration.

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action, nondepolarizing neuromuscular blocking drug, d-tubocurarine (d-Tc, Wako, Osaka, Japan), and a depolarizing neuromuscular blocking drug, succinylcholine (SCC, Wako, Osaka, Japan). d-Tc is an antagonist of nicotinic neuromuscular acetylcholine receptors, and provides muscle relaxation by competitive inhibition of acetylcholine. SCC is binding to the nicotinic acetylcholine receptor, and is opening of the receptor's nicotinic sodium channel; a disorganized depolarization of the motor end plate occurs. SCC is not hydrolyzed by acetylcholinesterase, and occurs to persistent depolarization. The receptor's nicotinic sodium channel is inactivated. When acetylcholine binds to an already depolarized receptor it cannot cause further depolarization. As a result, SCC provides muscle relaxation. So, d-Tc and SCC are relaxed muscle by neuromuscular transmission inhibitory as botulinum toxin. d-Tc and SCC were serially diluted 3-fold to yield 0.1–0.9 and 1–9 mg/mL, respectively. Each drug dilution (0.1 mL) was injected into caudal vein of five animals. Electrodes were attached as described above, and the CMAP amplitude was measured for the left hind leg of each rat at 3 and 2 min after d-Tc and SCC administration, respectively. The rats underwent the insertion of a tracheal tube to maintain respiration after drug administration. The respirator was delivered by SN-480-7 (Shinano manufacturing Co., Tokyo, Japan). Tidal volume was set on the respirator at 2 mL and respiratory frequency at 70 breaths/min.

2.5. Digit abduction scoring assay (DAS assay)

The different types of NTX were compared using the DAS assay (Aoki, 2001), which has been reported to assess the muscle flaccidity-inducing effect of botulinum toxin preparations. In the assay, mice received toxin injection into the gastrocnemius muscle, and the muscle flaccidity-inducing effect of the toxin was determined by the degree of digit abduction. The peak DAS responses were observed on Day 2 or 3 post-injection. The DAS assay was modified for rats, and carried out two days after NTX administration. For negative control, rats were injected with dilution solution (physiological saline containing 0.5% human serum albumin). Rats were suspended by the tail, and the degree of digit abduction in the toxin-treated leg was scored on a 5-point scale by an observer who was masked as to the treatment: score 0 = normal leg extension, and digit abduction equivalent to the contralateral side; score 1 = normal leg extension, but digit abduction differed from the contralateral side or two digits in contact while the other digits completely abducted; score 2 = leg extended, but weak abduction of all digits or three digits in contact; score 3 = leg extended without digit abduction, or leg bent with four digits in contact; score 4 = leg bent with no digit abduction.

2.6. Statistical analysis

The waveforms of a single CMAP were converted to 2000 dots using electromyograph software, and the coordinates of the dots were converted to numbers. The distance between the top and bottom of the waveform was measured as the CMAP amplitude. Statistical analysis for neurotoxin (SAN, ver. 1.07, self made soft) was used to analyze the CMAP amplitude. SAN was created to store raw data of the CMAP amplitude and perform various statistical analyses (i.e., *t*-test, regression analysis, parallel line analysis, and correlation coefficient).

For time course of CMAP amplitude, the MULTTEST procedure of contrast statement was performed using SAS (ver. 9.1).

To determine whether the inhibitory effect on the neuromuscular transmission of each NTX was quantifiable, ip LD_{50} data were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis.

To evaluate the efficacy of each NTX, the regression line of each NTX was calculated for the peak of the effect, as identified above. Regression lines were used to calculate the doses causing 50% (injection site) and 20% (contralateral site) reductions in the CMAP amplitude, and these values were termed the Effective Dose 50 (ED_{50}) and Toxic Dose 20 (TD_{20}), respectively.

For d-Tc and SCh of the data, the Jonckheere–Terpstra trend test was performed using SAS (ver. 9.1).

3. Results

3.1. Dose-dependent effects of the different NTX types on the CMAP amplitude

The CMAP amplitude was measured to compare the inhibition of neuromuscular transmission at the site of toxin administration. In all types of NTX, the CMAP amplitude decreased depending on the concentration of the ip LD_{50}. Following the administration of type B, C, C/D,

and D NTX, the CMAP amplitude in the left hind leg was reduced for four days at all dose, and was recovered thereafter at type B of or more 30 ip LD_{50}, type C and type C/D of all dose and type D of 100–10,000 ip LD_{50}. Following type A, E, and F NTX administration, the CMAP amplitude was reduced for two days and recovered thereafter at type A of 0.03–3 ip LD_{50}, type E of or more 1 ip LD_{50}, and type F of or more 10 ip LD_{50} (Fig. 1). After the administration of type A, B, C, C/D, D, and F NTX, the CMAP amplitude in the contralateral leg was reduced for four days at type A of more 3 ip LD_{50}, type B of 10,000 ip LD_{50}, type C of or more 30 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of or more 3000 ip LD_{50}, and subsequently recovered at type A of 10 ip LD_{50}, type B of or more 3000 ip LD_{50}, type C of 300 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of 10,000 ip LD_{50}, respectively. Following type E NTX administration, the CMAP amplitude was reduced for two days at or more 100 ip LD_{50} and recovered thereafter at 300 ip LD_{50} (Fig. 2).

3.2. Analysis of the CMAP amplitude after the different NTX types were administered

Type A, C, C/D, and E NTX reduced the CMAP amplitude in the toxin-injected limb below 1 ip LD_{50} one day after administration. Regression analysis revealed that the CMAP amplitude for type A, B, C, C/D, and D of NTX on days 1, 2, 4, 7, and 14 and type E and F of NTX on days 1, 2, and 4 following administration was related to the LD_{50} concentrations dose-dependently, and linearity was noted when the logit-transformed CMAP amplitude was plotted against the log ip LD_{50}. Although each NTX varied regarding its LD_{50} value for the reduction of the CMAP amplitude, the linearity range of all types of NTX was from the minimum ip LD_{50} to about 10^3 fold (Table 1). Parallel line analysis of the regression lines was performed for each NTX on days 1, 2 and 4, followed by Tukey's test. The regression lines for all types of NTX showed parallelism, except for type C NTX (data not shown).

3.3. Inhibitory effect of the different NTX types on neuromuscular transmission

To compare the inhibition of neuromuscular transmission by the different types of NTX, the ED_{50} was defined as the dose that decreased the CMAP amplitude to 50% of the pre-toxin level, and was calculated for the peak effect at each dose. The ED_{50} rank order of NTX was type $D > B > F > E > C > C/D > A$. Type A NTX was most potent to reduce the CMAP amplitude, and type D NTX was lowest potent (Table 2).

3.4. Comparison of diffusion to the contralateral site and safety index of the different types of NTX

To compare diffusion to the contralateral site of the different types of NTX, the CMAP amplitude was measured in the right hind leg. The TD_{20} was defined as the dose that decreased the CMAP amplitude to 20% of the pre-toxin level and was calculated to assess the peak effect at each dose. The TD_{20} value rank order of NTX was type $D > F > B > C > E > A > C/D$. Type C/D NTX was most potent to diffuse to the contralateral site, and type D NTX the lowest potent. The ratio of TD_{20}/ED_{50} was calculated for each NTX, and is expressed as the safety index. The safety index rank order of NTX was type $F > C > D > E > A > C/D > B$. The results revealed that type F NTX showed the widest safety index and was hard to diffuse, and type B NTX the narrowest index and was prone to diffuse (Table 2).

3.5. Changes in CMAP amplitude induced by muscle relaxants (d-Tc and SCh)

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action. The average CMAP amplitudes in groups treated with 0.01, 0.03, and 0.09 mg d-Tc were reduced to about 78, 15, and 2% of that in the vehicle group, whereas those in the groups treated with 0.1, 0.3, and 0.9 mg SCh were reduced to about 68, 12, and 1%, respectively. Differences were significant for both d-Tc and SCh ($p < 0.0001$; Jonckheere–Terpstra trend test). The muscle relaxants induced a dose-related decrease in the CMAP amplitude, similar to botulinum toxin, indicating that the CMAP test can be used to evaluate the inhibition of neuromuscular transmission.

3.6. Comparison of muscle flaccidity induced by the different types of NTX

To compare the muscle flaccidity-inducing effects of the different types of NTX, we evaluated them using the DAS assay. When the log of the ip LD_{50} and median DAS score were plotted on the horizontal and vertical axes, respectively, the reaction curves for each NTX showed dose-dependent linearity. The required toxin value rank order of NTX for muscle flaccidity was type $B = D > F > E > A = C = C/D$. Type A, C, and C/D NTX were most potent to exhibit a muscle flaccidity-inducing effect, and type B NTX was lowest potent (Fig. 3). The ip LD_{50} dose necessary to achieve a score of 1 in the DAS assay was greater than that required to reduce the CMAP amplitude.

4. Discussion

The neuromuscular transmission inhibitor (d-Tc and SCC) with different mechanisms of action to the botulinum toxin induced a dose-related decrease in the CMAP amplitude. This result indicated that the CMAP amplitude reflected the inhibition of neuromuscular transmission caused by the muscle flaccidly-inducing effect of the drug. The results generated by the CMAP test are continuous data, whereas those of the mouse ip LD_{50} method and scores are discrete data.

We used the rat CMAP test to determine the toxin activity of different NTX types. Type A and C/D NTX reduced the CMAP amplitude one day after administration at 0.01 and 0.03 ip LD_{50}, respectively. In contrast, 10 ip LD_{50} of type B NTX were needed to reduce the CMAP amplitude. Type A, C, C/D, and E NTX required a dose of 1 ip LD_{50} or below, and the CMAP method was more sensitive than the mouse ip

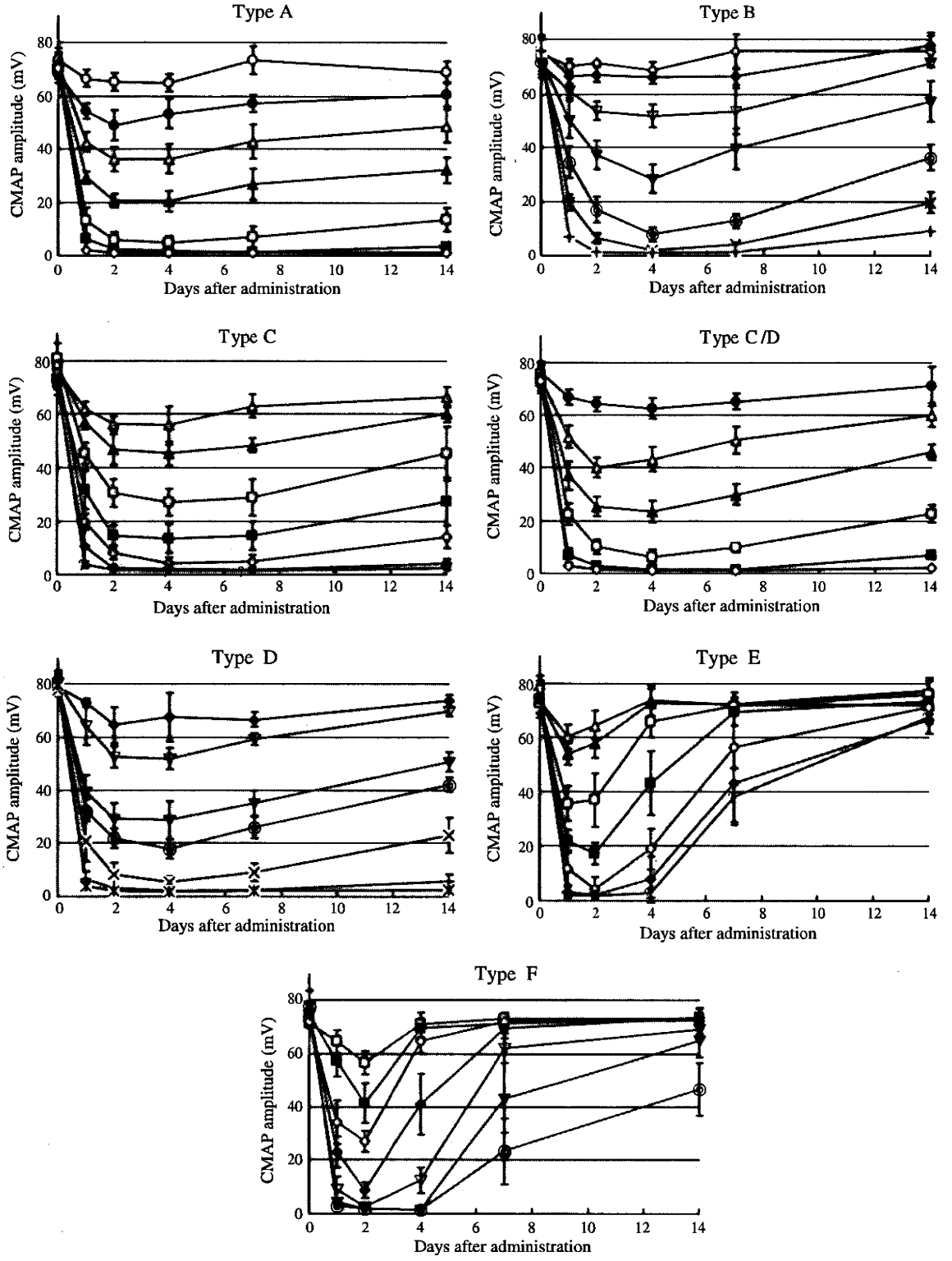

Fig. 1. Dose–response of the CMAP amplitude with the different NTX types at the injection site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50} and ⋆: 30,000 ip LD_{50}). CMAP amplitude was measured for the left hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

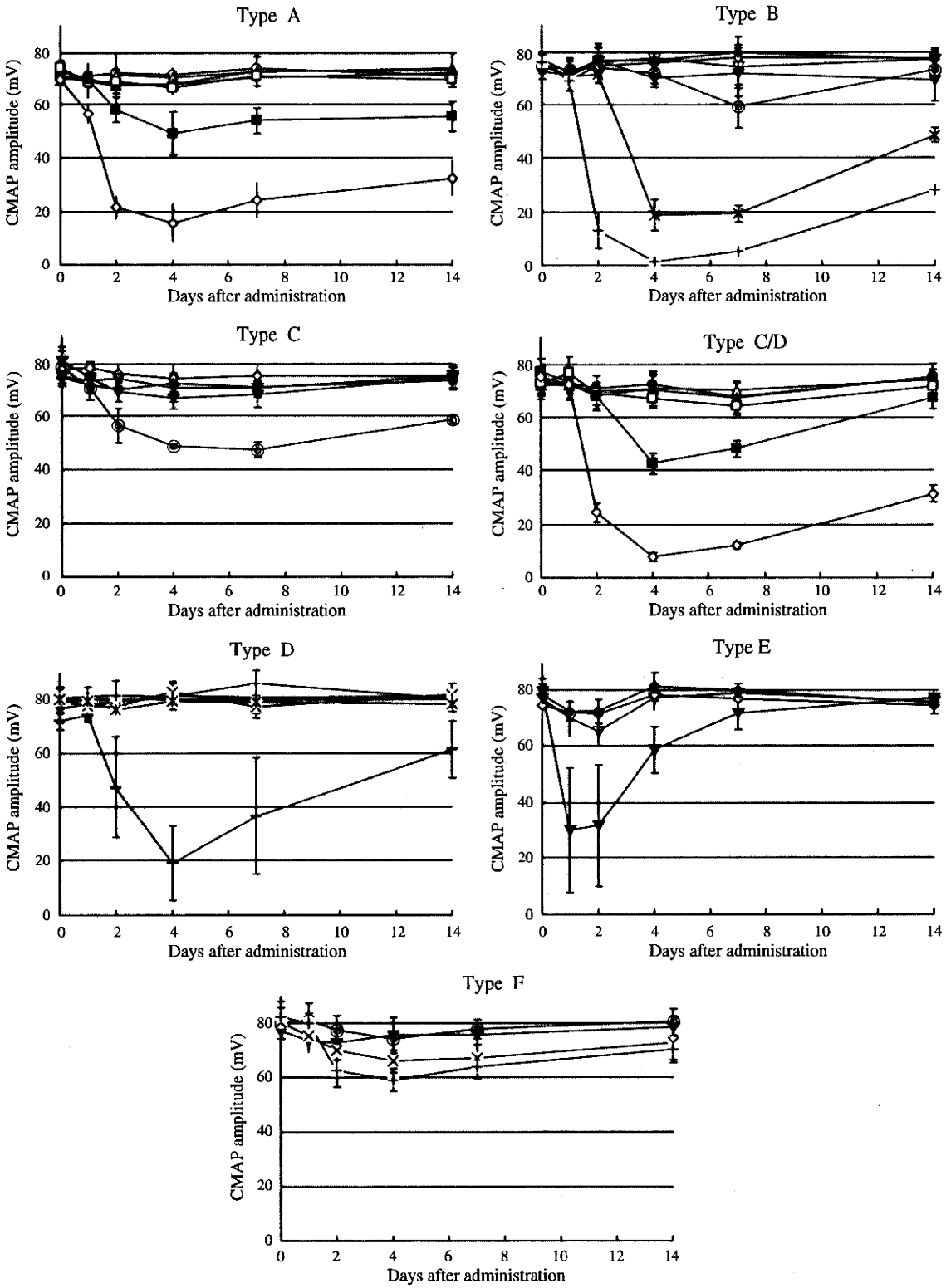

Fig. 2. Dose–response of the CMAP amplitude with the different NTX types at the contralateral site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50}; ⋆: 30,000 ip LD_{50} and –: 100,000 ip LD_{50}). CMAP amplitude was measured for the right hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

Table 1
Linearity ranges on regression analysis of the CMAP amplitude after the administration of type A to F NTX.

Type	Linearity range (ip LD_{50})				
	1 day (R^2)[a]	2 day (R^2)	4 day (R^2)	7 day (R^2)	14 day (R^2)
A	0.01–30 (0.979)	0.01–10 (0.971)	0.01–10 (0.966)	0.01–10 (0.954)	0.01–10 (0.959)
B	10–10,000 (0.932)	10–10,000 (0.954)	10–10,000 (0.949)	10–10,000 (0.950)	10–10,000 (0.915)
C	0.1–100 (0.957)	0.1–30 (0.957)	0.1–30 (0.955)	0.1–30 (0.968)	0.1–100 (0.946)
C/D	0.03–10 (0.964)	0.03–10 (0.980)	0.03–3 (0.968)	0.03–3 (0.962)	0.03–10 (0.963)
D	30–30,000 (0.942)	30–30,000 (0.953)	30–10,000 (0.958)	30–10,000 (0.949)	30–30,000 (0.937)
E	0.1–30 (0.930)	0.1–30 (0.928)	0.3–100 (0.915)	–	–
F	1–300 (09.37)	1–300 (0.954)	10–300 (0.956)	–	–

[a] R^2: multiple correlation coefficient.

Table 2
ED_{50}, TD_{20}, and safety index values for type A to F NTX.

Type	ED_{50}[a] (ip LD_{50})	TD_{20}[b] (ip LD_{50})	Safety index (TD_{20}/ED_{50})
A	0.09	1.57	18
B	167	1226	7
C	0.54	385	718
C/D	0.13	1.38	11
D	206	36,433	177
E	0.85	50	59
F	4.67	3772	808

[a] ED_{50}, dose which caused a 50% reduction of the CMAP amplitude.
[b] TD_{20}, dose which caused a 20% reduction of the CMAP amplitude.

LD_{50} assay. However, in type B, D, and F NTX, the CMAP method was less sensitive. These results suggest that mice and rats show a different sensitivity to toxins. The results indicated an advantage whereby the method can be used to measure a broad range of toxin activities on the day following administration.

The rat CMAP test is useful not only for measuring toxin activity, but also for evaluating the characteristics of the neuromuscular transmission-inhibitory effect of different NTX types. To assess whether the neuromuscular transmission-inhibitory effect was correlated with the muscle flaccidity-inducing effect, the latter effect of the different NTX types was compared using the DAS assay. The neuromuscular transmission-inhibitory effect (ED_{50}) using the CMAP test was compared to that using the DAS assay, and all types of NTX showed a correlation between the effect of the inhibition of neuromuscular transmission and the potency of muscular flaccidly, except type C NTX. The ED_{50} of type C NTX showed a higher dose than that of type A and C/D NTX; however, the effect of flaccid muscle paralysis was the same. The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by inhibiting the release of acetylcholine from synaptic vesicles. This suggested the possibility that the muscle-relaxing effect of type C NTX is caused not only by the inhibition of neuromuscular transmission through the cleavage of SNARE proteins, but also by other action mechanisms (i.e., the effect on the muscle).

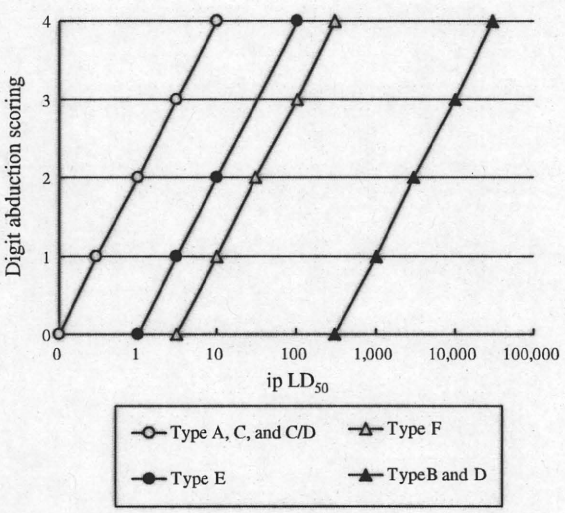

Fig. 3. Comparison of the muscle flaccidity-inducing effect of type A to F NTX on digit abduction in rats two days after administration. The scores indicate the median of each dose, $n = 5$.

Assuming that the findings can be extrapolated to humans, type A and C NTX might show a higher efficacy and safety than other types of NTX as muscle relaxants. Type A NTX showed the strongest effect on the inhibition of neuromuscular transmission and muscle flaccidity, having longer-lasting effects than type B NTX. Type A NTX showed a higher sensitivity than the other types of NTX in humans, and so might be the most suitable as a muscle relaxant. However, the results suggested that type A NTX has the disadvantage that it is prone to diffuse compared to the other types, except for type B and C/D NTX. Type C NTX might be the most suitable for relaxing a particular muscle, because it showed a potent muscle flaccidity-inducing effect and diffuses less than the other types. A dose inhalation toxicity study in monkeys showed equivalent effects between type A and C NTX (LeClaire and Pitt, 2005). In a clinical study, it was reported that type C had a muscle-relaxing effect equivalent to that of type A NTX (Eleopra et al., 1997; Eleopra et al. 2004).

Recently, type C, E, and F toxins have been tried as treatments for various diseases. The inhibitory effect of type E NTX on neuromuscular transmission was the fourth strongest after type A, C, and C/D NTX. The safety index of type E NTX was ranked fourth after type F, C, and D, and the duration of the effect of type E NTX was the shortest. It was reported that the muscular flaccidity-inducing effect of type E and F toxins was of a short duration in humans, similar to the result in this study. Since an effect of type E toxin on the central nervous system was reported (Bozzi

et al., 2006), the toxin may be of use in diseases of the central nervous system. In the future, the other NTX except types A and B may be applied to treatment, and they might be approved as new drugs.

In this study, the rat CMAP test was able to quantify the toxin activity of types A–F toxin. This method is applicable to different types of botulinum toxin preparation which might be marketed, and aids in quality control. This method is useful for evaluating the pharmacological effects of muscle relaxants.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Short communication

Quantification of potency of neutralizing antibodies to botulinum toxin using compound muscle action potential (CMAP)

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ABSTRACT

We evaluated a method for quantifying botulinum toxin-neutralizing antibodies which utilizes the CMAP. This method can be used just one day after administration, and the detection sensitivity was higher than that of the mouse neutralization test. The CMAP neutralization test detected neutralizing antibodies in patients who were resistant to treatment with the botulinum LL toxin. These results indicate that the CMAP neutralization test is useful for detecting low levels of antitoxin.

Botulinum toxins have recently been developed and used in the treatment of blepharospasm, spasmodic torticollis, dystonia, pain, and urological disorders (Jankovic, 2004; Truong and Jost, 2006; Casale and Tugnoli, 2008). The toxins show a high-level efficacy at very low doses, and are widely used in medical treatment. Resistance to the toxin was reported in some patients who received repeat high-dose (>100 mouse ip LD_{50} per injection cycle) toxin therapy over a long period of time (Borodic et al., 1996; Sesardic et al., 2004; Dressler, 2004). This reduced therapeutic response was reported to lead to the development of neutralizing antibodies in the patients. The potency of neutralizing antibodies in the serum of patients is measured using the mouse neutralization test (Hatheway et al., 1984). This method is based on the LD_{50} assay, which, in turn, is based on the number of surviving mice typically 96 h after the intraperitoneal (ip) injection of a fixed lethal dose of toxin premixed with different amounts of antitoxin. The antitoxin titer in the sample is expressed relative to that of the standard botulinum antitoxin. The detection limit of this method is reported to be about 10–100 mU/mL (Sesardic et al., 2004; Byrne et al., 1998). A reduced therapeutic response in certain patients was reported to be caused by minute amounts of antibodies which could not be detected by the mouse neutralization test (Sesardic et al., 2004). To detect neutralizing antibody presence in patients, a highly sensitive assay for neutralizing antibodies is needed. We reported quantitative of biological activity of botulinum toxin using the compound muscle action potential (CMAP) (Torii et al., submitted for

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publication). CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which qualitatively assesses the response to the toxin before treatment in patients who might possess antibodies against the botulinum toxin (Kessler and Benecke, 1997). Based on the EDB test, we investigated a highly sensitive quantification method for botulinum toxin-neutralizing antibodies in animal and human sera. In addition, we investigated whether the CMAP neutralization test was able to detect antitoxin in patient's serum, and we compared detection capability of this method, mouse neutralization test and ELISA.

Botulinum neurotoxin types A, B, E, and F (150 kDa, NTX) were cultured and purified using a previously reported method (Sakaguchi et al., 1981; Torii et al., submitted for publication). Equine-derived Japanese standard botulinum antitoxin types A, B, E, and F (National Institute of Infectious Diseases, Tokyo, Japan) were each used as a standard. One unit (U) of corresponding type of standard botulinum antitoxin neutralizes 10,000 mouse intraperitoneally (ip) LD_{50} of toxin types A, B, F or 1000 mouse ip LD_{50} of toxin type E (Jones et al., 2006). The sera of seven patients resistant to treatment with the botulinum LL toxin (BOTOX®, Allergan, Irvine, U.S.) were collected after informed consent was obtained. For the negative control, sera were collected from one volunteer who did not receive any treatment of botulinum toxin. For the positive control, sera were collected from one volunteer who had been immunized three times with the botulinum tetravalent (A, B, E, and F) toxoid. CMAP neutralization test was performed using a modification of a previously report method of CMAP test using female S/D rats (8 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., submitted for publication). Modification was using mixture which botulinum antitoxins and test toxins in place of toxins. Mixtures were prepared as follows: the standard botulinum antitoxin and various sera were serially diluted with physiological saline containing 0.5% human serum albumin. The test toxins comprised NTX of each type, at quantities whereby the CMAP amplitudes on day 1 after injection were decreased to one quarter of those before administration (type A: 10 mouse ip LD_{50}/mL, B: 60,000 mouse ip LD_{50}/mL, E: 60 mouse ip LD_{50}/mL, F: 600 mouse ip LD_{50}/mL). The type A of test toxin dose also was set at 1 mouse ip LD_{50}/mL to increase the measurement sensitivity. Equal volumes of the antitoxin or serum and test toxin were mixed and reacted for 1 h at room temperature. The anesthetized rats were injected 0.1 mL of a mixture into the left gastrocnemius muscle. The CMAP amplitude of the left hind leg was measured before (0) and 24 h after injection. The mouse neutralization test was performed using a previously reported method using female ICR/CD-1 mice (4 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., 2002). ELISA was performed using a modification of a previously reported method (Torii et al., 2002). Modifications were buffer using Tris Buffer containing 0.15 M NaCl, secondary antibody using peroxidase-conjugated goat anti-human IgG, IgA and IgM (Sigma, Tokyo, Japan) and substrate using TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories Inc, Gaithersburg, U.S.). ELISA titers were expressed in multiples of absorbance of the negative control, and antibodies were considered to be detected by ELISA when the absorbance of the sample was more than twice that of the negative control. To determine whether the neutralizing antibody of each type was quantifiable, antibody potencies were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis using Statistical Analysis for Neurotoxin (SAN, ver. 2.1, self made soft). To determine the antibody titers of patients' sera, the amplitude data of standard botulinum antitoxin were calculated by regression analysis, and the regression line was used as the calibration curve using SAN.

The CMAP amplitude of each mixture of antitoxin and test toxin (types A, B, E, and F) decreased along with the antitoxin titer. For types A and E, regression analysis was performed by plotting the logarithmic values of the CMAP amplitude and antitoxin titer on the vertical and horizontal axes, respectively, and linearity was noted within a range of 3–100 mU/mL in type A ($R^2 = 0.983$) and 1–50 mU/mL in type E ($R^2 = 0.989$). For types B and F, the CMAP amplitudes were plotted on the vertical axis, and the log values of the antitoxin titer on the horizontal axis, and linearity was noted within a range of 25–100 mU/mL in type B ($R^2 = 0.953$) and 3–50 mU/mL in type F ($R^2 = 0.974$) (Fig. 1). To increase the measurement sensitivity, the test toxin dose was set at 1 mouse ip LD_{50}/mL in type A, and linearity was noted within a range of 1–6 mU/mL (data not shown). This method can be used to measure a broad range of neutralizing antibodies titers the day after administration. In this study, the CMAP neutralization test demonstrated six advantages over the standard technique. 1) The CMAP is more sensitive than the mouse neutralization test. 2) The CMAP neutralization test incorporates a concise procedure. 3) The CMAP neutralization test can determine the neutralizing antibody titer within 24 h; whereas, the mouse neutralization test requires 4 days to obtain the same results. 4) The CMAP neutralization test is highly reproducible. 5) Only 20–30 animals are used in the CMAP neutralization test, whereas more than 100 animals are necessary in a single mouse neutralization test. In addition, the rats are anesthetized during the test, and the amounts of injected test toxin do not completely block neuromuscular transmission nor paralyze the muscles. 6) The CMAP amplitude values obtained by the CMAP neutralization test are a continuous quantity. Taken together, these advantages indicate that this method is a useful substitute for the mouse neutralization test.

We then investigated whether minute amounts of neutralizing antitoxin present in patients' sera could be detected by the CMAP neutralization test. The neutralization antibodies in seven patients who showed a reduced therapeutic effect after repeated treatment with type A botulinum LL toxin were determined by the CMAP neutralization test, mouse neutralization test, and ELISA. Using the CMAP neutralization test, all sera showed a neutralizing antibody level of 3–50 mU/mL (Table 1). This suggests that the cause of the reduced therapeutic effect was the production of neutralizing antibody against botulinum LL toxin. In contrast, the mouse neutralization test detected neutralizing antibodies in sera from 1

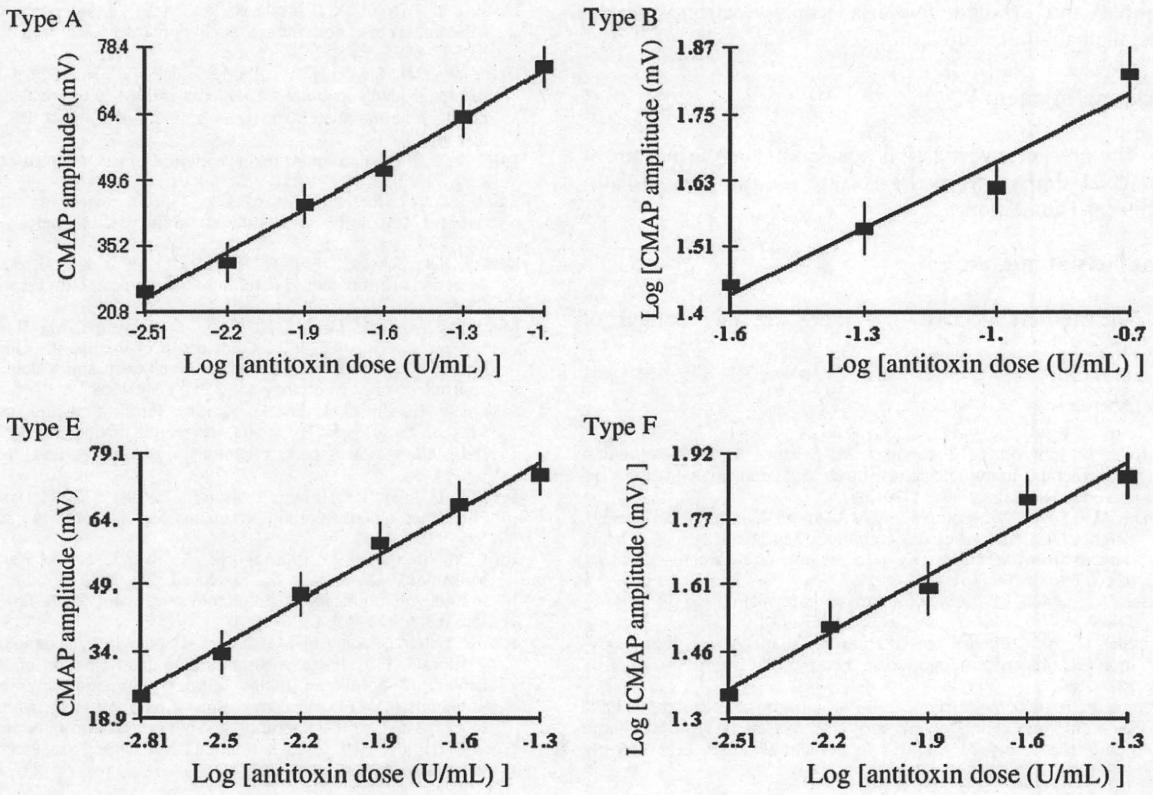

Fig. 1. Dose–response of CMAP amplitude of the test toxin mixed with standard botulinum antitoxin of each type. Rats received mixture of the antitoxin and test toxin into left gastrocnemius muscle. CMAP amplitude was measured for the left hind leg of each rat on day 1 after administration. Each point is the mean ± 95% confidence intervals, $n = 5$.

patient, showing a lower sensitivity than the CMAP neutralization test. ELISA detected neutralizing antibody presence in sera from 2 patients; however, ELISA titers were not correlated with the potency of neutralizing antibodies. This was because ELISA detected all antibodies (including non-neutralizing antibodies) against type A toxin. No antibodies were detected by these methods in the negative control. The antibody titers in serum No. 7 and the positive control detected using the CMAP and mouse neutralization tests showed similar values. Serum No. 7 was also antibody-positive on ELISA (Table 1). Comparing ELISA and the CMAP neutralization test, the correlation coefficient between the two assays was $R^2 = 0.056$ in all sera. No correlation could be identified between these titers.

Table 1
Antibody titers of sera from patients and volunteers measured using the mouse neutralization test, CMAP neutralization test, and ELISA.

Serum No.	Mouse neutralization test (mU/mL)	CMAP neutralization test (mU/mL)	ELISA[a]
1	<100	3	<2
2	<100	4	6
3	<100	4	<2
4	<100	4	<2
5	<100	4	<2
6	<100	5	<2
7	ca)100	50	3
8 (Positive control)	200	190	13
9 (Negative control)	<100	<1	<2

[a] ELISA titer expressed in multiples of the measurement from the negative control value.

As mentioned above, the CMAP neutralization test can be used for the detection of neutralizing antibodies in patients who have received treatment with type A botulinum toxin. Botulinum toxin preparations for treatment are used for various diseases in many patients. Since the therapeutic dose of botulinum toxin is very low, its therapeutic effect may be lost by only minute amounts of antibodies. For patients who show antibody presence, it may be necessary to treat them with increasing toxin doses or to change toxin types. The CMAP neutralization test may be useful to assist in such a diagnosis.

The CMAP neutralization test is capable of detecting minute amounts of neutralizing antibodies, not only against type A toxin, but also against types B, E, and F. Type A and B toxins have already been approved as formulations for the treatment of various disorders, and are currently being used in clinics. However, the effects of type E and F toxins have only recently begun to be investigated in clinical studies, and, in the future, these toxins may therefore be approved as new drugs (Mezaki et al., 1995; Eleopra et al., 1998, 2004). Thus, the ability of the CMAP test to also detect small amounts of neutralizing antibodies against type E and F toxins may be potentially useful for such toxin formulations to be developed in the future. This method is clinically applicable and useful as the measurement is

simple and straight forward, using electromyographs installed at clinical sites.

Acknowledgment

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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特集 脳深部刺激療法

ジストニアに対する脳深部刺激の効果*

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Key Words：deep brain stimulation (DBS), globus pallidus internus (GPi), thalamus, dystonia

はじめに

ジストニアは持続的な筋緊張により，しばしば捻転性または反復性の運動や異常な姿勢をきたす病態と定義され，その罹患部位の広がりに応じて局所性から全身性に分類されている[1]．さらにその発症原因により一次性と二次性に分けられている．一次性ジストニアはDYT3[2]のような特殊な遺伝性ジストニアを除き明確な神経変性所見がなく，神経の結合性，可塑性あるいはシナプス制御などの微細な異常に基づく機能異常が原因と考えられているが[3]，その詳細な病態生理はいまだ明らかになっていない．また，二次性ジストニアは遺伝性ジストニアを除く明らかな発症要因が存在する疾患群と考えられている．

ジストニアの治療として薬物療法が試みられるが抵抗性の症例も多く明確な治療体系はいまだ確立されていない．A型ボツリヌス毒素の使用が日本でも認められ普及しているが，現時点ではその適用範囲は頭頸部領域に限定されている．これらのことを背景に全身性ジストニアなどの広範囲に罹患筋を有する症例に対する治療オプションとして登場したのが淡蒼球手術である．本稿では，淡蒼球内節(globus pallidus internus：GPi)の脳深部刺激(deep brain stimulation：DBS)術を中心にジストニアに対する定位脳手術の現況を紹介する．

標的神経核の選択

定位脳手術の歴史をふり返ると，ジストニア治療では視床と淡蒼球が標的神経核とされ淡蒼球手術が1990年代に復活を遂げるまでは視床手術がその主役を演じてきた[4]．しかし，現在では両者をジストニアのサブタイプによって使い分けることが必要である．図1に簡略化した大脳基底核を中心とした神経回路機能モデルを示す．視床からの出力は大脳皮質に投射しそれを介して主に体肢末梢部の筋活動を調節しているが，GPiからの出力は上行性に視床へ投射するのと同時に，下降性に中脳被蓋野に連動する脚橋被蓋核(pedunculo-pontine nucleus：PPN)などの脳幹運動中枢へ直接投射し，体幹および体肢近位部の筋群の動きをも調節しうると考えられている[5][6]．したがって，上肢などの末梢性のジストニアは視床手術で治療可能であるが，体軸を含む広範な分布を示すジストニアには淡蒼球手術が推奨

* The effect of deep brain stimulation for dystonia.

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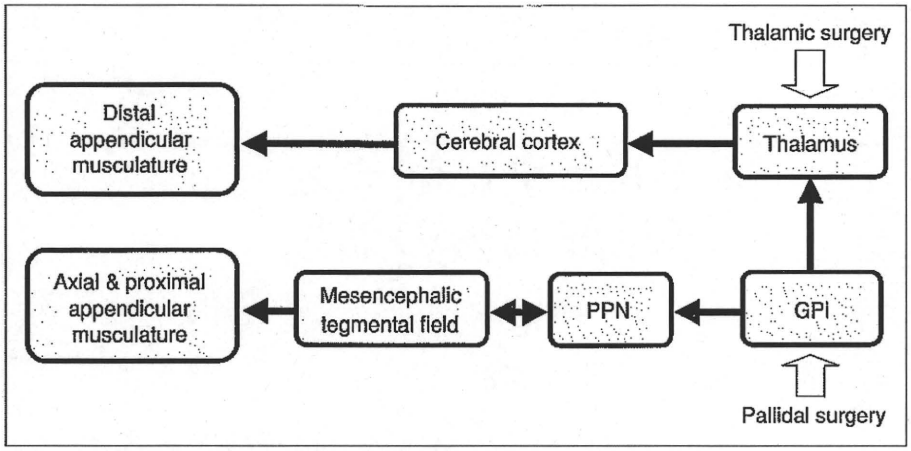

図1　大脳基底核を中心とした神経回路機能モデル
GPiからの出力系は上行性に視床へ投射し，大脳皮質を介して対側の上下肢末梢の運動を調節するとともに，下降性にPPNを中心とする脳幹の領域へも投射し，体幹や下肢の運動やバランスを制御している．したがって，上肢などの末梢性のジストニアは視床手術で治療可能であるが，体軸を含む広範な分布を示すジストニアには淡蒼球手術が推奨される．

される．実際，全身性，分節性，局所性問わず上肢末梢を除く一次性ジストニアの多くはGPiの刺激が効果を示し，上肢にみられる局所性ジストニアの代表であるfocal hand dystonia(FHD)では視床外腹側核(n. ventralis lateralis：VL)の刺激が有効である．視床VL核はVo-complex(Voa＋Vop)と相同である．

逆に，視床手術で体軸のジストニアを改善するのは一般に困難である．Grenoble大学のグループの経験では，12例の全身性ジストニア患者に対して視床中間腹側核(n. ventralis intermedius：Vim)刺激術を行ったところ，わずか5例にのみ有効であり，それも体肢ジストニアだけに軽度から中等度の改善がみられるものの体軸症状にはまったく効果がなかったとされている[7]．同様のことは視床凝固術を用いた1976年のCooperの報告[4]にすでに記載されている．視床下核(subthalamic nucleus：STN)のDBSが全身性ジストニアに対して有効であったという症例報告が散見される．STN-DBSがParkinson病(PD)に付随するオフ期ジストニアを改善しうること[8]を考慮すれば理解できるが，いまだ経験が乏しく，STN-DBSのジストニア治療への応用については慎重であるべきであろう．以下，ジストニアに対する定位脳手術の現在の主役であるGPi-DBSを中心に詳述する．

GPi-DBSの刺激部位および刺激条件

GPiにはDBSに関連した機能分画が存在するが，これはPD治療で最初に見出されたものである(図2)．PD症例では(図2-左)，後腹側部の刺激で固縮(rigidity)の著明な改善とレボドパ誘発性ジスキネジアの完全抑制が得られるが，レボドパの無動(akinesia)に対する効果は阻害され患者は著明な無動状態に陥るとされ，一方，前背側部の刺激ではオフ期無動の中等度の改善とジスキネジアの誘発がみられると報告されている[9]．筆者は頸部ジストニアや他のタイプのジストニア症例において類似した経験をしている(図2-右)．つまり，後腹側部の刺激ではジストニア症状の改善をみるが，前背側部の刺激では逆にジストニアの増悪および舞踏運動を主体とするジスキネジアの誘発がみられた．したがって，ジストニア治療では視索に近接した後腹側部の刺激がもっとも効果的であると考えられる．

DBSの効果を十分に得るためには上記の適切な部位に電極を留置することは当然であるが，その後の刺激条件調節もその手術手技と同等の意義をもつ．

刺激条件に関しては絶対的な指針などはないが，これまでの報告とわれわれの施設の経験をまとめると，一次性全身性・分節性ジストニア

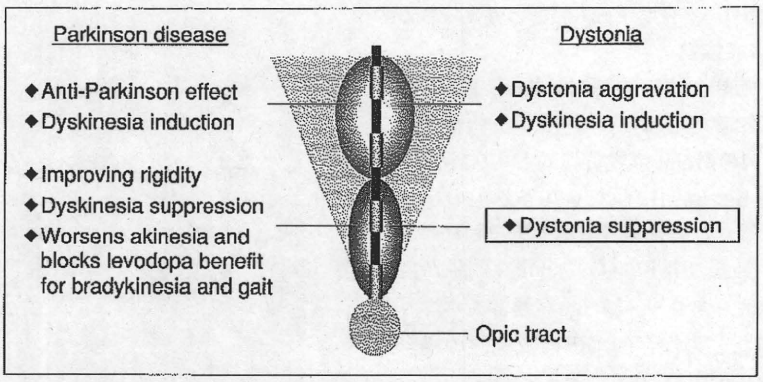

図2　GPi-DBSに関連するGPiの機能分画
PDでは後腹側部の刺激で固縮(rigidity)の著明な改善とレボドパ誘発性ジスキネジアの完全抑制が得られるが，レボドパの無動(akinesia)に対する効果は阻害され患者は著明な無動状態に陥るとされ，一方，前背側部の刺激ではオフ期無動の中等度の改善とジスキネジアの誘発がみられると報告されている．ジストニアでは，後腹側部の刺激ではジストニア症状の改善をみるが，前背側部の刺激では逆にジストニアの増悪および舞踏運動を主体とするジスキネジアの誘発がみられる．

では1～2個の電極を用いた単極かつ，刺激頻度130～185Hz，電圧0.8～3.8V，刺激幅120～450μsecの間の刺激が用いられている．一方でMeige症候群では，60～145Hzの比較的低頻度刺激，1.0～5.2V，120～450μsecで時には双極刺激も使用されうる．

二次性ジストニアも概ね上記刺激範囲内であることが多いが，pantothenate kinase-associated neurodegeneration(PKAN)に代表される脳内鉄蓄積性神経変性疾患(neurodegeneration with brain iron accumulation：NBIA)は60～215Hz，1.0～5.0Vとより低頻度，高電圧が用いられることもあり，ジストニア-舞踏アテトーゼ型脳性麻痺60～150μsecと刺激幅はより小さく，刺激頻度は130Hzが使用されている．

痙性斜頸に関しては刺激条件の検討を目的とした系統的な研究が行われている．Moroらの報告によると，60Hzを超える高頻度刺激と高電圧が症状の改善と有意な関連があり，130Hzの刺激がもっともよい効果をもたらしたが，刺激幅を上げても有意な改善は得られなかった[10]．

すべてのタイプのジストニアにおいていえることであるが，症例によってはジストニア症状の刺激頻度依存性反応がみられる場合があるので注意を要する．刺激強度は最大のジストニア抑制効果が得られ，刺激による耐えがたい副作用が出現しない程度に設定する．個々の症例ごとの刺激条件の最適化は当然必要であるが，最後腹側部を中心とした単極刺激，130Hz，210μsec，2.0～3.0Vほどがもっとも汎用性が高い刺激設定かもしれない．

一次性ジストニア

1．一次性全身性・分節性ジストニア

*DYT1*遺伝子異常に基づくジストニアは最初にGPi-DBSの有効性が示された一次性全身性ジストニアであり[11]，その原型と考えられている．Vidailhetらは，22人のDYT1を含む一次性全身性ジストニアにGPi-DBSを施行し，1年後には，Burke-Fahn-Marsden Dystonia Scale(BFMDRS)のMovement Score(MS)，Disability Score(DS)がそれぞれ術前の55%，44%の改善率であったと報告した[12]．翌年にはKupschらにより，DYT1を含む一次性全身性・分節性ジストニアに対するGPi-DBSのランダム化試験の報告がなされ，シャム刺激群に比べ神経刺激群に有意な症状改善が認められた[13]．さらにVidailhetらは，上記の症例群をさらに追跡し，運動症状改善率は1年後に51%であったが，3年後には58%まで上昇したことを示し[14]，GPi-DBSの一次性全身性・分節性ジストニアに対する有効性はほぼ確立された．また，術後10年経過しても効果が持続している症