Comparison between botulinum neurotoxin type A2 and type A1 by electrophysiological study in healthy individuals

Authors: Yohei, Mukai [ab], Yoshimitsu, Shimatani [a], Wataru, Sako [a], Kotaro, Asanuma [a], Hiroyuki, Nodera [a], Takashi, Sakamoto [b], Yuishin, Izumi [a], Tomoko, Kohda [c], Shunji, Kozaki [c], Ryuji, Kaji [a]

Author affiliations:

[a] Department of Clinical Neuroscience, Institute of Health Biosciences, Graduate School of Medicine, University of Tokushima, 18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan

[b] Department of Neurology, National Center Hospital of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira-shi, Tokyo 187-8551, Japan

[c] Department of Veterinary Sciences, School of Life and Environmental Sciences, Osaka Prefecture University, 1-18 Rinku-oraikita, Izumisano-shi, Osaka 599-8531,Japan

Corresponding author: Yohei Mukai

Postal number: 770-8503

Postal address: Department of Clinical Neuroscience, Institute of Health Biosciences, Graduate

School of Medicine, University of Tokushima, Tokushima, Japan

E-mail: ymukai@ncnp.go.jp

Fax: +81886337208

Telephone: +81886337207

Abstract

Botulinum neurotoxin type A1 (BoNTs/A1) and type B (BoNT/B) have been used for treating hyperactive muscle contractions. In the present study, we compared the effect of botulinum neurotoxin subtype A2 (6.5 mouse LD50 units A2 neurotoxin, A2NTX) and onabotulinumtoxinA (10 mouse LD50 units BoNT/A1 product) by measuring the compound muscle action potentials (CMAPs) before and after administration.

In total, 8 healthy subjects were examined in the present study. A2NTX was injected into the extensor digitorum brevis (EDB) muscle, followed by onabotulinumtoxinA injection into the contralateral EDB muscle after 16 weeks. The CMAP amplitudes from the EDB, abductor hallucis (AH), and abductor digiti minimi pedis (ADM) muscles were measured after each BoNT injection on days 1, 3, 7, 14, 28, 56, 84, and 112 to assess the effect of the toxin. On day 14, both A2NTX and onabotulinumtoxinA produced an approximately 70% decline in EDB CMAP amplitude compared to the baseline values; significant reduction of the CMAP continued through day 112. The CMAP

amplitudes from neighboring muscles (AH and ADM) remained intact throughout the study period, except for a slight but significant drop at day 28 after onabotulinumtoxinA injection compared to A2NTX. The current findings indicate that small doses (6.5 units and 10 units) of A2NTX and onabotulinumtoxinA have at least comparable onset and duration of action, although similar clinical effects were obtained with lower dose using A2NTX.

Keywords: Botulinum neurotoxin, A2NTX, EDB test, CMAP

1. Introduction

Botulinum neurotoxins (BoNTs) are produced by *Clostridium botulinum*, which is a Gram-positive anaerobic bacterium. The toxins reportedly inhibit exocytosis of acetylcholine from motor nerve terminals and cause muscle weakness. BoNTs have been widely used to treat muscle hyperactivity disorders such as blepharospasm, hemifacial spasm, dystonia, and spasticity. BoNTs are grouped antigenically into 7 serotypes (A–G), with type A BoNTs further classified into 5 subtypes (A1–A5) based on the amino acid sequence. Only BoNT/A and BoNT/B are currently clinically used. Commercially available preparations of BoNT/A belong to the subtype A1 (BoNT/A1) only, and at present, no reports have described the use of other BoNT/A subtypes (A2–A5) in humans.

Botulinum neurotoxin subtype A2 (BoNT/A2) was first identified from an infant botulism case in 1990 in Japan (Sakaguchi et al., 1990). Further studies showed that this subtype differed both immunologically and biologically from BoNT/A1 (Kozaki et al., 1995; Pier et al., 2011; Torii et al., 2011). In the present study, we compared BoNT/A2 to BoNT/A1 in terms of their effect on electrophysiologic properties of the muscle, diffusion characteristics, and duration of effectiveness in 8 healthy participants through electrophysiological testing. The limitation of this report is that the comparison was made using different mouse LD50 unit doses (10 units for onabotulinumtoxinA or BoNT/A1, and 6.5 units for BoNT/A2).

2. Materials and Methods

2.1 Participants

Individuals were screened electrophysiologically at the Department of Neurology, University of Tokushima, School of Medicine, Tokushima, Japan. Those with extensor digitorum brevis (EDB) compound muscle action potential (CMAP) amplitudes (baseline to negative peak) of <1 mV after stimulation of the fibular nerve at the ankle were excluded from the study. Individuals with abductor hallucis (AH) or abductor digiti minimi pedis (ADM) CMAPs of <5 mV were also excluded from the study. Individuals without a history of botulism, peripheral nerve disorder, or prior treatment with BoNTs were selected for this study. In total, 8 participants (7 males and 1 female; mean age, 34.3 years; range, 27–46 years) were recruited for the study. All the participants provided written informed consent prior to enrolling in the study. The study period was from October 2009-November 2010.

2.2 Preparation of neurotoxins

Botulinum toxin is produced by *Clostridium botulinum*, in the form of a complex comprising a neurotoxin (150 kDa, NTX) and nontoxic component. The botulinum toxin causes muscle weakness, whereas the nontoxic component protects the NTX in the intestinal tract and stomach. According to the difference in molecular weight, the toxin complexes can be divided into

the M toxin (300 kDa,), L toxin (500 kDa), and LL toxin (900kDa) (Sugii et al., 1975). Most of *Clostridium botulinum* strains produce only one type of botulinum toxin. Based on this classification, onabotulinumtoxinA is one of the LL toxins.

Botulinum neurotoxin subtype A2 free from nontoxic component (150 kDa, A2NTX) was prepared using modified culture and purification methods (Sakaguchi et al., 1981; Sakamoto et al., 2009; Akaike et al., 2010) and stored at -70° C until use. Commercial progenitor BoNT/A1 (900 kDa, onabotulinumtoxinA) was used as a control. OnabotulinumtoxinA belongs to the A1 subtype and it includes the nontoxic component. A2NTX and onabotulinumtoxinA toxin activities were determined using the mouse intraperitoneal (IP) LD50 (median lethal dose) potency assay. The mouse LD50 for BoNT/A2 was defined as 1 unit (U) of activity, as previously reported (Pearce et al., 1994). The study started to use the same 10 unit for both A1 and A2 preparations, but a later evaluation showed that 10 units of A1 and 6.5 units of A2 toxins were used in this study. Prior to EDB injections, toxins were diluted to 100 U/mL using sterile 0.9% NaCl.

2.3 Experimental protocol

The present study was designed as an open-label, single-center trial. Because the participants of the present study were healthy volunteers, we tried to avoid producing muscle weakness; intramuscular injection of a small amount of botulinum toxin produces an

electrophysiological change, but muscle weakness is not clinically manifested. Participants were injected with 6.5 units of A2NTX in the EDB muscle, followed by injection of 10 units of onabotulinumtoxinA in the contralateral EDB muscle 16 weeks later. The CMAP amplitudes of the EDB, AH, and ADM muscles on both sides were measured to evaluate the electrophysiological effect, diffusion characteristics, and start of the recovery process.

A Nicolet Viking Select Version 11 (VIASYS Healthcare, Tokyo, Japan) software was used to measure CMAP amplitudes. All responses were recorded following supramaximal electrical stimulation. Adhesive electrodes with identical surface sizes were used for all measurements (22 mm × 32 mm; Item#:019-435300, VIASYS Healthcare, Tokyo, Japan). The recording and reference electrodes were placed over the muscle belly and tendon, and the stimulation distance was maintained constant throughout the course of the study. We took pictures of the participants' foot at the first time when we pasted the adhesive electrodes. In order to accomplish reproducible electrode positions, we thereafter placed the electrodes by checking the positions in the pictures. An electromyograph filter was set between 2 and 10,000 Hz, and the skin surface temperature was maintained between 32°C and 34°C. We identified the muscle belly by palpation, placed the electrode over the muscle belly, and measured the CMAP value, as per the standard methodology (Kimura, 2001). The evaluation days were set as follows: day 1, day 2, and day 3 prior to the injection of A2NTX as well as day 1 (after 23–25 hours), day 3 (after 71–73 hours), day 7 (after

167–169 hours), day 14, day 28, day 56, day 84, and day 112 after the injection of A2NTX. These evaluation days were selected based on the data from previously published research papers (Wohlfarth et al., 2007).

On each evaluation day, the CMAP values on both sides of the ADM, AH, and EDB muscles were measured 5 times each. The recording electrode was affixed and re-affixed once every 5 evaluations, but the position of the electrode was left unchanged. To avoid underestimation, the 2 lowest CMAP values obtained from the 5 measurements were excluded, and the mean of the remaining 3 values was considered as the final measurement value for that evaluation day.

The mean of the measured values obtained on day 1, day 2, and day 3 prior to the injection were defined as the baseline CMAP values. On day 112, 10 units of onabotulinumtoxinA were injected at the EDB on the opposite side, and the same evaluations as those performed for A2NTX, were conducted for 16 weeks thereafter.

We evaluated the clinical weakness of the EDB, AH, and ADM muscle using the Manual Muscle Test proposed by the Medical Research Council (MRC). This evaluation was performed at each visit.

2.4 Statistics

The mean CMAP baseline values were defined as the average of 3 independent

measurements obtained prior to BoNT administration (day 1, day 2, and day 3). The percent CMAP (%CMAP) amplitude was defined as the value obtained by dividing the CMAP amplitude after administration by the baseline value and multiplying it by 100. Significant decline in the CMAP was defined as a reduction to less than 80% in %CMAP amplitude. SPSS (Statistical Package for Social Science) was used to analyze CMAP amplitudes and conduct descriptive statistics including means and standard deviations (±SD). Student's two-sided *t*-test was used to compare %CMAP, and p values of <0.05 were considered to be statistically significant.

3. Results

3.1 CMAP amplitude measurements and clinical muscle strength

Figure 1 illustrates the variation of the baseline CMAP amplitudes over the 3-day period. The baseline CMAP coefficients of variation ranged from 0.1% to 20.5%. The changes in mean %CMAP of EDB are shown in Figure 2. The mean baseline CMAP amplitude values of EDB were 5.2 ± 2.1 mV and 4.1 ± 2.1 mV for A2NTX and onabotulinumtoxinA, respectively ($p = 0.30$). The onset of CMAP decline of the EDB was similar for both A2NTX and onabotulinumtoxinA administration. A %CMAP value of <80% was observed on day 1 in 7 participants injected with A2NTX and in 6 participants injected with onabotulinumtoxinA. On day 3, the %CMAP of the EDB was <70% in all the participants. The maximum reduction in the mean %CMAP of the EDB was

observed on day 14 with both A2NTX (32.9% ± 19.5%) and onabotulinumtoxinA (30.9% ± 12.3%) administration. No statistically significant difference was present between the data on the day 14 and those on other days (3, 7, 28, 56, 84(both toxins) and 112(A2NTX)). The CMAP reduction was still present on day 112 after BoNT administration (A2NTX, 46.5% ± 23.9%; onabotulinumtoxinA, 46.8% ± 12.6%). Differences in the %CMAP of the EDB following A2NTX administration and following onabotulinumtoxinA administration were not statistically significant across any of the time-points ($p > 0.05$). The %CMAP of the EDB on the side receiving the A2NTX injection was 60.0% ± 18.2% at 224 days after A2NTX administration (not shown in graph).

No significant changes were noted in mean %CMAP from adjacent AH and ADM muscles, except for a sight but significant drop at day 28 after onabotulinumtoxinA injection compared to A2NTX (Fig,3). The mean %CMAP values of the contralateral EDB, AH, and ADM muscles were also evaluated, but no significant changes were noted among these values (data not shown). We noted that the Manual Muscle Test values of the EDB, AH, and ADM on both the sides did not indicate any clinical muscle weakness on any of the evaluation days during the study period in any of the participants.

3.2 Safety

The side effects of botulinum toxin formulation, such as allergic reactions, rash, itching, and pain or tenderness at the injection site, are well known. However, in the present study, we did

not note any adverse events that could reasonably be attributed to BoNT throughout the study period.

4. Discussion

To our knowledge, no reports describing the effectiveness or tolerance of A2NTX administration in humans have been published. The present study is the first to assess A2NTX administration in humans and to evaluate its effects electrophysiologically. The parameters were compared between those following 6.5 units of A2NTX and 10 units of onabotulinumtoxinA administration, and showed equivalent effects in CMAP decline, time of onset, start of the recovery process, and diffusion, except for a slight diffusion to AH after onabotulinumtoxinA at day 28 compared to A2NTX.

The present study did not compare the clinical potencies of reducing CMAPs using the same mouse LD50 units, and it is not precisely concluded that A2NTX has more clinical effectiveness than A1LL toxin in humans. A previous report (Torii et al,. 2011) demonstrated significantly higher potency of A2NTX compared to the A1LL toxin (onabotulinumtoxinA) in rats, and it is likely that this is the case also for humans.

The fact that there was no marked diffusion to neighboring muscles in both toxins indicates that the current dose was not large enough to observe the difference. In fact, A2NTX was demonstrated to have less diffusion to homonymous muscles on the other side using non-lethal doses

in the rat (Torii et al,. 2011).

At present, several commercial BoNT products are used for clinical or cosmetic purposes. The toxin activities of these products are defined by the mouse IP LD50 assay (Pearce et al., 1994). However, some reports (Odergren et al., 1998; Rosales et al. 2006; Chapman et al., 2007) have indicated considerable differences across different formulations. The EDB test was first reported by Hamjian and Walker (1994), and is commonly used to evaluate and compare the effect of BoNTs and may be used to presumptively detect and compare antibody titers against BoNT (Sloop et al., 1996; Kessler et al., 1997; Jost et al., 2005; Wohlfarth et al., 2007; Wohlfarth et al., 2008). Injection of small doses of BoNT to the EDB muscle does not produce clinical weakness, and the risk of systemic side effects is minimal.

The amino acid sequence of A2NTX differs from that of onabotulinumtoxinA, and it also lacks the nontoxic component. By contrast, onabotulinumtoxinA is composed of toxic and nontoxic components, the latter of which lacks therapeutic effect. Previous reports have indicated that the nontoxic components have immunogenic potential, and may elicit the production of neutralizing antibodies (Lee et al., 2005; Wohlfarth et al., 2007). Therefore, the nontoxic components may potentially affect the therapeutic uses of the BoNT product. The toxic component separates from the nontoxic component after injection into the muscle, and is taken up by the nerve endings. The onset of action was believed to be quicker if the BoNT does not contain the nontoxic component. However,

a clinical use report comparing onabotulinumtoxinA (BoNT/A1) and Xeomin® (Botulinum neurotoxin subtype A1 free from nontoxic component) did not indicate any differences in the onset of action (Dressler, 2009). In the present study, the onset of action between 6.5 units of A2NTX and 10 units of BoNT/A1 was also similar.

The present study has certain limitations, which may be considered and avoided in future studies. First, we injected only small doses of BoNT into healthy participants in the present study. In such cases, muscle weakness was not observed in either the EDB or adjacent muscles (AH and ADM). In addition, we did not observe any changes in the CMAP amplitudes of neighboring muscles. Only 10 units of BoNT/A were used, which may not be sufficient to evaluate the electrophysiological spread range using the EDB test. Therefore, larger doses of BoNT/A may yield differences in clinical muscle weakness or reduction in the CMAP amplitudes between A2NTX and BoNT/A1.

Second, an electrophysiological evaluation method was used in the present study. Although this is an established method, the potential for errors cannot be completely excluded. Moreover, the evaluations were performed using the same settings for the measuring instruments, types of electrodes, positions of electrodes, and examiners, but it was difficult to ensure that the participants' body temperature and skin tension were exactly similar. In fact, lower temperatures have been reported to augment the CMAP amplitude (Kimura, 1984). In addition, identification of

the muscle belly by palpation for recording electrode placement, which is a commonly used method by examiners, may lead to differences in results. Kong et al. (2009) performed nerve conduction studies twice within 10 minutes on 30 healthy volunteers, and noted that the CMAP coefficients of variation ranged from 15.6% to 19.8%. However, Kohara et al. (2000) performed nerve conduction studies twice with a time interval of 1–4 weeks, and reported more reproducible results. In the present study, the baseline CMAP coefficients of variation ranged from 0.1% to 20.5%.

Third, the results in the present study may have been affected by the individual differences in muscle volume, slight deviation of the injection site, and the left-right difference of the foot muscle.

Fourth, the 2 toxins were injected at different times (at an interval of 16 weeks), and therefore, the series of CMAPs following injection with the 2 toxins were not obtained simultaneously.

Fifth, this study was not a placebo-controlled study. However, potential bias appears to be minimal, given the objective measures of evaluation.

In conclusion, the present study using different doses of 6.5 units of A2NTX and 10 units of A1LL indicates that there are little differences in the electrophysiological effect, onset, or response up to 112 days following administration of small doses of A2NTX and onabotulinumtoxinA.

5. Acknowledgments

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6. Conflict of interest

RK and SK have patent pending for A2NTX (WO2008/050866).

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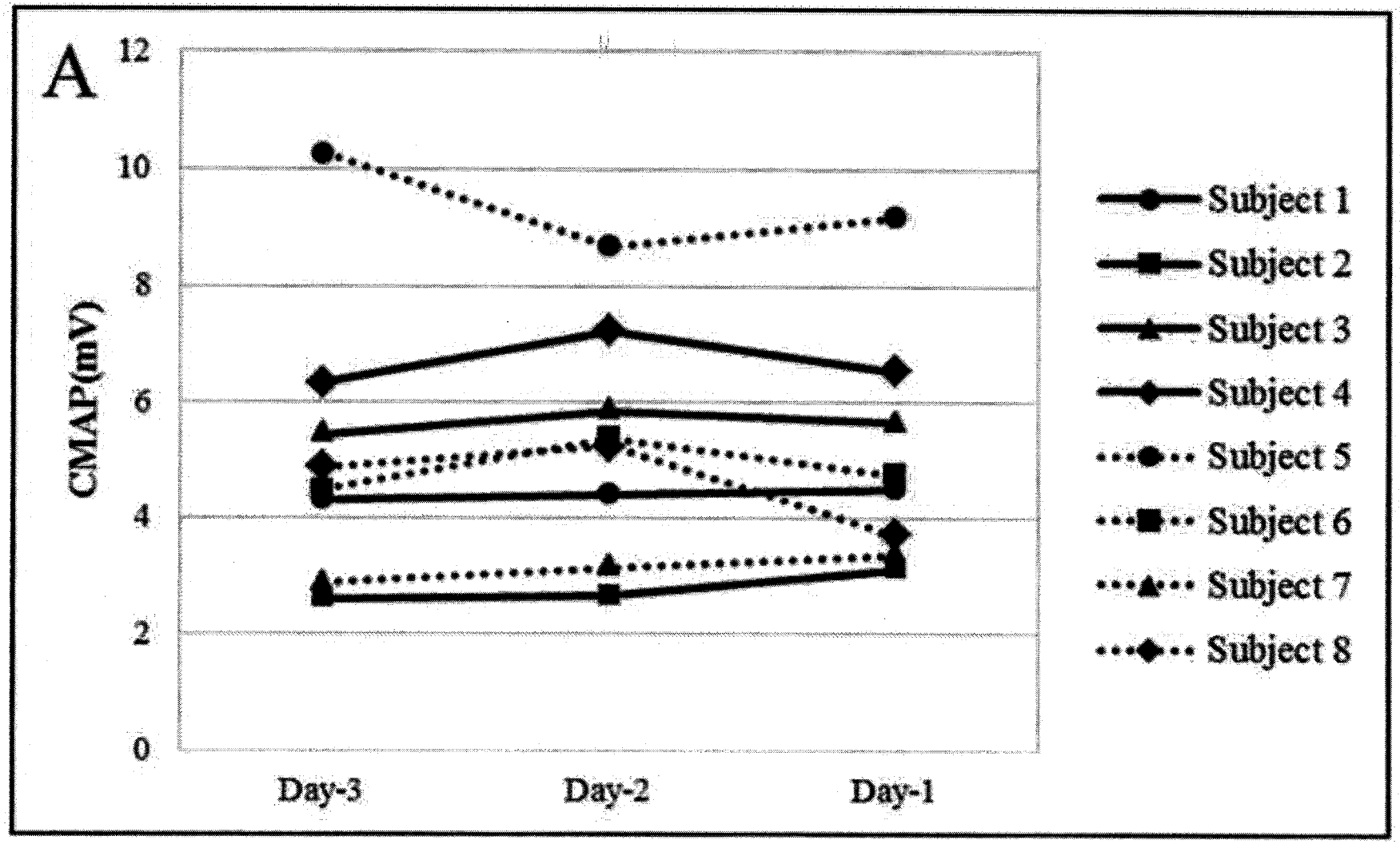

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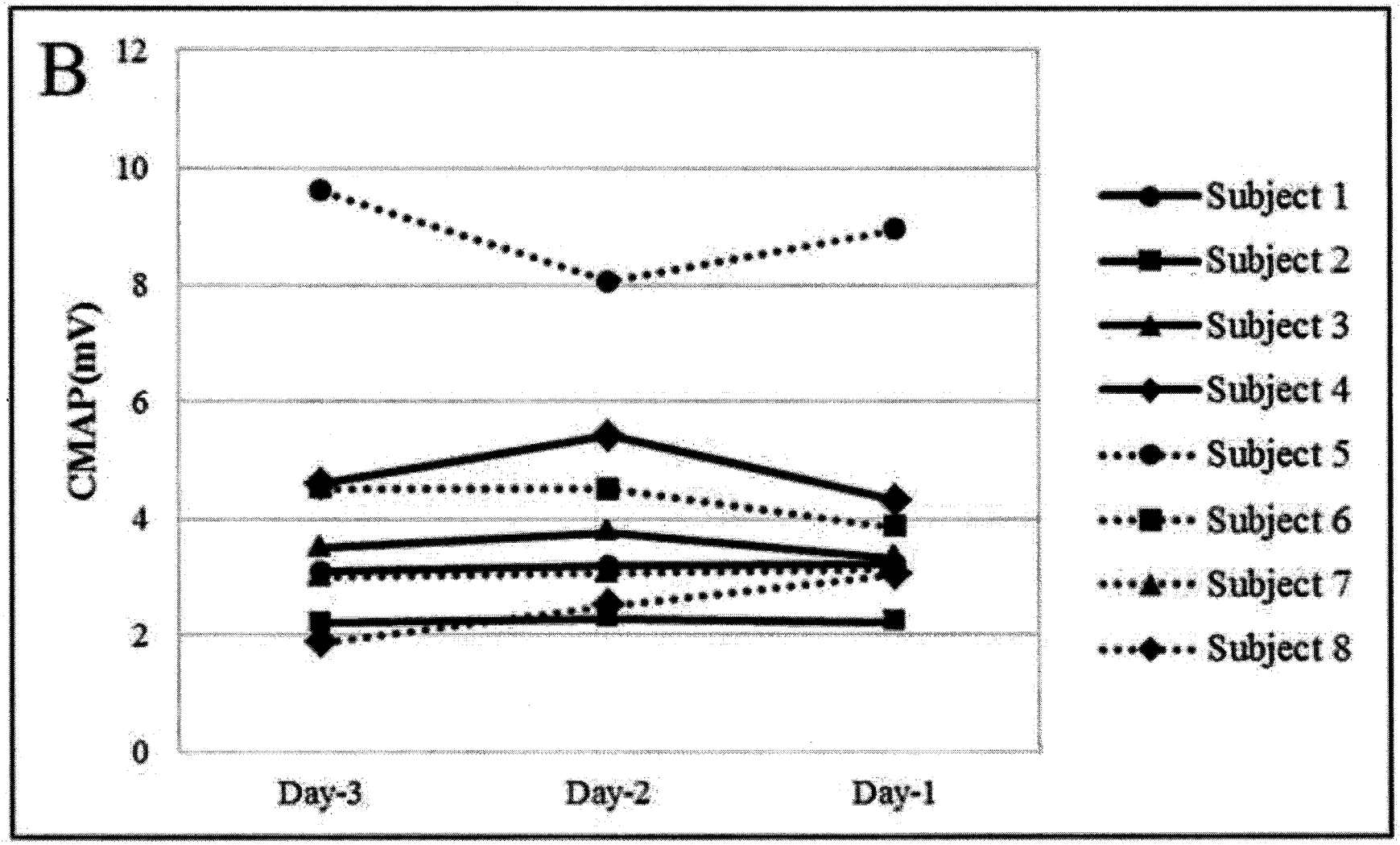

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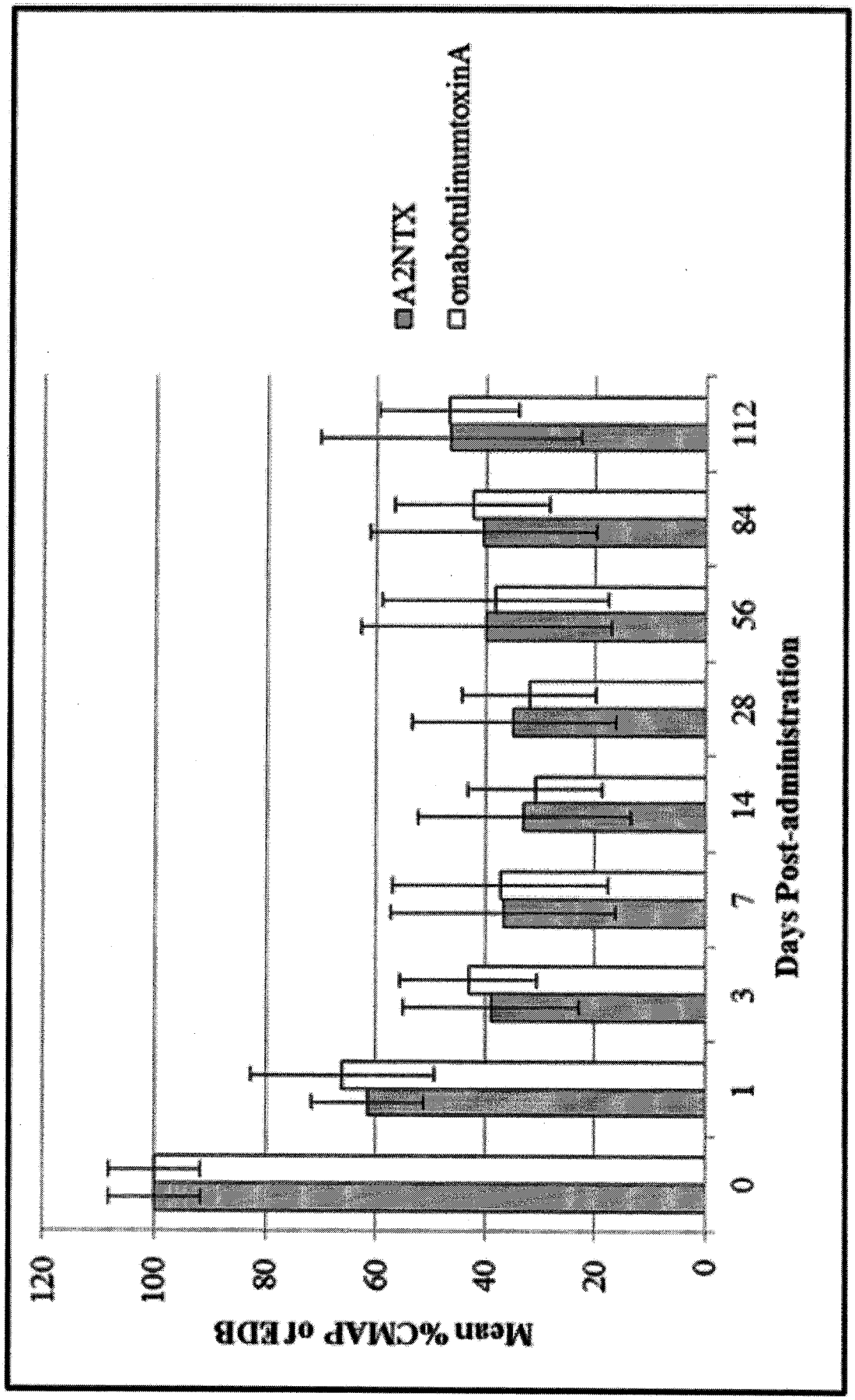

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Fig.3

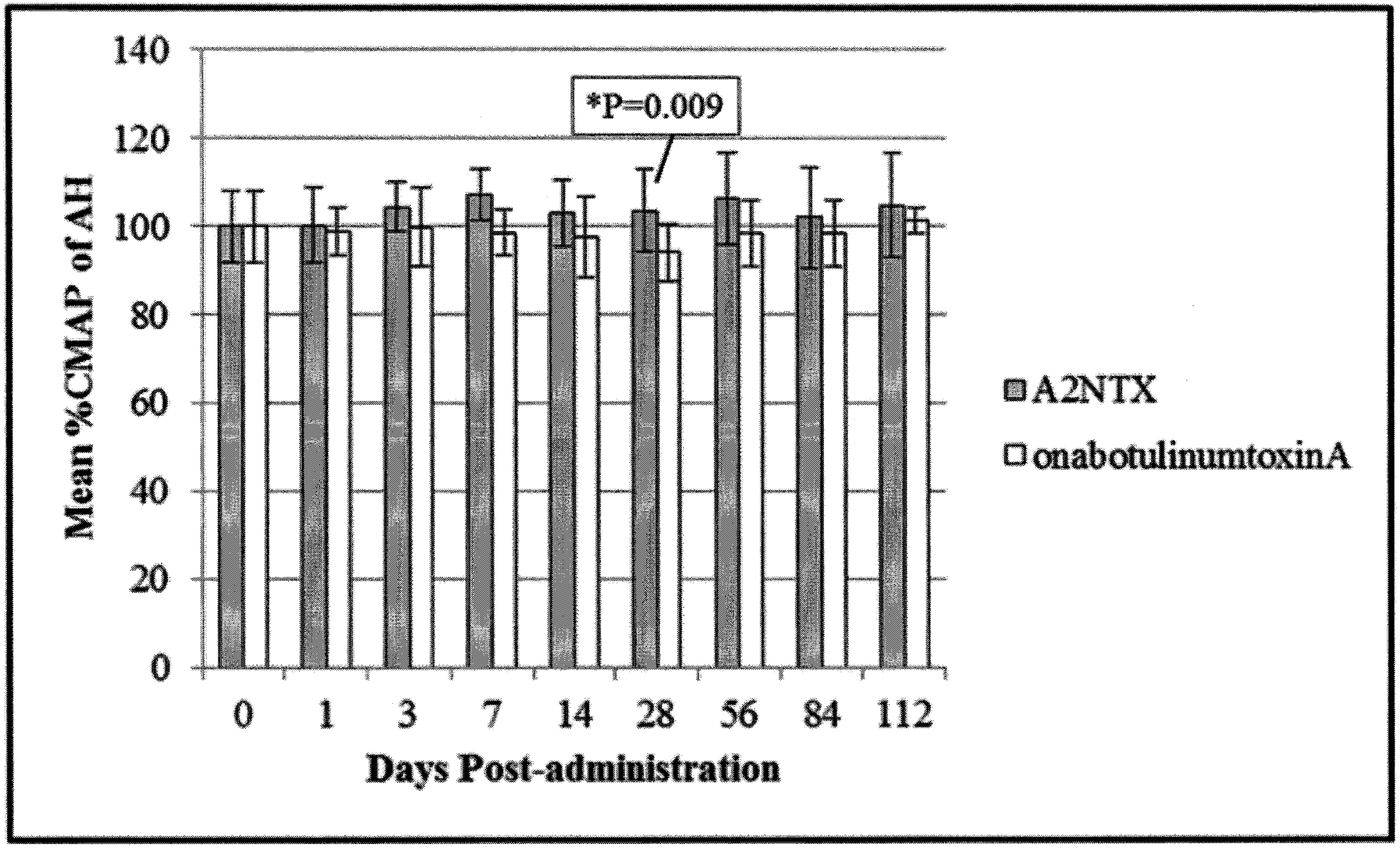

Figure legends

Figure 1

The variation of the baseline CMAP amplitudes of the EDB muscle over the 3-day period.

A) A2NTX injection site and B) onabotulinumtoxinA injection site.

EDB, extensor digitorum brevis muscle; CMAP, compound muscle action potential; A2NTX, botulinum neurotoxin subtype A2

Figure 2

The mean %CMAP after the injection of A2NTX (gray bar) and onabotulinumtoxinA (white bar) into the EDB muscle. Data are expressed as mean ± SD.

Mean %CMAP, mean compound muscle action potential, expressed as percentages of the baseline values

EDB, extensor digitorum brevis muscle; A2NTX, botulinum neurotoxin subtype A2

Figure 3

The mean %CMAP of ipsilateral AH muscle after the injection of A2NTX (gray bar) and onabotulinumtoxinA (white bar) into the EDB muscle. Data are expressed as mean ± SD.

AH, abductor halluces muscle; EDB, extensor digitorum brevis muscle; A2NTX, botulinum

neurotoxin subtype A2