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A comparison study of prabotulinumtoxinA vs onabotulinumtoxinA in myostatin-deficient mice with muscle hypertrophy

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Abstract

Botulinum toxin A (BoNT-A) is used clinically for various muscle disorders and acts by preventing the release of the neurotransmitter acetylcholine into the synapse space. Here, we compared the efficacy of prabotulinumtoxinA (PRA) and onabotulinumtoxinA (ONA) for the reduction in hypertrophy in myostatin-deficient (*Mstn*$^{-/-}$) mice. Two different BoNT-A products (2.5, 10 and 25 U/kg) were injected to paralyse the hindlimb for 2 months, after which sciatic nerve conduction study, 3D micro-CT, haematoxylin and eosin (H&E) and dystrophin staining were conducted. Administration of BoNT-A products induced denervation-mediated atrophy and alleviated muscle hypertrophy generated in *Mstn*$^{-/-}$ mice. The present study revealed that each BoNT-A regulates skeletal muscle size, myofibre number and myofibre diameter in *Mstn*$^{-/-}$ mice. The potential applicability of BoNT-A for the treatment of rare muscle hypertrophic diseases was demonstrated. Compared with ONA, PRA had a comparable ability to act in the local area.

1 | INTRODUCTION

Botulinum toxin A (BoNT-A) is a neurotoxin produced by *Clostridium botulinum* that causes flaccid paralysis and botulism by preventing the release of acetylcholine at the neuromuscular junction.[1] There are seven serotypes of BoNT, named type A-G. Types A and B have been studied extensively and used clinically to treat various disorders characterized by overactive muscle, including blepharospasm, cervical dystonia, facial spasms, post-stroke spasticity, spasms of the limbs, jaw and oesophagus.[2-8] BoNT-A also has therapeutic indications for migraines, refractory overactive bladder and decubitus ulcer.[3,9,10] BoNT-A exerts its main effects by cleaving the proteins required for nerve activation at the axon terminal in a process consisting of several stages.[11] Firstly, the toxin binds specifically to the presynaptic nerve terminal. After binding, the toxin is internalized into a vesicle via endocytosis. The

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vesicle acidifies, and the light-chain portion of the toxin translocates into the cell. Once inside the cytoplasm, the toxin enzymatically cleaves intracellular synaptosomal-associated protein of 25 kDa (SNAP25), thereby blocking neurotransmitter vesicle release and vesicular recycling. This process inhibits nerve signalling, leading to transient paralysis.[12] Injecting BoNT-A into muscle tissue prevents the extracellular release of acetylcholine due to protein degradation of SNAP25 and effectively leads to muscle paralysis.[13]

Several studies have shown that BoNT-A induces muscle atrophy and can be used to successfully treat muscle hypertrophy, including masseter[14] and gastrocnemius.[15,16] However, BoNT-A has never been used to treat chronic increased muscle bulk resulting from a combination of muscle hypertrophy and hyperplasia. Myostatin (MSTN), a highly conserved negative regulator of skeletal muscle mass, is produced and released by myocytes and inhibits muscle growth and differentiation. Mice depleted of MSTN show approximately 2 to 3-fold more skeletal muscle mass than wild-type mice.[17] The absence of MSTN increases the number of myofibres as well as their diameter during development[18] and alteration in fibre type, and fibre contractile properties have been reported in $Mstn^{-/-}$ mice model.[19] Several studies have also demonstrated that blockage of MSTN using the antagonist follistatin[20] or antibodies[21] induces myotube hypertrophy in vitro and post-natal myofibres.[22]

Prabotulinumtoxina is manufactured by a new method of producing high-purity BoNT-A, precipitating the BoNT-A with acid and purifying the BoNT-A by anion exchange chromatography, yielding a purity of 98% or higher.[23] PRA showed comparable safety and efficacy to onabotulinumtoxinA (ONA) in a study using a rat model[24] as well as in a clinical study of patients with glabellar frown lines.[25] Other studies have shown that PRA improves post-stroke upper limb spasticity[26] and reduces masseter muscle hypertrophy.[27,28]

To elucidate the potential actions of PRA, we compared the effect of PRA with that of ONA on skeletal hypertrophic muscle atrophy induced by the absence of MSTN. Our aims were to determine whether administration of PRA decreases skeletal muscle mass in MSTN-depleted mice and to determine what effect (if any) PRA has on myofibre number and size in the hypertrophied muscle. In addition, we set out to compare the efficacy and effect duration of PRA to those of ONA.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical approval

The original C57BL/6 and $Mstn^{Ln}$ breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The generation of $Mstn^{-/-}$ mice has been previously described.[17] The animals were fed standard rodent food and water ad libitum and in sawdust-lined cages housed (maximum of three per cage) in an air-conditioned environment with a 12-hour light/dark cycle. Male (8 weeks) wild-type mice that express MSTN ($Mstn^{+/+}$) and $Mstn^{-/-}$ mice were used in this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (2017-00028) and conformed to all applicable National Institutes of Health guidelines. The study was conducted in accordance with the journal's policy for experimental and clinical studies.[29]

2.2 | Toxin preparation and administration

Each vial was diluted and administered as previously described.[30] The two preparations of botulinum toxin type A (prabotulinumtoxinA, Nabota®; Daewoong Pharmaceutical, Seoul, Korea, and onabotulinumtoxinA, Botox®; Allergan Inc, Irvine, CA, USA) were reconstituted in the same volume of 0.9% saline and administered for each study. For BoNT-A injection, 30 μL of BoNT-A (2.5, 10, 25 U/kg) in 0.9% saline was injected into the mid-belly of the right lateral gastrocnemius muscle using a 30-gauge needle attached to a sterile 250 μL Hamilton syringe. For each experiment, six mice were injected per dose. Based on previous studies, muscle tissue was harvested at 56 days after BoNT-A injection for 3D CT imaging and histology.[31,32] Separate control animals received saline injections into the right lateral gastrocnemius muscle.

2.3 | Nerve conduction study—Electrophysiology

A Dantec™ Keypoint® Focus (Natus Neurology, Middleton, WI, USA) instrument was used for nerve conduction recording of the gastrocnemius muscle; data were automatically analysed and averaged. Three surface disc electrodes (recording anode, cathode and ground electrode) were used. An incision was made from the gluteus muscles to the popliteal region to expose the sciatic nerve with standard settings (electric potential = 5.8 mA; stimulus duration = 0.1 ms). The proximal side of the nerve was stimulated with a supramaximal electric stimulation at the same position each time. The compound muscle action potential (CMAP) amplitude (mV) (peak-to-peak), distal latency (ms) and area (mm^2) were recorded each time. The protocol was repeated three times for each mouse during all individual nerve conduction study (NCS) experiments. Amplitude was chosen as the principae variable in the data analysis because it was the best predictor of the physiological changes at the muscle motor unit level.

2.4 | Micro-CT scanning

Mice were scanned with a Viva CT 80 (Scanco Medical, Bassersdorf, Switzerland) at the Micro-CT Core Laboratory at KNOTUS Corporation (Guri, Korea). Each specimen was aligned horizontally and separated by soft sponges. The holders were placed vertically on the turntable of the micro-CT system. The following settings were used: 45 kVp energy, 117 μA X-ray intensity, 79.9 mm FOV/diameter and 78 μm voxel size. The micro-CT Evaluation Program V6.6 (Sanco Medical software) was used to display 3D muscle rendering. To render 3D-based muscle structures, the whole structure was selected based on the 3D projection of the entire lower leg. The following selection criteria were used: the threshold of difference between the bone and muscle was selected, after which the signal detected from the bone structure was removed from that belonging to the whole structure (criteria for muscle structure: Gauss sigma: 5.0, Gauss support: 9 and threshold: 53). Muscle volume was calculated automatically and is expressed in cubic metres.

2.5 | Muscle histology

Muscle tissue biopsy specimens were collected, immediately fixed with 10% paraformaldehyde in PBS and incubated overnight at 4°C. Next, the samples were dehydrated, embedded in paraffin wax and sectioned with a microtome into 5-μm serial transverse sections. The sections were then transferred to treated slides (Thermo Fisher Scientific, Pittsburgh, PA, USA), de-paraffinized, stained with haematoxylin and eosin (H&E; Dako Japan Co., Tokyo, Japan) and then stained immunohistochemically with an antibody against dystrophin (ab15277; Abcam, Cambridge, UK). For immunohistochemical analysis, the sections were heated in pH 6.0 citrate buffer and were incubated with a primary antibody overnight at 4°C followed by incubation with a secondary antibody for 1 hour at room temperature in the dark. After rinsing, the sections were covered with a mounting medium (Thermo Fisher Scientific, Waltham, MA, USA). H&E-stained sections were then examined by light microscopy (DM750; Leica, Wetzlar, Germany) to assess histological changes. Data were analysed using ImageJ 1.38 software (NIH, Bethesda, MD, USA). Fluorescence images were acquired by using a confocal microscope (LSM700; Zeiss, Jena, Germany).

2.6 | Statistical analysis

All quantitative data are presented as the mean ± standard error of the mean (SEM) for three independent experiments. Statistical analyses were performed using SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA) program. Analysis of variance (ANOVA) was used for multiple comparisons, followed by Tukey's multiple comparisons test. The significance of differences between two groups was evaluated by a paired *t* test. Significant values were $*P < 0.05$ and $**P < 0.01$.

3 | RESULTS

3.1 | Myostatin deficiency induces skeletal muscle hypertrophy and impairs neuromuscular functions

We compared the phenotype in 8-week-old myostatin null ($Mstn^{-/-}$) mice to that in wild-type ($Mstn^{+/+}$) control animals. $Mstn^{-/-}$ mice are approximately 30% heavier than $Mstn^{+/+}$ mice (Figure 1A). $Mstn^{-/-}$ mice exhibited overproduction of skeletal muscle, centralized nuclei and enlarged myofibre cross-sectional area (CSA) in the hindlimb muscle (Figure 1B,C). Notably, in the NCS, there was no statistical difference in muscle contraction time (duration) between $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (Figure 1D). However, despite the significantly greater muscle mass and total body mass in $Mstn^{-/-}$ mice compared to wild-type mice, the amount of evoked muscle (amplitude) was decreased in $Mstn^{-/-}$ mice compared to wild-type mice (Figure 1E). Muscle hyperplasia in MSTN-deficient mice increased axon diameter and myelin thickness in the fore- and hindlimbs without affecting internode length.[33] Consistently, it has been reported previously that generalized muscle hypertrophy decreases nerve conduction,[34] the structural changes underlying this decrease can be observed histologically.[35,36] These data indicate that the nerve conduction is not increased in proportion to skeletal muscle mass in adult $Mstn^{-/-}$ mice.

3.2 | Both botulinum toxin A products prevent nerve transmission in MSTN-deficient mice

The effect of BoNT-A (ONA and PRA) on skeletal hypertrophic muscle was determined in $Mstn^{-/-}$ mice. To evaluate the blocking of neurotransmitter release, nerve conduction studies were performed on the right lateral gastrocnemius muscle for each mouse before each BoNT-A injection and every week thereafter.[37] In addition, the body-weight was measured in order to check the long-term toxicity after BoNT-A administration. Two preparations had similar recovery patterns until 56 days post-injection in a dose-dependent manner. Overall, mice injected with all dose of PRA or ONA recovered their body-weight in a similar pattern (Figure 2A). In addition, the gastrocnemius muscle weight comparison after 56 days showed that the muscle weight reduced in a BoNT dose-dependent manner

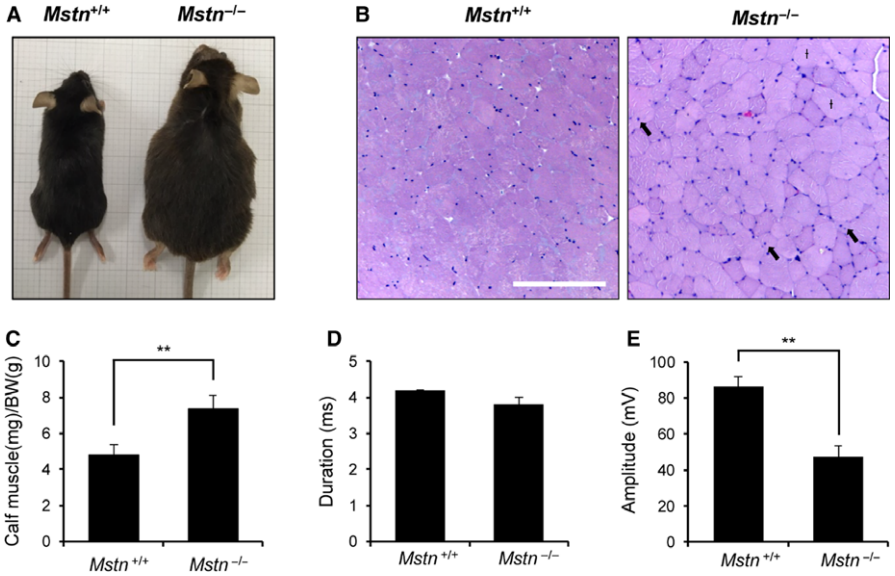

FIGURE 1 Biological differences between $Mstn^{+/+}$ and $Mstn^{-/-}$ mice. Myostatin gene deficiency induces muscle hyperplasia; the $Mstn^{-/-}$ mice showed enhanced skeletal muscle. A, Gross images of $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (n = 6 for each genotype). B, H&E-stained cross sections of the right hindlimb muscles from 8-wk-old male mice (crosses: swollen fibres; arrows: multiple centralized nuclei). Scale bar, 100 μm. Motor nerve conduction study results in $Mstn^{+/+}$ and $Mstn^{-/-}$ mice (n = 6 for each genotype). Muscle weight (C), duration (D) and amplitude (E) measured by compound muscle action potential. Data are presented as means ± standard errors of the mean for three independent experiments. $^{**}P < 0.01$ compared with the $Mstn^{+/+}$ mice

(Figure 2B). The NCS data showed a faster recovery with lower doses of each BoNT-A (Figure 2C,D). Mice treated with 2.5 U/kg BoNT-A tended to return to baseline first, followed by mice treated with 10 U/kg BoNT-A. The administration of 25 U/kg BoNT-A yielded a consistent effect until 35 days, indicating that BoNT-A potency depends on the concentration (Figure 2C). At 56 days after BoNT-A administration, CMAP values in the 2.5 U/kg group were recovered to about 42% (ONA) and 46% (PRA), respectively (Figure 2D). In the 10 and 25 U/kg groups, the amplitude value was about 11%-30% (saline = 59.8 mV, ABO 10 U/kg = 23.2 mV, PRA 10 U/kg = 30.8 mV, ABO 25 U/kg = 16.9 mV, PRA 25 U/kg = 11.5 mV) compared to control group, indicating that the electrical signal transmission blocking effect is maintained (Figure 2C,D). The results suggested that the acetylcholine release was inhibited for a longer time with higher doses of BoNT-A and that ONA and PRA had similar effects (Figure 2C-F). However, the dose-response relationship between two BoNT preparations did not vary in terms of muscle-weakening effects. Moreover, the inhibition effect of nerve conduction was higher in $Mstn^{-/-}$ mice compared to wild-type mice at 56 days after both BoNT-A preparation injection (Figure 2E).

3.3 | Botulinum toxin A administration into the hindlimb reduces muscle volume in MSTN-deficient mice

3D-reconstructed micro-CT images showed the longitudinal and transverse views of the right lower hindlimb of each animal, enabling muscle volume to be determined (Figures 3 and S1). The muscle volume of the gastrocnemius, which is located on the posterior side of the lower hindlimb, was reduced by BoNT-A administration. Muscle volume was taken to be the isolated soft tissue volume, which was calculated as the total volume minus the hard tissue (skeletal mass). The calculated muscle volumes of the lower limb ranged between 300 and 500 mm^3 (saline, 502 ± 8.3; ONA, 2.5 U/kg = 446 ± 5.5, 10 U/kg = 387 ± 35.3, 25 U/kg = 363 ± 32.4; PRA, 2.5 U/kg = 421 ± 24.9, 10 U/kg = 400 ± 11.3, 25 U/kg = 305 ± 29.2) as assessed by micro-CT (Figure 3). BoNT-A decreased muscle volume in a dose-dependent manner. Moreover, the soft tissue volume of the lower limb, including the gastrocnemius, was significantly reduced by almost 39% (PRA) and 28% (ONA) in the 25 U/kg-injected groups ($^{*}P < 0.05$) compared with the normal control limb at 56 days after BoNT-A injection (Figure 3). However, the dose-dependent effects of the two BoNT-A preparations were not statistically significant.

3.4 | Botulinum toxin A administration in the hindlimb induces muscle fibre contraction in MSTN-deficient mice

Muscle fibre diameter change is a useful measure of denervation after BoNT-A administration.[38] We first confirmed that $Mstn^{-/-}$ mice have increased myofibre CSA (Figure 1B). Although the hypertrophic muscle and enlarged CSA result in high force generation capacity, this did not involve a proportional increase in contraction strength in $Mstn^{-/-}$ mice.[19,35,39] Therefore, the unnecessary hypertrophy of muscle and muscle fibres should be ameliorated for potential therapeutic and cosmetic applications. Muscle fibre diameter was significantly reduced in all BoNT-A

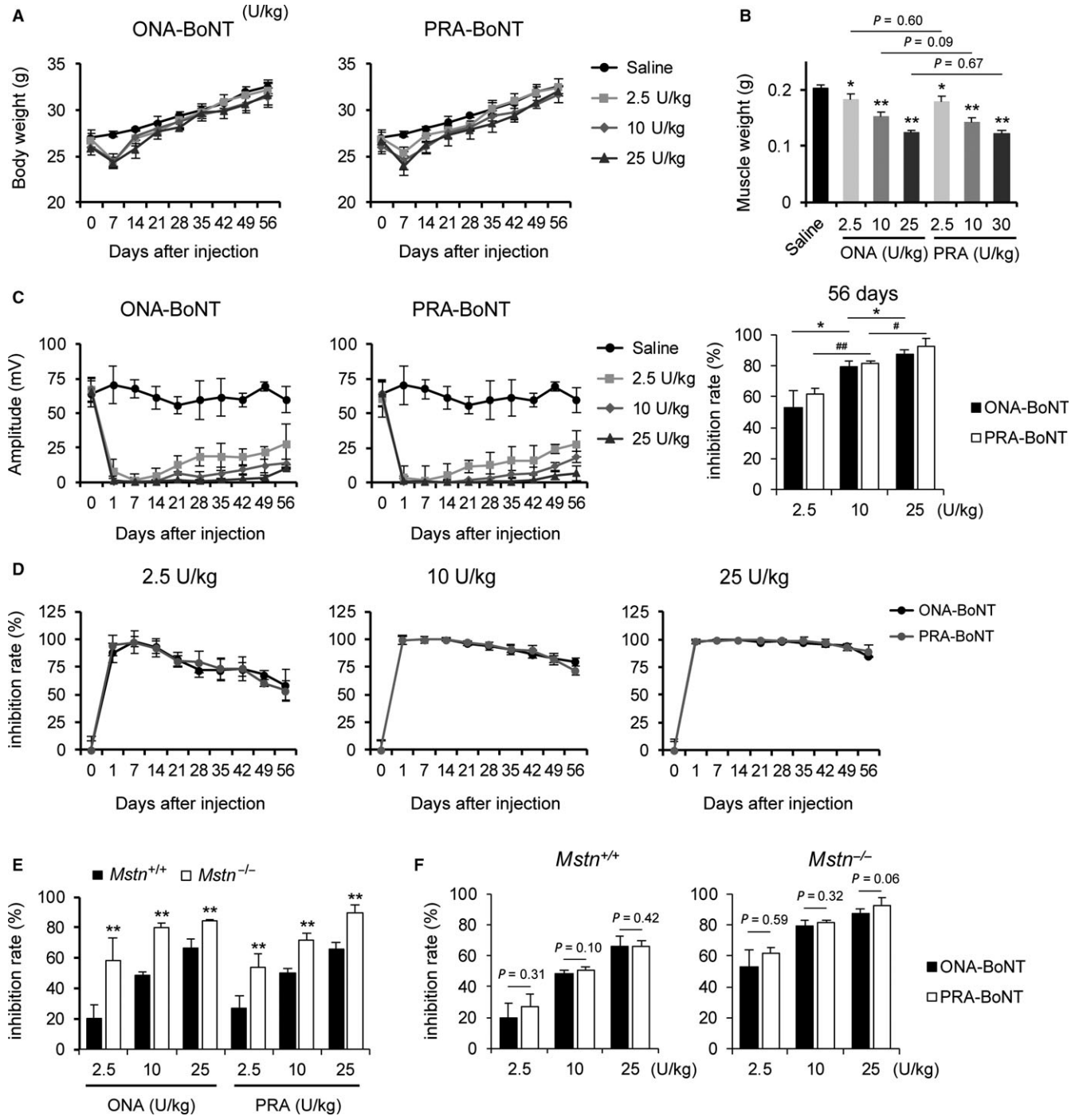

FIGURE 2 Sciatic nerve conduction study (NCS) results in $Mstn^{-/-}$ mice until 56 d after botulinum toxin A (BoNT-A) injection. The graph displays (A) body-weight recovery and (B) muscle weight at 56 d (n = 4 for each group). *$P < 0.05$ and **$P < 0.01$ compared with the saline-treated group. C, Amplitude changes in NCS on the mouse hindlimb (n = 4 for each group). Mean compound muscle action potential amplitudes in the 2.5, 10 and 25 U/kg BoNT-A injection groups. OnabotulinumtoxinA (ONA) (left); prabotulinumtoxinA (PRA) (right). *$P < 0.05$, #$P < 0.05$, and ##$P < 0.01$. D, Inhibition rate changes between the ONA and PRA groups. 2.5 U/kg (left); 10 U/kg (middle); 25 U/kg (right). E, F, Inhibition rate in $Mstn^{+/+}$ mice and $Mstn^{-/-}$ mice at 56 d after BoNT-A administration. **$P < 0.01$ compared with $Mstn^{+/+}$ mice. Data are presented as means ± standard errors of the mean for three independent experiments

groups (ONA, 2.5 U/kg = 781 ± 114.2, 10 U/kg = 512 ± 278.7, 25 U/kg = 481 ± 104.1; PRA, 2.5 U/kg = 766 ± 85.3, 10 U/kg = 566 ± 105.2, 25 U/kg = 441 ± 106.4) compared with the control group (saline, 1036 ± 130.7) at 56 days after BoNT administration. In addition, the changes in the expression of dystrophin, a muscle

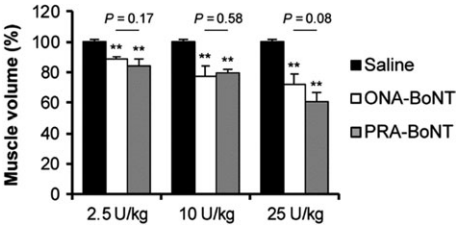

FIGURE 3 Volumes of Nabota®- and Botox®-injected muscles at 56 d after botulinum toxin A (BoNT-A) injection in *Mstn*$^{-/-}$ mice. X-ray micro-CT (CT)-reconstructed 3D rendering of a mouse injected with BoNT-A (n = 4 for each group). The 3D images are presented in Figure S1. Muscle volumes were calculated in cubic metres and presented percentage. Data are presented as means ± standard errors of the mean for three independent experiments. $^{**}P < 0.01$ compared with saline-treated mice

membrane marker, were observed in conjunction with the changes in muscle fibres after 56 days. The PRA- and ONA-treated groups exhibited a decrease in dystrophin expression compared with the control group (Figure 4A). The effects of ONA and PRA did not differ significantly (Figure 4A,B). Together, these results indicate that BoNT-A-induced muscle fibre shrinkage was maintained during the first 2 months of muscle recovery and that the rate of muscle fibre shrinkage was not correlated with the BoNT-A dose for either ONA or PRA.

4 | DISCUSSION

The BoNT-A preparations have diverse differences, including complex protein structures, purity and molecular weights, and can affect their pharmacological profiles and efficacy. In this study, we have explored the potential applicability of BoNT-A for uncommon muscle hypertrophic diseases such as *Mstn*$^{-/-}$ disease through the comparison of two BoNT-A preparations. We identified the atrophic actions of PRA in *Mstn*$^{-/-}$ mice and compared the results with those of ONA. Two BoNT-A preparations tested showed comparable effects on neuronal transmission, muscle volume and myofibre contraction in *Mstn*$^{-/-}$ mice following intramuscular injection. Thus, BoNT-A is effective for the treatment of muscle hypertrophy in *Mstn*$^{-/-}$ mice, and the dose between ONA and PRA may be converted into a one-to-one ratio.

Although several studies have shown that BoNT-A formulations are successful for treating muscle hypertrophy such as masseter hypertrophy[14,40] and gastrocnemius hypertrophy,[15,16] no report has focused on the chronic increased muscle bulk resulting from the combination of muscle fibre hypertrophy and hyperplasia. MSTN, also known as growth differentiation factor 8 (GDF-8), is expressed in skeletal muscle myocytes and acts as a negative regulator of muscle growth and differentiation.[17,41] MSTN-related muscle hypertrophy is an unusual genetic disorder with incomplete autosomal dominance characterized by increased skeletal muscle size and reduced body fat.[17] Mutation or deletion of MSTN is associated with muscle hypertrophy in animal models as well as in children. MSTN-deficient mice have about twice the muscle mass of normal mice and have thus been termed "mighty mice".[17] In humans, one study reported that a child with muscle hypertrophy had a loss-of-function mutation in MSTN.[42] *Mstn*$^{-/-}$ mice exhibit a different phenotype compared to wild-type mice. Specifically, *Mstn*$^{-/-}$ mice have heavier skeletal muscle, lower total adiposity and increased myofibre cross-sectional area in the soleus and gastrocnemius.[18,35] Additionally, generalized muscle hypertrophy has decreased nerve conduction[34]; the structural changes underlying this decrease can be observed histologically.[35,36] Our findings are consistent with the results of previous studies.

Using electrophysiological experiments, we confirmed that BoNT-A inhibits acetylcholine release at the neuromuscular junction, thereby reducing muscular contractions. The CMAP was significantly reduced after administration of the two BoNT-A preparations, ONA and PRA; moreover, BoNT-A potency depended on its concentration. In a similar study, CMAP measurements were used to quantitatively assess the biological activity of BoNT-A.[43] Kim and colleagues also used the rat-CMAP test to compare the paralytic effect induced by ONA and PRA on the rat tibialis anterior muscle by the split-body method.[24] Compared with *Mstn*$^{+/+}$ mice, inhibition of nerve conduction lasted longer. The enlarged muscles exert the effects on glycolysis for energy production, but the enlarged muscles that occur in the absence of MSTN impair force generation of skeletal muscle.[19,35,39] In addition, the muscles of *Mstn*$^{-/-}$ mice are related to decreased mitochondrial numbers, capillary density and the mitochondrial enzymes.[39,44] Nerve conduction study also showed normal conduction velocity and decreased amplitude in chronic myopathy.[34] Thus, the application of botulinum toxin for the improvement of unnecessary hypertrophy of muscles and muscle fibres is important, not only for potential therapeutic applications, but also for cosmetics.

The inhibitory effect of BoNT-A led us to investigate the muscle volume changes using 3D CT image analysis in *Mstn*$^{-/-}$ mice. The 3D CT images revealed decreased muscle volume of the right lower hindlimb after BoNT-A administration. Consistently, BoNT-A-induced muscle volume reduction occurred in a dose-dependent fashion; moreover, muscle volume loss was similar in the PRA-treated group compared with the ONA-treated group. Our results further demonstrated the effect of PRA on muscle fibre shrinkage. Interestingly, muscle fibre shrinkage was not dose-dependent and did not differ between the two

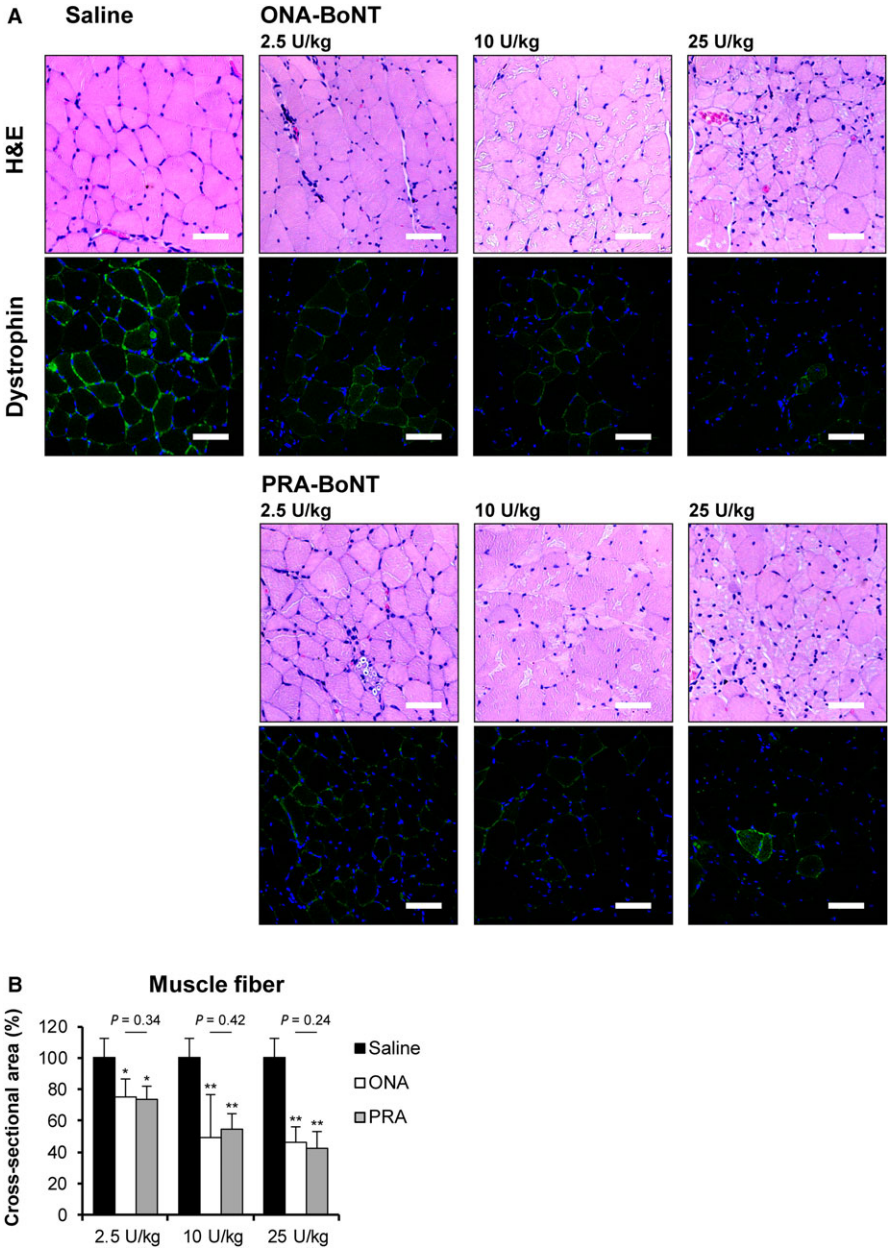

FIGURE 4 Comparison of muscle fibre contraction between ONA- and PRA-injected muscles at 56 d after botulinum toxin A injection in *Mstn*$^{-/-}$ mice. A, Representative H&E and anti-dystrophin staining images. Twenty fibres per images in three sections for each animal were included in the analysis. The gastrocnemius was fixed, and the thickest muscle belly was used as the transverse section site. Scale bar, 50 μm. B, Muscle fibre areas. Data are presented as means ± standard errors of the mean for three independent experiments. *$P < 0.05$ and **$P < 0.01$ compared with saline-treated fibres. ONA, OnabotulinumtoxinA; PRA, prabotulinumtoxinA

BoNT-A-treated groups during muscle atrophy in *Mstn*$^{-/-}$ mice. Consistent with our results, classical myofibre atrophy was observed in BoNT-injected gastrocnemius muscle, resulting in fibre diameter focal reduction as assessed by histological characteristics.[38,45]

Further studies are needed to determine the long-term safety and efficacy following intramuscular injection in *Mstn*$^{-/-}$ mice. Previous reports showed that BoNT injection into the muscle-induced muscle atrophy, regardless of the dose, but the recovery of muscle volume varied with the dose and time.[46-48] In addition, dose-dependent and injection number-dependent antigenicity studies are required for these commercially available BoNT-A formulations. Patients could be unresponsive to BoNT due to the generation of neutralizing antibodies against active BoNT.[49,50]

In conclusion, PRA exhibited similar muscle-weakening efficacy and duration as ONA in MSTN-deficient mice. We demonstrated the potential applicability of BoNT-A for the treatment of rare muscle hypertrophic diseases through a comparison of PRA and ONA BoNT-A formulations. Overall, muscle volume, nerve conduction, myofibre size and dystrophin expression were affected in the MSTN null mice. Various muscular hypertrophies may occur as a genetic factor in childhood; adults may also develop muscle rigidity and hypertrophy owing to a poor lifestyle. Thus, the applications of BoNT have recently been extended to treat muscle hypertrophy for cosmetic purposes. It remains to be determined whether repeat or discontinuation of BoNT administration may reverse these functional adaptations and re-establish the hypertrophy phenotype.

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CONFLICT OF INTERESTS

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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