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Original Article

None detectable retrograde transport of Chinese botulinum toxin type A in mice by single intramuscular injection

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Abstract: Botulinum toxin type A (BoNT/A) can specifically cleave synaptosomal associated protein of 25 kDa (SNAP-25) into cleaved SNAP-25 (cl.SNAP-25), thus blocking the synaptic transmission in motor end plate and resulting in paralysis. It has been widely applied in clinical for treatment of various conditions characterized by muscle hyperactivity, such as dystonia and spasticity. BoNT/A is used locally, with little diffusion. Its paralyzing role is considered to be restricted to the nerve muscle junction, or close to the injection site. Recently, more and more studies, however, have suggested that BoNT/A also has central effects. In addition, some investigators have demonstrated that BoNT/A enters into central nervous system via retrograde transport after local intramuscular administration. The retrograde axonal transport of Chinese BoNT/A (CBoNT/A) was studied in this paper, which was rare in report. And the results showed that cl.SNAP-25 appeared not only at the injection site but also in contralateral muscle. Retrograde transport, however, was non-existent or too little to be detected in our study.

Keywords: Botulinum toxin type A, remote effect, retrograde transport, cleaved SNAP-25, gastrocnemius

Introduction

Botulinum toxins, produced by clostridium botulinum-a kind of gram-positive rod-shaped anaerobic bacterium, have been classified into seven different serotypes (A-G) based on their immunological characteristics [1]. Among these seven serotypes, botulinum toxin type A (BoNT/A), not only plays an inhibitory role on fusion of vesicular and plasma membrane, but also specifically cleaves synaptosomal associated protein of 25 kDa (SNAP-25), which is a part of the soluble N-ethyl-maleimide-sensitive fusion protein attachment receptor (SNARE) [2]. Therefore, BoNT/A has a potential to be approved by FDA as a therapeutic agent for a number of hypercholinergic disorders. Moreover, it has been widely applied in treatment for skin wrinkles and cerebral palsy [3, 4]. Paralyzing role of BoNT/A is supposed to be restricted to the nerve muscle junction (NMJ) or to be close to the injection area. However, it has been reported that when BoNT/A was administrated in neck muscles to treat torticollis, the subsequent single fiber electromyography (SFEMG) in a limb muscle suggested subclinical effects of BoNT/A on distant muscles [5]. Besides, unexpected central effects of BoNT/A have also been reported. Wohlfarth et al. found changes of F-wave in remote area after local application of BoNT/A [6] and Kim et al. suggested modification effects of BoNT/A injection on cortical excitability in normal humans [7]. And in bilateral pain models, unilateral administration was observed to cause bilateral effects [8, 9]. Peripheral effect, obviously, is not enough to explain these distant contralateral effects.

Theoretically, these unexpected remote effects of BoNT/A in clinical may depend either on a direct action of the toxin that is transported via the hematogenous route and neural retrograde transport [9-13], or an indirect action which may lead to changes such as “reorganization” of central nerve system (CNS) [14-16]. BoNT/A is a protein (150 kDa), which is too large to cross the blood brain barrier (BBB). Retrograde transport of purified BoNT/A has been observed

in some tissues (e.g. hippocampus, visual system, facial motoneurons and dorsal root ganglion (DRG)) currently via immunofluorescence stain detection on expression and distribution of cleaved SNAP-25 (cl.SNAP-25) [13, 17]. This axon transport, however, has been considered non-existent or very limited up to now. And moreover, its activity in the brain after peripheral delivery is also questionable.

Experimental findings in this paper suggested that intramuscular injection of Chinese BoNT/A (CBoNT/A) at relative low dose (1 U/kg) showed enzymatic activity in remote uninjected neuromuscular junction, but not in peripheral nerves or central nervous system; similar trend was also observed in even high dose group (30 U/Kg). Unlike other studies, in which cl.SNAP-25 was detected by immunofluorescence stain, western blot was used in our study for measurement of SNAP-25 and cl.SNAP-25 to support the specificity.

Materials and methods

Animals

Adult male ICR mice (25-30 g) were raised in our animal laboratory (conditions: 22 intained a, 12 h/12 h light-dark cycle) and inhibited from food and water. The mice were treated in accordance with the guidelines of the Animal Advisory Committee at Zhejiang University and the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

CBoNT/A injection

Three different dosages (1 U/kg, 5 U/kg, 30 U/kg in volume of 25 μL) of CBoNT/A (Lanzhou Biological Products Institute, China), diluted in 0.9% saline were injected intramuscularly (i.m.) into the gastrocnemius of the mice. And the positive control group was given CBoNT/A via intrathecal injection (i.t. 1 U/kg in volume of 5 μL). Mice were restrained and injected (31-gauge needle, 25 μL Hamilton syringe) consciously. While for i.t, tail flick reflex indicated a correct placement of needle when CBoNT/A could be slowly injected [18].

Western blot

Western blot was used to analyze SNAP-25 and cl.SNAP-25 in gastrocnemius, sciatic nerve, and spinal cord of three different dose groups on 3rd, 7th, 14th, and 28th day after injection, respectively. First, mice were anesthetized with chloral hydrate decapitated to take tissue specimens. Then, gastrocnemius was added to liquid nitrogen in a mortar mill and mashed immediately with a pestle until the tissue was ground to a homogenate; while lumbar cord and bilateral sciatic nerve were removed and immediately homogenized in solubilization buffer (50 mm HEPES, containing 1 mM EDTA, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/ml aprotinin, 2 mM benzamidine and 0.01 mM PMSF, pH 8). After that, proteins were rocked (at 4°C for 30 min) and centrifuged (12,000 rpm at 4°C for 5 min). Dissolved material was recovered in the supernatant, which was used for Western blot analysis. Proteins were loaded on 15% acrylamide SDS-PAGE gels, and transferred onto polyvinylidenefluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The primary antibodies were mouse anti-SNAP25 (Covance, Berkeley, CA), which were visualized after incubation of the membranes with either horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibody (Sigma, St. Louis, MO). Signals were developed with enhanced chemiluminescence reagent (ECL-Plus; Perkin-Elmer, Waltham, MA) and detected on X-ray film. For densitometric quantification, immunoblots were digitized on a flatbed scanner and digital images were measured with Image J.

Results

Effects of unilateral injection of CBoNT/A (1 U/kg)

Firstly, in order to explore whether CBoNT/A could spread to the contralateral muscles or not, CBoNT/A (1 U/kg) was injected into left gastrocnemius, and bilateral SNAP-25 and cl.SNAP-25 were measured. cl.SNAP-25 failed to be observed in control group; while in CBoNT/A administered group (**Figure 1A**), however, cl.SNAP-25 was appeared at ipsilateral side (7%) on the 3rd day after injection; it was increased and reached the peak on the first week (~25%), and then declined on the fourth week (18%). Unexpectedly, cl.SNAP-25 was detected at contralateral side on the 3rd post-injection day (0.42%); it was raised to 8.1% on first week; and the proportion was maintained on the second and fourth week after injection. SNAP-25 at ipsilateral side rose from 10% to a peak of 20% on the 3rd day after injection and

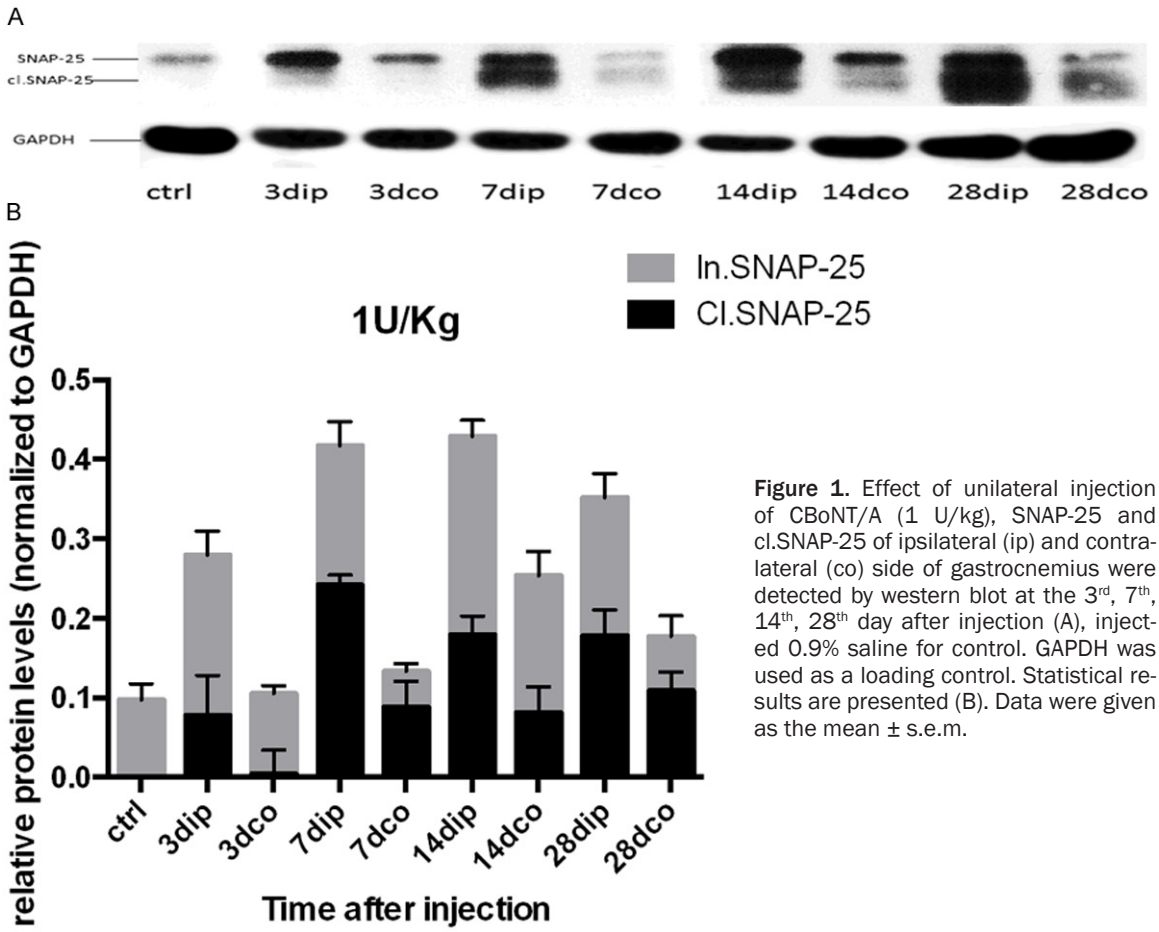

Figure 1. Effect of unilateral injection of CBoNT/A (1 U/kg), SNAP-25 and cl.SNAP-25 of ipsilateral (ip) and contralateral (co) side of gastrocnemius were detected by western blot at the 3rd, 7th, 14th, 28th day after injection (A), injected 0.9% saline for control. GAPDH was used as a loading control. Statistical results are presented (B). Data were given as the mean ± s.e.m.

fluctuated between 17%~20% in the second week. It was continued to rise till 24% on the second week, and then fell to 17.8% finally on the fourth week. SNAP-25 on contralateral side, however, maintained 10% after injection; though declined to 4.4% on the first week, it was rebound to 17%, and then decreased to 6.8% by the fourth week (**Figure 1B**).

Effects of unilateral injection of CBoNT/A (5 U/kg)

cl.SNAP-25 was found detectable in contralateral muscle even under very low dose of BoNT/A (1 U/Kg). Regular therapeutic dosage (5 U/kg) was also tried. It was found that on ipsilateral side (**Figure 2A**), cl.SNAP-25 was increased from 0 to 23% and reached the peak of 35% at second week after injection; and it finally fell to 22% on the fourth week. However, on contralateral side, cl.SNAP-25 was visible on the 3rd day (19.6%) and the proportion (16%~20%) was maintained by the fourth week. In terms of SNAP-25, its proportion (10%) at ipsilateral side was maintained within three days after injection; then it was increased to 15%, peaked at 20% and restored to normal proportion (10%). on contralateral side, SNAP-25 was increased and reached the maximum of 20% on the first week after injection. Then it fell to normal state at the second week (**Figure 2B**).

Effects of unilateral injection of CBoNT/A (30 U/kg)

High therapeutic dose (30 U/kg) of BoNT/A was injected into left gastrocnemius to investigate whether toxins are transported in an axonal route to the contralateral muscle. Bilateral SNAP-25 and cl.SNAP-25 in spinal cord, muscle and sciatic nerve were analyzed. As expected, cl.SNAP-25 in gastrocnemius of both sides was detected (**Figure 3A**). cl.SNAP-25 at ipsilateral side emerged at 3rd day (30%). It was increased and peaked at 38.5% on second week after injection, and finally fell to 35.6%. cl.SNAP-25 at contralateral side appeared at 3rd day (18.9%) and rose to 29% by the fourth week. In

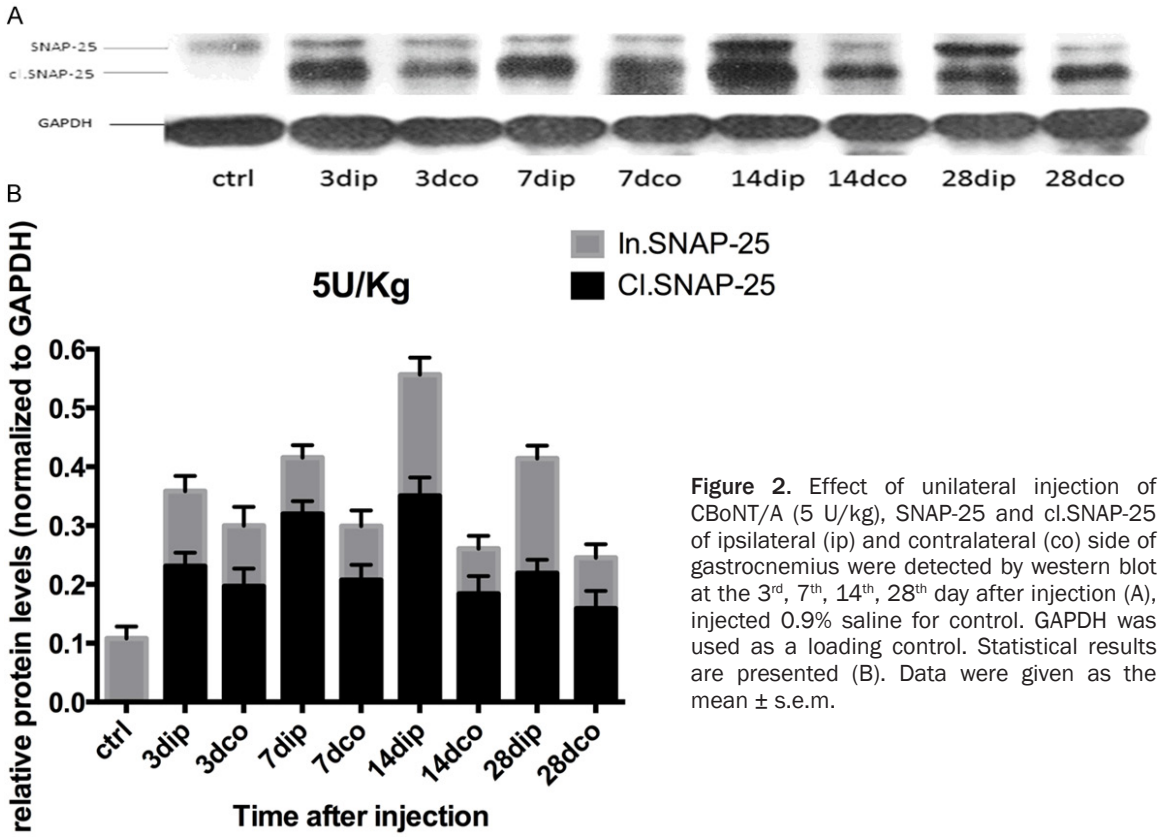

Figure 2. Effect of unilateral injection of CBoNT/A (5 U/kg), SNAP-25 and cl.SNAP-25 of ipsilateral (ip) and contralateral (co) side of gastrocnemius were detected by western blot at the 3rd, 7th, 14th, 28th day after injection (A), injected 0.9% saline for control. GAPDH was used as a loading control. Statistical results are presented (B). Data were given as the mean ± s.e.m.

terms of SNAP-25, SNAP-25 at ipsilateral side was declined from 10% to 7.8%; then it rose to 22.6% by the second week, but fell to 20.4% in the end (**Figure 3B**). Although SNAP-25 at contralateral muscles showed similar tendency, it ended up with 4.7%.

Except in the intrathecal injected group, Cl. SNAP-25 was not observed in both sides of sciatic nerve and spinal cord no matter at any time point (**Figure 3C**, **3D**). The baseline expression of SNAP-25 at ipsilateral side of sciatic nerve was 37%, which was lower than control. But it was raised to 137% on the second week after injection, and then restored to 107%. The expression of SNAP-25 at contralateral side indicated similar tendency as that at ipsilateral side, but the changes started from one week after injection (from 40% to 98%) (**Figure 3E**). No cl.SNAP-25 was observed in spinal cord under high therapeutic dose, and no significant differences were found between four different time points in expression of SNAP-25.

Discussion

Recently, there have been several reports about the unexpected effects of botulinum toxin preparations. Among them, an adverse effect includes the subclinical effects of botulinum toxin on non-target muscle, such as changes of SFEMG and F wave. On the other hand, behavioral data obtained from bilateral pain models suggest that local effects alone are hard to explain the effects of BoNT/A on pain. Some CNS changes in human and animals treated with BoNT/A intramuscularly have been described [6, 7, 16, 19, 20] and the central effects of the toxin have also been demonstrated. From theoretical aspect, the effects of BoNT/A may depend either on a direct action of the toxin transported via the hematogenic route and neural transport, or an indirect action causing CNS “reorganization”. This so called “reorganization” is resulted from action of BoNT/A on the intrafusal region, which reduces muscle afferent input from the injected muscle, thus leading to a temporary reorganization of the altered sensorimotor interaction [15].

The distribution of cl.SNAP-25 after injection of CBoNT/A was investigated in this paper. It was found that under three different dosages of CBoNT/A, Cl.SNAP-25 was visible not only at the injection area, but also in contralateral

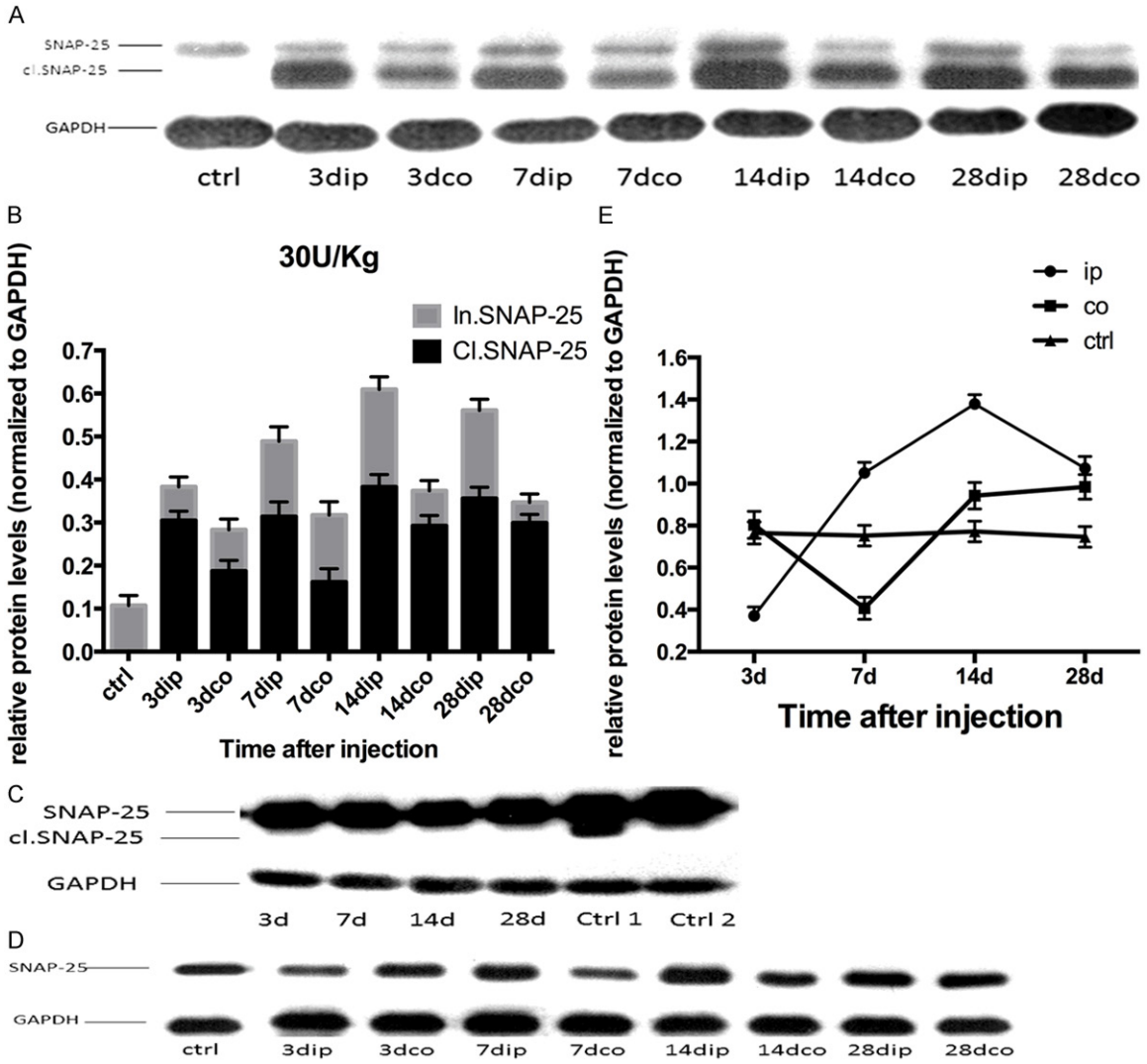

Figure 3. Effect of unilateral injection of CBoNT/A (30 U/kg), SNAP-25 and cl.SNAP-25 of ipsilateral (ip) and contralateral (co) side of gastrocnemius, sciatic nerve and spinal cord were detected by western blot at the 3rd, 7th, 14th, 28th day after injection (A), injected 0.9% saline for control and statistical results are presented (B). Results from spinal cord were shown in (C). Ctrl1 served as positive control by intrathecal injection (1 U/kg), and ctrl 2 served as negative control by intramuscular injection saline. SNAP-25 in sciatic nerve fluctuated after injection (D, E), but no cl.SNAP-25 were observed. GAPDH was used as a loading control. Data were given as the mean ± s.e.m.

muscle. But no axonal transport can be detected by western blot in this study even under the high dosage of CBoNT/A. This suggested that retrograde transport of CBoNT/A might be too little to be detected by western blot under therapeutic dose, or such transport might be absent.

Recently, axonal transport of BoNT/A, especially retrograde transport, is the crux of controversy. Caleo and Matteo et al. provided evidences for retrograde transport by which purified BoNT/A cleaved SNAP-25 that was distinct from the site of injection [13]. Purified BoNT/A was injected into the rat superior colliculus, and cl.SNAP-25 in the contralateral retina was detected finally. Purified BoNT/A (135 pg) was also tried to be injected into whisker muscles and found cl.SNAP-25 in facial motoneurons. Because this dose was lower than clinical therapeutic dose for torticollis spasmodicus (2-22 ng), retrograde transport was considered a common occurrence. On the contrary Oliver Dolly et al. indicated that except at concentrations far exceeding clinical doses, when the culture cells were exposed to BoNTA, peripheral

application of BoNTA failed to block neurotransmission at cell bodies [21]. Z. Lacković et al., who had done lots of work in central antinociceptive of BoNT/A. found cl.SNAP-25 in ipsilateral trigeminal nucleus caudalis (TNC) and spinal cord after peripheral application of BoNT/A (botox) (3.5 U/Kg-15 U/Kg) [11, 22]. Nevertheless, Ryuji Kaji et al. found cl. SNAP25 in the bilateral ventral and dorsal horns 4 days after injection of purified BoNT/A (10U) [23], and suggested BoNT/A spread not only via axonal transport, but also via transcytosis. Whereas, the immunohistochemical results of Z. Lacković and Ryuji Kaji's researches showed that almost all cl.SNAP25 were localized in neurite rather than soma. This distribution was not in accordance with normal situation of retrograde transport. Meanwhile, Oliver Dolly, and Jet al. also [24, 25] suggested that only recombinant proteins composed of TeTx subdomains and BoNTs (light chain) were capable proteins for retrograde transport. Retrograde transport of CBoNT/A was examined by western bolt in our study, and the results were in contrast with some previous researches. Different kinds of BoNT/A might be the reason for this. We are the first group to test CBoNT/A, which might be different from other brand of BoNT/A (Botox®, Allergan Inc., Irvine, CA, USA) and purified BoNT/A. Although they are the same toxin, the preparations are different in many regards, such as chemical properties, biological activities, and mouse ED_{50} and LD_{50} units, which result in different outcomes in clinical treatment [26, 27].

Finally we suggest that cl.SNAP-25 is detected in remote neuromuscular junction after single intramuscular injection of CBoNT/A. However, retrograde transport is non-existent or too little to be detected. BoNT/A's activity in the central nerve system following peripheral delivery has been questionable till now. As a result, a lot more evidence is needed to support this hypothesis.

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Disclosure of conflict of interest

None.

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