Clinical safety and tolerability of A2NTX, a novel low molecular weight neurotoxin derived from botulinum toxin subtype A2, in comparison with subtype A1 toxins

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Abbreviations

A1LL: onabotulintoxinA (BOTOX®)

A1NTX: low-molecular weight subtype A1 botulinum toxin

A2NTX: low-molecular weight subtype A2 botulinum toxin

Authors Roles

TT, TO, AM conducted clinical research, TK and SK prepared the toxins, RM and YI recruited patients, RK supervised the study and wrote the manuscript.

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Abstract: All the available botulinum type A neurotoxins for clinical uses are of A1 subtype. We developed a subtype A2 low molecular weight (150kD) neurotoxin (A2NTX), with less spread and faster entry into the motor nerve terminal than A1 *in vitro* and *in vivo*. Preliminary clinical studies showed its efficacy superior to A1 toxins. We conducted an open study exploring its safety and tolerability profile in comparison with A1LL (onabotulinumtoxinA) and low molecular weight (150kD) A1 neurotoxin (A1NTX). Those who had been using A1LL (n=90; 50-360 mouse LD50 units) or A1NTX (n=30; 50-580 units) were switched to A2NTX (n=120; 25-600 units) from 2010 till 2018 (number of sessions ~ 27, cumulative doses ~11,640 units per patient). Adverse events for A2NTX included weakness (n=1, ascribed to alcoholic polyneuropathy), dysphagia (1), local weakness (4), spread to other muscles (1), whereas those for A1LL or A1NTX comprised weakness (n=2, A1NTX), dysphagia (8), ptosis (6), local weakness (7) and spread to other muscles (15). After injections, 89 out of 120 patients preferred A2NTX to A1 for the successive sessions. The present study demonstrated that A2NTX had the clinical safety up to the dose of 500 units, and was well tolerated compared to A1 toxins.

Word count:199

Keywords: botulinum toxin, subtype A2, clinical tolerability, safety

Key Contribution: This study is the first to report the clinical safety and tolerability of botulinum neurotoxin subtype A2.

Short title: Clinical safety of A2NTX

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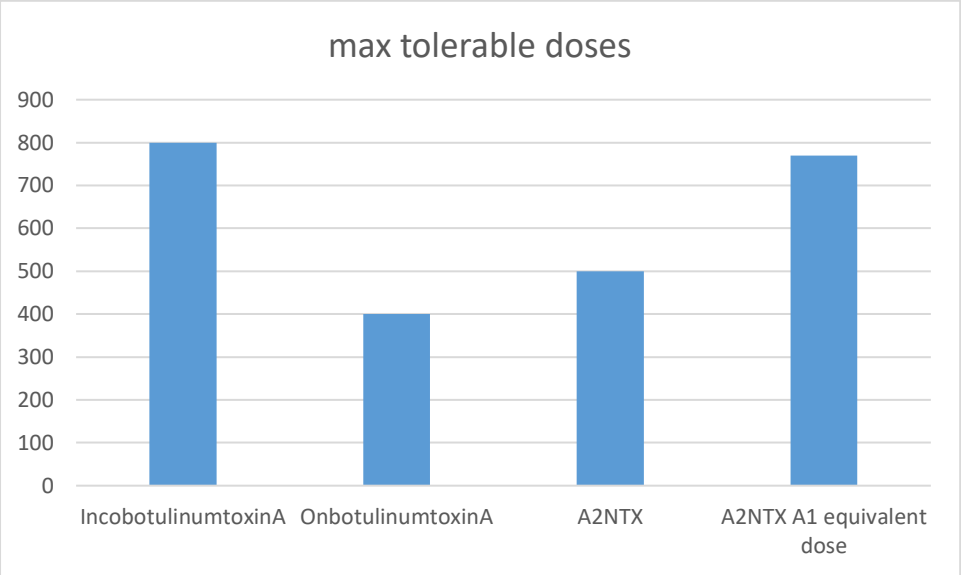
Graphic Abstract

Comparison of tolerability among A1 and A2 toxins



Comparison of maximum tolerable dose among IncobotulinumtoxinA and OnabotulinumtoxinA in Japan in comparison with A2NTX and its equivalent dose to A1 toxins.

Note: IncobotulinumtoxinA is only approved for use up to 400u for upper and lower extremities at the same injection session. OnabotulinumtoxinA is approved for lower extremity up to 300u

A2NTX is also represented in A1 toxin equivalent dose (1.54x) on the right column.

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Introduction

Botulinum neurotoxins (BoNT) are known as the most potent biological substance to risk people to death. Many attempts to use BoNT as a biological weapon were made in the past. In US Army, investigators of Camp Detrick conducted researches on BoNT from 1943 to 1956 to develop toxins and effective vaccines during and after World War II. The isolation and purification for BoNT originated from Hall strain of *Clostridium botulinum*[1], which made it possible to produce botulinum toxins and toxoid vaccines in large-scale. In the 1960's, when the treaty for biological weapon banned wartime uses of BoNTs, Dr. Edward J. Schantz, a basic scientist who had worked at Camp Detrick, supplied the toxin for scientific purposes[2]. Dr. Alan B. Scott, an opthalmologist, came across an idea of its clinical uses at small doses to reduce muscle hyperactivity. He first tested *botulinum toxin* type A (BoNT/A) from the Hall strain from Dr Schantz in humans in 1978, after he received permission from the FDA. Ten years later, Allergan Inc. acquired the rights to distribute the drug or onabotulinumtoxinA (BOTOX®). The company extended clinical researches to obtain FDA approvals for its use in dystonia, spasticity, migraine and others.

Botulinum neurotoxins (BoNTs) are categorized into immunologically distinct 7 serotypes BoNT/A to /G. BoNT/A, which exist in various molecular weights from LL (900kD), L (500kD), M (300kD), and S (150kD). LL and L toxins contain hemagglutinin (HA) component and NTNH (non-toxin, non-hemagglutinin) component, which are not essential for the action of neurotoxin (S toxin), although LL toxin may have advantages over S because of its less diffusibility due to larger molecular weight[3]. M toxin is composed of NTNH and S toxin. Neurotoxin or S toxin comprises the heavy chain (HC), which has binding and translocation domains, and the light chain (LC), having a catalytic domain.

Detailed researches on amino acid sequences on each serotype revealed subtypes among type A toxins (A1, A2) [4]. These subtypes have been increasingly recognized on each serotype.

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By now, serotype A is divided into subtypes A1-A8, and only A1 toxin from the Hall strain (OnabotulinumtoxinA or BOTOX®, AbobotulinumtoxinA or Dysport®, IncobotulinumtoxinA or Xeomin®) have been clinically available in US [5] , except for type B toxin (RimabotulinumtoxinB or Myobloc®/ Neurobloc®). Type F was once used for clinical researches, but was found to have shorter duration of action than type A [6]. Sakaguchi and colleagues [7] have reported unique strains of C botulinum obtained from cases of infant botulism in Japan, and found the strains (Chiba-H and Kyoto-F) only producing M-sized type A toxins, which later were categorized as subtype A2. Because of the high yield and purity of the M toxin produced by Chiba-H strain, BoNT/A2 preparation was easily cleaved and converted into highly purified S toxin (neurotoxin) for clinical uses (A2NTX). The latter was found to be less diffusible and more efficacious per mouse LD50 unit in vitro and in vivo. The first-in-man clinical study indicated that A2NTX is around 1.54 times as potent as the same unit of A1LL[8].

Here we report a long-term clinical study of A2NTX exploring its safety and tolerability in comparison with A1LL and A1 S toxins (A1NTX). A part of this study was published as a proceeding.[9]

Materials and Methods

Toxins

Neurotoxins (molecular weight: 150k Dal) were produced and purified from Chiba-H strain of *Clostridium Botulinum*, isolated from honey associated with cases of infant botulism, for subtupe A2 (A2NTX) and from strain 62A for subtype A1 (A1NTX) as described elsewhere.[10] The toxicity of purified neurotoxin A2NTX, titrated by serial 2-fold dilution intraperitoneal injection measured as a mean 50% lethal dose (LD50), was 5.2 X 10'

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LD50/mg protein, which was nearly the same as that of 62A neurotoxin (A1NTX) (5.3 X 10' LD50/mg protein). We also compared the safety of these small molecule type A neurotoxins with A1LL or onabotulinumtoxinA (BOTOX®, Allergan Inc.), whose approved doses were up to 360 mouse LD50 units for spasticity in Japan then (as of 2018, up to 400u). Those preparations of A1NTX and A2NTX were stored in a deep freezer (<-70 degrees Celsius), and thawed immediately before use.

Criteria for entry

Patients with spasticity, dystonia or tremor, being treated with A1LL or A1NTX whose responses fell short of the subject's need, or their benefits disappeared as judged by frontalis test (secondary non-responder). The age should be between 10 and 95.

Written consent was obtained from all the subjects or their parents if they were under the age of 20, after giving full information on the exploratory nature of the study, and the data being published for the research purpose.

Clinical test periods (**Fig.1**)

During the period of Oct 2006 to Nov 2010, we used A1NTX (up to 580 units) mainly for spasticity and dystonia. Since there had been no BoNTs available for spasticity in Japan, this study started as a proof-of-concept clinical study of A1NTX for spasticity. A2NTX was developed by 2010 after *in vivo* and *in vitro* studies [11-14], Institutional Review Board (IRB) of Tokushima University approved its use up to 600 mouse LD50 units for spasticity or dystonia in 2010 (approval number TU-214) after the first-in-man study using 6.5 units in the extensor digitorum brevis (EDB) muscle in healthy subjects [8], and 300 units in post-stroke-spasticity (published as a proceeding in 2015 [9]). The previous study showed the potency of A2NTX being equivalent to 1.54 times as much as that of A1LL in humans[8].

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After A1LL (onabotulinumtoxinA) was registered for spasticity in Japan using the doses up to 360 units in Oct 2010, those treated with A1LL who wished to try A2NTX were entered to A2NTX study (n=90) after informed consent at any time until March 2018. Those who had been treated with A1NTX from Oct 2006 until December 2018 (n=89) were also allowed to switch to A2NTX or A1LL at any time after December 2010. A total of 120 patients received A2NTX injections (**Fig.1**).

[Fig. 1 near here]

Injection Protocol

All the subjects of A2NTX group had an initial dosing of 50 units, followed by 4-6 weeks with the second dosing of up to 400units. The maximum doses were increased to 500 units in some spasticity patients who required further benefits from the injections (**Fig.2**). Injection session intervals thereafter ranged from 8 to 12 weeks until the plateau of clinical benefit was reached. After the second dosing, the patients were asked on their predilections of the BoNT preparations, and they were allowed to switch back to the original regimen (A1NTX or A1LL) at any time after the start of dosing A2NTX.

[Fig.2 near here]

Blood samples including those for serum electrolytes, liver, renal and haematological routines were obtained before and after the initial dosing of 50 units, as well as when they were needed.

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Adverse events

The adverse events were analyzed and categorized as follows:

Mild: An event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities.

Moderate: An event that causes sufficient discomfort and interferes with normal everyday activities.

Severe: An event that prevents normal everyday activities, and may require hospitalization.

Serious: Any untoward medical occurrence that at any dose, results in death, is life-threatening, requires inpatient hospitalization or prolongation of existing hospitalization, or results in persistent or significant disability/incapacity.

When serious or any adverse events in question arose, the independent committee for safety was called, and members were asked to assess whether the event was causal or not.

Since all the complete medical records of A1LL- or A1NTX-treated groups were not available due to their time (A1NTX: 2006-2010) or referral from other medical institutions (A1LL), a thorough review of any adverse events were limited for A1 toxins.

Protocol approval

The entire protocol was approved by IRB of Tokushima University (No.2005-216, with revisions in 2006 and 2010). The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board

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Results

Patients

Those who had A1LL, A1NTX, and A2NTX were diagnosed as having spasticity or dystonia/tremor. Total number of patients for each injection group were 90 (34 female 56 male) for A1LL, 89 (23 female 66 male) for A1NTX, and 120 for A2NTX (53 female 67 male) (**Table 1**), which included patients with spasticity (21 for A1LL, 29 for A1NTX and 42 for A2NTX) and dystonia (69 for A1LL, 60 for A1NTX and 78 for A2NTX). The breakdown of spasticity patients was post-stroke (17 for A1LL, 10 for A1NTX and 25 for A2NTX), hereditary spastic paraplegia (HSP) (2 for A1LL, 11 for A1NTX and 12 for A2NTX), acquired spinal cord injuries (1 for A1LL, 4 for A1NTX and 4 for A2NTX), and others including cerebral palsy. Because of its promise in efficacy, A1NTX (n=9) and A2NTX (n=28) were also used for generalized and other dystonias or tremor involving larger parts of the body than cervical dystonia or blepharospasm. The maximum dose was 600 units of A2NTX in one patient with generalized dystonia, who eventually chose the surgery (deep brain stimulation), whereas the rest being 500 units of A2NTX (**Fig.2 A, B**). The maximum dose per kg body weight was 13.54 units in a subject who received 600 units, and was up to 11.57 units in one who had 500 units per session. The ceiling of 500 units for A2 seemed to be appropriate because two patients who underwent injections of 580 units of A1NTX developed generalized weakness (*vide infra*). Those who switched from A1LL to A2NTX included 3 patients who became unresponsive to A1LL as diagnosed by frontalis test (secondary non-responders).

As for those who had A2NTX, the age was 14-93 years (mean 54.5 years), the duration of treatment 1-8 years (mean 5.15 years), the cumulative dose 25-11640 units (mean 2527 units), the number of injection sessions 1-27 (mean 9.5). In A1NTX-injected group, the age

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was 19-79 years (mean 53 years), the duration of treatment 1-10 years (mean 3.0 years), the cumulative dose 50-8200 units (mean 2653 units), the number of injection sessions 1-25 (mean 9.0). Overall, the patients' background and dosing did not differ significantly between A1NTX- and A2NTX-treated groups.

Adverse events

Table 1 summarizes the adverse events among A1LL-, A1NTX-, and A2NTX-treated groups.

[Table 1 near here]

Mild adverse events

Routine blood samples showed no major changes after the initial dosing of 50 units, except for 3 patients, whose liver function data (AST, ALT, γ -GTP) deteriorated slightly. These were the only mild adverse effect except for local pain or ecchymosis.

Moderate adverse events

Patients were specifically asked and checked for the presence of unwanted weakness in the injected muscle (local weakness), in the un-injected neighboring muscles (spread to other muscles), in the muscles of distant body parts (generalized weakness), dysphagia (in case of cervical dystonia), and ptosis (in case of blepharospasm). **Table 1** depicts that the incidence of these adverse events were higher in A1NTX-injected group than in A2NTX. Despite the different doses, those who had A1LL also showed high incidence of these parameters.

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Serious adverse events

There were 3 cases with serious adverse events of generalized muscle weakness: one from A2NTX group (**Table 2**), and 2 from A1NTX.

Case A2-53 is a 60 years old man who had right putaminal haemorrhage 5 years prior to dosing 400 units of A2NTX into left upper and lower extremity muscles. He had been diagnosed as having alcoholism, and his food intake was decreased from 1 month before the dosing. He developed weakness of lower limbs and became unable to walk 2 weeks after the dosing. Neurological findings were compatible with subacute alcoholic polyneuropathy, and he recovered fully after vitamin B1 supplements. Repetitive motor nerve stimulation did not show waxing at 30Hz, typically seen in botulism. The report of the independent safety committee was that the event is not causal to the dosing.

The other case (A1-13) was a 44 years old woman with generalized dystonia, who developed generalized weakness of limbs after 2 repeated injections of 580 units of A1NTX into lower limb muscles at 10 weeks interval. She became unable to walk, and was hospitalized for 4 weeks with full recovery. Electrophysiological studies showed increased jitter using single-fibre EMG and more than 30% incremental response (waxing) after 30 Hz motor nerve stimulation tested in the hand muscle, suggesting the effect of the botulinum toxin at distant muscles. The safety committee judged this case as having a side effect.

Another case of cerebral palsy (Case A1-42), a 42 years old woman, developed generalized weakness including the hand grip at 2 weeks after having injections of A1NTX (580 units) into lower limb muscles. She remained ambulatory and the weakness recovered completely within 8 weeks.

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[Table 2 near here]

Table 2 depicts the rest of adverse events in A2NTX-treated group, picked up from all the medical records for more than 8 years (2010-2018). Falls were reported in 3 cases. One (case 50) had a hip bone fracture at the peak effect of A2NTX injected into the leg (4 weeks after the injection), which was judged as causal, because the patient became ambulatory and had a chance to walk for a long distance after a period of inactivity. This patient recovered completely after the joint replacement. There were 2 deaths (all with generalized dystonia), which were judged not directly related to the injection, one from a suicide and the other from an accident.

Secondary non-responders to A1LL

Those with resistance to A1LL tested by frontalis test included a 46 years old man (Case A2-62) , a 35 years old woman (Case A2-89) and a 35 years old woman (Case A2-91), all suffering from cervical dystonia. All of them, who had been treated with 240 units of A1LL with no benefits responded to a total of 400 units A2NTX injection. The frontalis test in one patient (A2-91) regained asymmetry of the frontal crease, suggesting the clinical response, using 50 units of A2NTX.

Patients' predilection

Eighty-nine of the 120 patients (74%), who had been treated with A1LL or A1NTX at intervals of 8-12 weeks, eventually chose A2NTX injections, and the intervals became 10-24 weeks. Nine patients (7.5%) switched back to A1LL injections at local neurologists, because

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they became unable to visit. Twenty-two (18.3%) discontinued BoNT injections, because they recovered fully or attained a plateau of clinical benefits.

Secondary non-responders to A2NTX

There was no individual that became unresponsive to A2NTX during our observation period.

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Discussion

The present study showed that A2NTX was well tolerable up to the dose of 500 units for spasticity and dystonia. As expected from the previous *in vitro* and *in vivo* studies[12,15-19], the spread of the toxin was significantly less than A1NTX. It was even less than those who had smaller doses of A1LL. The margin of safety would be even higher considering that 1 unit of A2 is 1.54 times as effective as A1[8]. Although this study was not designed as testing the efficacies, A2NTX seemed to be more efficacious than A1 toxins with regards to the patients' predilection, and was useful even in secondary non-responders to A1LL if used at relatively high doses of A2.

Detailed analyses of the adverse events revealed 3 cases with falls, which obviously an underestimate, considering the disability, the age and the number of subjects. Bone fracture was reported in 3 cases, which also seemed to be less than expected. The relapses of stroke was seen in 2 cases, which is probably an underestimate, because the stroke relapse rate in Japan is around 5% *per annum* [20]. One patient with alcoholism developed full-blown polyneuropathy with generalized weakness after A2NTX, which was reversed with vitamin B1 injections. It is important however that those with other potential risks of developing weakness should be carefully screened before the use of A2NTX, but this caution would apply to other preparations of BoNTs. As such, A2NTX at doses up to 500 units does not seem to carry extra-risks compared to other neurotoxins.

Concerning the treatment-related side effects (**Table 1**), A2NTX seems to be less spreading not only in animals[12,18,19], but also in clinical settings. Interestingly, a similarly prepared A1NTX showed more spreading to neighbouring or distant muscles than A2NTX or A1LL (**Table 1**). The large molecular weight of A1LL is possibly related to its immunogenicity, resulting in antibody development, but could be beneficial in limiting its diffusion to other

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muscles locally or through CNS[3]. Despite its low molecular weight (150kD), A2NTX seems to be less spreading not only than A1NTX but also A1LL in the present study. Our previous report also suggested its less spreading than A1LL in lower limb post-stroke spasticity[9]. The exact reason is unknown, but it may be related to its higher affinity and faster binding to the receptor SV2 possibly because of the difference of the amino acid residues in the heavy chain[16]. If so, it is reasonable to observe its efficacy in secondary non-responders to A1LL, who could have antibodies to A1 toxins, since A2 might find its way to the receptor faster than the antibody in high doses. It is also conceivable that A1 and A2 have different immunogenicity[21].

Conclusion

The present study underscored the safety and tolerability of A2NTX, a low molecular weight neurotoxin, up to 500 mouse LD50 units per 10-12 weeks, in the long-term condition without eliciting antibodies. A2 is not suited for its use as a biological weapon like A1 because of its less diffusibility or spread, but is a promising therapeutic agent in the clinical settings where the reduction in tone can be expected in targeted muscles.

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Legends for the figures

Fig.1

Flow chart of the subjects with respect to the toxin preparations

Fig.2

A: Dosing of A2NTX among 120 subjects Individuals are depicted with the same color.

B: Number of subjects per each dose.

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Protocol Approval

Tokushima University IRB 2005-216, with revisions in 2006 and 2010.

Conflict of Interest

RK has a patent on A2NTX (WO 2008/050866).

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Fig. 1

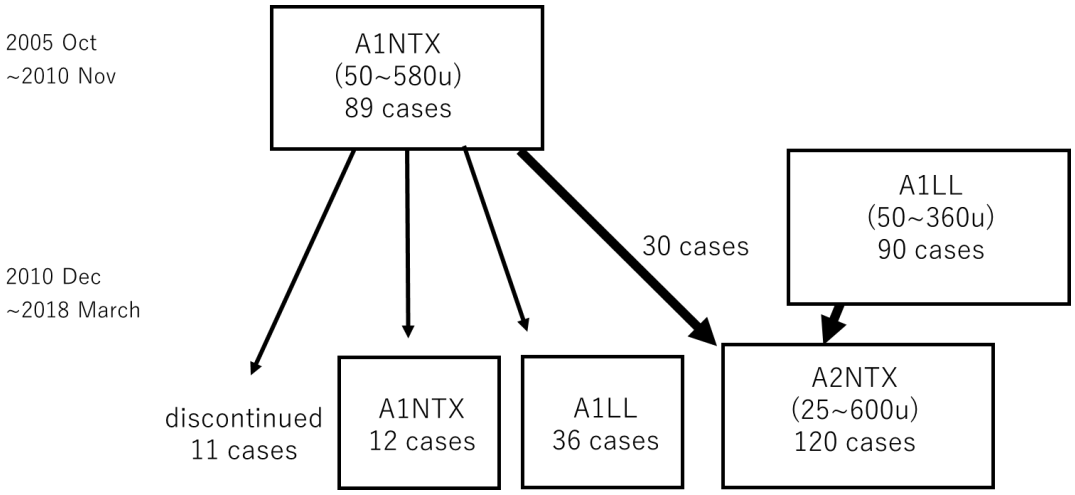

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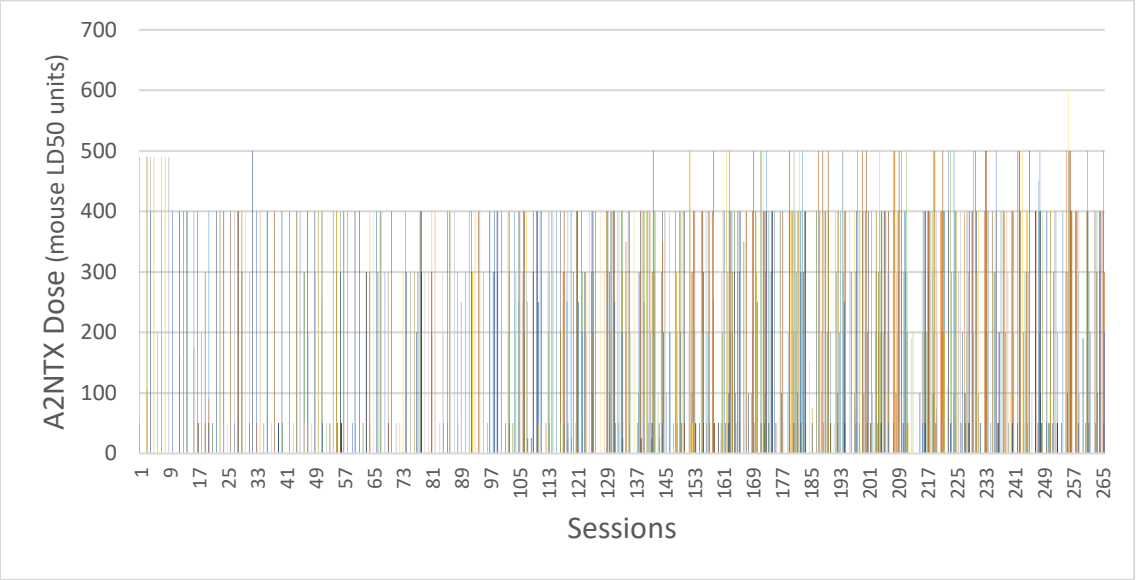
Fig. 2

A Individually colored doses per session among A2NTX-injected group



B **Number of individuals with maximum doses among A2NTX-injected subjects**

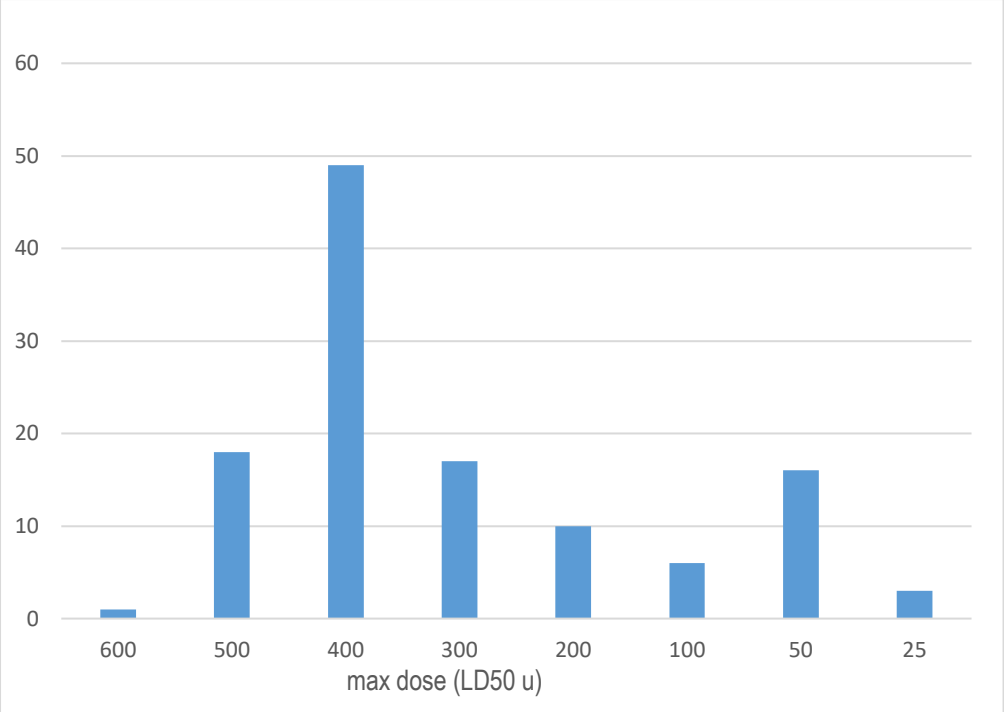


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Table 1

Cumulative occurrence of treatment-related side effects per individual among A1LL, A1NTX and A2NTX-injected groups

	A1LL	A1NTX	A2NTX
No, of total subjects	91	89	120
total months of observation (max)	50	50	87
Spasticity (dose)	*300-360u*	*290-580u*	*300-500u*
number of subjects (*age*)	21 (28-96y)	29 (28-79y)	43 (*41-96y*)
generalized weakness	**0**	**2 (6.9%)**	**1 (2.3%)**
local weakness	0	3 (10.3%)	3 (7.0%)
spread to other muscles	**3 (14.3%)**	**12 (41.4%)**	**1 (2.3%)**
Cervical/truncal dystonia (dose)	*100-240u*	*290-580u*	*300-500u*
number of subjects (*age*)	19 (25-81y)	30 (26-73y)	31 (*25-81y*)
local weakness	1 (5.3%)	3 (10.0%)	1 (3.2%)
dysphagia	**3 (15.8%)**	**5 (16.7%)**	**1 (3.2%)**
Blepharospasm (dose)	*50u*	*50u*	*50-100u*
number of subjects (*age*)	51 (30-90y)	21 (34-78y)	19 (*30-90y*)
ptosis	**4 (7.8%)**	**2 (9.5%)**	**0**
Others (incl. tremor and other dystonia)		29-580u	25-500u
number of subjects		9	28
spread to other muscles		**3**	**0**

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Table 2

Summary of major adverse events among A2NTX-injected group

moderate	severe	serious
falls	***bone fracture***	***generalized weakness***
case 5	case 2	case 53
case 17	case 50*	***relapses of stroke***
case 47*	case 101	case 43
aggravation of tremor		case 118
case 11*		***death***
		case 10 (suicide)
		case 35 (asphyxia in an accident)

*considered as causal