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Optimization of the rat digit abduction score (DAS) assay: Evaluation of botulinum neurotoxin activity in the gastrocnemius lateralis, peronei, and extensor digitorum longus

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ABSTRACT

The mouse digit abduction score (DAS) assay is commonly used to measure muscle flaccidity-inducing effects of botulinum neurotoxin (BoNT) *in vivo*. Adapting the assay to rats has been challenging, as injection of onabotulinumtoxinA (onaBoNT-A) into the gastrocnemius muscle, as performed in mice, or into the tibialis anterior leads to sub-optimal sensitivity of the test (Broide et al., 2013). To optimize the experimental design of the rat DAS assay, we evaluated the effects of research-grade, purified, native BoNT serotype A1 (BoNT-A) in three muscles: the gastrocnemius lateralis, peronei, and extensor digitorum longus using female animals. Following injection, animals were tested daily for the digit abduction and body weight.

BoNT-A caused dose-dependent inhibition of digit abduction when injected into the gastrocnemius lateralis or peronei. BoNT-A was six-fold more potent when injected into the peronei in comparison to the gastrocnemius lateralis. As injection of BoNT-A into the extensor digitorum longus muscle resulted in an all-or-none digit abduction response and therefore prevented calculation of the ED_{50}, it was considered unsuitable for the rat DAS assay. At equipotent doses, peronei- and extensor digitorum longus-injected animals showed normal body weight gain, while those injected with BoNT-A into the gastrocnemius lateralis gained less weight in comparison to vehicle-treated controls.

Thus, injecting the peronei muscles of female rats offers optimized conditions for evaluating the biological properties of BoNTs in the rat DAS assay; for assessing the potency, onset, and duration of action across natural and recombinant BoNT in a robust and reproducible manner.

1. Introduction

Botulinum neurotoxin (BoNT), produced by *Clostridium botulinum* anaerobic bacteria, causes transient muscle flaccidity by preventing the presynaptic release of acetylcholine at the neuromuscular junction (Rossetto et al., 2014). BoNT is a diverse group of proteins, which includes at least seven serotypes and 40 sub-types (Peck et al., 2017). The duration of muscle paralysis depends on the serotype and the dose used. Products of BoNT serotypes A and B have been used in the clinic to treat a range of neurological conditions associated with muscle hyperactivity and pain (Fonfria et al., 2018).

The mouse LD_{50} test has historically been used to measure potency of BoNT (Pearce et al., 1994). However, its clinical relevance and ethics are questionable, as the test requires intraperitoneal route of administration and assesses the lethality caused by BoNT intoxication. The mouse digit abduction score (DAS) assay offers an alternative approach, in which BoNT is administered intramuscularly (i.m.) into the gastrocnemius complex of the mouse hindlimb and its flaccidity-mediated reduction in the reflexive spread of digits is measured in conscious animals over time (Aoki, 1999).

While the mouse DAS assay is widely used to study BoNT (Aoki, 1999, 2001; 2003; Morbiato et al., 2007; Chung et al., 2013; Duregotti et al., 2015; Vazquez-Cintron et al., 2016; Elliot et al., 2019), the rat DAS studies are less common (Adler et al., 2001; Rosales et al., 2006; Broide et al., 2013). Although the DAS assay performed in either mice or rats are more humane than the mouse lethality bioassay, there are some advantages of using rats instead of mice in this assay. Because of their size, it is easier to inject BoNT into one of several hindlimb muscles. This can help in understanding the mechanisms mediating BoNT diffusion and spread from the injection site. In addition, as the rat is the species of

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choice for toxicity studies, assessing BoNT activity in the rat DAS offers an opportunity to measure the efficacy and toxicity of the compound in the same species.

Previously, Broide et al. (2013) investigated the effects of onabotulinumtoxinA (onaBoNT-A) in the rat DAS assay, where the toxin was injected in either tibialis anterior or gastrocnemius. In the rat hindlimb, the tibialis anterior and gastrocnemius are the two largest, superficial muscles, responsible for ankle flexion and extension, respectively (Armstrong and Phelps, 1984). In fact, the gastrocnemius muscle is composed of two large heads, the gastrocnemius lateralis and gastrocnemius medialis, each being the size of the tibialis anterior (Wang and Kernell, 2001). Although Broide et al., (2013) did not specify the subunit of the gastrocnemius that was injected in their study, neither of the two muscles investigated by the authors appear to be ideal for the rat DAS assay. Firstly, the patterns of digit abduction inhibition seen in rats injected with onaBoNT-A in the gastrocnemius and the tibialis anterior were distinct and differed from those seen in toxin-treated female CD-1 mice (Broide et al., 2013). Secondly, when onaBoNT-A was injected into the rat tibialis anterior or the gastrocnemius muscle, its maximum efficacy, expressed as a full inhibition of the digit abduction (DAS 4), was obtained only at doses that were also linked with significant suppression of spontaneous locomotor activity and reduced body weight gain (Broide et al., 2013).

The objective of the current study was to optimize the experimental design of the DAS assay, in order to better adapt it to rats. Specifically, in order to identify the most suitable muscle for this assay, we assessed the following hindlimb muscles: gastrocnemius lateralis, extensor digitorum longus, and a closely positioned group of muscles, including the peroneus longus, peroneus brevis, and peroneus digiti 4,5, that, for the purpose of this study, were treated as a single muscle: the peronei.

2. Materials and methods

2.1. Ethics

All experimental procedures were approved by the Ethics Committee of Ipsen Innovation (C2EA; registration number 32) and were performed in full compliance with the ARRIVE guidelines, EU Directive 2010/63/EU for animal experiments and the 2013 French Regulatory Decree.

2.2. Animals

All experiments were performed on adult, female, Sprague-Dawley rats (Janvier Labs, Saint Berthevin, France). Animals were housed 3–4 per cage and maintained on a 12 h light/dark cycle (lights on from 07:00 to 19:00 h) under a constant temperature (22 ± 1 °C) and humidity (55 ± 5%) with food and water available *ad libitum*. Animals were acclimatized for at least 7 days prior to experimentation. Animals weighed 160–200 g on the day of injection and were in free estrous cycle when tested in these experiments.

2.3. Study design

The effects of BoNT-A on digit abduction and body weight were assessed following injection into each of three muscles of the lower limb: the gastrocnemius lateralis, peronei, or extensor digitorum longus. The effects in each muscle were investigated in independent experiments, which allowed us to test a wider range of doses in each experiment and more precisely estimate the ED_{50} value, as well as the doses resulting in half-maximal and maximal responses in the DAS (see below).

In the initial set of pilot experiments we evaluated expression of the digit abduction reflex in male Sprague-Dawley rats when animals were tested repeatedly over time. We noticed that as male rats grew older and heavier, the digit abduction reflex became more difficult to elicit. In contrast, in female rats, the digit abduction reflex remained stable over time. Because female rats did not gain as much body weight as males, they were also easier to test, especially in longitudinal experiments. Therefore, the main study was performed only in female Sprague-Dawley rats.

2.4. BoNT administration

Research-grade, purified, native BoNT serotype A1 (BoNT-A; 150 kDa) was purchased from List Biological Laboratories (Campbell, CA, USA). It was reconstituted in 1 mg/ml phosphate buffer saline/bovine serum albumin (0.1%) to obtain a stock solution, which was aliquoted and kept frozen at −80 °C. Dilutions of aliquots were performed with 0.2% gelatin phosphate buffer (GPB) to obtain the final concentrations (see below).

Before starting the experiments, animals were weighed, pre-screened for normal digit abduction responses (DAS 0) and randomized to obtain a comparable mean body weight in each group. Before the injection, animals were anaesthetized with 3% isoflurane in oxygen.

All injections were made using a 30-gauge needle attached to a 100 μL syringe (SGE Analytical Science, Interchim; Montluçon, France). We performed a series of pilot experiments involving the intramuscular (i.m.) administration of Evans blue to optimize the injection technique and volume for each muscle. Additionally, several pilot experiments were performed to establish the dose range for BoNT-A in each muscle.

To perform an i. m. injection in the left peronei or extensor digitorum longus, the rat was placed on its right side, the distal part of the left leg was shaved, and the skin was opened to visualize tendons, while the fascia was left intact. The needle was inserted into the peronei or extensor digitorum longus at tendon level in the axis of the muscle and a fixed volume of 10 μL of the toxin was injected. After the injection, the skin was closed using surgical staples.

To perform an i. m. injection into the right gastrocnemius lateralis, the rat was placed in a ventral position and the distal part of the right leg was shaved. The needle was inserted perpendicularly through the skin into the center of the head of the gastrocnemius lateralis and a fixed volume of 40 μL of the toxin was injected. The injection volumes were within the recommended range for intramuscular injections in rats (up to 50 μL per injection site; see Turner et al., 2011). We did not observe any signs of pain following the injection or throughout the experiment.

In the initial set of experiments, animals received an i. m. injection of BoNT-A (0.5, 1, 2.5, 5, 10 pg/rat; n = 6/group/muscle) or its vehicle (GPB) into the peronei or extensor digitorum longus. To assess BoNT-A effect following injection into the gastrocnemius lateralis, we used a wider dose range of BoNT-A (0.5, 1, 2.5, 5, 10, 15, 20, 25, 40, 60 pg/rat; n = 6/group/muscle). We used a range of doses that were significantly lower compared with the doses causing early signs of intoxication, such as marked reduction in body weight gain or body weight loss. Animals were closely monitored for clinical signs but none were observed throughout the study. The experiments were performed in duplicate for each muscle. In all experiments, the contralateral muscle was kept intact (injection-free).

2.5. DAS assay

The hind limb digit abduction reflex in the rat was induced by grasping the animal lightly around the torso and lifting it swiftly into the air or by lifting it with the nose pointing downwards. Animals were pre-screened for a normal digit abduction response before the experiment and those showing abnormal digit abduction responses or hind paw deformities were excluded from the study. Typically, the percentage of animals with abnormal digit abduction responses is less than 1%. The digit abduction response of each rat was scored live using a five-point scale, from normal reflex/no inhibition (DAS 0) to full inhibition of the reflex (DAS 4) as described in female CD-1 mice (Aoki, 1999). Rats were scored for digit abduction response 8 h following BoNT injection, twice a day during the first week post-dosing, and once daily for 25 days, following injection of BoNT-A. We also measured body weight daily

immediately after assessment of the digit abduction. All scoring was done by the same experimenter, who was blind to treatment.

2.6. Data analysis and statistics

In each experiment, a mean DAS was calculated for each dose and time point. The mean peak digit abduction response of each dose was plotted and analyzed to calculate an ED_{50}, defined as a theoretical dose inducing a DAS 2 value. The statistical difference between the ED_{50} of BoNT-A following injections into the peronei versus gastrocnemius lateralis was determined using a Student's t-test.

The BoNT-induced body weight changes in rats were expressed as a percentage of weight change at each timepoint compared to initial body weight (Day 0). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* analysis.

3. Results

3.1. Effects of BoNT-A on digit abduction and body weight

Vehicle (GPB) injected into either the peronei or gastrocnemius lateralis had no effect on digit abduction responses (Fig. 2). BoNT-A injected into either the peronei or gastrocnemius lateralis resulted in dose-dependent increases in DAS (Figs. 1 and 2).

The mean ED_{50} in the peronei (0.8 pg/rat) was more than six-fold ($p < 0.05$) lower than that in the gastrocnemius lateralis (5.2 pg/rat; Table 1; Fig. 1).

The lowest dose of BoNT-A inducing maximal inhibition of digit abduction (i.e. DAS 4 dose) was lower in peronei-injected animals (10 pg/rat) compared with those injected in the gastrocnemius lateralis (20 pg/rat; Table 1). The lowest dose of BoNT-A inducing a half-maximal inhibition of digit abduction (i.e. DAS 2 dose) was lower in peronei-injected animals (1 pg/rat) compared with those injected in the gastrocnemius lateralis (5 pg/rat; Table 1).

To compare the effects of BoNT-A in the DAS following injections into the peronei and the gastrocnemius lateralis, the time-course of activity of DAS 2 and DAS 4 doses was plotted (Fig. 2). BoNT-A injected at 1 or 5 pg/rat in the peronei or the gastrocnemius lateralis, respectively, resulted in DAS 2 in 2 days, followed by a reduction in DAS values and full recovery to DAS 0 in 10–15 days post-injection (Fig. 2). BoNT-A injected at 10 or 20 pg/rat in the peronei or the gastrocnemius lateralis respectively, resulted in DAS 4 the day after injection, followed by recovery to DAS 2 approximately 13 days post-injection. In these groups we did not observe a full recovery of digit abduction responses (i.e. DAS 0) when animals were tested up to 25 days post-injection (Fig. 2).

Vehicle (GPB) injected into the extensor digitorum longus had no effect on digit abduction, whereas BoNT-A injected into this muscle at the highest dose (10 pg/rat) resulted in an almost complete suppression of the digit abduction (i.e. DAS being between 3 and 4; data not shown). The maximal responses were obtained two days post-administration. However, effects of lower doses of BoNT-A lacked dose-dependency, causing either no change or a weak inhibition in digit abduction that were difficult to quantify (data not shown).

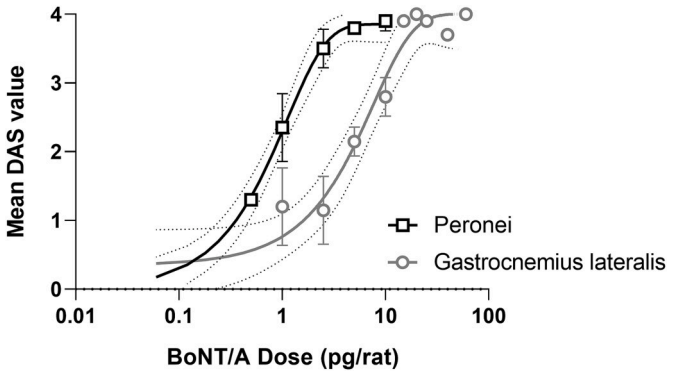

Fig. 1. BoNT-A dose-response in the mean peak digit abduction score (DAS) following injection into the peronei or gastrocnemius lateralis. The curves correspond to mean peak DAS responses observed 1 or 2 days post-administration. All values are means ± standard error of the mean from two independent experiments (n = 6–8 animals dose/experiment).

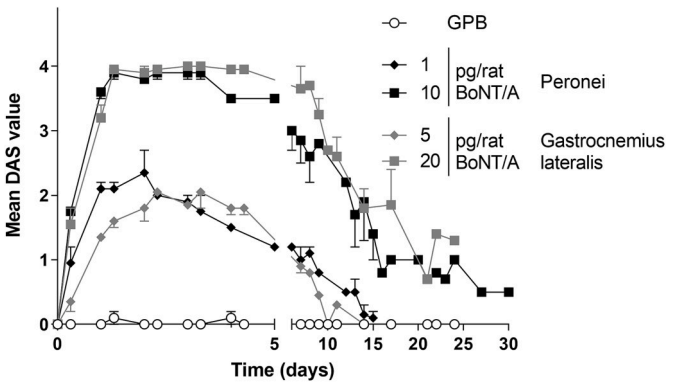

Fig. 2. The time course of DAS following injection of BoNT-A at the closest dose to ED_{50} (diamond) and the first dose reaching DAS 4 (square) for the peronei and the gastrocnemius lateralis in comparison to that following injection of vehicle, gelatin phosphate buffer (GPB; circle) in either of the two muscles. All values are means ± standard error of the mean from two independent experiments (n = 6–8 dose/experiment).

Table 1
Table summarizing the effects of BoNT-A in the rat digit abduction score (DAS) assay following its injection into the peronei, gastrocnemius lateralis, or extensor digitorum longus muscles.

Muscle Injected	ED_{50}[a] pg/rat [individual values]	DAS 2[b] dose (pg/rat)	DAS 4[b] dose (pg/rat)	Dose affecting body weight gain[c] (pg/rat)
Peronei	0.8* [0.70, 0.93]	1	10	>10
Gastrocnemius lateralis	5.2 [2.60, 7.8]	5	20	1
Extensor digitorum longus	ND	ND	~10	>10

*$p < 0.05$ in comparison to ED_{50} value following gastrocnemius injections.
ND – not determined.
[a] Calculated mean ED_{50}.
[b] the closest experimental dose reaching half-maximal (DAS 2) or maximal (DAS 4) responses, as stated.
[c] first dose with significant effect on body weight gain.

Animals treated with vehicle (GPB) either into the peronei, gastrocnemius lateralis, or extensor digitorum longus gained approximately 30% of their body weight by post-injection day 14 (Fig. 3A and B).

Administration of BoNT-A in the peronei at 1 and 5 pg/rat, resulting in the half-maximal (DAS 2) or maximal (DAS 4) inhibition of digit abduction, respectively, had no effect on body weight gain over 14 days post injection (Fig. 3A). Additionally, administration of BoNT-A into the extensor digitorum longus at 10 pg/rat resulting in near-maximal inhibition of digit abduction had no effect on body weight gain (data not presented). However, administration of BoNT-A into the gastrocnemius lateralis, resulted in a significantly reduced body weight gain. Specifically, 10 pg/rat BoNT-A injected into the gastrocnemius lateralis, linked to a half-maximal inhibition of digit abduction (DAS 2), caused less body weight gain in comparison with vehicle-treated controls on post-injection days 2, 3, and 7 (all $p < 0.05$), but not on day 14 (Fig. 3B). Similarly, 20 pg/rat, BoNT-A linked to maximal inhibition of digit

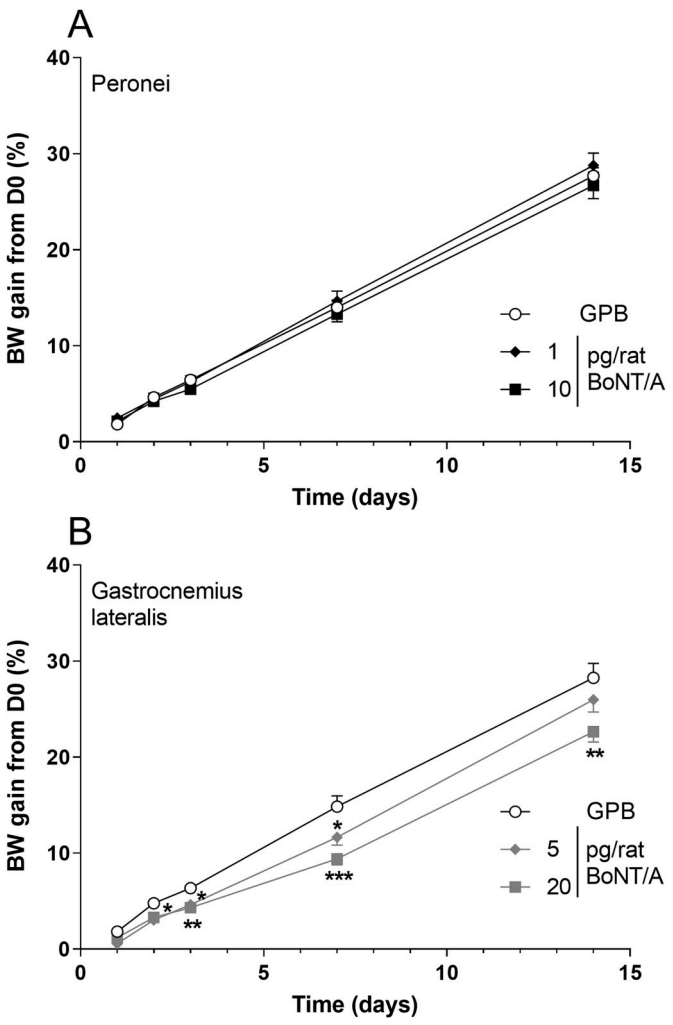

Fig. 3. Effects of BoNT-A and vehicle (gelatin phosphate buffer; GPB) on body weight gain in the rat digit abduction score (DAS) assay. The effects were assessed at the half-maximal (DAS 2) and maximal (DAS 4) doses injected into the peronei muscles (A) or the gastrocnemius lateralis (B). All values are means ± standard error of the mean from two independent experiments with 6–8 animals per dose. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ significantly different from the GPB-injected control group by one-way ANOVA followed by Dunnett's *post-hoc* analysis.

abduction (DAS 4), caused less body weight gain in comparison with vehicle-treated controls on post-injection days 3 ($p < 0.01$), 7 ($p < 0.001$), and 14 ($p < 0.01$; Fig. 3B). The maximal difference in body weight gain between BoNT-A- versus vehicle-treated animals was 5%.

4. Discussion

The objective of the current study was to optimize BoNT-A administration conditions in the rat DAS assay, originally proposed by Broide et al. (2013), in order to address some of the issues in sensitivity and specificity raised by the authors. Specifically, Broide et al. (2013), evaluated the sensitivity of two hindlimb muscles, the tibialis anterior and gastrocnemius to onaBoNT-A, in male Sprague-Dawley rats. They concluded that the abduction responses were different from each other and from those seen in onaBoNT-A-injected mice. In the same study, a full suppression of digit abduction was accompanied by significant inhibition of locomotor activity and reduced body weight gain.

With the objective of optimizing the experimental design of the rat DAS assay proposed by Broide et al., (2013), we evaluated the effects of native, purified BoNT-A in three hindlimb muscles: the gastrocnemius lateralis, peronei, and extensor digitorum longus. The desirable characteristics of the digit abduction response, following injection of BoNT-A into a muscle, that would support a robust assay were (1) a dose-dependent reduction in digit abduction (enabling calculation of ED_{50}), (2) ability to use the same scoring methods of digit abduction in rats and in mice, (3) lack of effect of BoNT-A on body weight gain at doses resulting in maximal inhibition of digit abduction (DAS 4 doses), and (4) evidence of good interexperimental reproducibility of the DAS data. Assessment of the sensitivity of each muscle in separate experiments allowed us to test a wider range of doses of BoNT-A in each experiment and more precisely estimate the ED_{50} value as well as the doses resulting in half-maximal and maximal responses in the DAS. However, as the muscles were not tested in parallel, in the same experiment, the statistical power to evaluate differences across the muscles was limited, which is a limitation of the study.

To our knowledge, our laboratory is the first evaluating activity of BoNT in the peronei muscles using the rat DAS assay. We investigated these muscles due to their proximity to the extensor digitorum longus and accessibility for i. m. injection. In the rat, the peronei consist of a group of deep, closely-bundled muscles including, the peroneus longus, peroneus brevis and peroneus digiti 4, 5 (Wang and Kernell, 2001), that are categorized as ankle evertors (Charles et al., 2016). Each peronei muscle is significantly smaller than the gastrocnemius lateralis. In fact, the weight of each peronei muscle is between 5- (for p. longus) and 14-fold (for p. digiti 4, 5) smaller in comparison to the weight of the gastrocnemius lateralis, whereas in total, all peronei muscles weigh 2.5-fold less than the gastrocnemius lateralis (Wang and Kernell, 2001). As it was technically virtually impossible to inject each peronei muscle without risking tissue damage, we chose to perform i. m. injections that did not differentiate between the peronei muscles. BoNT-A injected into the peronei at the same dose range, but at a lower volume (10 versus 40 μL) than into the gastrocnemius lateralis, elicited a dose-dependent decrease in digit abduction, enabling us to calculate the ED_{50} value. The activity of BoNT-A injected into the peronei was characterized by a fast onset and long duration, as maximal activity (DAS 4 responses) was seen 1 day following the injection and lasted for more than 25 days. The activity of BoNT-A following peronei injection was accompanied with normal body weight gain and the absence of clinical signs. We were also able to apply the same scoring method to the digit abduction of female rats as the one used in female mice injected in the gastrocnemius-soleus complex (Aoki, 1999). The effect of peronei-injected BoNT-A in the DAS assay was characterized by good reproducibility across experiments. In the current study, the experiments involving injections of BoNT-A into peronei were repeated twice (6–8 animals per group in each experiment) and yielded similar results. Also, using the current study design, we tested native BoNT-A in the rat DAS assay in set of experiments with the objective of comparing *in vivo* properties of native BoNT serotypes A through F (Donald et al., 2018). The ED_{50} values of BoNT-A in the rat DAS obtained in two experiments of the current study, 0.7 and 0.9 pg/rat, are well aligned with those obtained in the study by Donald et al. (2018), 0.7 pg/rat. In this study all BoNT serotypes, except D, resulted in dose-related increases in DAS values; the maximal score of DAS 4 being reached 1–3 days post-administration and the rank order of BoNT potency, from the most to the least potent serotypes being A, C, F, E, and B (Donald et al., 2018). The effects of BoNT serotypes in the DAS assay were seen without any effect of body weight gain (Donald et al., 2018).

BoNT-A injected into the gastrocnemius lateralis of female Sprague-Dawley rats resulted in a dose-dependent inhibition of digit abduction responses, causing a half-maximal inhibition of digit abduction (DAS 2) at 10 pg/rat and a maximal inhibition (DAS 4) at 20 pg/rat (Table 1; Fig. 1). The ED_{50} values in two experiments involving the gastrocnemius were 2.6 and 7.8 pg/rat, suggesting that inter-experimental variability in digit abduction might be higher following gastrocnemius lateralis injection in comparison to peronei. In accordance with our findings, a dose-related inhibition of digit abduction responses was observed in female Sprague-Dawley rats injected with BoNT-A in the gastrocnemius

(Torii et al., 2010), despite the fact that the method used by the authors to elicit the digit abduction reflex, suspending animals by the tail, was different from the one used in our study (picking up by the torso). A dose-dependent inhibition of digit abduction responses was also observed in male Sprague-Dawley rats injected with onaBoNT-A in the gastrocnemius muscle (Broide et al., 2013).

Here, animals injected with BoNT-A into the gastrocnemius lateralis at doses linked to half-maximal or maximal digit abduction inhibition (DAS 2 and DAS 4) showed no observable changes in behavior or locomotor activity and continued showing body weight gain, albeit at mildly reduced rate (up to 5% difference) in comparison to vehicle-injected controls. In contrast, the effect of onaBoNT-A injected into the gastrocnemius of male Sprague-Dawley rats at doses linked to maximal effect in DAS (38.2 U/kg) caused near complete (15-fold) inhibition of locomotor activity and a near full cessation of body weight gain (0.38 versus 13% growth in toxin versus vehicle-treated animals Broide et al., 2013). While Broide et al. (2013) argue that robust reduction in locomotor activity and cessation of body weight gain in onaBoNT-A-injected animals is the result of systemic spread, generalization of this conclusion on more milder effects on weight gain, needs to be taken with a caution, as a local muscle relaxation can also impact locomotor activity, reduce food and water intake and subsequently impact body weight gain.

Unlike the scoring method used by Broide et al. (2013), here we were able to apply the same scoring method as the one used in mice (Aoki, 1999). At this point we can only hypothesize about specific experimental factors or their combination that can explain this discrepancy in the pattern of digit abduction inhibition seen between these two studies. Methodological differences, including the use of males versus females, potential differences in exact site of the injection (undefined by Broide et al., 2013), or the total volume of BoNT injection (40 μL here versus 20 μL used by Broide et al., 2013), may have contributed to this discrepancy.

The finding that administration of BoNT-A into the gastrocnemius lateralis or peronei can reduce digit abduction is perplexing, as neither of the two muscle groups mediates digit abduction in a normal animal. We can hypothesize that when injected into the gastrocnemius lateralis or peronei, BoNT-A is diffusing into an adjacent muscle or muscle groups that are actually responsible for digit abduction. The muscle that is responsible for digit extension in the rat is the extensor digitorum longus (Jaspers et al., 2002). Here, for the first time, a range of doses of BoNT-A were tested in the rat extensor digitorum longus in order to estimate an ED_{50} values. We found that in response to a high dose of BoNT-A (10 pg/rat), there were near-maximal inhibition of digit abduction responses (DAS 3–4) in female rats. These findings are well aligned with those of Adler et al. (2001) which show a full inhibition of digit abduction in male Sprague-Dawley rats injected with BoNT-A in the extensor digitorum longus. Furthermore, a near-maximal inhibition of digit abduction obtained in BoNT-A-injected animals in our study was not accompanied by any change in body weight gain. However, in response to a lower dose range, while mild effects on digit abduction were detected, the responses lacked dose-dependency and were difficult to quantify. As the response profile of BoNT-A in the extensor digitorum longus made it impossible to calculate the ED_{50} values, the muscle was considered to be unsuitable for the DAS assay in rats. Thus, while direct injections of BoNT-A into the extensor digitorum longus are not suitable for the rat DAS assay, the muscle may still play a leading role in the digit abduction inhibition when BoNT-A is injected into either the gastrocnemius lateralis or peronei. Alternatively, since all muscles are mechanically coupled by myofascial connections it is possible that the gastrocnemius lateralis and peronei can exert force via the extramuscular connective tissue to the epimysium and perimysium of the extensor digitorum longus (Huijing, 2003, 2009).

In conclusion, the rat DAS assay is a useful tool for evaluation of biological properties of BoNT *in vivo*. It is used to measure functional effects of BoNT in the skeletal muscle and is markedly easier to perform than other *in vivo* tests, such as CMAP (Torii et al., 2010) or the muscle force test in rats (Pickett et al., 2008), as it does not require equipment and involves testing conscious animals over time. Here, we described optimized experimental conditions for assessing the biological properties of BoNT in the rat DAS assay. Our data suggest that the rat can indeed be used to evaluate *in vivo* activity of BoNT in the DAS assay. BoNT injected into the peronei of female rats results in dose-dependent inhibition of digit abduction responses without signs of BoNT toxicity, characterized by good sensitivity, specificity and reproducibility. The optimized experimental conditions of the rat DAS assay can be used to assess and compare potency, onset, and duration of action of the different BoNT in the same species where evaluation of compound toxicity is usually performed, and thus better estimate the compound's therapeutic window. Also, as the optimized design offers higher sensitivity of the assay, it can be performed with fewer animals, thus contributing into reduction, refinement and replacement principals of animal welfare. Future studies should explore the mechanisms behind the inhibition of digit abduction responses in animals injected with BoNT-A in the gastrocnemius lateralis, peronei and extensor digitorum longus, including immunohistochemical detection of the cleaved section of its target protein, SNAP-25, after injection into each muscle.

Ethical statement

All experimental procedures were approved by the Ethics Committee of Ipsen Innovation (C2EA; registration number 32) and were performed in full compliance with the ARRIVE guidelines, EU Directive 2010/63/EU for animal experiments and the 2013 French Regulatory Decree.

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Declaration of competing interest

SC, CP, and MK are all employees of Ipsen Innovation, France.

CRediT authorship contribution statement

Sylvie Cornet: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft. **Cindy Périer:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft. **Mikhail Kalinichev:** Visualization, Supervision, Writing - original draft, Writing - review & editing.

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