ORIGINAL ARTICLE

Botulinum Neurotoxins Serotypes A and B induce paralysis of mouse striated and smooth muscles with different potencies

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Abstract

To address the scarcity of direct comparison of botulinum neurotoxin serotypes activity on smooth versus striatal muscle, we have studied the action of BoNT/A1 and BoNT/B1 on ex vivo preparations of both muscle types. We have set up and characterized a model of neurogenic contractions in the isolated mouse bladder, and used this model to explore the effects of the two serotypes on contractions evoked by electrical field stimulation. Both toxins were also tested in the mouse phrenic nerve hemidiaphragm assay, to compare their potency in smooth versus striated muscle. The characterization of the model of neurogenic contractions in the isolated mouse bladder indicates that about half of the activity is driven by purinergic signaling, and about half by cholinergic signaling. Furthermore, we find that BoNT/B1 is more potent than BoNT/A1 in inhibiting activity in the mouse detrusor smooth muscle preparation, but that both toxins have comparable potency on the striated muscle activity of the phrenic nerve hemidiaphragm model. We also show that these findings are mouse strain independent. In conclusion, the established mouse bladder detrusor smooth muscle model is able to discriminate between different botulinum neurotoxin serotypes and could be a useful preclinical tool to explore the pathophysiology of bladder overactivity, as well as the effects of new therapeutic candidates. It is interesting to note that the high proportion of purinergic transmission driving detrusor contractions in this model is similar to that seen in neurodetrusor overactivity disease, making this model relevant with regard to pathophysiological interest.

Abbreviations
ACh, acetylcholine; BoNT, botulinum neurotoxin; EFS, electrical field stimulation; FGFR, fibroblast growth factor receptor; HCC, C terminal portion of the HC; HC, heavy chain; HCN, N terminal portion of the HC; KHB, Krebs-Henseleit buffer; LC, light chain; NANC, nonadrenergic noncholinergic; NDO, neurogenic detrusor overactivity; NMJ, neuromuscular junction; PKC, protein kinase C; PNHD, phrenic nerve-hemidiaphragm; SNAP-25, SyNaptosome-Associated Protein of molecular weight 25 kDa; SNARE, Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor; Syt, synaptotagmin; t_{50}, time to half amplitude contraction; VAMP, Vesicle Associated Membrane Protein.

Introduction

Botulinum toxin is a highly active poison produced by the gram-positive anaerobic bacteria *Clostridum* *botulinum* that causes botulism, an often lethal disease characterized by flaccid paralysis due to inhibition of release of neurotransmitters, primarily acetylcholine (ACh), at neuromuscular junctions. Botulinum

neurotoxins (BoNTs) block neurotransmission by cleaving specific cytoplasmic actors of neurotransmitter release after having selectively targeted neuronal receptors. BoNTs are used as therapeutics to restore the activity of hyperactive nerve endings to normal levels. In the early 1980s, BoNT/A was first used to treat strabism (Scott 1981), and since then, therapeutic and esthetic applications have been developed. Seven antigenically distinct BoNTs serotypes, divided into 42 subtypes, have been described so far (Rossetto et al. 2014). Despite this diversity, BoNT/A1 is the main serotype used in patients, with three commonly available commercial preparations, while only one BoNT/B1 product is available on the market (Dressler 2012).

The BoNT structure comprises a heavy (HC) and a light chain (LC) linked together by a disulfide bond. BoNTs mode of action relies on three distinct steps: docking onto the cell membrane, internalization into the cytoplasm, and enzymatic cleavage of a target SNARE (Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor). In this cascade of events, the C terminal portion of the HC (HCC) is responsible for binding of BoNT to specific receptor proteins, the N terminal portion of the HC (HCN) allows internalization, while the LC bears the endopeptidase activity. BoNT/A1 and BoNT/B1 have distinct modes of action, regarding receptor recognition, as well as the SNARE they target. Whereas BoNT/A1 needs Synaptic Vesicle protein-2 (SV2) to attach onto the neuronal cell surface, BoNT/B1 requires synaptotagmin (Syt). Ganglioside binding is also a pre-requisite for both serotypes to enter into neurons (Rummel 2013). Intracellularly, BoNT/A1 cleaves SNAP-25 (SyNaptosome-Associated Protein of molecular weight 25 kDa), while BoNT/B1 targets VAMP (Vesicle Associated Membrane Protein)/synaptobrevin (Rossetto et al. 2014). Although the *modus operandi* of these two neurotoxins is thus different (Lam et al. 2015), the physiological effect is the same: the neurotransmitter is not released into the synaptic cleft, resulting in paralysis.

The bladder consists of three layers termed detrusor, the smooth muscle responsible for bladder contractions, the lamina propria, mainly consisting of myofibroblasts and fibroblasts, and the urothelium with basal, intermediate and umbrella cells. The bladder is innervated by the pelvic and the hypogastric nerves, the former containing parasympathetic postganglionic axons responsible for the release of ACh and nonadrenergic noncholinergic (NANC) transmitters producing, in nonpathological conditions, bladder contractions to achieve voiding (Fowler et al. 2008). In neurogenic detrusor overactivity (NDO), contractions occur during the filling phase because of the abnormal activity of the parasympathetic system and the increased coupling of the muscle bundles (Parsons and

Drake 2011). BoNT/A1 use by urologists has increased dramatically in the last 15–20 years to treat NDO, and BoNT/A1 is now well-established as a second-line therapy when anticholinergic strategies fail. BoNT/A1 injections reduce incontinence episodes and pain, through a multi-focal action on afferent and efferent innervations (Dolly and Lawrence 2014).

BoNTs effects on muscle activity can be studied in organ bath preparations (Smith et al. 2003; Takahashi et al. 2012). The action of BoNTs on smooth muscle is often explored using rats or guinea-pigs tissues (MacKenzie et al. 1982; Lawrence et al. 2010). Likewise, phrenic nerve-hemidiaphragm (PNHD) preparations, from mice and rats, have been used for decades to study the neuromuscular junction (NMJ) at the striated muscle level (Simpson and Tapp 1967; Dolly et al. 1987; Rummel et al. 2009). The latter assay closely mimics breathing muscle paralysis, with a very low variability. However, direct comparative studies of toxin action on smooth versus striatal muscle are sparse.

To address this scarcity of direct comparative data we have studied here the action of BoNT/A1 and BoNT/B1 on both muscle types. Because the rat has a mutation at the VAMP1 cleavage site that makes rat VAMP1 insensitive to BoNT/B (Schiavo et al. 1992; Peng et al. 2014), we chose the mouse as the most appropriate species for these assays. We have set up a model of neurogenic contractions in the isolated mouse bladder, to focus on the efferent signaling of the detrusor muscle, and used this model to explore the effects of the two toxins on bladder contractions evoked by electrical field stimulation (EFS). Both toxins were also tested in the mouse phrenic nerve hemidiaphragm assay, to compare their potency in smooth versus striated muscle preparations. We made the comparisons in two different mouse strains to confirm that potential differences were not confined to just one strain.

Materials and Methods

Animals

This study was conducted in compliance with the relevant animal health regulation in place in France (Council Directive No. 2010/63/UE of September, 22nd, 2010 on the protection of animals used for scientific purpose). Female CD1 and C57Bl6 mice weighing 18–25 g were purchased from Charles River (Lyon, France). They were allowed to acclimatize for at least 5 days before the experiments, with free access to food and water. On the day of experiment, mice were deeply anaesthetized with isoflurane (3.5% in 2% O_2) and exsanguinated before tissue collection.

Reagents

All chemicals were purchased from Sigma-Aldrich (St Louis, MO) and solubilized in distilled water.

Natural Botulinum toxins A and B were purchased from List Biologicals Laboratories, Campbell, CA. They were prepared as stock solutions (666 nmol/L) in PBS (Gibco, Invitrogen, Cergy-Pontoise, France); 1 mmol/L KH_2PO_4, 155 mmol/L NaCl and 3 mmol/L Na_2HPO_4) supplemented with 1 mg/mL BSA (Sigma-Aldrich) and stored at $-80°C$. Dilutions were prepared as needed, in Krebs–Henseleit buffer (KHB; mmol/L: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11) supplemented with 0.5% gelatin type A (Sigma-Aldrich).

The activity of the toxins was confirmed in cell-free assays using BoTest® (Botulinum Neurotoxin Detection Kit; BioSentinel, Madison, WI) and in cell-based assays using rat spinal cord neuronal cultures.

Detrusor strip assay

Mouse bladders were collected and cleaned of surrounding adipose and connective tissue. After the dome and base of the organ were removed, the urothelium and most of the lamina propria were dissected to leave the detrusor muscle. Two strips measuring about 6×2 mm were cut out from one bladder, fixed onto a custom electrode tissue holder and tensed to 0.5 g in organ baths (Emkabath4; Emka Technologies, Paris, France) filled with KHB and bubbled with carbogen at 37°C and pH of 7.4. Bladder contraction force was measured with isometric transducers (Emka Technologies). After about 45 min of equilibrium period, with renewal of the buffer every 15 min, 70 mmol/L KCl was applied to check the smooth muscle integrity after the dissection. After several washings and return to baseline, 10 μmol/L carbachol was used to test postsynaptic muscarinic receptors.

Subsequently, contractions of the detrusor muscle were evoked by electrical field stimulation using trains of 20 pulses (intratrain frequency 10 Hz, single pulse duration 20 μsec) separated by 1 min intervals. This stimulation was generated with the use of two platinum electrodes placed a few millimeters on each side of the strip. This protocol was specifically designed to depolarize autonomic nerves in the mouse detrusor plexus, and to generate release of cholinergic and NANC transmitters (ATP, SP, etc.) which could be quantitatively antagonized by BoNTs (Palea et al. 2012; Takahashi et al. 2012).

To validate the model, 1 μmol/L tetrodotoxin (voltage-gated sodium channel blocker) was added into the bath to check that the contractions were of neurogenic origin. Atropine (muscarinic receptor antagonist) and α-β-methylene-ATP (P2X receptors desensitizer) were tested at 1 and 10 μmol/L respectively.

Study of the paralytic effect of BoNTs on detrusor contractions

After intensive washings and after at least 20 min of stable contractions, BoNTs were added to the baths, with final concentrations of 0.1 to 5 nmol/L. Each strip was exposed to only one concentration of BoNT. Amplitude of contractions was measured until 90% of the signal was abolished, or for 4 h if this value was not reached (for low concentrations).

At the end of the experiment a final addition of 10 μmol/L carbachol was performed to assess the viability of the tissue. Experiments were rejected when poststimulation carbachol responses were <80% of the prestimulation carbachol response.

Phrenic nerve hemidiaphragm assay (PNHD)

The left phrenic nerve and hemidiaphragm were removed from the thoracic cage. The muscles were fixed onto a custom electrode tissue holder and suspended (1 g passive tension) in organ baths (Emkabath4; Emka Technologies) containing 10 mL of KHB at 37°C and gassed with carbogen. The phrenic nerve was continuously and directly stimulated with the use of two ring electrodes into which the nerve was gently fixed. The phrenic nerve was stimulated continuously with 20 μsec pulses delivered at 1 Hz. Contraction force was measured with isometric transducers (Emka Technologies). The preparations were allowed to equilibrate for 45 min in medium renewed every 15 min. Following equilibration, each tissue was subjected to a maximal concentration of tubocurarine (10 μmol/L). The full inhibition of the signal was considered as an indication that the contractile response was mainly due to ACh released by nerve stimulation (preparations with inhibition lower than 95% were discarded). The preparations were then washed extensively. After a washout period of 20–30 min, when the contractile response was stable, BoNTs were tested with final concentrations of 1 to 100 pmol/L. Each preparation was exposed to only one concentration of BoNT. This range of concentrations is much lower than the one used for bladder experiments, the neuromuscular junction being directly accessible to the toxin, in contrast with the detrusor where the toxin needs to penetrate the tissue to reach the parasympathetic postganglionic nerves in the bladder wall.

Expression of neurotoxins potency

Experimental data from both preparations were recorded with software IOX v 2.9 from Emka Technologies. Data processing was done by Datanalyst software. A nonlinear regression analysis was performed, to calculate the time to half-amplitude contraction (t_{50}) as compared to control contraction amplitude. This parameter allows expressing the potency of a neurotoxin, with the smaller value indicating a higher potency. For concentrations where t_{50} was not reached, the relative amplitude remaining after 100 min was used. In these cases, results are expressed as a percentage of the initial control contraction just prior to addition of toxin. Data are expressed as individual data or as mean ± standard error of the mean. As previously described (Lawrence et al. 2010; Weisemann et al. 2015), concentration-response curves were plotted for each serotype. For statistical analysis, an unpaired Student's t test was performed, with $P < 0.05$ considered as significant.

Results

Characterization of EFS-induced contractions of mouse detrusor strips

As shown in Figure 1A, 1 μmol/L tetrodotoxin (which inhibits action potentials by blocking voltage gated sodium channels) completely blocked the contractions induced by our protocol, demonstrating their neurogenic origin
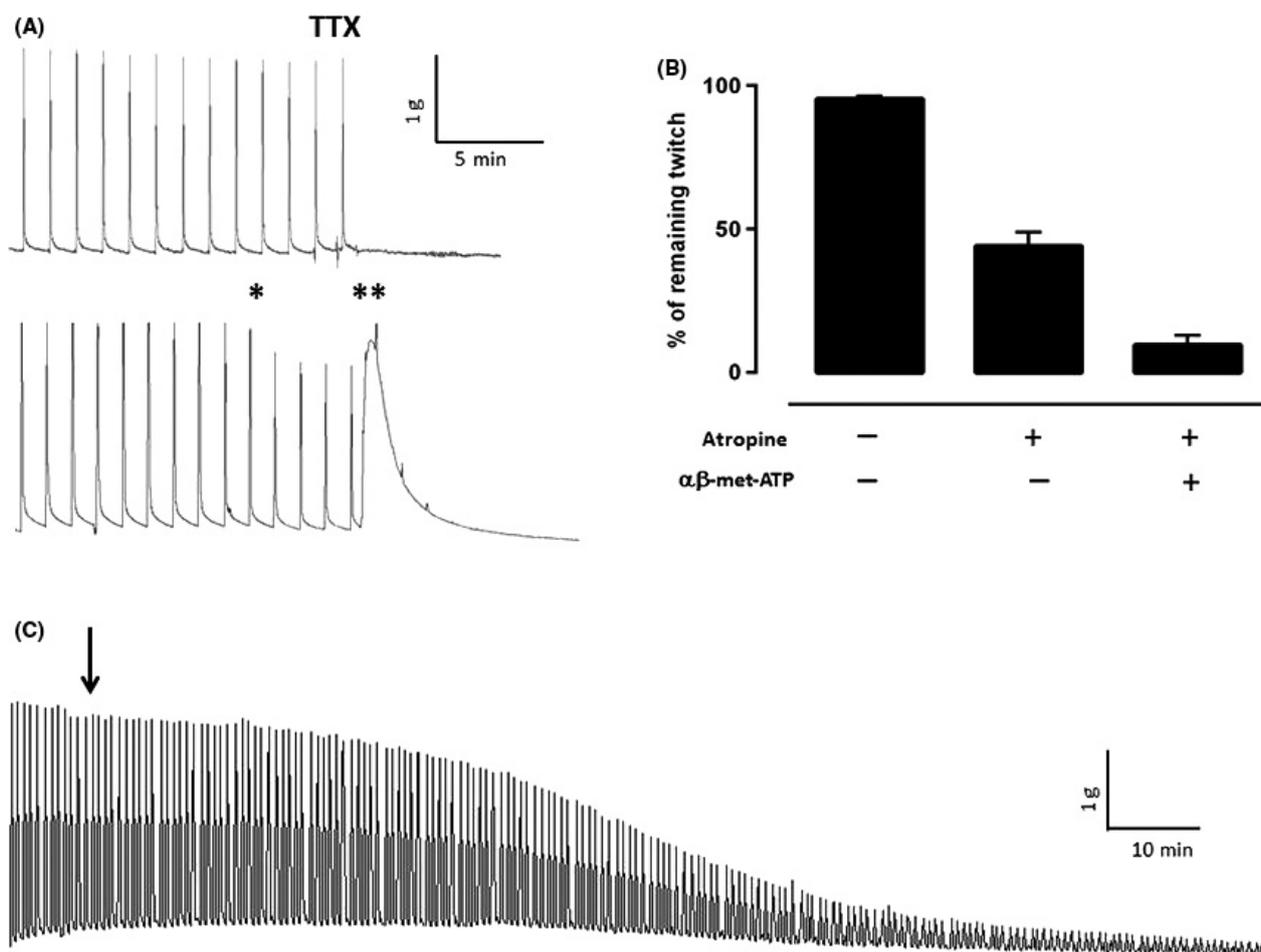

Figure 1. Characterization of the isolated mouse bladder strip preparation. (A) Typical recording illustrating the effect of 1 μmol/L tetrodotoxin (TTX) on electrical field stimulation (EFS)-induced contractions of the isolated mouse bladder strips, resulting in complete inhibition of EFS-induced contractions. 1 μmol/L atropine (*) partially inhibited the contractions, while the subsequent addition of 10 μmol/L α-β-met-ATP (**) completely abolished the remaining signal after a peak in amplitude due to the overstimulation of purinergic receptors. (B) Calculated mean values of experiments as shown in A ($n = 3$–6/group). (C) Recording illustrating the effect of addition of 1 nmol/L BoNT/A1 (arrow) on EFS-induced detrusor strips contractions.

($n = 3$). Moreover, 1 μmol/L atropine (which inhibits muscarinic ACh receptors) ($n = 6$) inhibited approximately 50% of EFS-evoked contractions, the remaining signal being abolished by 10 μmol/L α-β-met-ATP which produced an overstimulation - of purinergic receptors - followed by a complete desensitization ($n = 4$; Figs. 1A and B). Thus, in this model muscarinic and purinergic receptors are involved in driving the smooth muscle contractions. This suggests that the electrical stimulation causes the o-release of excitatory ACh and ATP from the neuronal plexus located in the mouse detrusor smooth muscle, as previously shown in the rat (Lawrence et al. 2010).

We next tested whether BoNT/A1 would be active in inhibiting the EFS-induced contractions in this model. As shown in Figure 1C, applying 1 nmol/L BoNT/A1 to the bath resulted in a gradual loss of muscle activity with an onset of action of paralysis that was even at this relatively high concentration of BoNT/A1 much slower as compared to atropine and α-β-met-ATP, for which the onset was nearly immediate.

BoNT/B1 inhibits activity in CD1 mouse smooth muscle detrusor strip preparations with higher potency than BoNT/A1

In order to establish whether this effect was dose-dependent we tested additional concentrations of BoNT/A1 in this model. While the potency of neurotoxins in organ bath models is typically expressed as the time to half paralysis of the muscle, i.e. t_{50}, only BoNT/A1 concentrations over 0.6 nmol/L reduced detrusor smooth muscle activity by more than 50% during 2 h long recording sessions. Thus, the activity of BoNT/A1 in this model is in the following expressed as percentage of muscle twitch remaining after a period of toxin bath application of 100 min (open circles in Fig. 2A).

Even after 100 min, detrusor smooth muscle contractions were still at 81 and 75% of initial muscle twitch when 100 pmol/L BoNT/A1 was tested. The highest tested concentration of BoNT/A1 (5 nmol/L) reduced the muscle twitch to $6.4 \pm 1.8\%$ of initial strength after 100 min in our protocol ($n = 4$). Two intermediate toxin concentrations had intermediate effects. In control strips, fatigue resulted in about $12 \pm 3\%$ ($n = 6$) loss in muscle twitch after 100 min of recording (approximately 88% of initial muscle twitch remaining). In some experiments, data in control strips were recorded for 4 h, with a maximal loss of 20% (80% of initial muscle twitches remaining) (data not shown). Overall, the data indicate that with the stimulation protocol used, BoNT/A1 has a rather low efficacy in the mouse smooth muscle detrusor model.

When BoNT/B1 was tested in the same conditions, the response profile was quite different (filled squares in Fig. 2A). Even with a concentration as low as 0.1 nmol/L, only $33.1 \pm 3.3\%$ ($n = 3$) of initial muscle twitch remained after 100 min. This effect was even more pronounced with higher BoNT/B1 concentrations ($19.5 \pm 6.3\%$ at 1 nmol/L ($n = 3$), $6.7 \pm 1.4\%$ at 3 nmol/L ($n = 4$), and $4.1 \pm 0.6\%$ at 5 nmol/L ($n = 3$)). Thus, while both serotypes induced a dose-dependent paralysis in isolated mouse detrusor strips, the effect of BoNT/B1 was stronger than that of BoNT/A1.

BoNT/A1 and BoNT/B1 have similar efficacy in inhibiting striatal muscle activity in CD1 mouse phrenic nerve hemidiaphragm preparation

After investigating the paralyzing effect of BoNT/A1 and BoNT/B1 in the mouse smooth muscle detrusor strips, the mouse phrenic nerve hemidiaphragm preparation was used to study their potencies at the neuromuscular junction of a striated muscle. This model has been studied intensively in the past and has been pivotal in the discovery of the mechanisms of action of neurotoxins. The diaphragm contractions rely only on ACh release which stimulates nicotinic receptors at the postsynaptic level (Rasetti-Escargueil et al. 2011). In contrast with what was seen in the bladder, but in agreement with previously described data for the phrenic nerve hemidiaphragm preparation (Wang et al. 2012), BoNT/A1 and BoNT/B1 profiles were similar (Fig. 2B).

The difference in efficacy between BoNT/A1 and BoNT/B1 on smooth versus striatal muscle preparations is not mice strain specific

All the data shown to this point were obtained from ex vivo preparations of CD1 mice. Given the apparent difference between BoNT/A1 and BoNT/B1 in efficacy on smooth versus striatal muscle, we wanted to know whether this effect was specific to this strain of mice. We thus decided to test both toxins also on preparations obtained from a different mice strain, C57Bl6N mice. To allow easier comparison between strains, we decided to compare t_{50} values at one concentration of both toxins for each preparation. For the bladder strip preparation we used 1 nmol/L of toxin, because BoNT/A1 and BoNT/B1 both inhibit detrusor smooth muscle activity after 100 min by more than 50% at this concentration, and thus a consistent t_{50} can be obtained for both toxins (see Fig. 2A). For the PNHD assay we used a 10 pmol/L concentration of toxin which matches the t_{50} values of around one hour in the detrusor strip preparation for BoNT/B1.
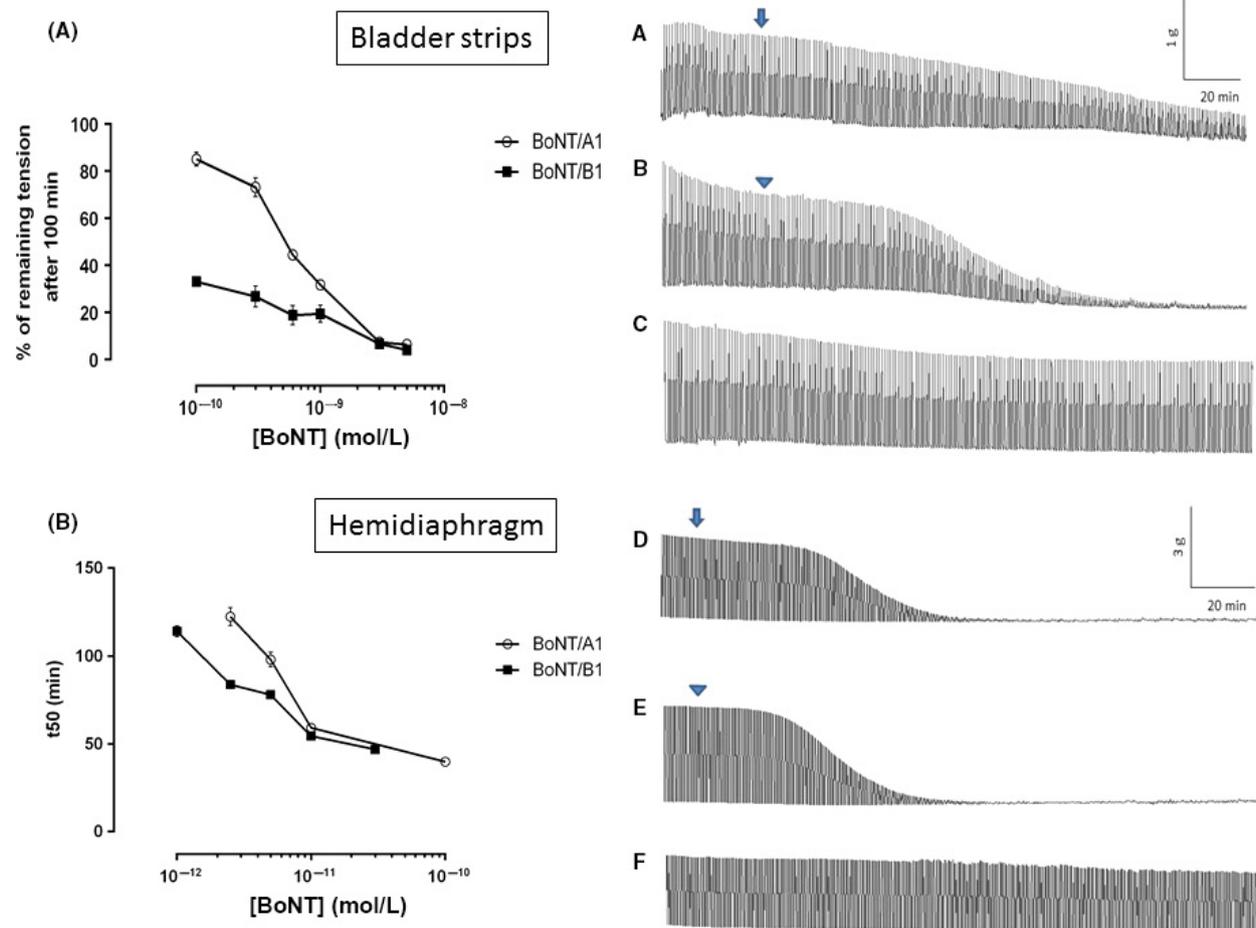
Figure 2. BoNT/A1 and BoNT/B1 inhibit smooth and skeletal muscles with different potencies. (A) BoNT serotypes A1 and B1 dose-dependently induce a reduction in electrical field stimulation (EFS)-evoked twitch in CD1 mouse bladders trips (n = 2–8/group), with a marked discrepancy in their potencies. Traces on the right illustrate the effect of 0.3 nmol/L BoNT/A1 (trace a; arrow indicates addition of BoNT/A1 to the bath) or BoNT/B1 (trace b; arrow head indicates addition of BoNT/B1 to the bath) on EFS-evoked contractions in the mouse detrusor strip. Trace c shows the stability of a control strip over a 3 h-recording (B). BoNT subtypes A1 and B1 were able to dose-dependently induce a reduction in nerve-evoked twitches in CD1 mouse phrenic nerve-hemidiaphragm (PNHD) preparation in a very similar way (n = 3–4/group). Traces on the right are representative of the effect of 10 pmol/L BoNT/A1 (trace d; arrow indicates addition of BoNT/A1 to the bath) or BoNT/B1 (trace e; arrow head indicates addition of BoNT/B1 to the bath) in the PNHD assay. Trace f shows the behavior of a control nontreated PNHD preparation.

As shown in Figure 3, BoNT/B1 was more potent than BoNT/A1 not only in the CD1 mouse detrusor bladder strips preparation in these conditions, but also in the preparations obtained from the C57Bl6 mouse. In both strains the difference between BoNT/A1 and BoNT/B1 was significant. In contrast, in the PNHD preparation, the activity of both toxins was similar and not significantly different.

Discussion

In this study, we have established a model in which electrical field stimulation of mouse bladder smooth muscle strips evokes neurogenic contractions that are due to release of ACh and ATP in about equal contribution. This model thus reproduces activity that is due to overactivity of the parasympathetic nerves, resulting in ACh and ATP secretion. Furthermore, we developed a stimulation protocol which correlates with the intense neural activity seen in NDO patients (i.e. 10 Hz stimulation), but producing relatively faint fatigue after 4 h, so that the paralytic effect of the neurotoxins was not masked and the viability of the tissues was ensured throughout the recordings.

Our ex vivo model was sensitive to BoNTs in a concentration-dependent manner. Previous studies were not all successful in inhibiting mouse detrusor muscle force after incubation with botulinum toxin (Howles et al. 2009), but this may be explained by the very low concentrations
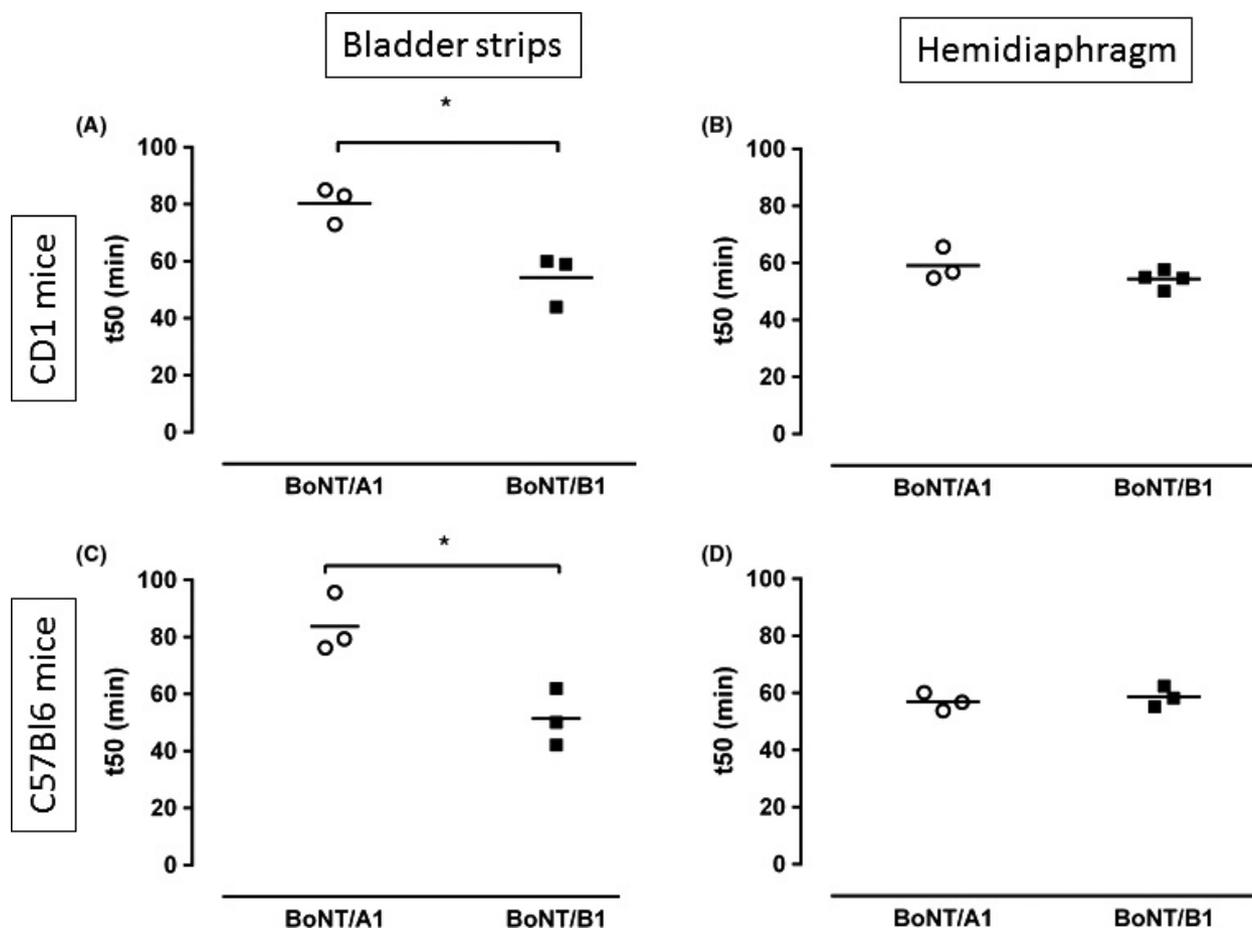
Figure 3. The difference between BoNT/A1 and BoNT/B1 is mouse strain independent. (A, C) Summary data from the bladder strips assay, at 1 nmol/L, illustrating the higher potency of BoNT/B1 compared to BoNT/A1 whether the source of tissue was mice of the CD1 (A) or C57Bl6 (C) strain. (B, D) Potencies of BoNT/A1 and BoNT/B1 were equal in the phrenic nerve-hemidiaphragm preparation (10 pmol/L), in CD1 (B) and C57Bl6 mice (D).*, $p < 0.05$ (unpaired Student's t test).

of toxin used in this study. In addition, toxins were incubated on resting, noncontracting tissues, which does not favor the penetration of the toxin, which is a challenge in isolated bladder strip experiments. It is interesting to note that our mouse bladder muscle strips model was at least as sensitive as the rat model described by van Uhm et al. (2014), with concentrations as low as 0.1 nmol/L inducing a paralysis.

Using this model we directly compared the activity of BoNT/A1 and BoNT/B1 in this preparation. We also compared these results from a smooth muscle to those from a striated (hemidiaphragm) muscle from the same species. An intriguing finding of our study is that under our experimental conditions, BoNT/B1 showed a different profile as compared to BoNT/A1 when it comes to inhibition of mouse striated versus mouse smooth muscle activity. Specifically, we find that BoNT/B1 is more potent than BoNT/A1 in inhibiting smooth muscle activity, but

that both toxins have comparable potency in striated muscle. This finding is not due to the provenance of the toxins used in this study, as we have confirmed the findings using recombinant toxins of both serotypes (data not shown). Our results correlate with previous data describing similar inhibition of phrenic nerve hemidiaphragm activity by both toxins (Wang et al. 2012). However, the discrepancy between subtypes in the bladder strip preparations was not described in this earlier study. This might be explained by a difference in stimulation protocols, which is pivotal in the mode of action of BoNTs. While the exact stimulation protocol used in the study by Wang et al. (2012) is not given, it has been shown that BoNT/A inhibits transmitter release at higher frequencies, possibly by the action of a prejunctional facilitation mechanism involving the phosphorylation of L-type Ca^{2+} channels by protein kinase C (PKC) (Smith et al. 2003). Our protocol uses a relatively high frequency of stimulation (20 Hz,

2 sec trains every 62 sec) and may activate these prejunctional M1-receptors, enhancing ACh release. Moreover, differences in tissue thickness may explain the difference between our results and those by Wang et al. (2012); in fact, our preparation was a pure smooth muscle detrusor preparation, whereas Wang and colleagues studied a preparation containing the urothelium. It should also be noted that in the study by Wang and colleagues, the two natural toxins were provided by two different suppliers and that the BoNT/B toxin needed to be proteolytically nicked with TrypZean. Differences in the toxin sources and the exact experimental conditions, might also explain that Weisemann et al. (2015) found a fairly similar profile for BoNT/B1 in the mouse hemidiaphragm assay, but a higher potency for BoNT/A1, compared to our study.

Because BoNT/A1 and BoNT/B1 use different receptor proteins to enter neurons and also cleave different substrates (Lam et al. 2015), the molecular basis for this difference between BoNT/A1 and BoNT/B1 at the bladder level may involve a difference in receptor and intracellular substrate populations. While studies directly comparing the expression of BoNT receptors and substrates at the bladder versus the diaphragm in situ are scarce, there is some evidence that synaptotagmin I is the predominant synaptotagmin isoform in autonomic and sensory neurons (Li et al. 1994). Synaptotagmin II might be the predominant synaptotagmin isoform at the neuromuscular junction of striated muscle (Juzans et al. 1996), although synaptotagmin I has a functional role also there (Pang et al. 2006). Of note, the reported tissue distribution of the two synaptotagmin isoforms would argue for a better activity of BoNT/B relative to BoNT/A at the hemidiaphragm preparation rather than the bladder, as synaptotagmin II is the higher affinity synaptotagmin receptor for BoNT/B (Nishiki et al. 1996). While this could potentially be offset by a much higher relative expression of synaptotagmin in the bladder tissue as compared to the hemidiaphragm tissue, the strength of expression of the two synaptotagmin isoforms at both preparations has to our knowledge not yet been compared. Concerning the distribution of the SV2 receptor and BoNT substrates, it has been shown that SV2 and SNAP 25 are expressed in the human bladder (Coelho et al. 2010). A gene expression study in porcine tissue indicates that only SV2B and SV2C, but not SV2A, are expressed in this tissue (Bahadory et al. 2014). This study also indicates that in the mucosa/suburothelium and the detrusor the relative gene expression of SNAP-25 versus VAMP is roughly equal with regard to the housekeeper gene GADPH, but that there is a lower expression of SNAP-25 in the urothelium itself. However, the urothelium was not present in the preparation used in our study. Overall, while a differential expression of the receptor and/or substrates might explain

some of our findings, it is likely that other differences between the two preparations also contribute. Nevertheless, it should be noted that another VAMP-targeting neurotoxin, BoNT/D, was shown to be more potent than BoNT/A in a neurally evoked bladder contraction model (Smith et al. 2003; but see Lawrence et al. 2010), which indeed may suggest a role of this SNARE protein in the difference seen between striated and smooth muscle preparations.

Another possible explanation for the difference in activity between BoNT/A1 and BoNT/B1 could be the presence of fibroblast growth factor receptor type 3 (FGFR3) in the bladder tissue. This receptor has been described to be a high affinity receptor for BoNT/A1 (Jacky et al. 2013). If BoNT/A1 binds to FGFR3 at the surface of non-contracting cells, such as fibroblasts or interstitial cells, FGFR3 could act as a 'biological sink' and thereby decrease the effective concentrations of BoNT/A1, resulting in an apparent higher activity of BoNT/B1.

While the relative contribution of cholinergic and purinergic signaling in the bladder tissue is species-specific, it is interesting to note that in NDO patients, the purinergic pathway is more active than in control patients, where the main contributor for bladder contractions is ACh. Given the fact that in our model we observed an almost equal contribution of cholinergic and purinergic signaling to the contractions, our model might mimic the pathological situation seen in bladder overactivity, where ATP signaling is one of the culprits for bladder contractions during the filling phase, resulting in incontinence and pain. Nonetheless, while previous results showed that the main mechanism for the action of BoNT in the bladder is through parasympathetic fiber impairment in the detrusor (Coelho et al. 2010), the impact of ATP delivery to and by the other layers must be kept in mind, especially the urothelium, which was not present in our model.

Interestingly, our data fit to clinical findings that BoNT/B has higher efficacy at blocking autonomic nervous activity (Birklein et al. 2003; Dressler and Eleopra 2006). However, due to species differences in the synaptotagmin II primary amino-acid sequence and the resulting low affinity of natural BoNT/B1 for human synaptotagmin II, the present commercial BoNT/B product needs to be administered at high doses to have therapeutic efficacy. This results in a high occurrence of immune reactions (Dressler and Hallett 2006).

In conclusion, the established mouse bladder detrusor smooth muscle model is able to discriminate between different neurotoxin subtypes and could be a useful preclinical tool to explore the pathophysiology of bladder overactivity, as well as the effects of new therapeutic candidates. It is interesting to note that the

higher implication of NANC players in detrusor contractions in rodents might make our model close to the scenario seen in NDO situations. Our finding that the mouse bladder smooth muscle strips have higher sensitivity to BoNT/B1 as compared to BoNT/A1, whereas the striated muscle hemidiaphragm has nearly equal sensitivity to both toxins is interesting and should be explored further. Nevertheless, this advantage of BoNT/B1 over A1 in smooth muscle might not be conserved from mice to humans. The human form of the BoNT/B protein receptor (synaptotagmin II) interacts with lower affinity in human tissue compared to in mouse (Peng et al. 2012; Strotmeier et al. 2012) and this difference might be enough to outweigh any relative advantage of BoNT/B1 over A1 in affecting human smooth muscles.

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Disclosures

None declared.

Author Contributions

Participated in research design: Maignel-Ludop, Huchet, Krupp. Conducted experiments: Maignel-Ludop. Performed data analysis: Maignel-Ludop. Wrote or contributed to the writing of the manuscript: Maignel-Ludop and Krupp.

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