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Botulinum neurotoxin serotype D – A potential treatment alternative for BoNT/A and B non-responding patients

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HIGHLIGHTS

- The murine *ex vivo* potency of BoNT/D is 3.7 times lower compared to BoNT/A.
- 110-fold higher protein dosage of BoNT/D achieved the same clinical effect as incobotulinumtoxinA.
- BoNT/D constitutes a potential treatment alternative for BoNT/A & B non-responder.

ABSTRACT

Objectives: Botulinum neurotoxin serotypes A and B (BoNT/A & B) are highly effective medicines to treat hyperactive cholinergic neurons. Due to neutralizing antibody formation, some patients may become non-responders. In these cases, the serotypes BoNT/C-G might become treatment alternatives. BoNT/D is genetically least related to BoNT/A & B and thereby circumventing neutralisation in A/B non-responders. We produced BoNT/D and compared its pharmacology with BoNT/A *ex vivo* in mice tissue and *in vivo* in human volunteers.
Methods: BoNT/D was expressed recombinantly in *E. coli*, isolated by chromatography and its *ex vivo* potency was determined at mouse phrenic nerve hemidiaphragm preparations. Different doses of BoNT/D or incobotulinumtoxinA were injected into the *extensor digitorum brevis* (EDB) muscles (n = 30) of human volunteers. Their compound muscle action potentials were measured 11 times by electroneurography within 220 days.
Results: Despite a 3.7-fold lower *ex vivo* potency in mice, a 110-fold higher dosage of BoNT/D achieved the same clinical effect as incobotulinumtoxinA while showing a 50% shortened duration of action.

Abbreviations: BoNT/A-H, X, J, Botulinum neurotoxin serotype A-H, X, J; CMAP, compound muscle action potential; EDB, *M. extensor digitorum brevis*; ENG, electroneurography; MPN hemidiaphragm assay, mouse phrenic nerve hemidiaphragm assay; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosome associated protein of 25 kDa; VAMP, vesicle associated membrane protein.

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Conclusions: BoNT/D blocks dose-dependently acetylcholine release in human motoneurons upon intramuscular administration, but its potency and duration of action is inferior to approved BoNT/A based drugs.
Significance: BoNT/D constitutes a potential treatment alternative for BoNT/A & B non-responders.

1. Introduction

Botulinum neurotoxins (BoNT) belong to the most toxic substances that nature ever created (Gill, 1982). They inhibit the neuronal release of acetylcholine resulting in flaccid paralysis. Therapeutically, BoNT is applied in patients with dystonia, spasticity, pain, hypersalivation, and hyperhydrosis (Bigalke, 2013; Ward et al., 2006).

Seven different BoNT serotypes have been characterised (BoNT/A-G), and three more serotypes (BoNT/HA aka H/FA, X and eBoNT/J) are suggested (Peck et al., 2017; Zhang et al., 2017; 2018; Brunt et al., 2018). Each serotype shows differences in the molecular intoxication mechanism, potency, and duration of action (Foran et al., 2003; Kutschenko et al., 2017; Rummel et al., 2011; Rummel, 2015). BoNTs are classical AB toxins and consist of a 100 kDa heavy chain and a 50 kDa light chain connected by a disulphide bond. The metalloproteolytic light chain cleaves one of the three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins that are part of the vesicle exocytosis process (Brunger and Rummel, 2009; Rummel, 2015). VAMP (vesicle associated membrane protein)/synaptobrevin-1/-2 are cleaved by BoNT/B, D, F, G, H, X and J whereas SNAP-25 (synaptosome associated protein of 25 kDa) is the substrate for BoNT/A, C, E and also eBoNT/J (Rossetto et al., 2014; Rummel, 2015; Zhang et al., 2017; 2018).

Only BoNT/A (subtype A1) and BoNT/B (subtype B1) are approved drugs in the European Union (EU) and North America (BoNT/A: onabotulinumtoxinA, incobotulinumtoxinA and abobotulinumtoxinA; BoNT/B: rimabotulinumtoxinB) (Bigalke, 2013; Ward et al., 2006). Due to a transient effect, patients have to be regularly injected with BoNT/A about every 3 months. However, a long-term treatment increases the risk of developing BoNT neutralizing antibodies. As a consequence, patients may become secondary non-responsive to the approved BoNT/A and B drugs (Dressler, 2002; Göschel et al., 1997; Lange et al., 2009). For these individuals, the remaining serotypes might be considered as treatment alternatives.

However, those potential therapeutic agents show different restrictions: Only a single case of infant botulism caused by BoNT/C has been reported so far (Oguma et al., 1990), presumably due to a species selectivity of the neurotoxin associated proteins (hemagglutinins) for birds and cattle (Jin et al., 2009). Nevertheless, BoNT/C was shown to exhibit a duration of action almost as long as BoNT/A in human extensor digitorum brevis muscle (EDB) (Eleopra et al., 1997). At pharmaceutically irrelevant concentrations it also induced neurodegeneration and apoptosis *in vitro* (Berliocchi et al., 2005; Igarashi et al., 1996; Kurokawa et al., 1987; Osen-Sand et al., 1996; Peng et al., 2013; Williamson et al., 1998; Zhao et al., 2010). Despite a similar maximal effect on day 7, intramuscular injections of BoNT/E into human EDB resulted in a much shorter duration of paresis (~6 weeks) compared to injections of BoNT/A (4–6 months) (Eleopra et al., 1998; Montecucco and Molgo, 2005). BoNT/F was also proven to be clinically effective in patients with BoNT/A secondary resistance, but its duration of effect lasted only 1–2 months (Sheean and Lees, 1995; Eleopra et al., 2004). Even higher doses of BoNT/F resulted in a shorter duration of neuromuscular junction blockade compared to BoNT/A and, besides an increased risk of undesired side effects, 3 of 5 patients lost the initial clinical benefit (Greene and Fahn, 1996; Houser et al., 1998). Up to now *C. botulinum* type G has been isolated only twice from soil samples from Argentina, for the first time in 1969 (Gimenez and Ciccarelli, 1970), and in necropsy specimens of unexplained death in adults and infants in Switzerland together with its associated neurotoxin (Sonnabend et al., 1981 and 1985). The limited potency of BoNT/G vs. BoNT/B e.g. in the mouse phrenic nerve hemidiaphragm assay restricts its pharmaceutical applications (Rummel et al., 2004 and 2007). The recently discovered mosaic serotype BoNT/HA displays 5-fold lower specific toxicity in mice than BoNT/A, but exhibits 4-fold higher potency in human induced pluripotent stem cell (hiPSC) derived neurons (Pellet et al., 2016) which needs confirmation in human neuromuscular tissue to justify its development as a therapeutic agent. However, due to its mosaic structure comprising a 50 kDa H_C fragment 84% identical to that of BoNT/A1, BoNT/HA is neutralised by anti-BoNT/A antitoxin (Maslanka et al., 2016) and therefore not suitable as treatment alternative for BoNT/A-secondary non-responders. The latest serotypes identified, BoNT/X and eBoNT/J, showed as enzymatically ligated BoNT/X only at a dosage of 0.5 μg hind limb paralysis in mice (Zhang et al., 2017) and as ligated eBoNT/J at a dosage of 1.0 μg no hind limb paralysis in mice (Zhang et al., 2018), respectively, which does not allow any conclusions about their therapeutic potential.

Only one patient with botulism due to intoxication with BoNT/D has been reported to date (Demarchi et al., 1958; Prevot and Sillioc, 1959). In contrast, many cases of botulism in cattle and horses have been caused by BoNT/D (Lindström et al., 2010). Although BoNT/D seems to be prevalent over the world, humans are not a natural target for BoNT/D (Carpenter, 1967; Coffield et al., 1997; Sakaguchi, 1983). In congruence to this, it was shown that doses of BoNT/D, equivalent to BoNT/A, did not block neuromuscular transmission in human pyramidalis muscle preparations (Coffield et al., 1997). Recently, Eleopra et al. (2013) demonstrated in a small (n = 10 EDBs) *in vivo* study that BoNT/D is almost ineffective in human EDB in a dose up to 10 mouse LD_{50} units (=160 pg BoNT/D). Along that line, by detecting VAMP2 and SNAP-25 cleavage as read-out for potency of BoNT/D and A, BoNT/D exhibited a 25-fold and 150-fold lower potency than BoNT/A in two cultured hiPSC-derived neuronal populations, respectively. The maximum effect of BoNT/D action in hiPSC-derived neurons occurred on day 2 and lasted for 2–3 weeks (Pellett et al., 2015). Noteworthy, while BoNT/D and A displayed similar specific toxicities upon i.p. injection in mice, BoNT/D was 20-fold less potent in primary mouse spinal cord neurons than BoNT/A. In contrast, intercostal nerves from human muscle biopsies were found to be similarly sensitive to BoNT/D as to BoNT/A, B, and E in an *ex vivo* twitch assay (Anderson et al., 2009).

The present study tested the *in vivo* effect of six escalating doses of BoNT/D compared to three increasing doses of BoNT/A over a period of 220 days following the human extensor digitorum brevis muscle (EDB) injections (n = 30). We were able to demonstrate that BoNT/D is effective in paralysing human EDB muscles. However, for the same maximal effect in comparison to BoNT/A, a 110-fold higher protein dose of BoNT/D had to be applied. Despite the identical maximal effect, the duration of the BoNT/D action was twice as short as the duration of BoNT/A.

2. Methods

2.1. Recombinant production of botulinum neurotoxin D

The plasmid encoding tag-free full length BoNT/D (pBoNTD; *C. botulinum* strain BVD/-3, acc. no. X54254) was generated by PCR using suitable primers and plasmid pBoNTDS described previously (Bade et al., 2004; Binz et al., 1990) as template DNA. Correctness of pBoNTD was verified by DNA sequencing.

Full length single chain BoNT/D was recombinantly expressed under biosafety level 2 containment (project number GAA A/Z 40654/3/123) in the *E. coli* strain M15[pREP4] (Qiagen), following 16 h of induction at 22 °C. Full length single chain BoNT/D was isolated from total cell lysate by negative ion exchange chromatography (HiPrep 16/10 Q FF, GE Healthcare) in 50 mM NaP pH 7.5, 50 mM NaCl and hydrophilic interaction chromatography (HiTrap Phenyl Sepharose HP, GE Healthcare) in 50 mM NaP pH 7.5, 500 mM ammonium sulfate. Subsequently, full length single chain BoNT/D was proteolysed into the active dichain BoNT/D by trypsin immobilized on agarose beads for 10 min at 37 °C (0.01 U/μg BoNT, 24 U/ml beads, T4019, Sigma Aldrich). Degree of proteolysis into 50 kDa LC and 100 kDa HC was verified by SDS-PAGE. The supernatant was subjected to size exclusion chromatography (Superdex 200 10/30, GE Healthcare) in 20 mM HEPES pH 7.5, 150 mM NaCl to remove any remaining trypsin and other impurities. Fractions containing BoNT/D were pooled, shock frozen in liquid nitrogen, and stored at −70 °C. Protein concentration of purified BoNT/D was determined with BSA as reference by SDS-PAGE, Coomassie staining and densitometry.

2.2. Potency of botulinum neurotoxin D

All experiments were performed in accordance with the German Tierschutzgesetz (TierSchG, 29th March 2017) and Tierschutz-Versuchstierverordnung (TierSchVersV, 1st August 2013) and with the guidelines established by the European Community Council Directive n° 2010/63/EU and approved by the local authority veterinary services (Veterinäramt Hannover, protocol file number §4 2018/209). The mouse phrenic nerve (MPN) hemidiaphragm assay was performed as described previously (Bigalke and Rummel, 2015; Kutschenko et al., 2017): "The tissue was dissected from euthanized 25–30 g NMRI mice (Janvier SAS, France). The phrenic nerve was continuously stimulated with 5–25 mA at a frequency of 1 Hz with a 0.1 ms pulse duration. Isometric contractions were measured using a force transducer and recorded with VitroDat Online software (FMI GmbH, Seeheim, Germany). The time required for the amplitude to decrease to 50% of the starting value (paralytic half-time) was determined."

To compare the potency of BoNT/D with BoNT/A (subtype BoNT/A1; List Laboratories Inc.), the drug substance of incobotulinumtoxinA, concentration-response curves were constructed from three data points, each determined at least in triplicate, fitted to the logarithmic functions $y(\text{BoNT/D}) = -25.548\ln(x) + 233.4$, $R^2 = 1.0$ and $y(\text{BoNT/A}) = -37.817\ln(x) + 259.88$, $R^2 = 0.9885$. Due to the similar slope of both curves the difference in potency was determined by converting an arbitrary paralytic half-time of 75 min into the corresponding concentrations of BoNT/D (493 pg/ml) and BoNT/A (133 pg/ml), respectively.

2.3. In vivo effect of botulinum neurotoxin D

For injection, the BoNT/D stock solution was sterile filtered in a class II safety cabinet, diluted into six different doses of BoNT/D with sterile 0.9% NaCl / 0.1% HSA solution and filled in 1 ml syringes ready to use. Control measurements immediately after dilution and filling as well as prior to injection into subjects revealed the expected paralysis times in the MPN hemidiaphragm assay and thus confirmed stability of biological activity of the filled material.

All 15 healthy male subjects (33.1 ± 4.9 years), scientists and neurologists at our departments, voluntarily joined the self-experiment and co-authored this study. The self-experiment was noted by the Ethics Committee of Hannover Medical School, but consultation / evaluation of a self-experiment by the members of the Ethics Committee was not required.

Both *extensor digitorum brevis* (EDB) muscles of these 15 volunteers were injected with different doses of the recombinant BoNT/D or the approved drug incobotulinumtoxinA (Xeomin®), respectively. The injection volume of 400 μl per EDB was fixed. Six different doses of BoNT/D were each injected into a group of n = 4 EDBs (280, 560, 1120, 2240, 4480, 8960 pg). Three different doses of incobotulinumtoxinA (4 U = 20 pg, 16 U = 80 pg, 32 U = 160 pg; 1 U = 1 MLD = ~5 pg BoNT/A; Frevert, 2015) were injected into 2 EDBs, respectively. Electroneurography (ENG) examinations, performed according to standard methodology (Kimura 2001), were used to assess the effects of dose and concentration on the target muscle (EDB). Examinations were performed (using surface electrodes) at day 0, 2, 6, 14, 28, 56, 84, 112, 140, 168, 220. At each time point, the peroneal nerve was supramaximally stimulated at the level of the ankle joint; recordings were then taken from the EDB and base-to-peak compound muscle action potential (CMAP) amplitude was measured (Nicolet EDX, Natus neurology). The stimulation distance depended on the size of the foot, but it was kept constant for each volunteer for the duration of the study.

2.4. Statistics

For evaluation of the clinical data the basic value before injection was set to 100% and relative reduction of CMAP was analyzed and given as mean ± standard error of the mean (SEM). To check for significance unpaired t-test was performed. $P < 0.05$ was set as significant, $p < 0.01$ was defined as very significant, and $p < 0.001$ was specified as highly significant. The statistics and figures were performed with Microsoft Excel 2016 and MATLAB R2017b.

3. Results

3.1. Production and characterization of recombinantly produced BoNT/D

BoNT/D devoid of any affinity tag was recombinantly expressed in *E. coli* K12 strain and isolated from cell lysate by ion exchange and hydrophilic interaction chromatography as single chain 150 kDa polypeptide (Fig. 1). To gain biological activity, the single chain BoNT/D had to be proteolysed into a 50 kDa LC and a 100 kDa HC which remained covalently linked by a disulphide bridge conserved in all BoNT serotypes. Time-limited trypsinization yielded >90% dichain BoNT/D (Fig. 1B) which was held together by an intact disulphide bridge according to non-reducing SDS-PAGE analysis (Fig. 1C). Overall yield of BoNT/D displaying a purity of >95% was 1.7 mg BoNT/D per liter of *E. coli* culture.

Potency of BoNT/D was determined employing an *ex vivo* animal replacement test, the MPN hemidiaphragm assay. The activated di-chain BoNT/D exhibited a dose-dependent, highly potent biological activity in the subnanogram/ml range (Fig. 2). Compared to the dose-response curve obtained for BoNT/A, the drug substance of incobotulinumtoxinA isolated from *C. botulinum* culture supernatant, the biological activity of the recombinantly produced BoNT/D is about 3.7-fold lower than BoNT/A in mouse muscle tissue.

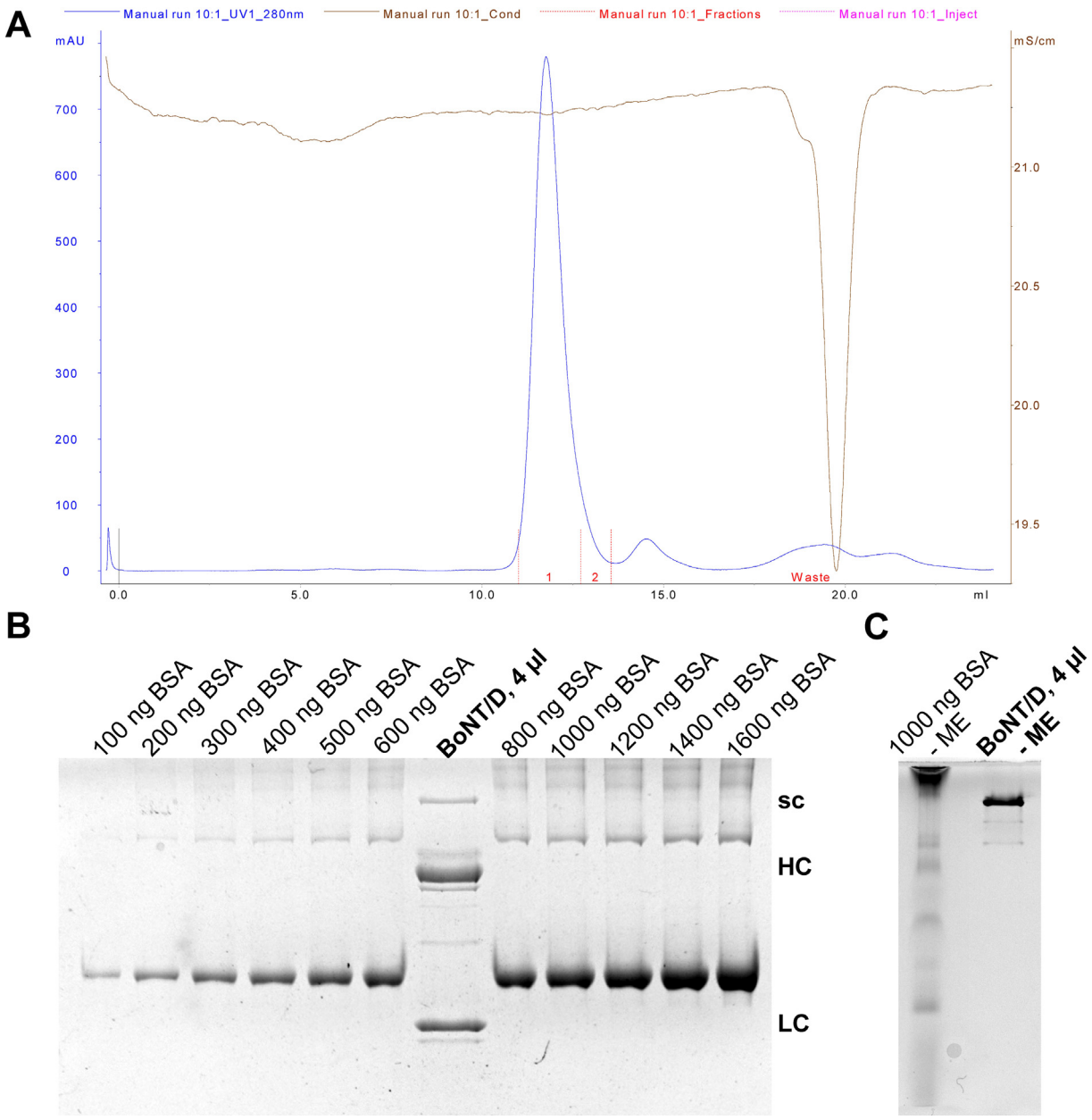

Fig. 1. A Size exclusion chromatography of BoNT/D nicked by immobilised trypsin. B 12.5% reducing SDS-PAGE analysis of peak fraction 1 and determination of BoNT/D protein concentration employing BSA as reference protein. sc, 150 kDa single chain; HC, 100 kDa heavy chain; LC, 50 kDa light chain of BoNT/D. C SDS-PAGE analysis of peak fraction 1 in the absence of β-mercaptoethanol (ME).

3.2. Injections of BoNT/D result in a dose-dependent reduction of CMAP amplitude in human EDB muscle

Six different doses of BoNT/D (280, 560, 1120, 2240, 4480, 8960 pg) and three different doses of incobotulinumtoxinA (20, 80, and 160 pg) in a fixed volume of 400 µl were injected into the EDB muscles of human volunteers. No adverse effects were observed in any subject. Second ENG-measurement was performed on day 2 post injection to monitor onset of action. The dose 8960 pg BoNT/D already caused a 30% reduction in CMAP amplitude. In the first week following the injections a relative decrease of CMAP amplitude occurred in each dose group. Related to basic value this decrease of CMAP amplitude was significant in the unpaired t-test after injections of the three highest doses of BoNT/D over a period of one to four weeks (2240 pg BoNT/D: $p < 0.05$ at day 6 and day 14, $p < 0.01$ at day 28; 4480 pg BoNT/D: $p < 0.01$ at day 6 and day 14; 8960 pg BoNT/D: $p < 0.05$ at day 2, $p < 0.001$ at day 6, day 14, and day 28). Subsequently, during the following weeks CMAP amplitude recovered until baseline level was reached again (Fig. 3A).

Fig. 3B illustrates that the effect of the highest dose of BoNT/D (8960 pg) was less than of the highest dose of incobotulinumtoxinA (160 pg). Compared to baseline level the decrease of CMAP amplitude after injection of 160 pg incobotulinumtoxinA was significant from day 6 to day 28 ($p < 0.05$ at day 6 and day 14, $p < 0.01$ at day 28) and after injection of 8960 pg BoNT/D from day 2 to day 28 ($p < 0.05$ at day 2, $p < 0.001$ at day 6, day 14, and day 28). Even the two lower doses of incobotulinumtoxinA (20 pg and 80 pg) induce a very distinct reduction of CMAP amplitude that is clearly more pronounced than the effect after injections of the lowest dose of BoNT/D (280 pg). Furthermore, subjects injected with BoNT/D recovered faster than subjects injected with incobotulinumtoxinA (Fig. 3B).

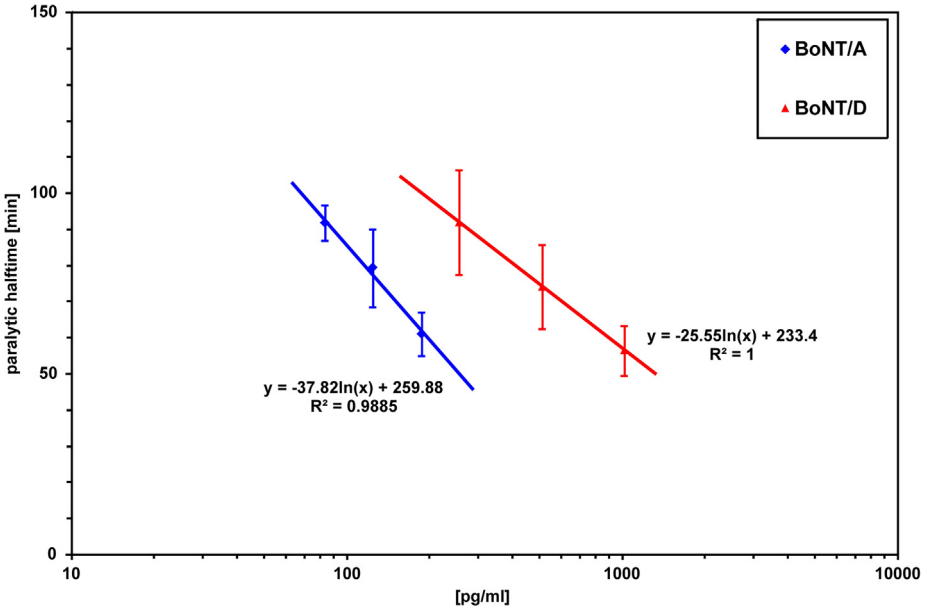

Fig. 2. Determination of biological activity of BoNT/D and BoNT/A, the drug substance of incobotulinumtoxinA, employing the *ex vivo* mouse phrenic nerve hemidiaphragm (MPN) assay, n = 3–5 ± SD.

3.3. Comparison of in vivo potency of BoNT/D vs. incobotulinumtoxinA

The clinical benefit of botulinum neurotoxins is based on the maximal effect and duration of action. The maximal effect at day six following the injections of BoNT/D and of incobotulinumtoxinA were compared (Fig. 4A). These data demonstrate that the maximal effect of CMAP amplitude reduction is dose-dependent for injection of incobotulinumtoxinA as well as of BoNT/D. Unpaired t-test was done to compare effect on day 6 with baseline CMAP amplitude. It declared significant reduction of CMAP amplitude for the three highest doses of BoNT/D ($^{*}p < 0.05$ for 2240 pg, $^{**}p < 0.01$ for 4480 pg, and $^{***}p < 0.001$ for 8960 pg) as well as for the highest dose of incobotulinumtoxinA ($^{*}p < 0.05$ for 160 pg).

The lowest dose of incobotulinumtoxinA (20 pg) results in a CMAP amplitude reduction up to 57.9 ± 23.5%. A similar CMAP amplitude reduction occurs after injections of 2240 pg BoNT/D (59.8 ± 12.8%) and 4480 pg BoNT/D (58.9 ± 9.9%), respectively. This suggests that a 110- to 220-fold higher dose of BoNT/D in comparison to incobotulinumtoxinA is required for the identical effect. Correlating with these data, a 110-fold higher dose of BoNT/D (8960 pg) is needed (43.6 ± 9.2%) to achieve a similar CMAP reduction as upon injection of 80 pg incobotulinumtoxinA (48.5 ± 19.9%).

Despite a similar maximal CMAP reduction following an injection with either incobotulinumtoxinA or with a 110-fold higher protein dose of BoNT/D, the duration of effect, defined as the period until >95% of baseline CMAP amplitude were reached again, was different (Fig. 4B). Our data demonstrate that increasing doses of incobotulinumtoxinA or of BoNT/D result in a longer duration of action until a ceiling effect is reached. But nonetheless, incobotulinumtoxinA displays a two-fold longer duration of action at similar maximal effect. Displaying a similar maximal effect on day six, the effect of 20 pg incobotulinumtoxinA was present for 150 days, whereas the effect of 2240 pg and 4480 pg BoNT/D was only detectable for 62 and 83 days, respectively. Injections of 80 pg incobotulinumtoxinA (180 days) resulted in a 1.9-fold longer duration of action in comparison to 8960 pg BoNT/D (96 days). Due to the small group sizes these effects were not calculated as significant by the unpaired t-test. But when the different dose groups of each serotype were pooled (2240, 44480, and 8960 pg BoNT/D vs. 20 and 80 pg BoNT/A) unpaired t-test revealed significant effect times ($p < 0.05$).

4. Discussion

Recently, Albrecht et al. studied 596 patients treated for 3–6 years with three different BoNT/A based pharmaceuticals for different indications: facial hemispasm, blepharospasm, cervical dystonia (CD), other dystonia, and spasticity. Altogether 83 patients (13.9%) displayed measurable anti-BoNT/A neutralizing antibodies (NABs). Already 15.7% of CD patients and 17.3% of other dystonia patients displayed NABs which means that in high dose indications a sixth of the patient population becomes untreatable within 3–6 years (Albrecht et al., 2019). Hence, long-term treatment side effects remain an important issue and treatment alternatives are heavily needed. Due to this secondary therapy failure by anti-BoNT/A and B neutralizing antibodies, efforts are made to find alternative BoNT injection therapies that avoid antibody cross reactivity. A promising alternative method could be injections of a BoNT serotype different from BoNT/A and B. Of the seven established serotypes, BoNT/D shares only 32.9% and 34.2% amino acid sequence identity with BoNT/A and B, respectively, thus being together with BoNT/C their least related serotype (Rummel, 2015). This low degree of similarity results in different antigenicity and likely circumvents their neutralisation in A/B non-responders (Bowmer 1963, Hansbauer et al., 2016). In addition, BoNT/D is highly potent in mice displaying a specific toxicity close to BoNT/A (1.15×10^8 i.p. LD_{50} Units/mg vs. 1.25×10^8 i.p. LD_{50} Units/mg) (Pellett et al., 2015).

Here, we successfully produced a recombinant tag-free BoNT/D displaying 3.7-fold lower potency in the *ex vivo* MPN hemidiaphragm assay than the reference BoNT/A (2×10^8 i.p. LD_{50}-Units/mg; Frevert, 2010; 2015). The 3.7-fold difference in potency is in accordance with previous results obtained for recombinant BoNT/A vs recombinant BoNT/D (Weisemann et al., 2015). Thus, the specific toxicity of our recombinant BoNT/D would be estimated as 0.55×10^8 i.p. LD_{50} Units/mg which is virtually identical to that of the native BoNT/D previously applied in human EDB (Eleopra et al., 2013).

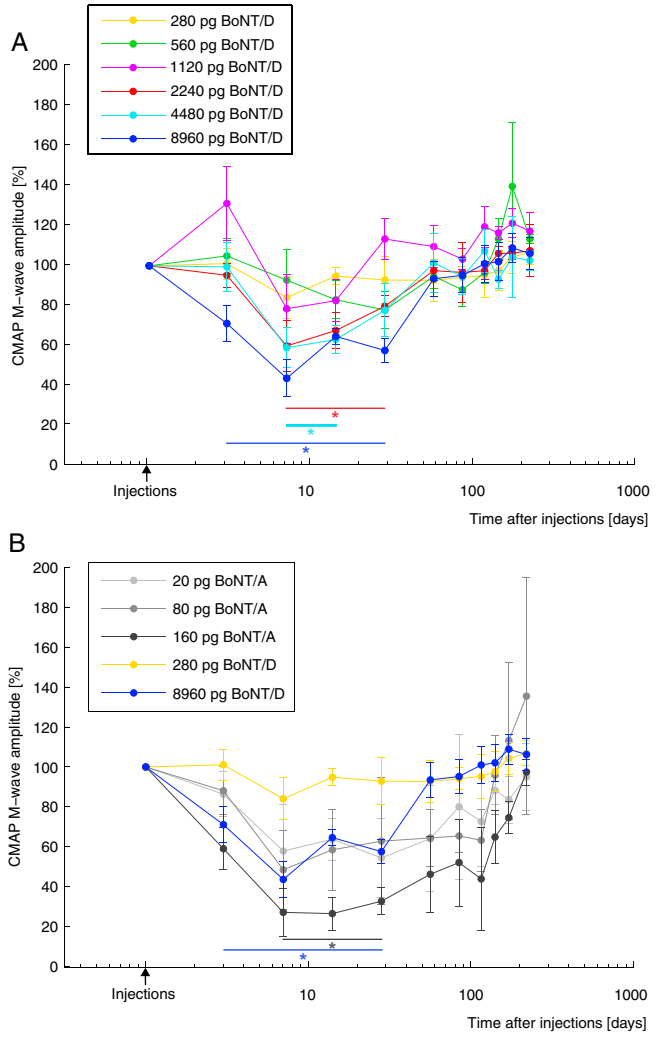

Fig. 3. Time course of mean values of EDB CMAP M-wave amplitude during the study relative to basic value before injection. Basic value was determined as 100%. A Effect of six doses of BoNT/D (n = 4 for each dose; 280 pg, 560 pg, 1120 pg, 2240 pg, 4480 pg, 8960 pg) during visit 1 to 10. Compared to basic value before injection, unpaired t-test indicated significant results (*p < 0.05) for 2240 pg BoNT/D (red) from day 6 to day 28 (p < 0.05 at day 6 and day 14, p < 0.01 at day 28), for 4480 pg BoNT/D (light blue) from day 2 to day 14 (p < 0.01 at day 6 and day 14), and for 8960 pg BoNT/D (dark blue) from day 2 to day 28 (p < 0.05 at day 2, p < 0.001 at day 6, day 14, and day 28). B Comparison of the highest and lowest dose of BoNT/D (280 pg and 8960 pg) vs. three doses of incobotulinumtoxinA (BoNT/A1; n = 2 for each dose; 20 pg, 80 pg, 160 pg) during visit 1 to 10. Decrease of CMAP amplitude was significant (*p < 0.05) in the unpaired t-test compared to baseline level before injection for 160 pg BoNT/A (dark grey) from day 6 to day 28 (p < 0.05 at day 6 and day 14, p < 0.01 at day 28) and for 8960 pg BoNT/D (dark blue) from day 2 to day 28 (p < 0.05 at day 2, p < 0.001 at day 6, day 14, and day 28). Data are expressed as mean ± SEM.

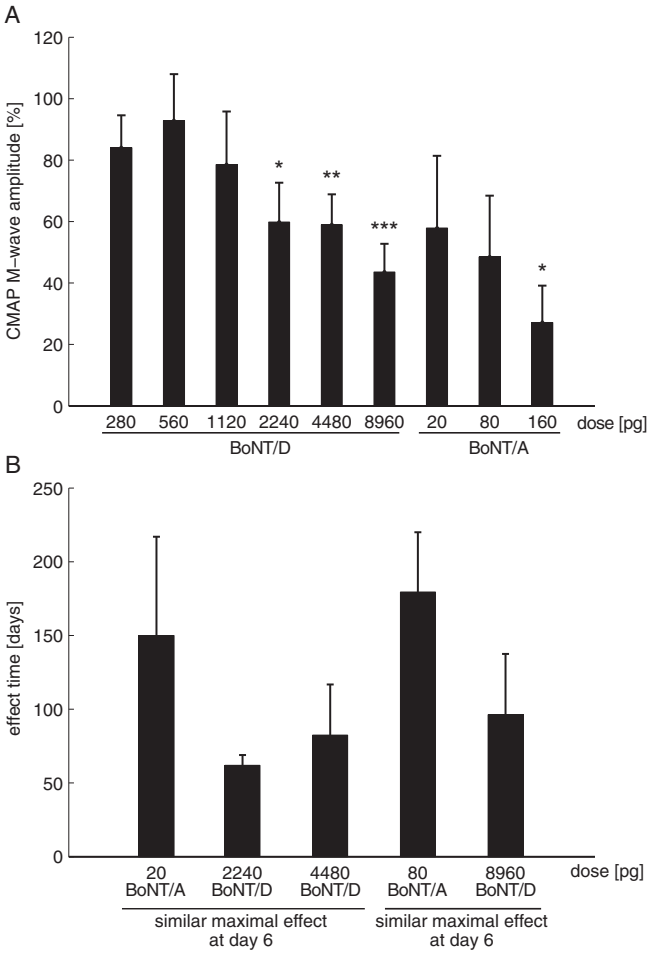

Fig. 4. A Comparison of maximal effect at day six post injections of six different doses of BoNT/D (n = 4 for each dose; 280 pg, 560 pg, 1120 pg, 2240 pg, 4480 pg, 8960 pg) and 3 different doses of incobotulinumtoxinA (n = 2 for each dose; 20 pg, 80 pg, 160 pg). Data are stated as mean values of EDB CMAP M-wave amplitude in percentage relative to basic value (basic value = 100%) ± SEM. Unpaired t-test revealed significant results at day 6 compared to baseline level for 2240 pg, 4480 pg, and 8960 pg BoNT/D as well as for 160 pg BoNT/A. * is set as p < 0.05, ** means p < 0.01 and *** reveals p < 0.001. B Time in days after BoNT injections until >95% recovery. BoNT doses showing the same maximal effect six days post injections (40–60% reduction of CMAP) are compared (2240 pg, 4480 pg and 8960 pg BoNT/D vs. 20 pg and 80 pg incobotulinumtoxinA, respectively). Data are given as mean ± SEM. Unpaired t-test of the pooled data of the three BoNT/D doses and the two BoNT/A doses revealed significant results (p < 0.05).

The present study demonstrates that BoNT/D is effective in inducing paresis of the human EDB muscle upon local injections. However, to evoke a similar decrease of CMAP amplitude compared to BoNT/A, a 110-fold higher BoNT/D protein dose is needed. Considering the 3.7-fold lower potency of BoNT/D in mice, BoNT/D is 30-fold less potent than BoNT/A in humans. For comparison, subjects with CD required ~40-fold higher dosage of rimabotulinumtoxinB (BoNT/B) as of onabotulinumtoxinA for equivalent benefit (205 ± 50 i.p. LD_{50} Units onabotulinumtoxinA vs 8520 ± 1892 i.p. LD_{50} Units rimabotulinumtoxinB) indicating the established use of higher BoNT protein dosage (Comella et al., 2005). In addition, the median time to loss of benefit was 14.0 weeks for onabotulinumtoxinA and 12.1 weeks for rimabotulinumtoxinB (Comella et al., 2005), while the duration of effect of BoNT/D in our study was twice as short as of BoNT/A.

Congruently to our results another study demonstrated *in vitro* in cultured human neurons and *in vivo* in mice a lower potency and a two-fold faster recovery after local injections with BoNT/D in comparison to injections with BoNT/A. Accordingly, a higher dose of BoNT/D (2 U) was needed to induce a maximal effect comparable to that of BoNT/A (0.75 U) and the BoNT/D maximal effect was delayed by ~24 h (Pellett et al., 2015). Furthermore, the time to death of intraperitoneally BoNT/D-injected mice (8.6 ng) was significantly longer compared to mice injected with BoNT/A (8 ng; Pellett et al., 2015). On the other hand, Eleopra's results showing that 0.1 nM BoNT/D with a specific toxicity of 0.6×10^8 i.p. LD_{50} Units/mg paralyzes the mouse hemidiaphragm more rapidly than 0.1 nM BoNT/A with a specific toxicity of 0.4×10^8 i.p. LD_{50} Units/mg are plausible (Eleopra et al., 2013). The serotype difference in occurrence of maximal effect is in contrast to our results which demonstrate a maximal effect of BoNT about six days

following the intramuscular injections of BoNT independent of the injected serotype. One explanation of the differently observed time courses of onset of paresis could be the diverging types of application of BoNT (intraperitoneal injection, intramuscular injection, or *ex vivo* application). Also the species dependent variations of VAMP1 isoforms could possibly affect the time course of BoNT/D-induced actions.

Recent studies have shown that BoNT/D is able to effectively cleave VAMP2 strictly conserved in mouse, rat and human, but not the human isoform of VAMP1 (Eleopra et al., 2013, Pellett et al., 2015, Yamamoto et al., 2012). Human VAMP1 varies from that of other species in the substitution of one amino acid at position 48 (I48 versus M48) resulting in a pronounced reduction of cleavability by BoNT/D (Peng et al., 2014, Yamasaki et al., 1994). This substitution could be one explanation aside species differences in intestinal absorption and neuronal target recognition why only one patient with mild botulism due to BoNT/D has been described in contrast to many cases in wild animals and livestock (Demarchi et al., 1958, Lindström et al., 2010).

Eleopra et al. (2013) demonstrated that injections of 48 pg and 160 pg BoNT/D in the EDB muscle of human volunteers induced no measurable paresis and a short (1 week) and slight (~10% CMAP reduction) paresis, respectively, suggesting absence of efficacy of BoNT/D in humans. A restriction of this study was that only 2 different doses of BoNT/D were tested distinguishing only by factor 3. Our lowest dose of 280 pg BoNT/D is just 1.75-fold larger than their highest dose and exhibits >15% reduction of CMAP thereby extending their findings. Further dose elevations up to 8960 pg enhanced CMAP reductions without observing adverse effects in our human volunteers. For safety reasons, higher dosages were not administered. Based on the safe outcome of our current study dosages above 8960 pg BoNT/D could be considered in a subsequent study to achieve maximal clinical benefit. It could be concluded that the hardly detectable effect after intramuscular injection of BoNT/D in the study by Eleopra et al. (2013) is due to a too low BoNT/D dose.

5. Conclusions

The results of our study present that BoNT/D is effective in inducing muscle paresis in humans. However, to achieve the same clinical therapeutic effect as by BoNT/A-based drugs at least 110-fold higher protein doses of BoNT/D and two-fold more injections per annum will be needed. Since the risk of inducing BoNT antibodies increases when the injected BoNT dose is higher and the interval is shorter (Lange et al., 2009), it can be assumed that a therapy with injections of high doses of BoNT/D enhances the risk of developing BoNT/D-neutralizing antibodies. Due to this assumption, a primary injection therapy with BoNT/D is not a treatment alternative to BoNT/A injections. However, for patients with an anti-BoNT/A & B antibody induced secondary therapy failure, BoNT/D injections constitute a promising therapeutic alternative due to its high sequence divergence and divergent antigenicity. In future, the effectiveness of BoNT/D injection therapy in BoNT/A & B secondary non-responding patients frequently associated with high-dose indications like CD, other dystonia and spasticity needs to be demonstrated.

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Competing financial interests

K.K. received travel grants and honoraria for lectures from Allergan, Biogen, Ipsen and Merz.

T.H.C.K. used to be members of the advisory board of Allergan (04/12-09/14). Michelle Magid and the employers of Tillmann H. C. Krüger and M. Axel Wollmer have filed a patent on the use of botulinum toxin in the treatment of personality disorders. Tillmann H. C. Krüger received honoraria from Allergan, Lilly, Lundbeck, Otsuka, Schwabe, Servier and Trommsdorf for lectures and advisory board activities. A.R. serves as key member of Revance's Scientific Advisory Board.

Author contributions

J.We. performed the cloning, protein expression and purification of BoNT/D. A.R. performed the MPN assay with support of Nadja Krez. K.W. injected T.F., G.A., S.A., S.B., C.E., N.G., S.G., S-B. K., R.K., T.H.C.K., T.S., S.S., P.T., F.W., J.W. K.K. and K.W. supervised electroneurography (ENG) measurements. A.K., F.W., H.B., K.W. and A.R. analysed the data and wrote the manuscript with input from all other authors.

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