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Replacing the mouse bioassay for diagnostics and potency testing of botulinum neurotoxins – progress and challenges

Ersatzmethoden für den Maus-Bioassay zur Diagnostik und Aktivitätsbestimmung von Botulinum Neurotoxinen – Fortschritte und Herausforderungen

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Summary

Botulinum neurotoxins (BoNTs) are the most potent toxins known and the causative agents of the rare but potentially life-threatening disease botulism. The elaborate mode of action of BoNTs at the molecular level, their exquisite specificity for peripheral motor neurons, and their ability to effectively inhibit neurotransmitter release led to the development of BoNTs into highly valued pharmaceutical products. Both diagnostics of botulism and potency testing of pharmaceutical BoNT preparations still employ the mouse bioassay as "gold standard assay". This animal experiment can pose a heavy burden on the animal, including a fatal outcome of testing. Additionally, several analytical disadvantages have been described. Consequently, the development of animal replacement methods is a long pursued goal which has been focused mainly on replacement methods for pharmaceutical potency testing so far. However, fundamentally different requirements and challenges apply for diagnostics of botulism and potency testing of BoNT pharmaceuticals, which necessitates the development of different assays tailored for each purpose. Here we review the underlying causes for this intricacy which are rooted in both the biological characteristics of the BoNTs as well as assay specific requirements. We review different functional assays that have been developed to replace the mouse bioassay. Despite significant progress in recent years, further substantial work is needed to pave the way for a fully validated replacement for the mouse bioassay for botulism diagnostics.

Keywords: botulinum neurotoxins, botulism, diagnostics, mouse bioassay, replacement methods, functional assays

Zusammenfassung

Botulinum Neurotoxine (BoNTs) sind die giftigsten bekannten Substanzen und ursächlich für die seltene, aber lebensbedrohliche Erkrankung Botulismus. Aufgrund ihres raffinierten molekularen Wirkmechanismus, ihrer exquisiten Spezifität für periphere Motorneuronen und ihrer Fähigkeit zur effektiven Blockade der Neurotransmitter-Ausschüttung wurden zudem auf BoNT basierende, hochwirksame pharmazeutische Produkte entwickelt. Sowohl in der Botulismus-Diagnostik als auch bei der Wirksamkeitsprüfung pharmazeutischer Präparate wird nach wie vor der Maus-Bioassay als „Goldstandardmethode" verwendet. Dieser Tierversuch kann mit schweren Belastungen bis hin zum Tod der Tiere einhergehen. Darüber hinaus weist der Assay verschiedene analytische Schwächen auf. Folglich stellt die Entwicklung einer Tierversuchsersatzmethode ein seit langem verfolgtes Unterfangen dar, welches bislang hauptsächlich auf die Entwicklung von Ersatzmethoden für die pharmazeutische Wirksamkeitsprüfung fokussiert war. Fundamental unterschiedliche Anforderungen an die Botulismus-Diagnostik und die Wirksamkeitsprüfung erfordern jedoch die Entwicklung verschiedener, zielgerichteter Nachweisverfahren für beide Zwecke. Der vorliegende Übersichtsartikel erläutert die zugrunde liegenden Ursachen, welche sowohl auf biologischen Eigenschaften der BoNTs als auch auf Assay-spezifischen Besonderheiten basieren, und stellt eine Übersicht verschiedener funktioneller Methoden zusammen, die zum

Ersatz des Maus-Bioassays entwickelt wurden. Trotz bedeutender Fortschritte in den letzten Jahren sind weitere umfassende Arbeiten notwendig, um zu einer vollständig validierten Tierversuchsersatzmethode für den Maus-Bioassay zur Botulismus-Diagnostik zu gelangen.

Schlüsselwörter: Botulinum Neurotoxine, Botulismus, Diagnostik, Maus-Bioassay, Tierversuchsersatzmethode, Funktionelle Methoden

Botulism and the family of botulinum neurotoxins

Botulism is a rare but potentially life-threatening disease affecting both humans and animals and is caused by botulinum neurotoxins (BoNTs) (Johnson and Montecucco 2008). The toxins are produced by anaerobic spore forming bacteria of the genus *Clostridium (C.)*, namely *C. botulinum, C. baratii* and *C. butyricum*, which occur ubiquitously in the environment, especially in soil or marine sediments (Dodds 1992, Peck 2009). Depending on the entry route, both the preformed toxins and clostridial spores can induce botulism, which occurs in three major forms (Fig. 1A): Food-borne botulism develops when improperly processed food is ingested, in which the bacteria found appropriate anaerobic growth conditions and produced the neurotoxins (Peck et al. 2011). This type of botulism is mostly caused by home-canned food (meat, fish, vegetables) containing preformed neurotoxins, but commercially available food can also be affected (Cowden 2011, Mad'arova et al. 2017, Mazuet et al. 2015, Peck 2006). Infant botulism can occur in infants under 12 months old when they ingest bacterial spores, which, in the absence of the competitive endogenous gut flora, may germinate and colonize the intestinal tract and start to produce neurotoxin. For infant botulism, uptake of honey has been identified as a risk factor since honey is a natural product that might contain *C. botulinum* spores (Grant et al. 2013, Ringe et al. 2014, Rosow and Strober 2015). Wound botulism occurs after entry of spores into deep wounds and is rare in the general population. However, clusters of wound botulism as well as sporadic cases among people who inject drugs have been reported in a number of European countries since 2000 (Akbulut et al. 2005, Alpers et al. 2005, Brett et al. 2005, Barry et al. 2009, Schroeter et al. 2009, Hope et al. 2012, MacDonald et al. 2013). In Germany, botulism is a notifiable disease in humans according to §6 and §7 of the Protection against Infection Act. Hereby a total number of 134 cases of botulism have been reported between 2001 and 2017, making up approximately 8 human cases on average per year. Of those, food-borne botulism represents the most frequent form with about 70% of all cases, followed by wound botulism (21%) and infant botulism (9%) (Robert Koch Institute 2018).

For animal botulism, numbers are not available due to the absence of a notification requirement for the veterinary disease. Animal botulism is usually caused by the ingestion of BoNT-containing feed, but sporadically cases of wound botulism may also occur (Bernard et al. 1987). The source of contamination is most frequently identified as contamination of the forage with dead animals or carcasses (Anniballi et al. 2013b). Less common are poisonings by direct osteophagia (Seddon 1922, Theiler and Robinson 1927, van der Lugt et al. 1995, Riet-Correa et al. 2012). Animal botulism can affect wildlife as well as husbandry or zoo animals. In contrast to human botulism, outbreaks among cattle, poultry, mink, but also wild birds and waterfowls can occur at a large scale, possibly affecting hundreds or thousands of animals (Grüll and Rauer 2000, Lindström et al. 2004, Popp et al. 2012, Włodarczyk et al. 2014, Dlabola et al. 2016, Relun et al. 2017). In Germany and Europe cattle and poultry farms are affected most often. More seldom horses or other animals are affected (Schettler 1979, Popp et al. 2012, Souillard et al. 2014, Souillard et al. 2015, Woudstra et al. 2015, Dlabola et al. 2016). According to current information botulism in farm animals is a rare disease. However a high number of individuals can be affected in a single outbreak, i.e. 115 animals (40%) of a cattle herd were lost during a recent outbreak (Dlabola et al. 2016). Hereby, botulism outbreaks can be associated with large economical losses.

In humans, the typical symptoms of botulism are characterized by flaccid descending paralysis. This paralysis first affects the ocular muscles (ptosis, double and blurred vision), facial and neck musculature (slurred speech, swallowing problems, dry mouth). It eventually leads to muscle weakness in the peripheral upper and lower limbs. In severe cases, if the paralysis reaches respiratory muscles, it may lead to death due to ventilatory failure unless supportive care is provided (Dembek et al. 2007). In animals, the symptoms observed are similar including weak tongue strength, sometimes protrusion and extensive salivation as hallmarks in cattle. Birds show paralysis of inner eyelids, droopy wings, weak legs, a floppy neck (limberneck) due to muscle weakness, and drowning, particularly in waterfowls (Critchley 1991, Dlabola et al. 2010, Anniballi et al. 2013b).

Those symptoms are caused by the uptake of BoNT released by the vegetative bacteria in a complexed form associated with different accessory proteins (non-toxic non-hemagglutinin, NTNH, and for some toxin serotypes additionally three different hemagglutinins [Lee et al. 2014, Lam and Jin 2015]) which protect the neurotoxin from the harsh conditions of the gastrointestinal tract (Fig. 1B). The associated complex proteins, especially hemagglutinins, are involved in binding of the neurotoxin complex to the intestinal epithelial barrier and transcytosis through the small intestine by a yet to be defined molecular pathway (Lee et al. 2014, Fujinaga and Popoff 2017). Once in the circulation, the BoNT, now stripped off its complex proteins, finally reaches the neuromuscular junction where it exerts its biological activity by a highly specific mode of action (Fig. 2 A). Here, individual domains of the 150 kDa BoNT molecule carry out different parts of the molecular mechanism (Rossetto et al. 2014). First, the 50 kDa C-terminal domain (H_C) of the 100 kDa large heavy chain binds to specific receptor molecules embedded in the presynaptic membrane before bound toxins are taken up into fused synaptic

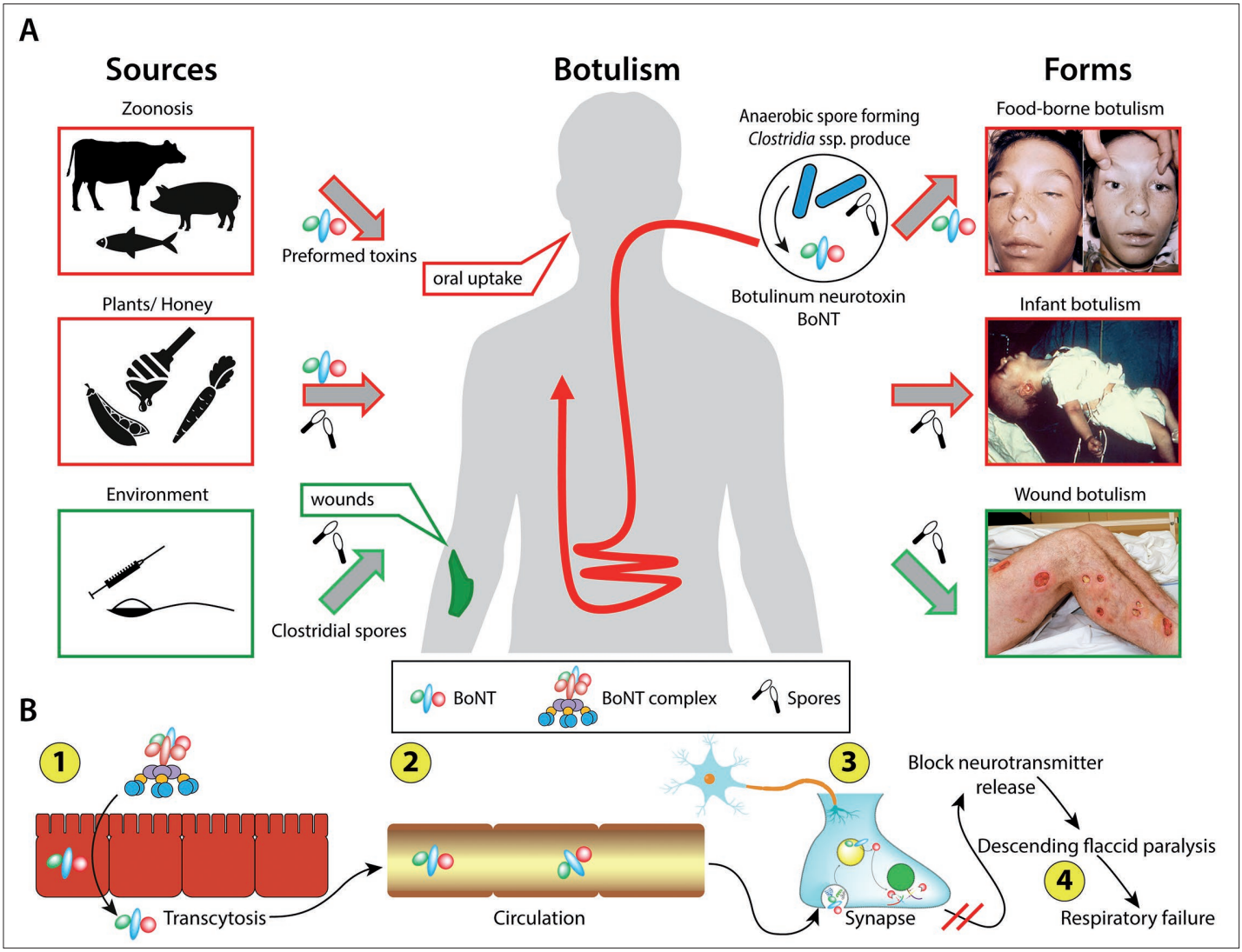

FIGURE 1: *A. Sources and forms of botulism in humans. Botulism is caused by botulinum neurotoxin (BoNT) producing, anaerobic spore forming bacteria of the Clostridia species (C. botulinum, C. baratii, C. butyricum). Either preformed toxin or spores are ingested from contaminated food (food-borne botulism), taken up orally by infants under 12 months old (infant botulism) or injected via contaminated drugs (wound botulism). B. Entry route of BoNTs and symptoms of botulism. BoNTs complexed with proteins mediating protection against low pH and protease in the stomach undergo transcytosis through the small intestine (1) and are released in the circulation (2) until they reach their site of action, synapses on the motor neuronal endplate (3). Here, by their specific mode of action (see Fig. 2) they block neurotransmitter release which leads to descending flaccid paralysis and, ultimately, death by respiratory failure (4).*

vesicles (Rummel 2013, Rummel 2016, Pirazzini et al. 2017). According to the current dual-receptor binding paradigm the interactions with a (glyco-)protein and the carbohydrate-groups of polysialo-gangliosides are needed for high affinity binding of BoNT to the presynaptic membrane (Montecucco 1986, Rummel 2016). Different serotypes employ different protein receptors, either synaptic vesicle protein 2 (SV2) or synaptotagmin (Syt, Fig. 2 B) (Rummel 2013, Lam et al. 2015, Rummel 2016). Recent work has shown that binding to the SV2 protein receptors is enhanced by glycosylation of SV2 (Mahrhold et al. 2016, Yao et al. 2016). It is likely that other BoNT require also glycosylation of their protein receptor for high-affinity binding. Noteworthy, both identified protein receptors (SV2 and Syt) are part of the neurotransmitter-loaded vesicles and thus only exposed to the cell surface during transmitter release. After binding to the receptors BoNT is internalized in endocytosed synaptic vesicles which are then acidified by vesicular ATPase (Pirazzini et al. 2017). Vesicle acidification induces integration of the 50 kDa N-terminal domain (H_N) of the heavy chain into the endosomal membrane, hereby forming a pore (Colasante et al. 2013, Fischer 2013). Finally, the enzymatically active 50 kDa light chain

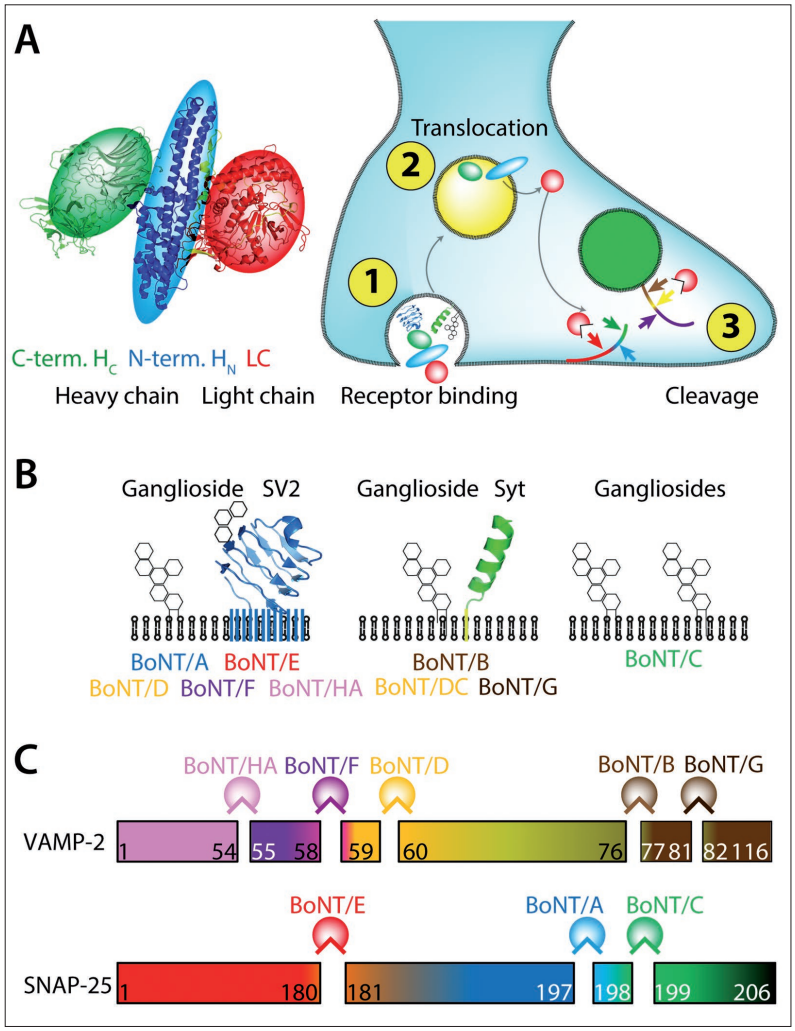

FIGURE 2: *A. Three domains of the 150 kDa proteotoxin mediate the mode of action of BoNTs. Receptor binding (1) on the presynaptic membrane is mediated by the 50 kDa large C-terminal domain of the 100 kDa heavy chain. After uptake and acidification, the 50 kDa N-terminal domain of the heavy chain inserts in the endosomal membrane, hereby forming a pore through which the 50 kDa light chain is translocated into the cytoplasm (2). Finally, the light chain cleaves different proteins of the SNARE complex (3) hereby inhibiting fusion of neurotransmitter filled synaptic vesicles with the presynaptic membrane, hereby blocking signal transduction from the peripheral neurons to the muscles leading to paralysis. B. Serotype-specific receptor binding for pathogenic BoNT serotypes. According to the dual-receptor binding model (Montecucco 1986) all BoNT serotypes need simultaneous interaction with both a ganglioside and a synaptic vesicle protein (either SV2 or Syt) for high affinity binding. However, serotype-specific differences exist in which protein receptors are employed (Rummel 2013, Lam et al. 2015, Rummel 2016). C. Cleavage sites on different substrate proteins for pathogenic BoNT serotypes. The BoNT serotypes differ in which SNARE protein (VAMP-2 or SNAP-25) is cleaved and the specific amino acid position targeted (Binz 2013).*

is transferred through this pore by a yet undetermined mechanism. In the cytosol, reduction of the disulfide bridge between light and heavy chain by the thioredoxin reductase/ thioredoxin system releases the light chain into the cytoplasm (Pirazzini et al. 2015, Pirazzini et al. 2016). Here, the light chain specifically cleaves proteins of the soluble NSF attachment protein receptor (SNARE) complex which mediates fusion of acetylcholine filled vesicles with the presynaptic membrane via its Zn^{2+}-dependent endopeptidase activity (Binz 2013). This leads to blockage of neurotransmitter release which inhibits signal transduction from peripheral nerves to muscle cells ultimately causing the paralytical symptoms of botulism.

The family of the botulinum neurotoxins comprises seven accepted serotypes, BoNT/A to BoNT/G. Botulism in humans can be caused by serotypes BoNT/A, B, E, and F, whereas serotypes C and D cause botulism in livestock, e.g. in cattle and poultry, and in wild animals (Montecucco and Rasotto 2015). In case of veterinary botulism, mosaic serotypes of BoNT/C and D (designated BoNT/CD and BoNT/DC) also exist which comprise the light chain and H_N-domain of one serotype and the receptor binding H_C domain resembling the other serotype (Sakaguchi et al. 2015, Hansbauer et al. 2016). Serotype G has not been clearly assigned to a natural outbreak in humans or animals (Peck et al. 2017). Some strains of *C. botulinum* can produce two or even three different BoNTs (Williamson et al. 2016). Recently, a novel, putatively 8th serotype termed BoNT/H was proposed (Barash and Arnon 2014, Dover et al. 2014). A more detailed analysis showed that the catalytic domain of BoNT/H is highly homologous to the unique catalytic domain of BoNT/F5 which is different from all other sero- and subtypes. The receptor binding domain of BoNT/H, however, shows a high degree of homology to BoNT/A. Moreover, the capability of BoNT/H to induce botulism can be blocked by antitoxins derived against BoNT/A (Maslanka et al. 2016). Interestingly, its light chain (as well as the light chain of BoNT/F5) represents a novel 'cleavotype' which cleaves its SNARE protein at a unique position (Kalb et al. 2012b, Kalb et al. 2015b). Therefore, BoNT/H is alternatively referred to as BoNT/FA or HA (the latter name is preferred by the authors and used in this manuscript) (Kalb et al. 2015b, Maslanka et al. 2016, Pellett et al. 2016, Yao et al. 2017). BoNT/HA is potentially also pathogenic to humans as it originates from a strain causing infant botulism, although its toxicity is clouded by the fact that the bivalent strain also produces BoNT/B (Barash and Arnon 2014, Dover et al. 2014).

Although all BoNT serotypes exert a similar mode of action, they differ by the receptor molecules employed for initial binding to the synaptic membrane. Furthermore the cleaved synaptic substrate as well as the individual cleavage site on the substrate proteins of the SNARE complex vary (Fig. 2B). Regarding the receptor molecules,

TABLE 1: *Different requirements for an animal replacement method for pharmaceutical potency testing as compared to diagnostic assays for botulism*

Requirement	Botulism diagnostics	Potency testing of pharmaceutical BoNT preparations
Different BoNT sero- and subtypes	Reliable detection of all >40 pathogenic sero- and subtypes	Detection of predefined sero- and subtypes known by production process, only BoNT/A1 and B1
Sample matrix	Detection from complex matrices: clinical, food and environmental matrices	Detection from physiological solutions, matrix not critical
Time to results	Critical due to time frame for therapeutic intervention	Not critical: dictated by length of production process
Precision	Approximate range of activity sufficient	Precise quantification of biological activity crucial
Mode of action	Positive detection more important than coverage of complete mode of action	Coverage of complete mode of action (binding, translocation, cleavage) crucial for reliable quantification of potency

BoNT/A, D, E, F and HA use the protein receptor SV2 plus gangliosides for high affinity binding, whereas BoNT/B, DC and G bind to Syt as protein receptor. Only for BoNT/C no protein receptor has been identified so far, instead two ganglioside molecules are used for binding (Rummel 2013). With respect to enzymatic activity, BoNT/A, C and E cleave the synaptic protein SNAP-25 whereas BoNT/B, D, F, G and HA target the substrate VAMP-2 at individual amino acid positions (Fig. 2C) (Binz 2013). BoNT/C additionally cleaves syntaxin as a second substrate (Blasi et al. 1993, Schiavo et al. 1995).

Adding further to this complexity on the molecular level is the fact that with the advent of the molecular biology age and the availability of more and more sequences, it became obvious, that differences within a given serotype exist for BoNT/A, B, E and F (Hill and Smith 2013). These so called subtypes can differ up to 36% on the amino acid level within a given serotype. So far, more than 40 subtypes have been described in the literature and even more subtypes are expected to be discovered in the future. Subtypes of a given serotype have been shown to differ in their biological activity, e.g. their kinetics of substrate cleavage, affinity to receptors and overall activity (Henkel et al. 2009, Kalb et al. 2012b, Wang et al. 2013b, Whitemarsh et al. 2013, Kull et al. 2015).

This high molecular variability poses a major challenge for the development of diagnostic assays covering all relevant sero- and subtypes (Dorner et al. 2013). Although a number of multiplexed detection methods targeting either the DNA (Lindström et al. 2001, De Medici et al. 2009, Fach et al. 2009, Kirchner et al. 2010, Fach et al. 2011, Fenicia et al. 2011, Anniballi et al. 2013a, Anniballi et al. 2013b, Woudstra et al. 2013) or the BoNT protein (Pauly et al. 2009, Zhang et al. 2012, Singh et al. 2015, Hansbauer et al. 2016, Rosen et al. 2017b) have been described, false negative results due to the high variability affecting PCR and immunological methods alike have been reported (Gibson et al. 1987, Fach et al. 1996, De Medici et al. 2009, Mad'arova et al. 2017). A feasible way to reduce this molecular complexity introduced by the different BoNT subtypes is the detection of either their full mode of action or parts of it. The rationale here lies within the consideration that point mutations that cause the failure of DNA- or protein-based assays might not necessarily impair functional detection as long as vital parts of the function such as receptor binding or substrate cleavage are not affected. If vital parts of the protein function are affected, the altered function truly reflects a different potency of the toxin variant. An example would be the subtype BoNT/A4 which has an about three orders of magnitude lower toxicity compared to the prototypic BoNT/A1 presumably due to a less efficient entry into neurons (Whitemarsh et al. 2013). The concept of functional BoNT detection by displaying endopeptidase activity and/or receptor binding in vitro has been used as a starting point for establishing animal replacement methods (see section "replacement methods for diagnostics of botulism").

Besides causing botulism, BoNTs are classical dual-use substances: Due to their high toxicity – the LD_{50} is only 1 ng per kg bodyweight in humans (Gill 1982) – early on efforts have been reported to make use of these potent toxins as biological weapons as reviewed by Arnon and colleagues (Arnon et al. 2001). On the other hand, their exquisite specificity is also harnessed in highly beneficial ways. BoNTs known under different trademarked brand names such as BOTOX®, Vistabel®, Dysport®, Azzalure®, Xeomin®, or Bocouture® (all BoNT/A) and Myobloc® (BoNT/B) are approved based on their paralyzing effects on muscles for the treatment of an ever increasing number of medical conditions ranging from strabismus, blepharospasmus, migraine, dystonia, spasticities, urological conditions, hyperhidrosis up to the newly evaluated treatment of depression (Bigalke 2013, Pirazzini et al. 2017). More popular in the public, however, is their use in aesthetic medicine to smooth facial wrinkles. For clinical use, only minute amounts of toxins are needed for the efficient treatment, making precise quantification of the biological potency a prerequisite for the safe application.

Challenges in BoNT detection

Generally, there are two different fields in BoNT research which require highly sensitive detection of BoNT molecules (Dorner et al. 2013):

- Potency testing of highly purified BoNT pharmaceuticals approved for medical / cosmetic application
- Diagnostics of suspect cases of botulism in humans and animals

Both fields have quite contrary requirements for diagnostic approaches (Tab. 1):

First, diagnostic assays for BoNTs have to detect all pathogenic sero- and subtypes reliably while only two specific sero- and subtypes, BoNT/A1 and BoNT/B1, are employed in pharmaceutical products (Pirazzini et al. 2017). Therefore, a much more comprehensive coverage of BoNT sero- and subtypes is needed for diagnostics as compared to potency testing – basically, all known and even the yet unknown BoNT subtypes have to be detected. A meaningful approach to reduce the analytical complexity is to develop separate assays for serotypes pathogenic to humans as compared to the serotypes pathogenic to animals only (Hansbauer et al. 2016), hereby separating serotypes A, B, E, F, and the novel HA from C, D, CD, and DC depending on the analytical focus. Though often practiced, it has to be taken into account that in rare cases serotypes usually pathogenic to humans can also occur in veterinary botulism (Johnson et al. 2010, Lamoureux et al. 2015).

Second, botulism diagnostics is always performed from complex sample matrices. Those could either be clinical sample materials such as sera, feces, organ extracts; complex food samples such as meat, fish or pickled vegetables; environmental matrices such as wipe samples, soil, or dust and finally cell culture supernatants from bacterial enrichment cultures (Dorner et al. 2013). Testing of BoNT production from food matrices also plays a role for food safety studies performed in the food industry, e.g. for testing of different storage conditions after artificial spiking of food with Clostridial spores. Here, similar challenges apply with respect to matrix interference and toxin recovery as for diagnostics of food samples in case of outbreak analysis, with the important difference that for food safety studies the BoNT serotype is known prior to analysis (Peck 2006). The analysis of complex matrices implies that the assays have to be robust and/or contain an enrichment step for extracting the toxins from the matrix. Additionally, the analytical procedure has to take into account that the toxin has to be detected in different molecular forms from different matrices. In food matrices and bacterial culture supernatants, the toxin is usually present in its complexed form. While the complex is stable at acidic pH, it dissociates spontaneously at physiological pH and high ionic strength (Sakaguchi 1982, Simpson 2004, Dorner et al. 2013) – this is of relevance for the analysis of different food samples. In serum, however, free BoNT is found and the exact fate of the associated complex proteins is not yet clear to date. Any diagnostic procedure has to ensure that both the free BoNT and the toxin in its complexed form can be detected from complex matrices. Contrary, BoNT pharmaceuticals are highly purified toxin preparations (either in its free or complexed form) in physiological buffer containing additives such as human serum albumin or stabilizers. Therefore, for testing BoNT pharmaceuticals the matrix does not pose an analytical challenge, although there has been a report on an unusual enzymatic activity of human serum albumin contained as a stabilizer (Jones et al. 2011).

Third, due to the short time frame during which administration of neutralizing antitoxins is effective, the time to results is highly relevant for botulism diagnostics (Tacket et al. 1984). Contrary, the time frame for potency testing is dictated by the much longer production process of the pharmaceutical formulations and is hence not critical.

Forth, there are high demands on the precision of assays for potency testing where exact dosing requires highly accurate, reproducible and quantitative results. On the contrary, the order of magnitude of the biological activity is usually sufficient for diagnostics.

Finally, there is a broad agreement that in order to accurately quantify the potency of BoNTs in pharmaceutical preparations, all steps critical in vivo to fulfill the complete mode of action have to be depicted (Adler et al. 2010). For botulism diagnostics the main issues are confident results and coverage of all sero- and subtypes from matrices without risking false negatives.

The mouse bioassay

Already in the 1920s, the mouse bioassay (MBA) was proposed for toxin detection and determination of its activity (Bengtson 1921). Until now it is still the most widely used "gold standard assay" to determine the potency of highly pure BoNT pharmaceuticals and to detect toxin from suspect samples of botulism (Dorner et al. 2013, Worbs et al. 2015). By way of example, for diagnostics of veterinary botulism a recent survey among 11 Western European countries, among them Germany, revealed that 81% of veterinary laboratories still apply the MBA for sample testing (Skarin et al. 2013). In fact, in Germany the only approved standard DIN 10102 on "microbiological analysis of meat and meat products and detection of *C. botulinum* and BoNT" relies on the MBA. In the absence of any alternatively accepted method, this procedure is also used for clinical specimens. Internationally, the AOAC Official Method 977.26 is the corresponding standard on "*C. botulinum* and its toxins in foods", effective since 1977 (AOAC International 1977).

The MBA requires different dilutions of particle-free sample materials (e.g. sera, culture supernatants, food extracts) to be injected intraperitoneally into mice. Subsequently the mice are monitored for typical botulism symptoms such as ruffled fur, labored breathing, wasp-like abdomen due to increased respiratory efforts, weakness of limbs and, finally, total paralysis and death by respiratory failure (Lindström and Korkeala 2006, Dorner et al. 2013). The assay can take up to four days as at low doses the development of neurotoxic effects can be delayed. For confirmation of specificity and to determine the disease causing serotype, toxin neutralization by specific antisera has to be performed in connection with further tests for specificity and activity (heat inactivation of the heat labile toxin; trypsin-activation for selected serotypes). Taking into account all assay controls suggested in DIN 10102, up to 44 mice are needed per single sample. Overall, conservative estimations indicate that in Germany between 8000 and 10,000 mice are needed each year for the purpose of BoNT diagnostics in all clinical and veterinary laboratories involved. This is remarkable in light of the low number of positive cases but reflects the high number of suspect cases which have to be analyzed to clarify the absence of botulism. For information, 10-20% of all suspect samples turn out to be positive in the German Consultant Laboratory for Neurotoxin-producing Clostridia at the Robert Koch Institute. This relatively high percentage of positive samples among all samples tested might reflect the fact that for suspect cases with initial positive results often numerous follow-up samples are analyzed of which several usually turn out to be positive (epidemiological investigation to identify the source of an outbreak). Even larger numbers of animals are needed for the potency testing of BoNT pharmaceuticals: Here, the European Pharmacopoeia mandates the LD_{50} test in mice, which precisely measures the toxin's dose lethal for half of the animals injected (European Pharmacopoeia Commission 2005, European Pharmacopoeia Commission 2011). For potency testing, it is estimated that in 2010, at least 600,000 mice were used worldwide for potency testing of BoNT/A pharmaceuticals (Bitz 2010). In Germany, only in 2014 approximately 60,000 and 90,000 mice were used for potency testing of pharmaceutical BoNT/B and BoNT/A preparations, respectively (German Federal Institute for Risk Assessment 2016, Wild et al. 2016).

Considering this high number of animals needed for potency testing and diagnostics, why is the MBA still in use to date? First, with an exquisite detection limit in the low pg per mL range, the assay offers the high sensitivity

necessary for both potency testing as well as detection of traces of the toxin from clinical, food and environmental samples for botulism diagnostics (Dorner et al. 2013). Second, the assay covers the complete mode of action of all functional domains of the neurotoxin hereby truly reflecting the activity of the molecule, a fact especially important for potency testing. Third, the MBA can detect all toxic sero- and subtypes (including any yet unknown subtypes) from complex matrices, which is crucial for diagnostic settings. Finally, it is technically straightforward to perform as it involves only basic sample preparation steps and injections into mice.

However, the mouse bioassay also has some grave disadvantages for both technical and ethical reasons. Generally, the MBA as an in vivo assay is very difficult to standardize due to the current absence of certified reference materials (Sesardic et al. 2003, Lindström and Korkeala 2006). Along this line, inter-laboratory comparisons have shown that MBA results may be variable depending on the age and strain of mice used and other experimental factors (McLellan et al. 1996, Sesardic et al. 2003). Indeed, in terms of quantitation of BoNT activity, the MBA has recently been shown to be more variable than other assays directly investigated in parallel in an international proficiency test (Worbs et al. 2015). Additionally, the need for an animal facility as well as trained and approved personnel significantly adds to the overall cost of the MBA and confines the circle of potential users to appropriately equipped laboratories. Furthermore, the long duration of up to four days can be critical, especially in a diagnostic setting. Here, a rapid laboratory confirmation of the clinical diagnosis allows for a timely administration of BoNT-neutralizing antisera – still a frequently used treatment option which is effective only during the early stages of the intoxication as long as as long as toxins are still circulating in the bloodstream (Kodihalli et al. 2017).

Besides that, species differences between mice and humans can lead to misinterpretation of results, as demonstrated for BoNT/B which is 40 times more potent in mice due to a single amino acid difference located in the protein receptor binding site (Peng et al. 2012, Strotmeier et al. 2012). Similarly, BoNT/D is functionally active in mice, but does obviously not affect humans (Coffield et al. 1997). Additionally, unspecific toxicity caused by other sample components can also lead to misinterpretation of results (Segner and Schmidt 1968, Solberg et al. 1985, Dezfulian 1989, Government Inquiry into the Whey Protein Concentrate Contamination Incident 2014). This is also the reason why toxin neutralization assays are routinely performed which further add to the timeframe required to perform the MBA.

Most importantly, however, in light of the high number of mice consumed and their high level of distress, there is ethical controversy about the employment of the MBA. This mostly affects its application for potency testing while its application in diagnostics is considered as ethically justifiable in the current absence of adequate internationally accepted alternative methods. Along this line, the effective Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes stipulates in Article 4 the strict adherence to the Russel's and Burch's 3R principles, reduction, refinement and replacement of animals, wherever possible (Russell and Burch 1959, The European Parliament and the Council of the European Union 2010). The Directive also prescribes the avoidance of death as an end-point to prevent severe suffering of animals. Wherever possible, it should be substituted by more humane end-points such as specific clinical signs and symptoms.

In this context, several assays have been established to fulfill Russel's and Burch's 3R principles for the potency testing of pharmaceutical BoNT preparations containing BoNT/A1 or BoNT/B1 (Sesardic and Gaines Das 2008, Bitz 2010). Of those, as humane endpoint of the MBA the measurement of local paralysis has been introduced as a refinement method (Sesardic et al. 1996, Wilder-Kofie et al. 2011, Broide et al. 2013, Cevc 2015). These assays measure e.g. flaccid paralysis (Sesardic et al. 1996, Jones et al. 2006), abdominal ptosis (Takahashi et al. 1990), hind limb paralysis (Sugiyama et al. 1975, Pearce et al. 1994, Aoki 2001), grip strength (Meyer et al. 1979, Torii et al. 2011), toe-spread reflex (Wilder-Kofie et al. 2011), rotarod (Pellett et al. 2015b), and running wheel assays (Kutschenko et al. 2011). These assays can be sublethal with known amounts of toxin applied, still they represent animal experiments, require several days to perform and are in vivo tests with a more or less subjective readout. Alternatively, an ex vivo replacement method measuring paralysis of an explanted hemidiaphragm has been developed (Rasetti-Escargueil et al. 2009, Bigalke and Rummel 2015). This mouse hemidiaphragm assay still relies on animals which are, however, sacrificed humanely. In essence, the assay employs the isolated *N. phrenicus*-hemidiaphragm tissue in an organ bath which, by incubation with BoNT undergoes a dose-dependent characteristic decrease of the contraction amplitude of the indirectly stimulated muscle. The assay can be well standardized and was shown to deliver superior qualitative and quantitative results when directly compared to the MBA in a recent proficiency test (Worbs et al. 2015). In the same proficiency test the hemidiaphragm assay also proved to be robust against matrix interference – this, however, has to be taken with care, since a comprehensive panel of matrices has not yet been tested (Bigalke and Rummel 2015). The main limitations of this assay lie within the relatively high instrument costs, the technical expertise needed to perform the assay reproducibly and the low throughput format.

Replacement methods for potency testing

In order to overcome the limitations of the MBA, intensive efforts have been put into the development of alternative replacement methods for potency testing of highly pure BoNT pharmaceuticals (Straughan 2006). In the last decade, significant progress has been made in this field worldwide by establishing cell-based assays which do not depend on animals any more. Cell-based assays employ cell lines which are sensitive for BoNT/A1 or B1 and measure the binding, uptake, translocation and intracellular substrate cleavage of the neurotoxins by different read-outs. They offer the advantage of covering the complete mode of action needed for a full replacement of potency testing (Pellett 2013).

Cell-based assays

In principle, cells from different sources can be employed for potency testing of BoNTs. Besides primary neuronal cells isolated from different sources, continuous cell lines and stem cell derived cells have all been evalu-

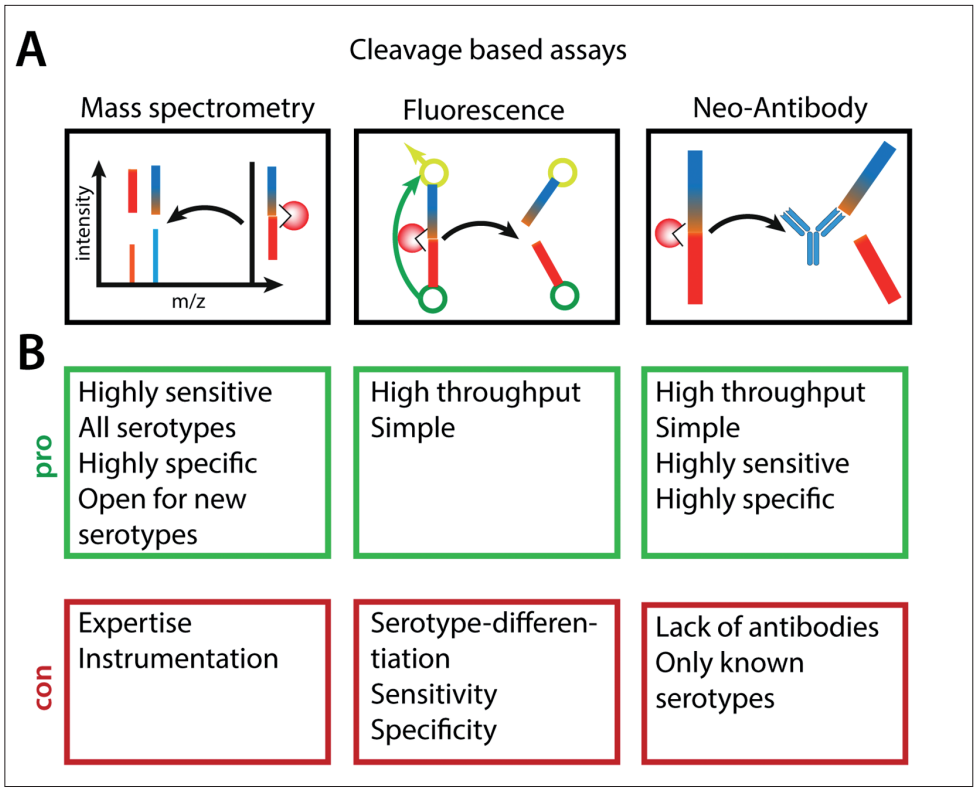

FIGURE 3: *A. Different readouts employed in assays based on enzymatic cleavage of substrate proteins. Short peptide substrates are detected by mass spectrometry in the so called Endopep-MS approach, while FRET or luminescence-based assays measure the change in fluorescence intensity after cleavage. Last, detection of cleaved substrate can also be based on neo-epitope specific antibodies which specifically bind to cleaved, but not uncleaved substrates on the newly exposed cleavage sites. B. Advantages and disadvantages of the different approaches.*

ated for detection of BoNTs with different challenges involved. Primary cells usually show high sensitivity towards BoNTs but still require the sacrifice of animals and prolonged cultivation by a skilled experimenter. Continuous cell lines derived from cancer cells, e.g. PC12, Neuro-2a or P19 cells, are easier to obtain and cultivate. The challenge here lies within the limited sensitivity of many cell lines, possibly due to different degrees of differentiation from a cancerous background as well as differential expression of receptor or substrate molecules compared to primary neuronal cells (Yowler et al. 2002, Dong et al. 2008, Tsukamoto et al. 2012, Pellett 2013, Rust et al. 2017). However, if suitable cell lines are identified, high sensitivity can be reached as exemplified by the first FDA approved replacement assay for the MBA which is based on a highly susceptible SiMa cell line (Fernández-Salas et al. 2012). Finally, stem cell-based cell lines offer a sensitive and well defined alternative to cancer derived cell lines (Whitemarsh et al. 2012, Pellett et al. 2015a). Additionally, human derived stem cells circumvent potential species specific differences in potency as has been observed for BoNT/B (Strotmeier et al. 2012). Those cell lines can be purchased cryopreserved from commercial suppliers and subsequently require only plating and relatively short cultivation before use. Hereby, despite initially higher costs the overall costs might be comparable to the cultivation of continuous cell lines including associated costs for quality control tests and personnel. Induced pluripotent human stem cells have also been implemented in the second approved replacement assay for the potency determination (Eisele and Mander 2015). This assay is based on commercially available hiPS stem cells which have been described before (Whitemarsh et al. 2012) and are cultivated under addition of GT1b to reduce variability and to increase sensitivity.

An important parameter to differentiate cell-based assays is the readout which is employed to quantify the action of BoNTs in the cells. Most often, cleavage of substrate proteins as the final step of intoxication is monitored. This can either be done directly using Western blotting or by cleavage-specific, so called neo-epitope specific antibodies. By definition, neo-epitope specific antibodies detect only the cleaved, but not uncleaved substrate (Bak et al. 2017, Pellett et al. 2017) (Fig. 3A). These assays can provide sensitive detection, especially if high affinity neo-epitope specific antibodies are implemented in an ELISA-based approach. This has the additional advantage to allow a high throughput (Fernández-Salas et al. 2012, Pellett et al. 2017). Alternative readouts based on fluorescence or luminescence and, more recently, electrochemical multi-electrode arrays have also been introduced (Dong et al. 2004, McNutt et al. 2011, Jenkinson et al. 2017).

Along these lines, a luminescence-based cell line with a novel readout has been developed (Pathe-Neuschafer-Rube et al. 2015). In this work, a SiMa cell line expressing a pH-stable firefly-luciferase, which was sorted into secretory vesicles by the inclusion of an N-terminal sorting sequence, was shown to enable sensitive inhibition of luciferase secretion into the supernatant after incubation with BoNT/A. Such an approach does circumvent the need for detection of the cleaved substrate by serotype-specific neo-epitope antibodies by directly measuring the inhibition of neurotransmitter release. Hereby the applicability of this cell line is theoretically broadened for the second therapeutically relevant serotype BoNT/B. However, this is currently not yet feasible without further assay modification, since the respective SiMa cell line used is not susceptible to BoNT/B due to the absence of the specific substrate VAMP-2. Further work would also be

needed to increase the sensitivity and reduce the variability of this promising approach.

The lack of VAMP-2 expression was a general obstacle standing in the way of full replacement of the MBA for potency testing for the detection of BoNT serotype B. Very recently, this could be overcome by an elegant approach by Rust and colleagues (Rust et al. 2017). In their work, they also employed the SiMa cell line which was used in the FDA approved replacement assay for BoNT/A (Fernández-Salas et al. 2012). The authors rendered the SiMa cell line susceptible to substrate cleavage by BoNT/B by introducing the VAMP-2 substrate. By generating a luciferase-VAMP-2 fusion protein they were able to capture cleaved VAMP-2 using a neo-epitope specific antibody and detect the captured molecule in a highly sensitive one-step ELISA via the luciferase activity. A remaining limitation was the usage of a polyclonal neo-epitope specific antibody that might be prone to lot-to-lot variations hereby complicating a comprehensive assay validation.

Finally, in a proof-of-principle study, the applicability of mouse embryonic stem cells grown on multi-electrode arrays to monitor the mode of action of BoNT/A by measuring the inhibition of electrochemical burst was shown in an interesting study (Jenkinson et al. 2017). An earlier study had already shown that mouse embryonic stem cells and an electrochemical readout of bursts by patch-clamp technique could be used for sensitive detection of BoNT/A, BoNT/B and tetanus toxin (Beske et al. 2015, Beske et al. 2016). Advancing the previous approach, Jenkinson et al. (2017) employed a multi-electrode array to record the neuronal network activity, hereby simplifying the experimental procedure. Although promising, it remains to be determined whether these rather sophisticated approaches offer true advantages over the technically simpler and broader applicable cell-based assays employing validated ELISA-based read-outs (Pellett et al. 2017, Yadirgi et al. 2017).

Binding and cleavage-based assays

Despite offering the only non-animal approach covering the complete mode of action consisting of binding, uptake, translocation, and intracellular substrate cleavage, cell-based assays also have technical limitations. These include long cultivation times ranging from few days to weeks to establish neuronal cell lines, a substantial level of expertise needed and significant assay costs associated especially with stem cell-based approaches, and a long incubation time for cleavage which is required to reach sensitive detection (Pathe-Neuschafer-Rube et al. 2015, Jenkinson et al. 2017). Consequently, simpler and more robust assays covering parts of the neurotoxins' mode of action, namely receptor binding and cleavage, have been developed. Although not appropriate for a complete replacement, such assays could potentially contribute to reducing the overall number of animals needed for potency determination of BoNT pharmaceuticals by replacing the MBA during individual production steps.

The concept of combining receptor binding and substrate cleavage was introduced by binding of BoNT/A, B, and F to synaptosomes, which represent isolated synaptic terminals from neurons and are prepared from rat brains, followed by subsequent cleavage of biotinylated substrate molecules SNAP-25 or VAMP1. Cleaved substrates were detected by polyclonal neo-epitope specific antibodies in an ELISA-based format (Evans et al. 2009). While testing both receptor-binding and enzymatic cleavage this assay has the disadvantage of relying on synptosomes from rat brains which are tedious to purify, not well defined and still require animals as organ donors. A more straightforward approach to detect correctly folded BoNT/A containing both the heavy- and light chain connected by the disulfide bond relied on dual-coated ELISA plates. Here, both a monoclonal antibody targeting the heavy chain and SNAP-25 as target of the enzymatically active light chain were immobilized to the microplates (Liu et al. 2012). However, good correlation with the biological activity as determined by the MBA required incubation with a chaotropic agent. This was done to prevent binding of partially denatured BoNT/A as both an endopeptidase assay as well as an ELISA based capture approach both overestimated the relative potency due to also measuring (partially) denatured BoNT molecules. Nevertheless, this assay was shown to be suitable for detection of BoNT/A from clinical and environmental food samples making it an interesting tool for botulism diagnostics (Jones and Marks 2013). However, as a monoclonal antibody was used for the binding step, this assay tested the enzymatic activity in combination with the presence of a disulfide-bonded heavy chain, but explicitly not the functional receptor binding.

The latter issue was pursued in another work where binding to the isolated protein receptor of BoNT/A, SV2C, was assayed in conjunction with detection of the enzymatic light chain activity by a recombinant commercially available SNAP-25 Förster resonance energy transfer (FRET) substrate (Gregory et al. 2014). FRET substrates are short peptides flanked by a donor and an acceptor fluorophore and can be used to monitor the cleavage of the synaptic substrates (Fig. 3). Here, a change in fluorescence upon incubation with a BoNT containing sample indicates cleavage activity. In the work by Gregory and colleagues, the assay turned out to be only moderately sensitive, despite a reasonable correlation with the MBA. This limited sensitivity was most probably caused by the incomplete presentation of the receptor complex as neither the dual-receptor binding was implemented (the addition of the ganglioside GT1b did not show improved sensitivity), nor the crucial N-glycan structure on the SV2C receptor was present which was recently shown to mediate high-affinity binding and toxicity of BoNT/A (Mahrhold et al. 2016, Yao et al. 2016).

Similarly, a bifunctional assay testing binding to a short peptide comprising the crucial amino acids in Syt for BoNT/B receptor recognition and cleavage of a VAMP-peptide to quantify BoNT/B neutralizing antibodies employed the protein receptor only (Rosen et al. 2016); again, this approach failed to mediate the high affinity dual-receptor binding (Weisemann et al. 2016, Desplantes et al. 2017). Finally, highly sensitive detection of the receptor binding and substrate cleavage of BoNT/B was achieved in a so called BINACLE assay (binding and cleavage assay) (Wild et al. 2016). Here, the dual-receptor binding was experimentally achieved by coating both ganglioside and the protein receptor Syt onto ELISA plates, before the cleavage of VAMP was detected by a cleavage specific polyclonal neo-epitope specific antibody. This assay seems to be a technically straightforward and highly sensitive approach, yet cor-

relation with the MBA and thorough validation studies will have to be performed in the future.

In conclusion, while cell-based assays offer the opportunity to fully replace the MBA for potency testing due to the depiction of the complete mode of action, binding and cleavage assays can contribute to reduce the overall number of animals used. Notably, the latter type of assays could also be implemented for diagnostics of botulism as they potentially overcome several of the challenges observed here.

Replacement methods for diagnostics of botulism

Most developments towards animal replacement methods have been focused on the potency testing of pharmaceutical BoNT preparations. This work has led to two FDA approved assays so far (Fernández-Salas et al. 2012, Merz Pharma GmbH & Co. KGaA 2015). Independent of these efforts, replacement methods for botulism diagnostics are also needed, but are less often addressed (Lindström and Korkeala 2006, Cai et al. 2007). Importantly, separate developments are needed in this field which take into account the fundamentally different requirements of diagnostic approaches as compared to potency testing (Tab. 1 and section on "Challenges in BoNT detection" (Straughan 2006)).

It is those prerequisites that have been dictating the different setups of animal replacement methods developed for either diagnostics or potency testing in the past. The requirement to depict the complete mode of action has pushed replacement assays for potency testing strongly towards cell-based assays (Pellett 2013). However, those assays are not well suited for the detection of BoNT from complex sample materials which is needed for diagnostics due to the potential interference of matrix components with cell viability and the need for sterile samples. Instead, in the field of diagnostics reducing the risk of false negative results paired with the high sensitivity of enzymatic assays has favored assays which detect the enzymatic activity of the BoNTs. Most critical, enrichment and purification steps have to precede such assays to remove unspecific proteases found in complex matrices (Jones et al. 2009) as well as to provide optimum buffer conditions for the *in vitro* cleavage reaction (Jones et al. 2008).

Enrichment to enable detection from complex matrices

Usually, in diagnostic settings antibodies coupled to magnetic beads are used for toxin enrichment from complex matrices (Dunning et al. 2012, Kalb et al. 2015c, Hansbauer et al. 2016). Hereby, enrichment from larger sample volumes enable sensitive detection while the optimization of washing steps and cleavage buffers enable the efficient removal of both inhibiting substances and unspecific proteases (Kalb et al. 2006).

Generally, there are some caveats that have to be considered when choosing antibodies for enrichment of BoNTs. Besides standard requirements such as high affinity and detection of native toxins from solution (Stern et al. 2016a, Stern et al. 2016b), great care has to be taken when choosing the antibodies' binding site on the toxins. Mostly, antibodies targeting the receptor binding domain are better suited as enrichment antibodies as they do not interfere with the subsequent enzymatic cleavage of the light chain (Kalb et al. 2009). However, there are notable examples where antibodies also targeting the light chain can lead to high sensitivity (Bagramyan et al. 2008). Therefore, for every experimental approach, an optimally matching antibody has to be selected for toxin extraction – there is no "one-for-all-purposes" antibody, and results obtained in one experimental setting cannot necessarily be transferred to a different experimental setup.

Additionally, it is highly crucial to select an antibody which is able to detect all relevant BoNT subtypes of a given serotype (see section "Challenges in BoNT detection") – a lack in binding to individual subtypes of a serotype would render the assay false negative. One possibility to circumvent this challenge is to implement pan-specific antibodies binding to conserved epitopes (Kalb et al. 2010) or to implement oligoclonal mixtures of antibodies complementing each other's sub- and serotype binding profiles.

Overall, high affinity monoclonal antibodies are better suited for extraction purposes compared to polyclonal antibodies, since their potential cross-reactivity with matrix components is usually limited and/or can be better controlled for. However, a current limitation in the field is that those high quality reagents are not commercially available and are restricted to few expert laboratories worldwide (Dorner et al. 2016). Along the same line, the testing of antibody specificity for binding of the known subtypes of the BoNT serotypes has been hampered by the fact that there is no single laboratory worldwide which has access to all relevant subtypes.

An alternative approach for toxin enrichment would lie within the coupling of the high-affinity endogenous receptors instead of antibodies to magnetic beads. However, this is not a trivial approach, since the BoNTs bind to two receptors of different molecular nature (transmembrane protein receptors, gangliosides, Fig. 2). Notably, very recent data indicate that BoNT/B, DC and G employ even ternary interactions – instead of dual interactions as previously anticipated – with a protein, ganglioside, and lipids (Stern et al. 2018). Additionally, at least for selected BoNTs, here BoNT/A, a highly specific post-translational modification of the protein receptor has been identified to be crucial for high-affinity binding (Yao et al. 2016). Furthermore, the individual subtypes of a given serotype can differ in their receptor binding affinities (Whitemarsh et al. 2013, Benoit et al. 2017, Davies et al. 2017) and as such have intrinsic difficulties similar to monoclonal antibodies. Regardless, subtypes with a lower affinity towards their receptors should also be less toxic in vivo so that a receptor-based enrichment step would give results more comparable to the MBA. In this context, novel technical approaches are needed to implement the dual-receptor binding concept appropriately.

Cleavage-based assays for sensitive detection

Cleavage-based assays suitable for diagnostic purposes as potential replacement methods for the MBA can be categorized by the different readouts used to detect the cleaved synaptic substrates (Fig. 3). While all assays can be used to detect the enzymatic activity of BoNTs, they are associated with different advantages and disadvantages, which are summarized below.

Mass spectrometry-based detection of BoNT activity by Endopep-MS

The mass spectrometry (MS)-based endopeptidase activity assay (Endopep-MS) is a functional method for rapid and sensitive detection, differentiation and quantification of all known BoNT serotypes (Barr et al. 2005, Boyer et al. 2005). The method combines the serotype-specific cleavage of a peptide substrate derived from the toxins' natural target in the SNARE complex with the highly sensitive, accurate detection and identification of the resulting two cleavage products by MS (Fig. 3). Either high-resolution matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) MS or liquid chromatography (LC) electrospray ionization (ESI) tandem MS (MS/MS) can be used to depict the two cleavage products of known mass. Serotypes BoNT/A, C and E can be distinguished by unique cleavage sites in SNAP-25, whereas BoNT/B, D, F, G and HA target VAMP-2 at specific amino acid positions (Fig. 2). Due to the fact that functional assays are sensitive towards interfering substances, e.g. endogenous proteases in stool, the method needs an immunoaffinity enrichment step prior to the cleavage reaction, combined with a high salt washing step to remove matrix components (Wang et al. 2011). The enrichment by serotype specific monoclonal antibodies coupled to magnetic beads (Kalb et al. 2006, Gaunt et al. 2007, Kalb et al. 2008, Kalb et al. 2009, Kalb et al. 2011b, Parks et al. 2011, Wang et al. 2011) made the Endopep-MS assay highly suitable for the detection of active BoNT from complex matrices like serum (Kalb et al. 2006, Parks et al. 2011), stool (Kalb et al. 2006), foods (Kalb et al. 2015a) and bacterial culture supernatants (Kalb et al. 2008, Wang et al. 2014) with limits of detection in the range of the MBA or below. Technical improvements comprised enhancing peptide substrate stability, reducing possible unspecific cleavage by endogenous proteases, and increasing the detection sensitivity by using artificial, optimized substrates to deliver limits of detection for BoNT/A at 0.1 mouse LD_{50}/mL (Wang et al. 2013a, Rosen et al. 2017a, Rosen et al. 2017b, Wang et al. 2017), for BoNT/B below 1 mouse LD_{50}/mL (Rosen et al. 2015, Rosen et al. 2017b), for BoNT/C at 0.5 mouse LD_{50}/mL (Wang et al. 2015a), for BoNT/E at 0.1 mouse LD_{50}/mL (Rosen et al. 2014, Wang et al. 2015b, Rosen et al. 2017b) and for BoNT/F at 0.1 mouse LD_{50}/mL (Kalb et al. 2011a).

Using different high quality antibodies for extraction, it was shown that the Endopep-MS assay can also detect veterinary BoNTs (Hedeland et al. 2011, Moura et al. 2011, Björnstad et al. 2014, Wang et al. 2015a, Hansbauer et al. 2016). The method was even able to differentiate the highly related veterinary BoNT serotypes C and D and their mosaic variants CD and DC (Björnstad et al. 2014, Hansbauer et al. 2016) by combining highly specific monoclonal antibodies targeting toxin subdomains plus appropriate cleavage conditions.

While the Endopep-MS assay was first introduced as a qualitative assay, it has been developed into a quantitative approach: For quantitative BoNT determination a known quantity of a stable isotope-labelled product peptide is added to the sample prior to analysis which can be differentiated from the cleavage reaction product by its slightly different mass. For quantitation, the peak area derived from the isotope-labelled product peptide is compared to a standard curve of known BoNT concentrations in the matrix (Kalb and Barr 2013, Björnstad et al. 2014, Wang et al. 2014, Kalb et al. 2015a, Kull et al. 2015). The method delivered excellent results in terms of qualitative and quantitative detection of known BoNTs in a recent international proficiency test. In fact, in this exercise it was among the three animal replacement methods giving superior results than the MBA (Kalb et al. 2015a, Weisemann et al. 2015, Worbs et al. 2015, Dorner et al. 2016).

Compared to the MBA the Endopep-MS assay is more rapid (6 hours instead of 1–4 days) and of similar sensitivity. A further benefit is that BoNT enriched by antibodies can be introduced into an MS-based identification and toxin subtyping procedure by high-resolution MS/MS amino acid sequencing (Kalb et al. 2012a, Kalb et al. 2015a, Hansbauer et al. 2016).

Independent of its application for diagnostics, the Endopep-MS assay turned out to be instrumental in identifying new BoNT sero- and subtypes by its ability to define new and unique cleavage sites in the SNARE substrates. This has been first shown for subtype BoNT/F5 (Kalb et al. 2012b) and confirmed for the newly identified BoNT/FA (HA) (Kalb et al. 2015b). However, conclusions about neurotoxicity cannot be drawn based on Endopep-MS data alone as only the enzymatic activity is depicted. Therefore, cell based assays or MBA have to complement the Endopep-MS assay for an in depth assessment of the toxic potential of new BoNT sero- or subtypes (Zornetta et al. 2016, Zhang et al. 2017, Zhang et al. 2018).

Up to now, the Endopep-MS assay was limited to expert laboratories with access to expensive high-resolution mass spectrometers and specialized technical expertise, but with rising interest for routine identification and typing of microorganisms by linear MALDI-TOFs coupled with an automated analysis software (*e.g.* Bruker MALDI Biotyper®, bioMérieux VITEK® MS or Andromas SAS), more and more clinical and public health laboratories use rapid and cost-effective mass spectrometers. In this context, it was recently shown that a Bruker MALDI Biotyper®, which is often available in modern clinical and public health laboratories, shows sufficient sensitivity and accuracy for BoNT detection by Endopep-MS assay (Perry et al. 2017).

Fluorescence or luminescence-based cleavage assays

For enzymatic detection of BoNT at lower instrumental levels than MS, fluorescence- or luminescence-based approaches have been developed. Here, peptide substrates are labelled with a fluorescent or luminescent reporter molecule which is released upon substrate cleavage by BoNT. To this end, an assay detecting all BoNTs pathogenic to humans (BoNT/A, B, E, and F) was developed by immobilizing serotype specific peptides flanked with fluorescein and measuring the fluorescence intensity of supernatants after substrate cleavage (Schmidt et al. 2001). For assay automatization this principle has been transferred into a microfluidic format, allowing semiautomatic detection of BoNT/A (Frisk et al. 2009, Frisk et al. 2011). Similarly, bioluminescence has been employed to detect BoNT/E from buffer and BoNT/A from buffer as well as from different sample matrices (Stevens et al. 2013). Here, a recombinant substrate protein consisting of two consecutive SNAP-25 sequences fused to luciferase was immobilized on magnetic beads. The release of luciferase after substrate cleavage and subsequent conversion of the luciferase substrate luciferin creates an amplified signal which could be monitored.

Another approach employing fluorescence intensity as signal read-out for BoNT detection are FRET-based assays which are also in use for potency testing (see section "Binding and cleavage-based assays", Fig. 3). The method is based on a substrate molecule equipped with a donor and an acceptor fluorophore. If donor and acceptor remain in close proximity, the fluorescence signal of the donor is quenched by the acceptor. Upon substrate cleavage by BoNT, donor and acceptor become separated which can be measured by an increase in fluorescence intensity as the donor is no longer quenched by the acceptor. Different variations of FRET-based assays for detection of BoNT/A and B have been developed. In respective assays, either commercially available peptide substrates were employed (Bagramyan et al. 2008, Rasooly and Do 2008, Rasooly et al. 2008) or – to push sensitivity – peptide substrates with special features such as the inclusion of important exosites or variations of donor and acceptor molecules were used (Anne et al. 2001, Dong et al. 2004, Pires-Alves et al. 2009, Poras et al. 2009). Akin to other enzymatic assays for BoNT detection, a stringent enrichment step was required to detect BoNT/A from complex matrices. This was facilitated by immuno-enrichment using anti-BoNT/A antibodies coupled to magnetic (Rasooly and Do 2008, Rasooly et al. 2008) or sepharose beads (Bagramyan et al. 2008). Here, BoNT could be detected with high sensitivity from different matrices, including serum and different foods.

Advantages of fluorescence/luminescence-based approaches are their high sensitivity combined with a simple and fast assay protocol. Furthermore, commonly available laboratory equipment is sufficient to perform respective assays ensuring a broad applicability in routine laboratories. A major drawback is, however, that serotypes targeting the same substrate cannot be distinguished by the method, as all cleavage sites are within the donor and acceptor molecule on the same substrate (SNAP-25: A, C, and E; VAMP-2: B, D, F, G, HA). Shorter peptide substrates enabling a discrimination of serotypes E or A/C and B or F/D may be applied, but presumably would lead to a loss in sensitivity due to the lack of important exosites (Ouimet et al. 2013). Still, the discrimination of cleavage by BoNT/A and C or BoNT/F and D would hardly be possible without further substrate modifications, since their respective cleavage sites on SNAP-25 or VAMP-2 are only one amino acid apart (Fig. 2).

Neo-epitope specific antibody-based assays

To develop simple assay systems detecting BoNT LC activity which can discriminate between the different BoNT serotypes, neo-epitope specific antibodies are employed. As already mentioned, these antibodies specifically recognize the newly exposed epitope on the cleaved substrate, while the intact substrate is not recognized. Regarding BoNT detection, such antibodies are employed to detect cleavage products of SNARE proteins after cleavage by the different BoNT serotypes (Fig. 3). Thus, different serotypes can be distinguished by detection of their specific cleavage site. Different approaches using different assay platforms – most of them employing polyclonal neo-epitope specific antibodies – have been developed for detection of BoNT/A, B, C, E, and F. Most commonly, neo-epitope specific antibodies were used in different variations of ELISA-based approaches. In a so-called Endopep-ELISA, the substrate molecule SNAP-25 or VAMP-2 is immobilized on microtiter plates and cleaved by addition of BoNT containing sample. The resulting neo-epitopes are then detected by neo-epitope specific antibodies and an enzyme-conjugated anti-species antibody. Using this method, detection of BoNT/A, B, or C with sensitivities similar to the MBA was facilitated (Hallis et al. 1996, Jones et al. 2008, Jones et al. 2009, Jones and Marks 2013). This approach was further extended by Liu et al. in a functional-dual-coating assay for BoNT/A detection (Liu et al. 2012): Here, in addition to SNAP-25, an anti-BoNT/A antibody was coated to accumulate the toxin on the microtiter plate surface achieving an excellent detection limit below the MBA. Detection of different toxin-contaminated foods was demonstrated for BoNT/B by employing immunoaffinity columns packed with a monoclonal anti-BoNT/B antibody for toxin enrichment. Subsequently, a biotinylated substrate was cleaved in solution and cleavage products were immobilized on streptavidin-coated plates and detected by neo-epitope specific antibodies (Wictome et al. 1999). Interestingly, an Endopep-ELISA format was among the three superior animal replacement methods in terms of qualitative and quantitative detection of BoNT in a recent international proficiency test (Simon et al. 2015, Worbs et al. 2015, Dorner et al. 2016).

In addition to Endopep-ELISA formats, an innovative surface plasmon resonance (SPR)-based approach for the detection of BoNT/A, and E has been developed (Lévêque et al. 2013, Lévêque et al. 2014, Lévêque et al. 2015a, Lévêque et al. 2015b). Here, SNAP-25 was immobilized on the surface of a sensor chip. Then, BoNT containing samples were injected over the sensor surface and cleavage was monitored by injecting neo-epitope specific antibodies. In contrast to the ELISA-based methods mentioned above, the SPR sensor employed monoclonal neo-epitope specific antibodies for detection of cleavage products, thereby making it suitable for comprehensive validation studies and a wide application. Impressive sensitivities greatly exceeding the MBA were achieved for detection from buffer as well as serum.

In summary, neo-epitope specific antibodies proved to be a highly suitable tool for detection of BoNT's enzymatic activity in different assay platforms, delivering sensitivities below the MBA even in selected complex matrices. Notably, cleavage activity for most BoNT serotypes (BoNT/B, C, F) on their respective substrates could be detected with polyclonal neo-epitope specific antibodies so far. Only for BoNT/A and E monoclonal neo-epitope specific antibodies have been published (Fernández-Salas et al. 2012, Lévêque et al. 2013, Lévêque et al. 2014, Lévêque et al. 2015b). Similar to polyclonal anti-toxin antibodies used for enrichment strategies, the use of polyclonal neo-epitope specific antibodies is often hampered by their tendency to interfere with matrix components, limiting their broad applicability in a variety of complex matrices. Thus, the investigation of monoclonal neo-epitope specific antibodies for detection of all disease-causing serotypes is highly desirable in the future.

Remaining challenges and conclusions

The development of animal replacement methods for testing the presence and activity of BoNTs is a long pursued goal which mainly focused on replacement

methods for pharmaceutical potency testing. The efforts in this area have resulted in two FDA approved replacement methods so far which are cell-based assays that depict the cellular uptake, translocation and substrate cleavage induced by BoNT/A1 (Fernández-Salas et al. 2012, Eisele and Mander 2015, Merz Pharma GmbH & Co. KGaA 2015). Because of the exquisite specificity of BoNT for neuronal cells, the main challenge in developing assays for potency testing is to identify or to establish suitable cell lines which are equipped with the necessary receptors and endogenous substrate molecules to allow for a highly sensitive and reproducible activity determination comprising all steps of the toxin's mode of action.

Due to fundamentally different requirements, the achievements in the field of potency testing cannot be transferred into the field of BoNT diagnostics. In the latter, detection of more than 40 different BoNT sero- and subtypes has to be achieved from complex clinical, food, and environmental matrices. To this end, several promising animal replacement methods have been developed depicting mostly the cleavage activity of BoNT either with or without demonstrating the presence of the receptor-binding domain. Several recent assays also explored the feasibility to display the binding of BoNT in vitro, followed by cleavage activity. This, however, turned out to be highly challenging due to the molecular structure of the BoNT receptors and their variability among serotypes – only the successful display of high-affinity binding would result in detection limits necessary to replace the MBA. Here, innovative approaches are necessary to display the endogenous receptor molecules containing all necessary post-translational modifications *in vitro*. If solutions to this can be found, this would enable functional enrichment of BoNT via the receptor-binding domain as described for binding and cleavage assays. However, before this can be a viable approach, further investigations of the precise molecular interaction of all BoNT serotypes with their receptors have to be pursued, especially for serotypes other than BoNT/A and B taking into account the very recent findings of tripartite toxin-receptor interactions (Stern et al. 2018, Yao et al. 2016).

For the time being, the majority of diagnostic laboratories use antibodies for enrichment of BoNTs from complex matrices. Here, the current limitation is that high affinity antibodies reactive against all subtypes of a given serotype are not commercially available and are restricted to few expert laboratories worldwide, if available at all (Dorner et al. 2016). Additionally, testing of antibody specificity for binding of all known BoNT subtypes requires access to all relevant subtypes which is currently not possible. An international effort is needed to work on this issue, to make available high quality detection tools and BoNT subtypes to authorized laboratories in order to enable comprehensive validation studies. As a starting point, the European Commission has recently launched a research project called EuroBioTox under the Horizon 2020 program to network toxin laboratories and to support international validation of diagnostic approaches (The EuroBioTox Consortium 2017). For BoNT detection, a first step in the establishment of quality assurance schemes has been undertaken in a previous European program (The EQuATox Consortium 2012). In this framework, the first international proficiency test for the detection of BoNT/A, B and E from differently spiked matrices has been organized (Worbs et al. 2015, Dorner et al. 2016). The exercise provided the valuable information that among several methods run in parallel on the same sample set, Endopep-MS and Endopep-ELISA approaches, which both do not require animals or animal tissues any more, delivered qualitative and/or quantitative results similar to or better than the MBA. Additionally, the mouse hemidiaphragm assay – still requiring animal tissue – delivered superior results. According to this information, those three functional approaches have the potential to significantly reduce the MBA for botulism diagnostics, provided that further extensive validation studies will be performed in the future on a comprehensive set of clinical, food and environmental matrices. A complete replacement of the MBA might be hard to obtain because of its pivotal role in research but also for detection and characterization of novel BoNT sero- and subtypes. Additionally, the MBA might still be needed as a supplementary or confirmatory assay to in vitro diagnostic assays and potency determinations. Further proficiency tests and method performance studies will be necessary to demonstrate the broad applicability, sensitivity, and robustness of the replacement methods under scrutiny. Finally, this work will be the basis for establishing internationally accepted recommended operating procedures to replace the current "gold standard" MBA, a process which requires further substantial work in the next decade.

Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript. The authors work in the German Consultant Laboratory for Neurotoxin-producing Clostridia (Botulism, Tetanus), simultaneously active as DVG Consultant Laboratory for *C. botulinum* and Botulinum Neurotoxins in Food.

Ethical approval of animal experiments

All animal experiments were performed at Robert Koch Institute in compliance with the German Animal Welfare Act and European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). Experiments were evaluated and approved by the State Office for Health and Social Affairs in Berlin (LaGeSo, Berlin, Germany) under registration numbers H109/03 (production of antibodies) and A0073/08 (mouse bioassay).

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Authors contribution

Daniel Stern and Brigitte G. Dorner drafted and wrote the manuscript with significant input from the other authors (Laura von Berg: chapter on Fluorescence or luminescence-based cleavage assays and Neo-epitope specific antibody-based detection. Martin Skiba: chapter on Mass spectrometry-based detection of BoNT activity by Endopep-MS. Martin B. Dorner: significant contributions to the introduction on animal botulism, the mouse bioassay and diagnostics of botulism). All authors corrected and approved the final version of the manuscript.

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