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The long journey of botulinum neurotoxins into the synapse

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ABSTRACT

Botulinum neurotoxins (BoNT) cause the disease botulism, a flaccid paralysis of the muscle. They are also very effective, widely used medicines applied locally in sub-nanogram quantities. BoNTs are released together with several non-toxic, associated proteins as progenitor toxin complexes (PCT) by *Clostridium botulinum* to become highly potent oral poisons ingested via contaminated food. They block the neurotransmission in susceptible animals and humans already in nanogram quantities due to their specific ability to enter motoneurons and to cleave only selected neuronal proteins involved in neuroexocytosis.

BoNTs have developed a sophisticated strategy to passage the gastrointestinal tract and to be absorbed in the intestine of the host to finally attack neurons. A non-toxic non-hemagglutinin (NTNHA) forms a binary complex with BoNT to protect it from gastrointestinal degradation. This binary M-PTC is one component of the bi-modular 14-subunit ~760 kDa large progenitor toxin complex. The other component is the structurally and functionally independent dodecameric hemagglutinin (HA) complex which facilitates the absorption on the intestinal epithelium by glycan binding. Subsequent to its transcytosis the HA complex disrupts the tight junction of the intestinal barrier from the basolateral side by binding to E–cadherin. Now, the L-PTC can also enter the circulation by paracellular routes in much larger quantities. From here, the dissociated BoNTs reach the neuromuscular junction and accumulate via interaction with polysialo gangliosides, complex glycolipids, on motoneurons at the neuromuscular junction. Subsequently, additional specific binding to luminal segments of synaptic vesicles proteins like SV2 and synaptotagmin leads to their uptake. Finally, the neurotoxins shut down the synaptic vesicle cycle, which they had exploited before to enter their target cells, via specific cleavage of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which constitute the core components of the cellular membrane fusion machinery.

1. The origin of BoNT

1.1. Diversity of BoNT: serotypes, mosaics and subtypes

Botulinum neurotoxins (BoNT) are closely related 150 kDa bacterial AB protein toxins which are postranslationally cleaved by specific clostridial or host proteases into a ~50 kDa light chain (LC), the Active part, and a ~100 kDa heavy chain (HC), the Binding part, which remain associated *via* a single interchain disulfide bond (Brunger and Rummel, 2009). They are grouped into the seven serotypes BoNT/A-G. The serotype designation defined by the absence of cross-neutralization in an animal bioassay by type-specific monovalent botulinum antitoxin (Leuchs, 1910) was first established by Georgina Burke in 1919 (Burke, 1919). In 1895, Emile van Ermengem was the first to isolate a *Clostridium botulinum* strain producing BoNT/A from an incompletely salted ham having caused a serious food-borne botulism outbreak in Ellezelles/Hennegau, Belgium (van Ermengem, 1897). Only nine years thereafter, Landmann isolated a *C. botulinum* strain producing BoNT/B from bean salad also having evoked a serious food-borne botulism outbreak in Darmstadt/Hessen, Germany (Landmann, 1904). Since then five

Abbreviations: BoNT, botulinum neurotoxin; GBS, ganglioside binding site; HA, hemagglutinin; HC, 100 kDa heavy chain; H_CX, 50 kDa C-terminal half of HC of CNTs; H_{CC}, 25 kDa C-terminal half of H_C; H_{CN}, 25 kDa N-terminal half of H_C; H_N, 50 kDa N-terminal half of HC; LC, 50 kDa light chain; L/M-PTC, large/medium progenitor toxin complex; MPN, mouse phrenic nerve; NAP, neurotoxin associated proteins; NMJ, neuromuscular junction; NTNHA, non-toxic non-hemagglutinin; SNAP-25, synaptosomal associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SV, synaptic vesicle; SV2, synaptic vesicle glycoprotein 2; Syt-X, isoform X of synaptotagmin; VAMP, vesicle associated membrane protein; TeNT, tetanus neurotoxin.

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further serotypes were discovered: BoNT/C in 1922 (Bengtson, 1922, 1923), BoNT/D in 1928 (Meyer and Gunnison, 1929), BoNT/E in 1937 (Hazel, 1937), BoNT/F in 1960 (Møller and Scheibel, 1960) and BoNT/G in 1970 (Gimenez and Ciccarelli, 1970). Of them, BoNT/A, B, E and F are predominantly causing food-borne (intoxication), infant (colonization of colon) and wound (infection) botulism in humans whereas BoNT/C and BoNT/D mainly evoke botulism in birds and cattle, respectively. The serotype designation was challenged when polyclonal antibodies generated against BoNT/D of strain South Africa (BoNT/D-SA) detected BoNT/C of strain Stockholm better than BoNT/D of strain 1873 while BoNT/C of strain 6813 was hardly detected. Furthermore, antibodies directed against the HC of BoNT/D-SA showed similar behaviour whereas antibodies directed against the LC of BoNT/D-SA detected BoNT/D-SA and BoNT/D-1873 equally well, but none of the two BoNT/C (Moriishi et al., 1989). This cross reactivity could only be explained by exchange of domains between the HCs of BoNT/C and D. Indeed, molecular cloning and sequencing of the coding sequences (CDS) of BoNT/C-6813 and BoNT/D-SA revealed that in both CDSs the terminal 1200 bp each encoded the converse 50 kDa H_C-fragment (Moriishi et al., 1996a, 1996b). Thereupon, BoNT/D-SA and BoNT/C-6813 were renamed to BoNT/DC and BoNT/CD, respectively, and are the first and best known examples for so called BoNT mosaics. Very recently, a novel toxinotype was identified. Its CDS revealed a close homology of its LC to BoNT/F5, the medium domain termed H_N is unique among the seven known serotypes whereas the C-terminal H_C-fragment is highly similar to that of BoNT/A (Dover et al., 2014b; Gonzalez-Escalona et al., 2014a). Therefore, also this novel toxinotype constitutes a mosaic BoNT and upon first functional studies is currently named BoNT/FA (Kalb et al., 2015; Maslanka et al., 2015).

The first BoNT CDS deciphered was that of BoNT/A in 1990 (Binz et al., 1990). In the following 4 years the CDS of the remaining six BoNT serotypes were determined (Niemann et al., 1994). In course of those studies also genetic variants of BoNT/A, B and F were identified (Thompson et al., 1993; Willems et al., 1993; Hutson et al., 1994); e.g. the BoNT/A genetic variant of strain Kyoto-F having caused an infant botulism case (Sakaguchi et al., 1990) displays a 89.8% amino acid (AA) sequence identity (132 of 1296 AA differ) with that of strain 62A having caused food-borne botulism (Willems et al., 1993). Interestingly, the AA exchanges are not evenly distributed; the LC of BoNT/A Kyoto-F is 95% identical to that of BoNT/A strain 62A whereas the corresponding HC only shows 87% identity. Since then further six BoNT/A genetic variants designated by addition of a number following the letter have been identified: BoNT/A1-A8 (Kull et al., 2015). Subsequent analysis of the primary structures of BoNT/A1-3 revealed that also BoNT/A2 is a mosaic toxin within serotype A comprising the LC of BoNT/A1 and the HC of BoNT/A3 (Arndt et al., 2006). Similarly, BoNT/F6 is a mosaic of BoNT/F1 and F2 and has been recombined in a different species, *Clostridium baratii* (Carter et al., 2013; Smith et al., 2015). Hence, DNA elements encoding BoNT are very mobile and can be exchanged horizontally even between different clostridia species (Skarin and Segerman, 2011).

Up to now more than 40 genetic BoNT variants have been identified due to the major progresses in sequencing technology (Hill and Smith, 2013). They can differ up to 36% in AA sequence as in case of BoNT/F variants. However, do these differences result in functional consequences? Indeed, already in 1977 the BoNT/B isolated from strain 111 displayed different antigenicity compared to BoNT/B1 produced by strain Okra (Kozaki et al., 1977). Analogously, BoNT/A2 differed in antigenicity towards monoclonal antibodies directed against BoNT/A1 and in addition, showed altered binding to synaptosomal membranes (Kozaki et al., 1995). Such differences in antigenicity might lead to a therapy failure employing neutralizing monoclonal antibodies (Smith et al., 2005; Mazuet et al., 2010) as well as lack of protection upon immunization with only one variant (Webb et al., 2009). These findings led to the introduction of the term *subtype*: a genetic BoNT variant with minimum 2.5% difference in AA sequence (Smith et al., 2005). An alternative phylogenetic approach defines subtypes as corresponding to clades formed by the clustering of bont sequences (Chen et al., 2007; Hill et al., 2007; Raphael et al., 2010). Applying either of these definitions, the currently identified genetic variants have been ascribed to the subtypes BoNT/A1-A8 differing in 2.9–15.6% in AA sequence, BoNT/B1-B9 (1.6–7.3%), BoNT/E1-E11 (0.9–10.9%) and BoNT/F1-F7 (3.0–36.2%; Fig. 1) (Kalb et al., 2012a; Raphael et al., 2012; Hill and Smith, 2013; Wangroongsarb et al., 2014; Weedmark et al., 2014; Kull et al., 2015). Apart from antigenicity, also functional differences predominantly of BoNT/A subtypes with respect to catalytic activity, neurotoxicity and interesting pharmacological properties like duration of action have been demonstrated (Henkel et al., 2009; Torii et al., 2011; Wang et al., 2013; Whitemarsh et al., 2013; Bradshaw et al., 2014; Torii et al., 2014; Whitemarsh et al., 2014; Kull et al., 2015; Torii et al., 2015). For BoNT/B2, differences in binding to its neuronal receptors were characterized in detail (Kozaki et al., 1998; Ihara et al., 2003; Kohda et al., 2007). Compared to BoNT/F1, BoNT/F7 (73.7% AA identity) and BoNT/F5 (69.9% AA identity) displayed drastic differences in catalytic properties (Kalb et al., 2011; Guo and Chen, 2015). Currently, all subtypes within each serotype are known to cleave their protein target in the same peptide bond, with the exception of BoNT/F5 (Kalb et al., 2012b). Here, LC/F5 is only 46.3–48.9% identical to the other LC/F1-7 which is much less than that between LC of differing serotypes (56% between LC/E and LC/F; 61% between LC/B and LC/G) whereas HC/F5 is 89.1% identical to HC/F2 (Raphael et al., 2010). Hence, sequence and functional data as well as 20-times higher dosage for BoNT/F5 neutralization suggests that also BoNT/F5 is a mosaic comprising a novel type of LC, termed e.g. LC/H and HC/F. No functional data is yet available for BoNT/E subtypes. In future, more genetic variants are expected, however, their classification as subtypes according to arbitrary dissimilarity in primary structure or defined functional differences is currently heavily debated in the field.

1.2. The BoNT host: Clostridium groups I–VI

Since Emile van Ermengem isolated a *C. botulinum* strain producing BoNT/A hundreds of strains have been isolated from soil, food and patients' samples like faeces. These anaerobic, rod-shaped, Gram+ bacteria were uniformly called *C. botulinum* due to its characteristic production of different BoNT serotypes. However, already microbiological and biochemical characterisation of *C. botulinum* isolates revealed that the bacteria within the species possessed different characteristics and belonged to four different groups, termed I–IV. Interestingly, group I strains only express BoNT/A, B and F whereas the non-proteolytic group II produces exclusively BoNT/E but also BoNT/B4 and BoNT/F6 (Table 1). Group III only hosts BoNT/C, D and their corresponding mosaics while group IV solely secretes BoNT/G (Sakaguchi, 1982; Hatheway, 1990; Popoff, 1995). Subtype BoNT/F7 is only produced by a non-*C. botulinum* species, *C. baratii*, which was isolated from faeces first of infants and later of adults due to intestinal colonization (Hall et al., 1985; McCroskey et al., 1991; Gimenez et al., 1992; Thompson et al., 1993). Subsequently, also neurotoxigenic *Clostridium butyricum* strains were identified as producer of BoNT/E4 and E5, first in infant botulism cases, later also associated with food-borne botulism (Aureli et al., 1986; McCroskey et al., 1986; Hauser et al., 1992; Poulet et al., 1992; Meng et al., 1997; Wang et al., 2000). Sequencing of the 16S rRNA gene in isolates within