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Current gaps in basic science knowledge of botulinum neurotoxin biological actions

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ABSTRACT

Botulinum neurotoxins are produced by anaerobic spore-forming bacteria of the genus *Clostridium* in several dozens of variants that inactivate neurotransmitter release owing to their metalloprotease activity. This results in a persistent paralysis of peripheral nerve terminals known as botulism. They are the most potent toxins known and are classified as one of the six highest-risk threat agents of bioterrorism. Despite their high toxicity, two of them are used as valuable pharmaceutical for the therapy of many neurological and non-neurological disorders. Notwithstanding the many advances in our understanding of the genetics and structure of botulinum neurotoxins, there are still many gaps in knowledge of toxin mechanism of action that will be discussed here.

1. Introduction

Neurotoxigenic clostridia belong to six phylogenetically distinct groups and produce seven serotypically different botulinum neurotoxins (denoted BoNT/A – G; Popoff and Bouvet, 2013; Hill and Smith, 2013). To date, the number of studies on the biology and ecology of toxigenic *Clostridia* is very limited, particularly in comparison with the extremely large cohort dedicated to the therapeutic uses of BoNTs or to the comprehension of BoNTs molecular action. However, thanks to the development of next generation sequencing, it has become recently clear that neurotoxigenic clostridia have considerable genetic heterogeneity in terms of genome organization, toxin gene clusters, and most importantly, toxin sequences variability (Hill and Smith, 2013). Accordingly, many toxin variants or subtypes within each serotype (with numerical designations following the toxin type, for example BoNT/A1 or BoNT/A2), have been identified and the number has dramatically grown reaching several dozens of BoNTs in a few years, and many others are predicted to be discovered (Montecucco and Rasotto, 2015). This is a potential goldmine for novel properties to be exploited for new clinical applications.

Despite of existence of a high number of isoforms, all BoNTs are structurally similar and consist of two chains linked by a unique disulphide bond: a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa). The complete crystallographic structures of three BoNTs (A1, B1 and E1) (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000; Kumaran et al., 2009; Montal, 2010) reveal different domains, which are functionally linked to the four steps of the mechanism of neuron intoxication by BoNTs.

1) Binding: the C-terminal domain of the heavy chain (HC) is responsible for the neurospecific binding. Notwithstanding the major effort of many different laboratories in the world, the receptors of BoNT/C (and many toxin subtypes) have not been yet identified.
2) Internalization: after binding to the presynaptic terminal via a double receptor mechanism, BoNTs enter into peripheral nerve terminals. In both cultured neurons and in vivo, BoNT/A1 have been shown to enter the synaptic vesicle lumen (Harper et al., 2011; Colasante et al., 2013), whereas the mechanism of internalization for other BoNTs remains to be formally established.
3) Translocation: in order to reach their intracellular targets, BoNTs translocate the L catalytic moiety across the membrane of endocytic vesicles into the neuronal cytosol and the N-terminal domain of the heavy chain (HN) is required for this step. Although studies carried out in the past decade have provided considerable insight on this pathway, the molecular aspects of

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BoNT translocation have been only partially elucidated (Montal, 2010; Fischer, 2013; Rossetto et al., 2014).

4) L chain catalytic activity: upon disulphide bond reduction, the L chain is released into the cytosol ready to exploit its enzymatic activity. The L chains of BoNTs are metalloproteases specific for one of the SNARE proteins: VAMP (vesicle associated membrane protein, also known as synaptobrevin), or SNAP-25 (synaptosomal-associated protein of 25 kDa) or syntaxin. Currently, the substrates of only a few of the BoNT subtypes have been determined and although it seems unlikely that novel BoNT substrates will be found, it is probable that novel cleavage sites within the SNARE proteins will be revealed. Therefore, another major challenge is to establish methods for the reliable comparison of the many BoNTs identified so far, and of those to come, which might reveal BoNTs of increased potency and duration of action in clinical applications.

The major open questions in the biology and pharmacology of botulinum neurotoxins are discussed below.

2. Gap 1. Diversity of neurotoxigenic clostridia and of botulinum neurotoxins

The neurotoxin-producing clostridia are genetically diverse by 16S ribosomal RNA sequences comparisons (Collins and East, 1998; Hill and Smith, 2013). They are organized into six clades (Groups I–VI) that also contain non neurotoxigenic species. Groups I–III are defined as *Clostridium botulinum* and include BoNT/A-/F producing *C. botulinum*, Group IV includes BoNT/G-producing *Clostridium argentinense*, Group V includes neurotoxigenic BoNT/F-producing and non-neurotoxigenic *Clostridium baratii* and Group VI includes neurotoxigenic BoNT/E-producing and non-neurotoxigenic *Clostridium butyricum* (Collins and East, 1998; Popoff and Bouvet, 2013; Hill and Smith, 2013). As genetic sequencing techniques became more efficient and economical, a considerable genetic heterogeneity of neurotoxigenic Clostridia and consequently high toxin sequences variability have been revealed. To date, nearly 40 toxin variants have been identified, mainly based on differences in genetic sequence (Hill and Smith, 2013), and this number is predicted to rapidly increase (Montecucco and Rasotto, 2015). They have been categorized as subserotypes (or subtypes), i.e. toxins immunogenically related to the parental serotypes, but with an amino acid composition difference comprised between 2.6 and 31.6% (indicated as BoNT/A1, …, BoNT/B1 …, etc.) (Hill and Smith, 2013; Rossetto et al., 2014). While the term “toxin subtypes” has been used extensively in the literature, several issues remain unclear including whether all nucleotide changes or just amino acid changes should be included, the number of differences that justify a new designation, whether changes must affect toxin function and how to designate these changes systematically. Moreover, the large number of BoNTs poses a major problem to current attempts to control the potential bioterrorist use via antibodies and vaccines because some of the novel BoNTs may be poorly neutralized by available antibodies. At the same time, a large pangenomic effort of next generation sequencing of soil samples from all over the world could lead to the identification of novel toxins endowed with novel therapeutic properties.

More efficient and accurate analysis methods are also being introduced, which are improving our knowledge of the mobility of neurotoxin genes among these bacteria. Neurotoxin gene localization on mobile DNA elements accounts for gene transfer between intra and inter Clostridium species (Popoff and Bouvet, 2013). It is noteworthy that tetanus neurotoxin (TeNT) produced by *Clostridium tetani* species is unique, whereas BoNT genes show a high degree of variability and are harboured by various *Clostridium* species and strains. Genetic and bioinformatic methods are providing the tools to expand our knowledge and understanding of the underlying mechanisms resulting in this diversity and *ad hoc* investigations will also throw light on the fundamental question of the origin and possible role(s) that BoNTs may have for *Clostridium* within their environments (Montecucco and Rasotto, 2015).

3. Gap 2. Neurospecific binding of BoNTs

To selectively target the presynaptic membrane of peripheral nerve terminals, BoNTs have evolved a unique binding mode via the C-terminal part of the HC domain, which is based on the use of two independent receptors: a polysialoganglioside (PSG) and a protein receptor located in the lumen of synaptic vesicles (SVs) (Montecucco, 1986; Rummel, 2013; Rossetto et al., 2014). The dual binding interaction with PSG and SV receptors increases the strength of BoNT interactions with the membrane as it is the product of the two binding affinities (Montecucco, 1986). It is also likely that additional low affinity, but selective interactions, mainly involving the N-terminal part of the HC binding domain, contribute to the neurospecificity (Montecucco et al., 2004; Muraro et al., 2009; Zhang et al., 2013) and this aspect should be further investigated.

The interaction with the protein receptors have been defined in molecular details for BoNT/B, BoNT/G and the hybrid BoNT/DC, which bind segment 40–60 of the luminal domain of the synaptic vesicle protein synaptotagmin (Syt), and for BoNT/A and BoNT/E, which in contrast bind specifically to two different segments of the fourth luminal loop of the SV transmembrane protein SV2 (for a complete list of references see Rummel, 2013 and Rossetto et al., 2014). Glycosylated residues are comprised within the toxin binding area of SV2 (Benoit et al., 2014) and the potential clinical relevance of this finding calls for appropriate investigations. In fact, a different pattern of glycosylation among individuals would provide a simple explanation for the variable sensitivity of different patients to BoNT/A1 injection, which is commonly observed in the clinic. Clearly, this consideration might also be applicable to different vertebrate species.

To date, the protein receptors of other BoNTs have not been characterized in similar details; furthermore, conflicting results have been reported, calling for further investigations. The mechanism underlying BoNT/C intoxication is particularly unclear. A protein receptor has not been identified for this serotype and it has been suggested that BoNT/C does not need a protein receptor (Tsukamoto et al., 2005). In addition to PSG, it has been shown to interact with phosphoinositides (Zhang and Varnum, 2012). Co-crystal structure of BoNT/C-HC with phosphoinositides would help to map the region of binding and to confirm these hypotheses.

4. Gap 3. Entry into the nerve terminal

After binding to the presynaptic receptors, BoNTs enter into the nerve terminal. At the mouse neuromuscular junction, BoNT/A1 was predominantly visualized within SVs and the number of toxin molecules (either 1 or 2) correlates with the number of SV2 molecules in the SV membrane (Colasante et al., 2013). A marginal amount of toxin was also found in early endosome and multivesicular bodies within hippocampal cultures (Harper et al., 2011), suggesting that in cultured CNS neurons other trafficking routes might contribute to toxin entry particularly at the very high concentrations that are frequently used in the laboratory.

The rate of entry for BoNT/A1 correlates with the rate of SV endocytosis (Saheki and De Camilli, 2012) and with the rate of paralysis of the mouse phrenic nerve hemidiaphragm. Also TeNT enters CNS neurons via SV endocytosis (Matteoli et al., 1996).