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Abstract: Tetanus (TeNT) and botulinum (BoNT) neurotoxins, the causative agents of tetanus and botulism, respectively, are the most potent toxic molecules known to mankind. This extreme potency is attributed to: i) their specificity for essential components of the neurotransmitter release machinery present at vertebrate synapses, and ii) their high-affinity targeting to motor neurons by binding to polysialogangliosides and protein receptors. Comprising the clostridial neurotoxin family, TeNT and BoNTs engage distinct surface receptors and intracellular sorting pathways in neurons. BoNTs bind to the intraluminal domain of specific synaptic vesicle proteins that are exposed to the extracellular milieu upon exocytosis, and are taken up by synaptic vesicle recycling. A sizeable proportion of BoNT molecules remain at the neuromuscular junction, where their protease moiety is released into the cytoplasm, blocking synaptic transmission and causing flaccid paralysis. In contrast, TeNT undergoes binding to specific components of the basal membrane at the neuromuscular junction, is endocytosed into motor neurons and sorted to axonal signalling endosomes. Following this, TeNT is transported to the soma of motor neurons located in the spinal cord or brainstem, and then transcytosed to inhibitory interneurons, where it blocks synaptic transmission. TeNT-induced impairment of inhibitory input leads to hyperactivity of motor neurons, causing spastic paralysis, which is the hallmark of tetanus. This review examines the molecular mechanisms leading to the entry, sorting and intracellular trafficking of TeNT and BoNTs.

1 **The travel diaries of tetanus and botulinum neurotoxins**

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29 **Abstract**

30 Tetanus (TeNT) and botulinum (BoNT) neurotoxins, the causative agents of tetanus and
31 botulism, respectively, are the most potent toxic molecules known to mankind. This extreme
32 potency is attributed to: i) their specificity for essential components of the neurotransmitter
33 release machinery present at vertebrate synapses, and ii) their high-affinity targeting to motor
34 neurons by binding to polysialogangliosides and protein receptors. Comprising the clostridial
35 neurotoxin family, TeNT and BoNTs engage distinct surface receptors and intracellular
36 sorting pathways in neurons. BoNTs bind to the intraluminal domain of specific synaptic
37 vesicle proteins that are exposed to the extracellular milieu upon exocytosis, and are taken
38 up by synaptic vesicle recycling. A sizeable proportion of BoNT molecules remain at the
39 neuromuscular junction, where their protease moiety is released into the cytoplasm, blocking
40 synaptic transmission and causing flaccid paralysis. In contrast, TeNT undergoes binding to
41 specific components of the basal membrane at the neuromuscular junction, is endocytosed
42 into motor neurons and sorted to axonal signalling endosomes. Following this, TeNT is
43 transported to the soma of motor neurons located in the spinal cord or brainstem, and then
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45 impairment of inhibitory input leads to hyperactivity of motor neurons, causing spastic
46 paralysis, which is the hallmark of tetanus. This review examines the molecular mechanisms
47 leading to the entry, sorting and intracellular trafficking of TeNT and BoNTs.

48

49 **Highlights**

- 50 • Tetanus and botulinum neurotoxins undergo long range traffic in mammalian neurons
- 51 • Signalling endosomes and autophagosomes mediate the transport of these neurotoxins
- 52 • The binding of tetanus toxin to the basal membrane is key for its uptake in neurons

53

54 **1. Historical background**

55 Tetanus (TeNT) and botulinum (BoNT) neurotoxins have been studied intensely over the last
56 century, while BoNTs have attracted worldwide attention in the last 25 years for their ever-
57 increasing medical applications. These neurotoxins are produced by *Clostridium tetani* and
58 various serotypes of *Clostridium botulinum*, which together form the clostridial neurotoxin
59 (CNT) family. The unmistakable clinical symptoms of tetanus toxicity were first reported in
60 Egyptian and Indian documents before 1500 BC. It was Hippocrates (460–370 BC) who
61 coined the term *τετανος* (translated to ‘tension’ in Ancient Greek) to describe these
62 symptoms when studying the progressive spastic paralysis developed by a sailor as a
63 consequence of an injury caused while handling the anchor of his boat (Udwadia, 1994).

64 However, the aetiology of tetanus remained a mystery until the end of the 19th century, when
65 the efforts of Carle and Rattone in Turin, Nicolaier in Göttingen and Kitasato in Berlin led to
66 the conclusion that tetanus was a transmissible disease caused by an anaerobic sporogenic
67 bacterium present in the soil (Udwadia, 1994). Although Nicolaier was able to report the
68 presence of a strychnine-like substance in the supernatant of these bacterial cultures, it was
69 Faber in 1890 who isolated TeNT and demonstrated its physiological role as the causative
70 agent of the spastic paralysis observed during tetanus (Udwadia, 1994). Importantly, the
71 availability of methods to isolate TeNT subsequently allowed Marie in 1897, and Meyer,
72 Ranson and others thereafter to demonstrate that TeNT was able to reach the central
73 nervous system (CNS), mediating its central effects after travelling along peripheral motor
74 nerves (Habermann, 1989; Marie, 1897; Udwadia, 1994). These findings, thus, set the stage
75 for the modern analyses of CNT trafficking in neurons.

76 Botulism, characterised by a general muscle weakness, was described independently in the
77 same period as TeNT by Kerner (1822), followed by the isolation of *C. botulinum* and the first
78 serotype of BoNT by van Ermengem in 1895 (van Ermengem, 1979). Traditionally seven
79 BoNT serotypes have been described in the literature including BoNT/A, BoNT/B, BoNT/C,
80 BoNT/D, BoNT/E, BoNT/F, and BoNT/G (Montal, 2010; Poulain et al., 2015; Pirazzini et al.,
81 2017). However, most recently, an eighth BoNT serotype has been discovered and named
82 BoNT/X (Zhang et al., 2017). Each individual serotype contains multiple subtypes of toxins
83 (e.g., BoNT/A1, BoNT/A2, etc.) (Poulain et al., 2015) with unique activities, synaptic targets
84 and downstream intracellular signalling (Pirazzini et al., 2017).

85 These discoveries, together with the isolation of different *C. botulinum* toxigenic strains and
86 studies on their intracellular activity and synaptic targets of TeNT and BoNTs in the 1990s
87 (Montal, 2010; Pirazzini et al., 2017), have revealed important insights into a complex protein
88 machinery responsible for the neuronal targeting, uptake and inhibition of synaptic
89 transmission by these neurotoxins. As a consequence, the study of the mode of action of
90 TeNT and BoNTs continue to have direct impact on several disciplines, including
91 microbiology, pharmacology, physiology, cell biology, biochemistry and molecular medicine.
92 TeNT and BoNTs have been used as tools of discovery in bioscience to dissect the
93 mechanisms of regulated secretion and intracellular trafficking, and as CNS-targeting
94 molecules for DNA vaccines and therapeutics (Behzadi et al., 2016; Toivonen et al., 2010).

95 The need for further in-depth characterisation of the mechanism of action of these
96 neurotoxins both *in vitro* and *in vivo* is further highlighted by the widespread use of BoNTs to
97 treat pathologies beyond the classical area of synaptic hyperactivity, such as chronic
98 migraine, depression and aesthetic/dermatological applications (Pirazzini et al., 2017). In
99 contrast, tetanus continues to claim the lives of thousands of individuals per year, including

100 many newborns affected by *tetanus neonatorum*
101 (http://apps.who.int/gho/data/view.main.1520_46) making the development of efficient
102 countermeasures an urgent priority.

103

104 **2. Mechanism of Action**

105 TeNT displays lethal dose, 50% (LD₅₀) ranging between 0.1 and 5 ng/kg of body weight in
106 mice (Gill, 1982), while the BoNT LD₅₀ lies between 0.1 and 500 ng/kg (Pirazzini et al.,
107 2017). The LD₅₀ for both neurotoxins, however, can greatly vary in different species (Gill,
108 1982). There are many factors that determine the precise time of symptom onset (i.e.,
109 paralysis) after CNT intoxication, including dose, route of application and species. For TeNT,
110 the incubation period between the initial injury and the onset of clinical symptoms is highly
111 variable (from 1-2 days to a couple of months) (Udwadia, 1994) and includes the time
112 needed for the spores to germinate into vegetative bacteria, which, after autolysis,
113 presumably release the neurotoxin into the bloodstream. Shorter incubation periods are
114 usually associated with TeNT of higher severity, in which the symptoms reach their peak in
115 7-10 days, plateau for 1-2 weeks and gradually decline in additional 1-2 weeks, although
116 muscle stiffness may persists for weeks or even months after recovery (Udwadia, 1994).
117 BoNTs, on the other hand, are typically released into the body via food contaminated by
118 spores, in which the storage conditions allowed their germination and the expression of the
119 progenitor toxin complex formed by BoNTs and non-toxic neurotoxin-associated proteins
120 (NAPs). NAPs comprise a non-toxic non-haemagglutinin component (NTNHA) that plays an
121 important role in protecting BoNTs from the harsh gastrointestinal tract, and other subunits
122 that enable binding to the surface of intestinal cells for subsequent transcytosis of the
123 neurotoxic complex from the apical membrane to the basolateral membrane of intestinal
124 epithelium (Amatsu et al., 2013; Gu et al., 2012; Lee et al., 2013; Lee et al., 2014; Sugawara
125 et al., 2014; Yao et al., 2014). Once released, the BoNT progenitor complex sequesters E-
126 cadherin in its monomeric form, blocking E-cadherin dimer formation, thus weakening the
127 trans-epithelial barrier (Lee et al., 2014; Sugawara et al., 2014). This process leads to bulk
128 entry of neurotoxin into the bloodstream and can accelerate intoxication.

129 After entering the general circulation, TeNT and BoNTs bind with high affinity to the
130 presynaptic membrane of the motor neuron at the neuromuscular junction (NMJ) where they
131 are rapidly internalised (Montal, 2010; Rummel, 2016) (**Figure 1A**). BoNTs mainly remain at
132 the NMJ and inhibit the release of the excitatory neurotransmitter acetylcholine (**Figure 1**),
133 blocking muscle excitation-contraction coupling and thus causing a flaccid paralysis. In
134 contrast, TeNT enters motor neuron axon terminals through endocytosis at the NMJ (**Figure**

135 **1B)** and is predominantly retrogradely transported in axonal signalling endosomes to the
136 soma of motor neurons in the spinal cord (Schmieg et al., 2014) (**Figure 1C**). TeNT is
137 subsequently transcytosed into inhibitory interneurons where it blocks neuroexocytosis
138 through the cleavage of the SNARE VAMP/synaptobrevin, thus inhibiting neurotransmitter
139 release from intoxicated interneurons to motor neurons (**Figure 1D**). As a consequence, the
140 balance between excitatory and inhibitory inputs to motor neurons is disrupted, eliciting
141 hyperactive motor neurons and spasticity. In addition to inhibitory interneurons (i.e.,
142 glycinergic and GABAergic), excitatory interneurons (i.e., glutamatergic and cholinergic) also
143 respond to TeNT application but with different sensitivity and effects (Bergey et al. 1987;
144 McMahon et al., 1992; Williamson et al. 1992; Shin et al., 2012). This preference for
145 inhibitory versus excitatory synapses is maintained when TeNT is applied directly into the
146 CNS and underlie the neurodegenerative and epileptogenic effects of TeNT (Bagetta et al.,
147 1990; Bowery et al., 1992; Ferecsko et al., 2015), which may result from unopposed release
148 of glutamate from excitatory central synapses.

149 Paradoxically, despite TeNT and BoNTs exert opposing influences on skeletal muscle (i.e.,
150 spasticity versus flaccidity), their modes of action are quite similar. Indeed, both CNT family
151 members block neurotransmitter release via specific cleavage of soluble NSF-attachment
152 protein receptor (SNARE) proteins involved in neuroexocytosis (Montecucco et al., 2005).
153 The differences in clinical symptoms arise from preferential site of action in different neurons
154 (Montal, 2010; Rummel, 2016) (**Figure 1**).

155 Interestingly, the hallmarks of TeNT and BoNT have also been observed in neurons other
156 than motor neurons, including cortical, sensory and sympathetic neurons (Blum et al., 2014;
157 Cordero-Erausquin et al., 2009).

158

159 **3. Multi-domain structure and function**

160 TeNT and BoNTs are remarkably similar in sequence and structure (Montal, 2010). The 150
161 kDa single-chain proteins are cleaved by proteases producing an active neurotoxin
162 comprising two chains of 100 kDa (heavy or H chain) and 50 kDa (light or L chain), which
163 remain associated via non-covalent interactions and a conserved inter-chain disulphide bond
164 essential for neurotoxicity (de Paiva et al., 1993; Pirazzini et al., 2014; Schiavo et al., 1990).
165 The heavy chain is further subdivided into two 50 kDa domains: the amino terminal (H_N) and
166 carboxy terminal (H_C) domains (Montal, 2010). X-ray crystallography of BoNT/A (Garcia-
167 Rodriguez et al., 2007; Lacy et al., 1998; Stevens et al., 1991), BoNT/B (Swaminathan and
168 Eswaramoorthy, 2000), BoNT/E (Kumaran et al., 2009) and TeNT (Masuyer et al., 2017) was
169 used to confirm the spatial orientation of these domains relative to each other. TeNT and

170 BoNT/E assume a more compact/closed arrangement, with the H_C domain interacting closely
171 with the L chain and H_N, although distinct interaction surfaces are employed by the two CNTs
172 (Kumaran et al., 2009; Masuyer et al., 2017). Conversely, BoNT/A and BoNT/B display an
173 elongated arrangement of the three domains, which are largely separated, with the exception
174 of an extended loop in the amino-terminus of the H chain (termed *belt*), which is wrapped
175 around the L chain.

176 The H_C domain of CNTs is responsible for their neuron-specific binding and is composed of
177 two sub-domains of roughly the same size (Pirazzini et al., 2017). While the amino-terminal
178 sub-domain (H_{CN}) is structurally similar to the carbohydrate-binding domain of the lectin
179 family, the carboxy-terminal sub-domain (H_{CC}) is homologous to domains involved in protein-
180 protein interactions (Montal, 2010; Pirazzini et al., 2017). It is in the H_{CC} loops of CNTs where
181 the highest degree of sequence and structural divergence lies (Lacy and Stevens, 1999),
182 which ultimately contributes to binding specificity. Crucially, in BoNT/A and BoNT/E, the H_C
183 domain is isolated from the remaining part of the molecule, allowing full access of all surface
184 loops for binding. The close conformation found in BoNT/E and TeNT may instead impose
185 some steric constraints to the full accessibility of H_C to protein and lipid receptors (Kumaran
186 et al., 2009; Masuyer et al., 2017). Moreover, other portions of TeNT may contribute to
187 enhanced clearance from the NMJ and wider spreading into spinal cord neurons (Ovsepian
188 et al., 2015).

189 The H_C domains of CNTs bind to polysialogangliosides on the plasma membrane, in
190 particular to G1b gangliosides, with high specificity and affinity (Montecucco, 1986), although
191 binding to other gangliosides series has been reported (e.g. BoNT/A interacts with GQ1b and
192 GT1b, but also to GD1a, albeit with lower affinity) (Kitamura et al., 1980; Takamizawa et al.,
193 1986). Binding to polysialogangliosides is facilitated by oligosaccharide-binding sites (one
194 and two, in BoNTs and TeNT, respectively) in the H_{CC} sub-domain of the heavy chain
195 (Rummel, 2016; Rummel et al., 2003). Mutations in the carbohydrate binding domain
196 abrogate binding of these toxins to neuronal plasma membranes, thus highlighting the
197 importance of this interaction (Rummel, 2016). Addition of the polysialoganglioside GT1b to
198 NMJs protects the neuron from the toxic effects of BoNT via competitive inhibition and
199 partially abolishes the retrograde transport of TeNT (Stoeckel et al., 1977). In addition,
200 removal of sialic acid residues from the plasma membrane by neuraminidase treatment
201 (Bigalke et al., 1986) or blocking ganglioside biosynthesis (Kitamura et al., 2005; Rummel,
202 2013; Williamson et al., 1999) inhibits CNT activity. Despite the strong requirement of surface
203 polygangliosides for uptake of CNTs, it is clear that they are not unique determinants of
204 binding since TeNT and BoNTs do not compete with each other for internalisation at the
205 NMJ. Additional protein receptor(s) have therefore been suggested to act in conjunction with

206 gangliosides, referred to as the dual receptor hypothesis (Montecucco, 1986; Rummel, 2016;
207 Rummel et al., 2007). According to this hypothesis, polysialogangliosides act in one of two
208 ways: i) recruit TeNT and BoNTs to specific regions of the plasma membrane, which are
209 locally enriched in a certain protein receptor, or ii) maintain a specific conformational state of
210 these toxins so as to enable the receptor to bind. In line with this hypothesis, specific protein
211 co-receptors have been identified for most CNTs (see section 4).

212 The pH-dependent translocation of the L-chain from the endocytic lumen into the cytosol is
213 mediated by the amino-terminal part of the H chain (H_N). H_N is composed of a belt closely
214 interacting with the L chain and a central portion containing two very long α -helices (Montal,
215 2010; Pirazzini et al., 2016). Although the function of this domain in membrane insertion was
216 first described in the 1980s, the exact mechanism underlying the transfer of the L chain to
217 the cytosol remains, at least in part, controversial (Montal, 2010; Pirazzini et al., 2016).
218 Recent findings have demonstrated that the reduction of the disulphide bridge linking the H
219 and L chains by the thioredoxin reductase-thioredoxin (TrxR-Trx) system is required for the
220 release of the L chain into the cytosol, and inhibition of TrxR-Trx activity prevents the
221 intoxication of neurons both *in vitro* and *in vivo* (Pirazzini et al., 2015; Pirazzini et al., 2014;
222 Zanetti et al., 2015). Reduction of the interchain disulphide bridge is strictly coupled to L
223 chain refolding, since the inhibition of cytosolic chaperone Hsp90 reduces the intracellular
224 activity of BoNTs (Azarnia Tehran et al., 2017). Interestingly, Hsp90 and TrxR-Trx physically
225 interact on the surface of SVs, where they orchestrate a chaperone-redox complex likely to
226 be involved in synaptic protein refolding, which is exploited by the L chains of CNTs to enter
227 the cytosol (Azarnia Tehran et al., 2017).

228 The L chain contains the catalytic zinc atom and is responsible for the intracellular
229 endopeptidase activity of CNTs, which is directed towards the SNARE proteins
230 VAMP/synaptobrevin 1-3 (BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/HA and BoNT/X,
231 TeNT), SNAP25 (BoNT/A, BoNT/C and BoNT/E) and syntaxin-1 (Montal, 2010; Pirazzini et
232 al., 2016; Zhang et al., 2017). BoNT/X also cleaves the non-canonical substrates VAMP4,
233 VAMP5 and Ykt6 (Zhang et al., 2017). The number of zinc atoms that bind to the L chain
234 varies among different CNTs; while the L chains of TeNT, BoNT/A, BoNT/B and BoNT/F
235 chelate one atom of zinc (Schiavo et al., 1992a; Schiavo et al., 1992b; Schiavo et al., 1993),
236 BoNT/C binds two atoms of zinc with different affinities (Breidenbach and Brunger, 2005;
237 Garcia-Rodriguez et al., 2007; Schiavo et al., 1995). The protease activity of the L chain can
238 be abolished by heavy metal chelators, such as ortho-phenantroline, thus generating inactive
239 apo-neurotoxins (Bhattacharyya and Sugiyama, 1989; Schiavo et al., 1992a). The zinc atom
240 is chelated by two histidines located in the endopeptidase motif (His-Glu-x-x-His); the
241 glutamic acid residue in this motif binds the water molecule necessary for the catalysis (third

242 ligand), with another glutamic acid (Glu261 in BoNT/A) acts as the fourth ligand (Montal,
243 2010; Pirazzini et al., 2016).

244

245 **4. Neuron-specific binding**

246 CNTs are exquisitely neuron-specific and able to bind neurons *in vivo* at concentrations in
247 the sub-nanomolar regime (Simpson, 2000). Both BoNTs and TeNT interact with the
248 peripheral cholinergic nerve terminals, while TeNT also binds to sympathetic and adrenergic
249 nerve fibres (Rossetto et al., 2001). The H_C domains are largely responsible for this high-
250 affinity binding, since paralysis caused by native toxins can be counteracted by recombinant
251 H_C proteins (Lalli et al., 1999; Rummel et al., 2009). Additional targeting information,
252 however, may be encoded elsewhere in the full length neurotoxin (Ovsepian et al., 2015).
253 The neuron-specificity of CNTs is also likely to reflect the complexity of their cellular
254 receptors, which are most probably composed of multiple lipid and protein components
255 forming arrays of presynaptic receptors (APRs) (Montecucco et al., 2004).
256 Polysialogangliosides play a key role in the binding and internalisation of CNTs at the
257 presynaptic membrane, presumably due to their high concentration at the NMJ and their
258 lateral mobility. Interaction of the toxin with polysialogangliosides allows its subsequent
259 interaction with other molecules in the APRs, thus leading to virtually irreversible binding. In
260 addition to polysialogangliosides, APRs contain lipids such as cholesterol and
261 sphingomyelin, GPI-anchored protein(s) and other membrane-bound protein(s) (Montecucco
262 et al., 2004). Interestingly, both TeNT and BoNT/A have been found to bind sphingomyelin-
263 enriched membrane microdomains (Herreros et al., 2001; Muraro et al., 2009); additionally,
264 BoNT/A and BoNT/C interact with phosphoinositol lipids (Muraro et al., 2009; Tsukamoto et
265 al., 2005; Zhang and Varnum, 2012). Since BoNTs and TeNT have been proposed to bind
266 distinct co-receptors, the APRs recognised by BoNTs would direct them inside vesicles that
267 are acidified within the NMJ, such as recycling synaptic vesicles (SVs), whereas the APRs
268 binding to TeNT would sort this neurotoxin into signalling endosomes undergoing axonal
269 retrograde transport towards the neuronal soma (Schmieg et al., 2014).

270 Several lines of evidence indicate that BoNTs enter the NMJ by exploiting the process of SV
271 recycling (Montal, 2010; Pirazzini et al., 2017). Accordingly, many CNTs bind to the
272 intraluminal domain of SV proteins, which are exposed to the extracellular milieu upon SV
273 exocytosis (**Figure 1B**). BoNT/B, BoNT/D, BoNT/C and BoNT/G interact with the calcium-
274 sensing proteins synaptotagmin-1 and/or -2 (reviewed in Rummel, 2016). Multiple isoforms of
275 the synaptic vesicle protein-2 (SV2) function as the protein receptors for BoNT/A, BoNT/E
276 and BoNT/F (Rummel, 2016), whilst BoNT/C and BoNT/D seem to utilize only gangliosides

277 as host cell receptors (Karalewitz et al., 2012). Due to its recent discovery, no protein
278 receptor has been described for BoNT/X (Zhang et al., 2017). Crucially, Harper et al. found
279 that BoNT/A is internalised in a SV subpopulation that is not destined for recycling,
280 highlighting the existence of functional heterogeneity between SV pools (Harper et al., 2016).

281 BoNT/A, similar to TeNT, is able to enter neurons when SV recycling is blocked (Restani et
282 al., 2012a), suggesting that BoNT/A could potentially use alternative entry route(s) targeting
283 this neurotoxin to sites other than the NMJ (**Figure 1B**). In agreement, BoNT/A has been
284 shown to be retrogradely transported in hippocampal, tectal and motor neurons and undergo
285 transcytosis in the visual system (Bomba-Warczak et al., 2016; Mazzocchio and Caleo,
286 2015). Additionally, BoNT/A accumulates in dorsal root ganglia upon injection in the bladder
287 (Papagiannopoulou et al., 2016). Although SV2A can potentially undergo long-range
288 transport in spinal cord motor neurons (Debaisieux et al., 2016), other protein receptors may
289 be involved in this process. One such protein whose endogenous trafficking route might be
290 exploited by BoNT/A is the fibroblast growth factor receptor-3 (FGFR3). Although
291 controversial (Weisemann et al., 2016), FGFR3 has been shown to bind BoNT/A (Jacky et
292 al., 2013). FGFR3 undergoes receptor-mediated endocytosis (Haugsten et al., 2011) and
293 has been identified in the proteome of axonal signalling endosomes (Debaisieux et al.,
294 2016), thus suggesting an alternative transport route for BoNT/A. On the other hand, BoNT/A
295 might bind to the basal membrane at the NMJ, as recently reported for TeNT (Bercsenyi et
296 al., 2014), leading to its sorting to axonal signalling endosomes and transcytosis.

297 To reach its final site of action, TeNT must enter two different types of neurons: a motor
298 neuron innervating skeletal muscle followed by an inhibitory interneuron of the spinal cord
299 (**Figure 1A,B,D**). Post-internalisation, TeNT is sorted to different intracellular pathways,
300 hence it is expected to bind to distinct receptors in these neurons. Several lines of evidence
301 indicate that TeNT and BoNTs are internalised via different routes. First, TeNT at
302 physiological concentrations does not block synaptic transmission at the NMJ, unlike BoNTs.
303 Second, if TeNT binding sites were present in recycling SVs, then an increase in the rate of
304 neuronal stimulation should lead to increased binding of the toxin to the membrane. This,
305 however, is not observed. While high frequency stimulation increases the rate of TeNT
306 intoxication, it does not enhance binding of the toxin to the NMJ (Schmitt et al., 1981). Third,
307 the abrogation of exocytosis and neurotransmitter release from NMJs by BoNT treatment
308 does not affect the uptake and retrograde axonal transport of TeNT (Habermann and
309 Erdmann, 1978). Fourth, TeNT exhibits temperature-sensitive binding and internalisation;
310 while fully functional at 25°C, it is inactive on NMJs at 18°C even in the presence of high-
311 frequency stimulation and massive neurotransmitter release (Schmitt et al., 1981).

312 Due to the presence of two ganglioside-binding sites in the H_C domain of TeNT, it was
313 proposed to rely solely on lipid binding for its cellular entry (Chen et al., 2009). Cis-
314 interactions of gangliosides have been suggested to play an important role in mediating
315 binding of the neurotoxin to target cells (Rinaldi et al., 2009). However, since
316 polysialogangliosides are not uniquely distributed at the NMJ and are not readily internalised
317 (Deinhardt et al., 2006a), TeNT would require additional factors to enter into motor neurons.

318 One of the proteins described to interact with TeNT is Thy-1, an abundant GPI-anchored
319 protein (Herrerros et al., 2001). However, Thy-1 is unlikely to be the main protein receptor on
320 motor neurons *in vivo* because mice lacking Thy-1 remain sensitive to the toxic effects of
321 TeNT (Herrerros et al., 2001). TeNT enters motor neurons together with the neurotrophin
322 receptors TrkB and p75^{NTR} (Deinhardt et al., 2006b; Terenzio et al., 2014a; Terenzio et al.,
323 2014b), and its internalisation is dependent on neurotrophin signalling. Interestingly, TeNT
324 interacts with specific basal membrane components at the NMJ to stimulate uptake of TrkB
325 and formation of signalling endosomes (Bercsenyi et al., 2014). In particular, the H_C domain
326 of TeNT (H_CT) directly binds to nidogen-1 and -2 (also known as entactin-1 and -2) and
327 selectively targets NMJs rich in nidogen-2. A small peptide derived from nidogen-1 blocks
328 TeNT uptake in motor neurons and at NMJs, and protects mice from TeNT-induced paralysis
329 (Bercsenyi et al., 2014). Nidogen-2 knockout mice are less sensitive to tetanus intoxication
330 and show TeNT-mediated botulism-like symptoms (Bercsenyi et al., 2014), which are also
331 observed when TeNT is injected in wild type animals at high doses (Matsuda et al., 1982).
332 Taken together, these results suggest that TeNT and BoNTs might share common entry
333 routes when key basal membrane components required by TeNT are absent or when its
334 preferred internalisation pathway is overloaded. Accordingly, addition of recombinant
335 nidogen-1 decreases the co-localisation of H_CT with SV2A and increases its rate of
336 internalisation, whilst at high concentrations, H_CT preferentially enters SV2A-positive
337 organelles (Bercsenyi et al., 2014). Although controversial (Blum et al., 2012), TeNT was
338 also shown to bind SV2 in hippocampal neurons and relied on this interaction for cell entry
339 (Yeh et al., 2010).

340 The identification of protein co-receptors for TeNT at the NMJ provides crucial information on
341 this trafficking pathway from the NMJ to spinal cord interneurons, offering new strategies for
342 the delivery of therapeutics into the spinal cord. Furthermore, it provides new insights into the
343 alternative trafficking pathway used by BoNT/A to elicit responses in the CNS (Caleo and
344 Schiavo, 2009). Although further studies are required to determine whether BoNTs engage
345 with basal membrane components, these findings open the possibility that extracellular
346 matrix-derived peptides might be used to mitigate some of the undesired long-range effects
347 of BoNT/A therapy in humans.

348

349 **5. Neuronal internalisation and axonal transport**

350 Endocytosis of CNTs is an active process: their cellular entry is temperature- and energy-
351 dependent and is differentially modulated by synaptic activity (Baldwin and Barbieri, 2007;
352 Blum et al., 2014; Pirazzini et al., 2017; Rummel et al., 2009). At physiological
353 concentrations, uptake of both CNTs occurs via distinct mechanisms with TeNT
354 internalisation predominantly occurring through clathrin-mediated endocytosis whilst BoNTs
355 exploit SV recycling (Blum et al., 2012; Deinhardt et al., 2006a; Montal, 2010). TeNT
356 internalisation is dependent on a specific subset of clathrin adaptors, which target the
357 neurotoxin to non-acidified endosomal compartments (Bohnert and Schiavo, 2005), thus
358 preventing the translocation of the L chain into the cytoplasm of the motor neuron and
359 enabling its arrival in a fully active form to spinal cord inhibitory interneurons. Internalisation
360 of BoNT/A and TeNT is partially abrogated by dynamin inhibitors (Deinhardt et al., 2006a;
361 Harper et al., 2011) or dynamin mutant overexpression (Deinhardt et al., 2006a), in
362 agreement with the established role of dynamins in the fission of clathrin-coated vesicles
363 from the plasma membrane.

364 Although TeNT and its atoxic H chain fragment (H_CT) uptake in motor neurons is largely
365 unaffected by membrane depolarisation (Deinhardt et al., 2006a), their mechanism of entry in
366 central neurons is likely to be dependent on SV recycling. Experiments by Blum *et al.*
367 indicate that H_CT entry in cortical neurons is stimulated by membrane depolarisation (Blum et
368 al., 2014), validating previous results that show TeNT internalisation in hippocampal neurons
369 follows SV re-uptake (Matteoli et al., 1996). However, subtle differences may exist between
370 H_CT and TeNT uptake and trafficking in cortical and spinal cord neurons, as recently reported
371 (Blum et al., 2014).

372 Post-internalisation, TeNT must undergo long-range transport to reach the soma of motor
373 neurons, from where it undergoes trans-synaptic transfer into inhibitory interneurons. In order
374 to achieve this, it exploits endogenous microtubule-based axonal transport pathways which
375 the neuron uses to communicate between the synapse and the soma (Goldstein and Yang,
376 2000) (**Figure 1C**). This highly regulated, long-range axonal transport is facilitated by two
377 classes of microtubule-dependent molecular motors: cytoplasmic dynein and kinesins.
378 Cytoplasmic dynein motor proteins are responsible for moving cargo in the retrograde
379 direction from axonal terminals to the cell body, where the minus ends of microtubules are
380 located. In contrast, kinesin motor proteins are responsible for delivering their cargo in the
381 anterograde direction toward the plus end of microtubules that are located in synaptic
382 terminals or growth cones (Hirokawa et al., 2010; Vale, 2003). Despite the majority of

383 transport dynamics involving microtubules (Hirokawa et al., 2010), actin-based motors (e.g.,
384 myosins) also contribute and hence, some form of interactions between the microtubule- and
385 actin-mediated transport systems has been suggested (Hirokawa et al., 2010; Vale, 2003).
386 Cytoplasmic dynein plays a particularly crucial role in the retrograde transport of TeNT to the
387 soma (Lalli et al., 2003; Schiavo et al., 2013) (**Figure 1C**). *In vivo* studies using mice carrying
388 a mutation in cytoplasmic dynein heavy chain showed deficits in axonal retrograde transport
389 of H_cT, which are associated with motor and sensory neuron degeneration (Hafezparast et
390 al., 2003). Functional axonal transport is crucial for the development and maintenance of the
391 nervous system, and impairments in this process are associated with neurodegenerative
392 conditions, such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease and
393 acquired peripheral neuropathies (De Vos and Hafezparast, 2017; Schiavo et al., 2013).
394 However, for cargo to bind, dynein must form a complex with dynactin and this formation is
395 dependent on the Bicaudal D (BICD) family of adaptor proteins that are enriched at the
396 minus-end of microtubules (Carter et al., 2016; Hoogenraad and Akhmanova, 2016).
397 Underpinning their importance, BICD1 is involved in the trafficking of TeNT and neurotrophin-
398 receptor complexes (Schmiege et al., 2014; Terenzio et al., 2014b) and mutations in the
399 homologous BICD2 have been shown to cause spinal muscular atrophy (Oates et al., 2013;
400 Rossor et al., 2015).

401 The retrograde transport of H_cT takes place in axonal signalling endosomes, which contain
402 neurotrophins, their receptors and other proteins (Deinhardt et al., 2006b; Lalli and Schiavo,
403 2002). To create a functional physical map of these organelles, our laboratory has developed
404 a method based on magnetic iron oxide nanoparticles coupled to H_cT, which enable the
405 purification of signalling endosomes from embryonic stem cell-derived motor neurons and
406 their quantitative mass spectrometry analysis (Debaisieux et al., 2016; Deinhardt et al.,
407 2006b; Wade et al., 2012). We found that H_cT-positive organelles undergo rapid maturation
408 with the acquisition of late endosomal markers, and are specifically enriched in proteins
409 known to be involved in neurodegenerative diseases and neuroinfection (Debaisieux et al.,
410 2016). The maturation of signalling endosomes is dependent upon Rab5, which is involved in
411 sorting after internalisation, followed by Rab7, which is involved in the fast retrograde
412 transport of H_cT (**Figure 1C**) as well as neurotrophin-receptor complexes (Deinhardt et al.,
413 2006b; Salinas et al., 2009). A functional cross-talk between H_cT and neurotrophins is
414 emerging, since the application of exogenous brain-derived neurotrophic factor (BDNF)
415 results in an increase in the internalisation of H_cT at the NMJ as well as accumulation of H_cT
416 in the sciatic nerve (Roux et al., 2006). However, the sharing of axonal signalling endosomes
417 by other virulence/pathological factors such as canine adenovirus-2, cholera toxin, poliovirus,
418 Borna virus and pseudotyped lentivirus with neurotrophin receptors suggests that despite

419 different methods of internalisation, a common mechanism for sorting and retrograde
420 transport may exist (Charlier et al., 2016; Hislop et al., 2014; Ohka et al., 2009; Salinas et al.,
421 2009).

422 In contrast to the acidic pH found in the lumen of the majority of endosomes, axonal transport
423 carriers containing H₃T display neutral pH. The pH of signalling endosomes is critical, as
424 acidification in TeNT and BoNTs carriers triggers the translocation of the enzymatically active
425 subunit (i.e., L chain) into the cytosol. Endosomal acidification also causes the dissociation of
426 neurotrophin-receptor complexes, terminates their en route signalling and targets the
427 endosome for degradation. Such tight regulation of the pH is dependent on the vacuolar
428 ATPase complex (Bohnert and Schiavo, 2005). Therefore, the maintenance of neutral pH of
429 TeNT carriers enables its presentation to interneurons in a fully active form to consequently
430 mediate the disruption of synaptic communication.

431 In contrast to the historical view that BoNTs only disrupt communication at the NMJ, several
432 studies provide evidence of long-range trafficking and CNS expression of BoNT after
433 intramuscular injections (reviewed in Caleo and Schiavo, 2009; Mazzocchio and Caleo,
434 2015). Indeed, BoNT/A was first detected in the spinal cord ventral horn after injections in the
435 gastrocnemius muscle (Wiegand et al., 1976). Its presence was also detected in diaphragms
436 after intraperitoneal injections of BoNT/A and /B (Black and Dolly, 1986). In this context,
437 higher BoNT/A levels were observed in the axoplasm of myelinated axons, suggestive of
438 differences in the uptake and sorting mechanisms of different BoNT serotypes (Black and
439 Dolly, 1986). Experiments comparing the effects of BoNT/A and /E applied to the distal
440 neurites of primary sympathetic neurons cultured in compartmentalised chambers revealed
441 that whilst most BoNT/A and /E cleaved SNAP25 near the sight of uptake, a small fraction
442 also cleaved SNAP25 in their soma, albeit at different rates (Lawrence et al., 2012). In
443 addition, Restani et al. have demonstrated that BoNT/A undergoes fast axonal retrograde
444 transport whereas BoNT/E exhibited slower axonal retrograde transport with a greater
445 frequency of pausing and short periods of anterograde transport in primary motor neurons
446 (Restani et al., 2012a). This study suggests that BoNT/E is coupled with a less efficient
447 mechanism of long-range trafficking and may explain, in part, why BoNT/E cannot mediate
448 similar effects in the CNS, despite having the same intracellular targets as BoNT/A.
449 Furthermore, these data also suggest that the serotype and concentration of BoNTs are also
450 key factors in local (i.e., NMJ) versus distant (i.e., soma) effects. These results were
451 confirmed in hippocampal neurons grown in microfluidic devices, where BoNT/A and BoNT/D
452 were found to be taken up into non-acidified organelles undergoing axonal retrograde
453 transport to the soma (Bomba-Warczak et al., 2016). After internalisation, their activities were
454 detected in upstream neurons, thus indicating that BoNT/A, BoNT/D and TeNT may undergo

455 interneuronal transfer in an active form *in vitro* (Bomba-Warczak et al., 2016). Interestingly,
456 Wang *et al.* found that a significant proportion of H_C fragment of BoNT/A (H_CA) was
457 incorporated into LC3-positive autophagosomes in hippocampal neurons, which then
458 underwent retrograde transport to the cell soma. Blocking autophagosome formation or
459 acidification inhibited the activity-dependent retrograde trafficking of H_CA, suggesting a role
460 for presynaptic autophagosomes in long distance transport of BoNT/A (Wang et al., 2015).
461 Elements of this process have been recapitulated *in vivo* by studies demonstrating the
462 retrograde transport of BoNT/A and H_CA in spinal cord motor neurons (Antonucci et al.,
463 2008; Restani et al., 2012a; Restani et al., 2012b; Wang et al., 2015) and sensory neurons
464 (Fan et al., 2017; Hong et al., 2017; Matak et al., 2014; Papagiannopoulou et al., 2016).
465 Taken altogether, these investigations provide evidence that BoNTs also undergo retrograde
466 transport to the CNS, the consequences of which are yet to be entirely understood.

467

468 **6.0 Future perspectives**

469 Since TeNT and BoNTs are capable of being sorted to the axonal retrograde trafficking route
470 and undergo interneuronal transfer *in vivo*, it has been proposed that non-toxic fragments of
471 CNTs may be used as targeting agents for the delivery of therapeutics, such as recombinant
472 proteins and/or DNA, into the CNS (Toivonen et al., 2010). Chimeras of H_CT and various
473 proteins have been shown to be successfully internalised and undergo axonal retrograde
474 transport, maintaining their enzymatic activity upon delivery to the targeted area (Francis et
475 al., 2004a). Importantly, these H_CT fusion proteins were shown to transfer across synapses
476 *in vivo* (Coen et al., 1997), access second and higher-order neurons (Miana-Mena et al.,
477 2003) and deliver their payload to the neuronal cytosol, when fused to translocation-
478 competent proteins (e.g. diphtheria toxin) (Francis et al., 2004b).

479 Due to their diverse biological activities, neuronal growth factors have frequently been used
480 as biological payloads. BDNF and glial cell line-derived neurotrophic factor (GDNF) fused
481 with H_CT have been found to have neuroprotective effects in animal models of ALS (Calvo et
482 al., 2011; Ciriza et al., 2008) and Parkinson's disease (Larsen et al., 2006). Fusion of
483 cardiotrophin-1 and H_CT also promoted motor neuron survival (Bordet et al., 2001), whilst a
484 chimera of the anti-apoptotic factor Bcl-XL and H_CT decreased apoptosis induced by
485 glutamate-mediated excitotoxicity (Carlton et al., 2008). BDNF has also been targeted to
486 neurons by nanoparticles made of polyethylene imine linked to H_CT (Oliveira et al., 2010).

487 Protein engineering has been explored to re-target BoNTs to different neuronal populations
488 by using a self-assembling 'protein stapling' technology (Ferrari et al., 2013). BoNT/A lacking
489 its H_CA domain as well as H_CT were produced separately and then linked by exploiting the

490 high-affinity interaction of paired SNARE motifs (Ferrari et al., 2013). The stapled chimera
491 was found to lack peripheral paralytic effects, and significantly reduce the enhanced
492 nociceptive sensitivity found in animal models of inflammatory, surgical, and neuropathic pain
493 (Mangione et al., 2016).

494 Whilst these studies have explored the potential of recombinant protein chimeras, a few
495 attempts have been made to directly express these fusion proteins by delivering exogenous
496 DNA. In particular, Moreno-Igoa *et al.* showed that a single intramuscular administration of
497 naked-DNA encoding GDNF-H_CT significantly delayed the onset of symptoms, ameliorate
498 the functional deficits and extended the lifespan of a mouse model of ALS (Moreno-Igoa et
499 al., 2012). H_CT might thus represent a valuable strategy to deliver therapeutics to the CNS by
500 exploiting its high tropism for motor neurons and its ability to undergo axonal retrograde
501 transport and transcytosis. In addition, DNA fusion vaccines encoding a portion of H_CT
502 coupled with tumour antigen sequences is highly immunogenic against colon carcinoma
503 (Behzadi et al., 2016).

504 Several studies have also highlighted the intrinsic ability of H_CT to protect neurons from
505 neurodegeneration in a variety of animal models, including chemically induced Parkinson's
506 disease (Mendieta et al., 2009), ALS (Moreno-Igoa et al., 2010) and spinal muscular atrophy
507 (Olivan et al., 2016). This property may be linked to the ability of H_CT to activate the
508 neurotrophin receptor signalling cascade, including ERK1/2 and Akt, via a mechanism still
509 not completely understood (Gil et al., 2003; Gil et al., 2001). H_CT co-localises with the
510 neurotrophin receptors TrkB and p75^{NTR} in axonal signalling endosomes (Deinhardt et al.,
511 2006a; Lalli and Schiavo, 2002), yet it is unclear whether H_CT signalling is physiologically
512 relevant and whether it would negatively or positively regulate axonal retrograde transport.
513 However, recent results from Wang *et al.* demonstrate that TrkB activation couples synaptic
514 activity with the retrograde flux of axonal signalling endosomes, thus suggesting that H_CT
515 and TeNT regulate their own sorting and/or retrograde transport (Wang et al., 2016).

516 In addition to their importance as virulence factors and biotherapeutics, BoNTs, TeNT and
517 their recombinant fragments are also becoming increasingly popular as key tools of
518 discovery to uncover deficits of axonal transport in animal models of neurological diseases
519 (Bilsland et al., 2010; LeRoux et al., 2014; Malik et al., 2011; Schafer et al., 2017; Sleigh et
520 al., 2017a; Sleigh et al., 2017b), ageing (Sleigh and Schiavo, 2016) and as flexible trans-
521 synaptic tracers (Coen et al., 1999; Kumar and Boehm, 2014).

522 Several important questions centred on the trafficking of BoNTs and TeNT are still
523 unaddressed. First and foremost, the nature of the receptor complex targeting TeNT and
524 BoNTs to axonal signalling endosomes at the NMJ need to be elucidated at the molecular

525 level, together with the exact role of neurotrophin signalling (or other signalling cascades) in
526 this process. This line of research would help the identification of the minimal requirements
527 for the efficient sorting of these neurotoxins to proximal and/or distal sites of action. This
528 information would be important for basic and clinical scientists to direct the *in vivo* activity of
529 BoNTs, thus improving their clinical specificity and limiting their side effects. Further research
530 is also necessary to define the neuronal receptors of the expanding family of BoNT subtypes
531 (Peck et al., 2017) and their preferential site of action *in vivo*. This in turn would allow the
532 selection of novel BoNT subtypes endowed with unique pharmacodynamics and
533 pharmacokinetics properties ideal for specific clinical applications (e.g. chronic pain, short
534 term treatment in post-operative management). In this way, the travel diaries of TeNT and
535 BoNTs would become not just a fascinating reading for molecular and cellular
536 neurobiologists, but a very useful roadmap for pharmacologists and clinical neuroscientists to
537 understand, navigate and treat the human nervous system.

538

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547

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- 926

927 **Figure legend**

928 **Figure 1. Trafficking of the tetanus (TeNT) and botulinum neurotoxins (BoNT) *in vivo***
929 **(adapted from Schmeig et al., 2014b).** **A. Anatomical connections between skeletal**
930 **muscles, spinal cord motor neurons and their afferent cells.** Motor neurons innervate
931 skeletal muscles via the neuromuscular junction (NMJ). The motor neuron axon is
932 myelinated and can reach over a meter in length in humans. The motor neuron soma is
933 located in the spinal cord, where it forms contacts with adjacent interneurons and upper
934 motor neurons. **B. Internalisation at the NMJ.** Both TeNT (T; in blue) and BoNTs (B; in
935 green) accumulate in the synaptic space of the NMJ, which is filled with basal lamina (in
936 yellow). TeNT binds to polysialogangliosides and nidogens, and this complex is targeted to
937 the axonal retrograde transport route (solid blue arrow). At higher doses or with the
938 unavailability of nidogens, TeNT is able to bind SV2 and can enter synaptic vesicle (SV)
939 recycling at the NMJ (thinner blue arrow) (Bercsenyi et al., 2014). The majority of BoNT
940 molecules remain at the NMJ (solid green arrow), where they cleave synaptic SNAREs,
941 thereby blocking the fusion of (SVs) containing acetylcholine and causing flaccid paralysis.
942 However, a fraction of BoNT/A may enter organelles targeted to the soma (thinner green
943 arrow), such as axonal signalling endosomes (Restani et al., 2012a) or autophagosomes
944 (Wang et al., 2015). **C. Axonal retrograde transport.** TeNT is transported to the soma via
945 axonal signalling endosomes, along with neurotrophins and their receptors. This long-range
946 retrograde axonal transport, which also requires the GTP-bound form of the small GTPase
947 Rab7 (in purple) (Deinhardt et al., 2006b), is dependent on the microtubule-based motor,
948 cytoplasmic dynein (in red). **D. Interneuronal transfer of TeNT into inhibitory**
949 **interneurons.** Once in the motor neuron soma in the spinal cord (Bilsland et al., 2010),
950 TeNT is released into the extracellular medium and is internalised by SV recycling into
951 inhibitory interneurons, where it cleaves VAMP/synaptobrevin, thereby blocking inhibitory
952 neurotransmission. This impairs the balance between inhibitory and excitatory afferents on
953 the motor neurons, leading to disruptions in co-ordinated muscle contraction and spastic
954 paralysis.

Figure 1

