The structure of the tetanus toxin reveals pH-mediated domain dynamics

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Abstract

The tetanus neurotoxin (TeNT) is a highly potent toxin produced by *Clostridium tetani* that inhibits neurotransmission of inhibitory interneurons, causing spastic paralysis in the tetanus disease. TeNT differs from the other clostridial neurotoxins by its unique ability to target the central nervous system by retrograde axonal transport. The crystal structure of the tetanus toxin reveals a “closed” domain arrangement stabilised by two disulphide bridges, and the molecular details of the toxin’s interaction with its polysaccharide receptor. An integrative analysis combining X-ray crystallography, solution scattering and single particle electron cryo-microscopy reveals pH-mediated domain rearrangements that may give TeNT the ability to adapt to the multiple environments encountered during intoxication, and facilitate binding to distinct receptors.

Keywords clostridial toxin; tentoxilysin; tetanospasmin; tetanus neurotoxin

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Structural Biology

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Introduction

The tetanus neurotoxin (TeNT) is one of most toxic proteins known to man, second only to the botulinum neurotoxins (BoNTs). It is produced by *Clostridium tetani*, a widespread spore-forming, Gram-positive, anaerobic bacillus commonly found in soil [1]. TeNT is the causative agent of tetanus, a paralytic disease that still inflicts high fatality worldwide, particularly in newborn infants and non-immunised adults, despite global vaccination programmes [2].

The tetanus neurotoxin is part of the clostridial neurotoxin (CNT) family that also includes seven confirmed and two potentially new BoNT serotypes [3,4], that all share approximately 35% sequence identity [5]. They are all expressed as a polypeptide of 150 kDa, which is post-translationally cleaved by a bacterial or host protease into an active di-chain form linked by a single disulphide bridge [1]. The light chain (LC) is composed of the N-terminal

catalytic domain, a 50-kDa zinc-protease that specifically cleaves members of the SNARE proteins [6,7]. These proteins form a complex that promotes cell secretion through fusion of vesicles with the plasma membrane [8]. Cleavage of pre-synaptic SNAREs prevents formation of the complex and results in inhibition of neurotransmission. The heavy chain (HC) consists of two domains of 50 kDa, each with a distinct function. The N-terminal translocation domain (H_N) is responsible for transport of LC through the membrane of synaptic vesicle into the cytoplasm by undergoing a pH-mediated conformational change [9]. The C-terminal domain (H_C) is responsible for binding of the toxin to peripheral neurons. Cell recognition varies among CNTs but is generally accepted to consist of a dual-receptor mechanism involving gangliosides and synaptic proteins [10]. Gangliosides are glycosphingolipids anchored to the cell membrane that contain sialic acid (N-acetylneuraminic acid) subunits. Variation on the position and number of these N-acetylneuraminic acids around a central polysaccharide assembly made up of Gal(β1-3)-GalNAc(β1-4)Gal(β1-4)Glc defines different classes of gangliosides [11,12]. Two carbohydrate-binding sites have been described for TeNT [13] that show varying degree of affinity [14]. Several protein receptors have also been proposed for the tetanus toxin: a glycosphosphatidylinositol (GPI)-anchored protein [15], SV2 [16] and more recently nidogen-1 [17], although molecular details of these potential interactions have yet to be determined.

Despite sharing similar structure and function, BoNT and TeNT differ by the clinical pathology they cause. BoNTs inhibit the release of acetylcholine in peripheral motorneurons thereby triggering flaccid paralysis [9]. On the other hand, TeNT prevents the transmission of glycine and γ-aminobutyric acid from inhibitory interneurons in the spinal cord, leading to spastic paralysis. Previously, the two toxins were thought to have distinct intracellular pathways. BoNTs undergo endocytosis during synaptic vesicle recycling, while TeNT is internalised in an endosome via a clathrin- and Rab5-dependent mechanism that is followed by its retrograde axonal transport towards the central nervous system (CNS) [18,19]. After transcytosis into the inhibitory interneurons, TeNT undergoes a structural change allowing translocation of the catalytic domain into the cytosol where it can specifically cleave its SNARE substrate VAMP2, at the Q76-F77 bond [6]. Recent assays have, however, shown that both TeNT and BoNT/A can cause concurrent local and distal effects in cultured neurons [20].

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The crystal structures of several BoNT serotypes have been determined and established a modular arrangement of the three functional domains [9]. Additionally, the structures of the individual catalytic and binding domains of TeNT have been characterised and presented a fold similar to those of BoNT, and hence, the full-length TeNT, including the translocation domain, was expected to resemble its closest homologue [21].

Here we report the crystal structures of TeNT in complex with its GD1a and GM1a polysaccharide receptors at 2.6 Å resolution. TeNT presents a unique conformation in a “closed” state, involving multi-domain interactions not seen before in clostridial neurotoxins. Evidence of a pH-dependent dynamism for the toxin is provided for the first time, using small-angle X-ray scattering (SAXS) analysis. Finally, single particle electron cryo-microscopy (cryo-EM) provides evidence of the toxin’s flexibility through an intermediate domain arrangement. The structural information provided here allow a better understanding of TeNT’s singular ability to adapt on its journey from peripheral neurons to the central nervous system.

Table 1. X-ray crystallography: data collection and refinement statistics.

	TeNT:GD1a	TeNT:GM1a
Data collection		
Space group	C2	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	152.7, 136.8, 92.6	92.2, 137.5, 152.4
α , β , γ (°)	90.0, 90.1, 90.0	90.0, 90.1, 90.0
Resolution (Å)	2.6–68.5 (2.60–2.67) ^a	2.6–68.5 (2.6–2.64) ^a
No. total/unique reflections	370,039/58,411	413,429/116,521
<i>R</i> _{merge}	0.134 (0.937) ^a	0.126 (0.729) ^a
<i>R</i> _{pim}	0.088 (0.644) ^a	0.116 (0.668) ^a
CC _{1/2}	0.996 (0.307) ^a	0.993 (0.281) ^a
<i>I</i> / σ <i>I</i>	10 (1.8) ^a	7.2 (1.6) ^a
Completeness (%)	99.8 (99.9) ^a	99.8 (99.7) ^a
Redundancy	6.3 (6.0) ^a	3.5 (3.5) ^a
Refinement		
<i>R</i> _{work} / <i>R</i> _{free}	21.0/25.3	22.1/25.2
No. atoms		
Protein	10,348	20,842
Ligand, Zn ²⁺	88, 1	136, 2
Water	136	452
<i>B</i> -factors		
Protein (Chains A/B)	66.7	47.0/53.1
Carbohydrates, Zn ²⁺ (Chains A/B)	65.2, 70.9	44.6/47.1, 42.7/52.2
Water	44.5	30.1
R.m.s. deviations		
Bond lengths (Å)	0.008	0.008
Bond angles (°)	1.17	1.12

^aValues in parentheses are for highest-resolution shell.

Results

Crystal structures of TeNT in complex with its ganglioside receptors

The recombinant protein produced for this study is a catalytically inactive and thus non-toxic variant, the previously described TeNT (RY) [22–24]. Crystal structures of the complex with the polysaccharides of GD1a and GM1a were obtained by co-crystallisation and solved at 2.6 Å. The two crystals were grown in different conditions with TeNT:GD1a solved in space group C2 and TeNT:GM1a in P2₁, with one and two molecules per asymmetric unit, respectively (Table 1). The structure of TeNT was identical in both crystal forms with the carbohydrates fully occupying the receptor-binding pocket. No electron density was observed for regions 441–465, loops 870–875 and 981–988 of the heavy chain, and the N-terminal poly-histidine tag, all in solvent-accessible areas.

The structure reveals a unique conformational arrangement with significant interactions between the three domains, LC, H_N and H_C (Fig 1). Individually, the LC and H_C have preserved the overall fold from their isolated crystal structures [25–28], with differences only observed in flexible loop regions. The double mutation in the catalytic pocket did not affect the global LC structure and only caused weakening of the zinc ion coordination at the active site with a change in orientation of the E271 side chain (Fig EV1). Zn²⁺ is, however, still present and stabilised by the classic binding motif H233, H237 and a water-coordinated by E234. Unsurprisingly, loops “210” and “250” that are involved in inter-domain interactions have moved significantly from their position in the free LC structure (Fig EV1). This confirms the flexibility of these two loops, which were suggested to undergo conformational change upon substrate binding [27]. Additionally, the C-terminal end of the light chain is well defined up to residue 440 where 435–438 are sandwiched by two H_N strands and form a β -sheet, which is further stabilised by the C439–C467 disulphide bond [29] (Fig EV2).

Light chain and H_N form a complex assembly covering a surface area over 5,000 Å² in an interface that is similar to what is observed for other clostridial neurotoxins (Fig 2). The region described as the “belt” in BoNT [30,31] intricately surrounds LC along a groove on its surface and includes the inter-domain β -sheet and disulphide bridge described above, the latter being essential for neurotoxicity [32]. Interestingly, residues 509–518 take on a short helical conformation in a similar position to the other CNTs. This site was also involved in SNAP25 binding in the complex-bound structure of LC/A [33], which resulted in the observation that belt and substrate overlap considerably around the catalytic domain. TeNT is known to have one of the longest substrate requirement among CNTs [34]. It is therefore likely that the belt follows the path of VAMP recognition by LC, involving multiple exosites, in a similar fashion to BoNT (Fig EV1).

The translocation domain presents coiled-coil α -helices of approximately 105 Å in length with a central bend making an angle of approximately 155° (Fig 1). Modelling the extremities of H_N, corresponding to the short loops linking the helices, was rendered difficult by weak electron density in these areas, and may be an indication of their inherent flexibility, as illustrated by high *B*-factor values (Fig EV3). Flanking the central coiled-coil region are two shorter α -helices also conserved in BoNTs [35]. The connection between the two domains relies on a flexible loop and results in the

Figure 1. Crystal structure of TeNT.

A Overall structure of TeNT with the catalytic domain (LC, cyan), the belt region (purple), the translocation domain (H_N , blue) and the binding domain (H_C , grey). The two disulphide bridges are marked with a black asterisk, and the corresponding cysteines are shown in sticks. The polysaccharide GD1a (orange) is bound to the primary binding site of H_C .

B Details of the novel inter-domain interfaces: residues involved in the interactions are shown as sticks. Hydrogen bonds are indicated in black dashed lines. The missing residues of loop 871–873 are schematised as a coloured dashed line.

Figure 2. TeNT presents a unique domain arrangement among clostridial neurotoxins.

Comparison of the crystal structure of TeNT with that of BoNT/B (PDB 1epw) and BoNT/E (PDB 3ffz). Upper panel is a schematic domain organisation in the corresponding toxins.

most striking structural difference compared to the previously described BoNTs. Here the binding domain interacts with both the translocation and catalytic domains, presenting a 90° shift and half-turn twist compared to its position in the linear arrangements seen in BoNT/A [36] and BoNT/B [37], and more analogous to the structure of BoNT/E [35] (Fig 2).

The newly found interface between the N-terminal subdomain of H_C and LC seen in TeNT is therefore of particular interest and covers 548 Å² over two main sites (Fig 1). The primary interaction shows residues 212–217 of LC taking on a short β -strand arrangement parallel to the first strand (residues 903–909) of a β -sheet from H_{CN} 's jelly roll motif and consists of three backbone-to-backbone hydrogen bonds. Furthermore, the hydrophobicity of this H_{CN} strand (VITYP) likely enhances the interaction with LC and its unusually hydrophobic sequence between residues 213–218 (ITSLTI). It should be noted that in the free LC structure (PDB 1z7h), residues 208–220 form a surface-accessible loop in a different position. The domain organisation in TeNT causes this loop to be sandwiched between H_C and H_N as illustrated by the electrostatic interactions of the N212 side chain with N903 and R798, these latter residues are further stabilising this multi-domain interface by directly interacting with each other. The secondary LC- H_C interaction site is located < 10 Å away and consists mainly of electrostatic bonds between loop 408–411 and the β -hairpin motif formed by residues 892–895 (Fig 1). The hydroxymethyl group of S409 makes a hydrogen bond with the carboxylate side chain of D894, while N893 and S410 present a hydrogen backbone-to-backbone bond. Additionally, the carboxamide of N893 can make hydrogen bond with the hydroxyl of Y411 and side chain of N368.

The binding domain not only interacts with LC but also has to overlay the translocation domain in a unique fashion to do so. The H_C - H_N interface created consists in a 793 Å² area and only involves the H_{CN} subdomain (Fig 1). It includes a salt bridge between R802 and D940, a hydrogen bond between S640 and H936, as well as extensive electrostatic interactions between the lysyl of K865 and the backbone of three surrounding residues (S900, N903 and D940) that are all part of a random coil.

The connection from H_N to H_C is marked by a gap in the electron density between residues 871 and 874 (Fig EV2). This loop region is, however, partly stabilised by the interactions of K865 mentioned above, and via a second cysteine bridge. This disulphide bridge between C869 and C1093 is unique to TeNT. Pioneering work by Krieglstein *et al* [29] had misidentified C1077 as making inter-chain bonds with C1093, these two residues are beyond bonding distance in the structure. The C869-C1093 bond was visible in several H_C crystal structures (PDBs 1a8d, 1fv2, 1fv3, 3hmY, 3hn1), but only recently investigated and suggested to be involved in H_C oligomerisation [38]. While multimer formation cannot be ruled out for the full-length toxin, none was observed during purification (Appendix Fig S1).

Receptor recognition

The crystal structure of TeNT was solved in complex with the polysaccharide moieties of the naturally occurring GD1a and GM1a. The two gangliosides only differ by a single N-acetylneuraminic acid group (Sia5) linked to Gal4 (α 2-3) in GD1a but absent in GM1a. TeNT possesses two ganglioside-binding sites. The first is a conserved binding pocket built around residues SxWY that recognises galactose (Fig 3). The secondary site involves R1226 and preferentially binds branched sialic acid subunits [39,40]. Clear electron density was visible for the entire polysaccharides in the two structures, where they occupy the galactose-binding site (Fig 3). Although previous studies showed it could synergistically bind both sites [40], GD1a was unfortunately not observed in the second position (Fig 3). It should be noted that the gangliosides are involved in crystal packing interactions and thus helped crystallisation in different space groups. In the two structures, the R1226 site is in an openly accessible area and is not involved in any contacts.

GD1a and GM1a offer identical hydrogen bond interactions with the toxin, which involve four residues detailed in Table 2 and Fig 3. The conserved tryptophan 1289 is a key element in receptor recognition by making a stacking interaction with Gal4, which is further stabilised by hydrophobic interactions with Y1290. Mutations of these two aromatic residues result in a loss of toxicity for TeNT and BoNTs [13,39]. The binding domain of TeNT has been extensively studied, and its crystal structure had been solved in complex with a GT1b analogue [26]. Superposition of the GD1a-complex structure with the GT1b derivative shows a common mode of binding centred around Gal4 and Gal-NAc3, but with the non-interacting subunits presenting different orientations (Fig 3). A direct comparison can also be made with the structure of BoNT/B, the closest TeNT homologue, in complex with GD1a [41] (Fig 3). The main difference is in the recognition of Sia5 with which BoNT/B makes four hydrogen bonds, whereas it mostly relies on hydrophobic interaction with TeNT, resulting in a considerably different position of the sugars. The TeNT:GD1a complex thus demonstrates the differences in receptor recognition across clostridial neurotoxins and clarifies their variation in ganglioside specificity.

Cryo-EM reveals a semi-open conformation

A cryo-EM structure for TeNT has been calculated to a nominal resolution of 4.5 Å, using a FSC criterion of 0.143 (Fig EV4). The

Figure 3. Ganglioside-receptor binding to TeNT.

Details of ganglioside carbohydrate (orange) recognition by TeNT are highlighted with residues involved in the interactions are shown as sticks and hydrogen bonds as dashed lines. The $2F_o - F_c$ electron density map counteracted at 2σ level is shown as a blue mesh.

A, B TeNT:GD1a complex. The secondary binding site (R1226) is indicated with an arrow.

C, D TeNT:GM1a complex.

E Superposition of the TeNT:GD1a structure (light grey, orange) with the H_c/Te:GT1b analogue complex (dark grey, pink; PDB 1fv2).

F Superposition of the TeNT:GD1a structure with the H_c/B:GD1a complex (blue, green; PDB 4kbb).

Table 2. Hydrogen bonds between TeNT and the polysaccharides GD1a and GM1a.

Protein atom	Distance (Å)	GD1a atom	Distance (Å)	GM1a atom
N1219 (ND2)	3.1	GalNAc3 (O4)	2.8	GalNAc3 (O4)
N1219 (ND2)	3.4	GalNAc3 (O5)	2.9	GalNAc3 (O5)
D1222 (OD1)	2.9	GalNAc3 (O6)	3.3	GalNAc3 (O6)
D1222 (OD2)	2.7	GalNAc3 (O4)	2.7	GalNAc3 (O4)
D1222 (OD2)	2.6	Gal4 (O6)	2.6	Gal4 (O6)
H1271 (ND1)	2.8	Gal4 (O4)	3.0	Gal4 (O4)
H1271 (ND1)	3.1	Gal4 (O5)	3.0	Gal4 (O5)
H1271 (ND1)	3.1	Gal4 (O6)	3.3	Gal4 (O6)
W1289 (NE1)	3.3	Sia6 (O1B)	3.3	Sia6 (O1B)

actual resolution of the cryo-EM map is lower than 4.5 Å, as the map suffers bias due to the preferred distribution of particle orientations. The α -helices of the catalytic domain are clearly resolved, the translocation domain suffers from the preferred orientation bias, and the C-terminal domain (H_C) is only poorly resolved due to structural heterogeneity. However, the domain positions are clearly determined allowing for structural comparison with the TeNT crystal structure. For this purpose, the fragments composed of LC+ H_N and H_C were docked separately within the map and adjusted with the Chimera “fit in map” algorithm [42] (Fig 4).

The cryo-EM map reveals an overall conformation different to that observed in the crystal structure, with a semi-open configuration. Remarkably, the intricate interaction between the translocation and catalytic domains appears conserved and provides the toxin with a stable frame. The main difference appears in the binding domain position with H_C being located away from LC but short of a linear structure seen in the X-ray crystal structures of BoNT/A and BoNT/B. In this intermediate arrangement, H_C makes almost no interaction with LC but instead slides approximately 20 Å on a lower position along H_N , pulled by the C-terminal end of H_N (residues 846–856), and creating a larger $H_N:H_C$ area (1,240 Å²) based mostly on hydrophobic interactions. Most of the secondary structure appears unchanged, and thus, the loop region linking H_N and H_C is expected to play a major role and likely drives the rotation of H_C by approximately 45° so that it makes a slightly obtuse angle (100°) with the LC+ H_N axis (Fig 4, Movie EV1).

Solution scattering of TeNT demonstrates a pH-dependent dynamism

The structures of TeNT resolved by X-ray crystallography and cryo-EM reveal a unique domain arrangement. SAXS experiments were carried out to investigate the potential effect of various environmental factors on the toxin's structure (Figs 5 and EV5). A first set of data collected at neutral pH indicated a radius of gyration (R_g) of 40 Å and the pair distribution function ($P(r)$) yielded a maximum intramolecular distance of 140 Å (D_{max}). This value is significantly longer than the 110 Å observed in the crystal structure. Furthermore, comparison to the theoretical scattering from the crystal structure highlights a clear divergence

Figure 4. Cryo-EM structure of TeNT.

- A Cryo-EM density map of TeNT fitted with the crystal structures of the toxin's domains. LC+ H_N and H_C were fitted separately in Chimera.
 B Comparison between the crystal (blue) and cryo-EM structures (purple), superposed with the LC+ H_N domains highlight the movement of H_C between the closed and semi-opened conformations.

between the two curves (Fig 5). A homology model of TeNT based on its closest homologue, BoNT/B, was built to assess the possibility of a linear conformation in solution and the corresponding curve correlated well with the data, thus confirming an extended configuration.

Subsequent experiments were performed over a physiological pH range 5.0–8.0 and provided the first evidence for pH-dependent structural changes in TeNT. Indeed, acidification of the sample resulted in a change of the scattering curves (Figs 5 and EV5) and a decrease in R_g , indicative of the toxin taking on a compact form (Fig 5). Comparison with the crystal structure and linear model indicates a flexible domain rearrangement from an extended to closed conformation. Remarkably, the structural modification seems to occur in a narrow pH range where TeNT has an extended structure above pH 6.3 and compact below pH 5.5. Additionally, the scattering of TeNT measured at pH 6.0 correlated well with the cryo-EM structure, providing evidence for the semi-opened conformation to be an intermediate arrangement (Fig 5).

Despite preparing the samples at near neutral pH for crystallisation and cryo-EM, the structures solved with these methods seem to correspond to the compact-acidic, and intermediate-pH 6.0 conformations observed by SAXS, respectively (Movie EV2). This is likely the result of changes in the experimental environment of the crystallisation drop and may be explained by a preferred orientation of the toxin on the cryo-EM grid.

Additional experiments in the presence of a reducing agent (2 mM TCEP) or with variation in the salt concentration (up to 0.5 M NaCl) showed these factors had little to no effect on the solution structure (Appendix Fig S2). Additional experiments may show that more stringent conditions could affect the structural stability of TeNT in solution.

Figure 5. Solution scattering analysis of TeNT.

- A Scattering curves of TeNT (2 mg/ml) at pH 8.0 (red), 6.0 (purple) and 5.0 (blue) with a close-up view highlighting the difference between the curves (I is an arbitrary unit).
- B pH-dependent variation of the radius of gyration (R_g).
- C Comparison between the experimental data (dotted lines) and the theoretical scattering curves (*, plain lines) from the crystal structure (blue), cryo-EM structure (purple) and a “linear” homology model based on BoNT/B (red).
- D Proportion of closed (blue) and extended (red) conformation as function of pH, calculated using OLIGOMER [83].
- E, F *Ab initio* model of the solution structure of TeNT at pH 8.0 (E) and 5.0 (F). The molecular envelope (grey surface) is compared to the linear homology model and the crystal structure.

Discussion

The crystal structure of TeNT presents a closed conformation unlike the linear domain arrangement seen in botulinum neurotoxins A and B. This novel layout is also different to the one observed in BoNT/E although they both show the binding domain in a different position. Indeed, the intricate ensemble composed of the light chain and translocation domain forms a rigid body

conserved across all clostridial neurotoxins [43]. The compact structure of TeNT also resembles the low resolution reconstruction from two-dimensional crystalline arrays proposed by Robinson *et al* [44].

In TeNT, H_C interacts extensively with the two other domains via its N-terminal subdomain (H_{CN}). It was recently demonstrated that H_{CN} of BoNT/A contributes to glycan binding of SV2C [45] and is essential for toxicity [46], in addition to some evidence that it binds

phosphatidylinositol phosphates (PIPs) on the plasma membrane [47]. H_{CN} does appear to make a central contribution to the structure of TeNT as an interacting partner to LC and H_N and allowing the toxin to orientate the C-terminal subdomain away from the rest of the toxin so that it can bind to its cell-surface receptors.

The connection between H_N and H_C is a long flexible loop (> 20 residues) in TeNT and BoNT/E, unlike BoNT/A and BoNT/B where a short loop leads to a α -helix taking H_C to the other side of H_N . Noticeably, the binding domain of BoNT/E was recently shown to be in a different position when the toxin is in complex with NTN1, a modification mediated by the linker taking on a α -helical conformation [48]. Additionally, in the progenitor toxin complex M, H_C of BoNT/A also reorients by 140° in a conformation mediated by the Hn-Hc helical linker. Mutations within the helix also altered BoNT/A neurotoxicity [49]. Thus, the adaptable nature of the inter-domain linker seems indicative of domain motion and may hold a key function during intoxication. In TeNT, this segment also presents a unique feature with the C869–C1093 disulphide bridge. The position of this cysteine bond suggests it could be influenced by the oxidation state surrounding the toxin. The importance of the other SS-bridge between heavy and light chains is well established as the toxin relies on an intact inter-chain bond until its translocation into the cytosol [32]. The C869–C1093 bond may play a role in stabilising the toxin's domain configuration and its reduction have structural consequences, although it is unlikely to occur in the endosomal compartments. Remarkably, early reports on partial proteolysis of the toxin by papain showed a cleavage site (S863–K864) between H_N and H_C [50]. Furthermore, TeNT cleavage was observed between residues D872–E873 in subcellular fractions of lymphoblastoid cells [51]. The disulphide bond may therefore allow the toxin to remain functional by keeping H_C and H_N connected after intracellular proteolytic degradation. Future experiment following the behaviour of a mutated cysteine bridge mutant in neurons should establish the role of this bond.

The first biophysical evidence for a pH-mediated conformational change was provided by solution scattering. In a neutral environment, TeNT presented an extended conformation similar to the BoNT/A and BoNT/B structures, while acidification led to the closed conformation seen in the crystal structure. The environment that surrounds TeNT during intoxication varies greatly from the point of infection to its final intracellular target in the cytosol of inhibitory interneurons. Overall, the results point towards the position of the binding domain being key to TeNT adapting through these various situations. Both pH and oxidative conditions are known to dramatically affect the toxin, particularly with their role in the metamorphosis of TeNT into a protein-conducting channel across the endosomal membrane [52]. Here we show that transition of TeNT from extended to closed conformation is mediated by acidification, and involves the creation of a multi-domain interface. The cryo-EM map of TeNT highlights the structural dynamism of the toxin by providing evidence of an intermediate conformation where H_C is the moving part thanks to an adequately flexible inter-domain loop. LC and H_N form a positively charged pocket (Figs 1 and EV3) that appears to favour complementary interaction with H_C upon acidification of the local environment.

Initial sorting of TeNT into its vesicular carrier is dependent on the activity of vacuolar(H^+)ATPase (vATPase), a key pH regulator of cellular compartments [19]. The retrograde transport organelles

lacking vATPase offer a neutral environment and thus promote the toxin's extended conformation (Fig 6). After transcytosis into inhibitory interneurons, TeNT encounters the acidic pH of the synaptic vesicle and adopts its closed configuration. This may itself represent a receptor bound intermediate state preceding pore-formation. The structural changes that clostridial neurotoxins undergo to allow translocation of LC into the cytosol remain mysterious. *In vitro* experiments with liposomes enriched in gangliosides have shown that TeNT forms a trans-membrane channel by interacting with the lipid bilayer at acidic pH below 5.0 [53–55]. Future experiments to investigate the structure of clostridial neurotoxins in a membrane environment will hopefully shed light on the pore-formation process.

The fact that TeNT can display multiple conformations has important consequences on its function and clearly demarks it from the botulinum neurotoxins. This is particularly relevant as TeNT is expressed on its own by *C. tetani*, whereas BoNTs are in a protective protein complex [56]. Noticeably, it was shown that the structures of the free BoNT/A [57] and BoNT/B [58] are not affected by pH, while progenitor toxin M assembly of BoNT/A is dependent on acid-mediated rearrangement of the binding domain in its NTN1 partner [57]. Similarly, acidic pH maintains the BoNT/E progenitor toxin complex, which dissociates in neutral condition [48].

In an experiment comparing chimeras composed of various TeNT and BoNT domains, Wang *et al* [59] demonstrated that only a molecule comprising all the TeNT components was able to cause spastic paralysis in mice, thus highlighting a structure–function relationship based on an integral TeNT scaffold. A recent study also determined that, while only the binding domain is responsible for cell recognition, sorting of TeNT in the right organelle for retrograde axonal transport depends on the full-length toxin, in a mechanism independent of synaptic vesicles. This supports that differential

Figure 6. The tetanus neurotoxin journey.

A Schematic of the different stages of TeNT neuronal pathway.
B Summary of the pH-mediated structural dynamism of TeNT.

entry of TeNT could be mediated by a novel interaction involving H_C and stabilised by H_N and LC [60].

The results presented here suggest domain flexibility is a prerequisite for the toxin's compartmentalisation in endosomes for neuronal trafficking. Any efforts to utilise TeNT as a delivery system to transport proteins to the CNS would therefore require adding component N-terminally of the full-length (and inactive) toxin while preserving its structural integrity. Such molecules have already been designed and used as biochemical tools to characterise the toxin's function [61,62].

For its activity, TeNT relies on activation into a di-chain molecule by a bacterial or host protease through cleavage of the LC-HC linker. This inter-chain connection is flexible and readily accessible. The rest of TeNT is especially resistant to proteolysis [50], and thus, the closed conformation of TeNT may provide additional resistance to enzymatic degradation in acidic environments. Additionally, the crystal structure provides the complete platform to understand the efficiency of the tetanus toxoid that has been used as a vaccine for almost a century [63,64]. The chemical modifications that neutralise the toxin have previously been fully mapped [65]. Interestingly, all of the epitopes are in surface exposed areas and none are present at the multi-domain interface (Fig EV3).

Recognition of gangliosides by the toxin is well documented and includes two sites [39]. In the crystal structure, a single pocket was occupied by GD1a or GM1a polysaccharides, at the conserved galactose-binding site. The two ligands were shown to have similar affinities [40], which correlate well with the identical binding profiles observed here around the Gal-GalNAc subunits. The secondary pocket, which prefers disialic acid [14] and is also a potential protein-binding site [66], remains freely accessible. The crystal structure is therefore compatible with the clostridial neurotoxins' dual-receptor paradigm [67]. Several candidates have been proposed as co-receptors for TeNT [17], it will be interesting to determine whether the various toxin arrangements play a role in recognition of other receptors.

Here we report a comprehensive structural characterisation of the TeNT dynamics through a combination of X-ray crystallography, SAXS and single particle electron cryo-microscopy, showing the unique domain arrangement of the toxin and its unexpected ability to undergo a pH mediated change from a closed to extended conformation. The singular organisation of the toxin offers useful information that complements our biochemical knowledge tracing the complex journey of the toxin from peripheral neurons to the CNS. It also provides a unique framework for the development of anti-toxin therapy and the engineering of biomolecules able to target central neurons.

Materials and Methods

Protein expression and purification

The TeNT construct used in this study corresponds to a catalytically inactive mutant (R372A/Y375F) [22–24] to prevent any safety issues. TeNT (UniProt P04958) was codon optimised for *E. coli* expression, synthesised and cloned into a pET-28a(+) expression vector (GenScript, NJ, USA) with a N-terminal 6×His-tag. Expression was carried out in *E. coli* BL21 (DE3) cells grown in terrific

broth medium at 37°C for approximately 3 h and induced with a 1 mM final concentration of IPTG, overnight at 16°C. Cells were harvested and frozen at –80°C. Cell lysis for protein extractions was performed with an Emulsiflex-C3 (Avestin, Germany) at 20 kPsi in 0.05 M HEPES pH 7.2 with 0.2 M NaCl and 40 mM imidazole. Cell debris was spun down via ultra-centrifugation at 4°C, 267,000 g for 45 min. The protein was purified by affinity chromatography (HisTrap FF, GE Healthcare, Sweden), dialysis and size exclusion (Superdex200, GE Healthcare, Sweden). Samples were kept at 12 mg/ml in 0.05 M HEPES pH 7.2 with 0.2 M NaCl and 5% glycerol.

X-ray crystallography

Samples for crystallisation were prepared by pre-incubation of the protein (10 mg/ml) with 5 mM of the GD1a or GM1a oligosaccharides (Elicityl, France). Crystals of TeNT:GD1a were grown with 2 µl of sample mixed with 1 µl of reservoir solution consisting of 20% v/v polyethylene glycol 3,350, 0.1 M Bis-Tris propane pH 6.5, 0.2 M potassium thiocyanate using a hanging drop set-up. Crystals grew within 2–3 weeks at 20°C. For TeNT:GM1a, crystals were obtained from a sitting-drop in 20% v/v polyethylene glycol 6,000, 0.1 M MES pH 6.0, 0.2 M sodium chloride (PACT screen, B7, Molecular Dimensions, UK). A drop of 200 nl of sample was mixed with equal amount of reservoir and incubated at 20°C, crystal grew within 5–6 weeks. Crystals were transferred briefly into a cryo-protectant solution, consisting of their respective growth condition supplemented with 25% glycerol, before freezing in liquid nitrogen.

Diffraction data were collected at station I04-1 of the Diamond Light Source (Oxon, UK), equipped with a PILATUS-6M detector (Dectris, Switzerland). Complete dataset to 2.6 Å was collected from single crystals at 100 K for each TeNT complexes. Raw data images were processed and scaled with Mosflm [68], and AIMLESS [69] using the CCP4 suite 7.0 [70]. The resolution cut-off chosen was based on the CC_{1/2} [71].

Initial molecular replacement was performed with the coordinates of LC and H_C of TeNT (PDB 1z7h and 1fv2, respectively) to determine initial phases for structure solution in PHASER [72]. The translocation domain was built in from a truncated poly-alanine model of the secondary structures of BoNT/B (PDB 1epw) and partially extended with BUCCANEER [73]. The working models were refined using REFMAC5 [74] and manually adjusted with COOT [75]. Water molecules were added at positions where Fo–Fc electron density peaks exceeded 3σ, and potential hydrogen bonds could be made. Validation was performed with MOLPROBITY [76]. Ramachandran statistics show that for both structures 97% of all residues are in the most favoured region, with a single outlier in the disallowed region. Crystallographic data statistics are summarised in Table 1. Figures were drawn with PyMOL (Schrödinger, LLC, New York) or Chimera [42].

Electron cryo-microscopy

TeNT was prepared by an additional size exclusion chromatography step (YARA SEC-3000, Phenomenex, Denmark) in 0.02 M HEPES pH 7.2, 0.2 M NaCl. TeNT eluted as a single sharp peak at 0.5 mg/ml, which was concentrated to a stock solution at 3.0 mg/ml (Vivaspin. 100 kDa cut-off, GE Healthcare, Sweden). Samples were pipetted onto

glow-discharged holey carbon cryo-EM grids (Quantifoil Cu R0.6/1) and frozen in liquid ethane using a Vitrobot (FEI, Hillsboro, OR).

The data were collected using a Titan Krios electron microscope (FEI) operated at 300 kV equipped with a K2 summit direct electron detector (Gatan, Pleasanton, CA). Images were collected in single-electron counting mode at a nominal magnification of $105,000 \times$ ($1.37 \text{ \AA/pixel}$), using a flux of $3.18 \text{ e/\AA}^2/\text{s}$ and a total dose of $47 \text{ e/\AA}^2$ over a total of 20 frames. Frames were aligned, averaged and dose-weighted with motioncor2 [77]. The contrast transfer functions for each micrograph were estimated using CTFFIND4 [78]. The subsequent particle picking and data processing were performed using Relion-2.0 [79]. A total of 1,024 micrographs were recorded from which $> 1,008,000$ particles were picked automatically in Relion. After 2D classification, a large number of spurious particles as well as particles of about $30 \text{ \AA}$ were removed, yielding a dataset of $\sim 197,000$ particles which was subsequently used for 3D refinement. The reported resolution is based on the gold-standard FSC-0.143 criterion [80], and the FSC-curve was corrected for the convolution effects of a soft mask using high-resolution noise substitution [81]. The density map was sharpened by applying a negative B-factor that was estimated using automated procedures [82].

Small-angle X-ray scattering

Samples of TeNT were prepared by an additional size exclusion chromatography step (Superdex200, GE Healthcare, Sweden) in 0.05 M HEPES pH 7.2, 0.2 M NaCl and 5% glycerol. TeNT eluted as a single sharp peak at 0.3 mg/ml , which was concentrated to 4.0 mg/ml (Vivaspin. 100 kDa cut-off, GE Healthcare, Sweden). Additional dialysis was performed on samples with various buffer conditions where HEPES was substituted with Tris at pH 8.0–7.5, MES at pH 6.7–5.5 and sodium acetate at pH 5.3–5.0. Sample was then diluted to 3, 2 and 1 mg/ml in corresponding buffer. Solution scattering (SAXS) data were collected at station B21 of the Diamond Light Source (Oxon, UK), equipped with a PILATUS-2M detector (Dectris, Switzerland). A buffer measurement was performed before and after each sample. Measurements were performed at 15°C and consisted of 18 10-s frames collected at 12.4 keV . The data were processed with PRIMUS [83] followed by particle distance distribution function $P(r)$ analysis using GNOM [84]. The theoretical curves were calculated with CRY SOL [85]. Data were further treated by using DAMMIF [86] to produce twenty *ab initio* models that were averaged with DAMAVER [87]. The level of closed against extended conformation was assessed using OLIGOMER [83].

Accession numbers

The atomic coordinates and structure factors (codes 5n0b and 5n0c) have been deposited in the Protein Data Bank (<http://www.wwpdb.org>). Cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under accession number EMD-3588.

Expanded View for this article is available online.

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Author contributions

GM performed all protein expression, purification, crystallography and SAXS experiments; analysed the data; and wrote the manuscript. JC performed the cryo-EM experiments and analysed the data. PS outlined and supervised the study, analysed the data and wrote the manuscript. All authors reviewed and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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