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REVIEW ARTICLE

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Tetanus and tetanus neurotoxin: From peripheral uptake to central nervous tissue targets

Aram Megighian[1,2] | Marco Pirazzini[1] | Federico Fabris[1] | Ornella Rossetto[1,3] | Cesare Montecucco[1,3]

[1]Dipartimento di scienze Biomediche, Università di Padova, Padova, Italy

[2]Padova Neuroscience Center, Università di Padova, Padova, Italy

[3]Istituto CNR di Neuroscienze, Università di Padova, Padova, Italy

Correspondence
Aram Megighian and Cesare Montecucco, Dipartimento di scienze Biomediche, Università di Padova Via Ugo Bassi 58/B, 35121 Padova, Italy.
Email: aram.megighian@gmail.com or cesare.montecucco@gmail.com

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Abstract

Tetanus is a deadly but preventable disease caused by a protein neurotoxin produced by *Clostridium tetani*. Spores of *C. tetani* may contaminate a necrotic wound and germinate into a vegetative bacterium that releases a toxin, termed tetanus neurotoxin (TeNT). TeNT enters the general circulation, binds to peripheral motor neurons and sensory neurons, and is transported retroaxonally to the spinal cord. It then enters inhibitory interneurons and blocks the release of glycine or GABA causing a spastic paralysis. This review attempts to correlate the metalloprotease activity of TeNT and its trafficking and localization into the vertebrate body to the nature and sequence of appearance of the symptoms of tetanus.

KEYWORDS

inhibitory interneurons, metalloprotease, retroaxonal transport, tetanus, tetanus neurotoxin

1 | INTRODUCTION

Tetanus is a life-threatening disease of vertebrates characterized by a spastic paralysis that has been adequately described and named by Hippocrates in the 4th century B.C. (Pappas et al. 2008). However, symptoms associated with a disease that can be identified retrospectively as tetanus were already reported around 3,000 B.C. Tetanus has been an untreatable syndrome believed to be of nervous etiology for many centuries until Carle and Rattone (1884) in Turin demonstrated it to be caused by an infectious agent. This agent was shown to be an elongated anaerobic bacterium capable of producing a terminal spore, therefore, named *Clostridium tetani*, which was shown to germinate under low oxygen tension (Kitasato, 1889; Tizzoni & Cattani, 1889). From pure cultures of *C. tetani*, a protein exotoxin termed tetanus neurotoxin (TeNT) was isolated and demonstrated to be the sole cause of all the symptoms of tetanus (Faber 1890; Tizzoni & Cattani, 1890). This finding was the basis for discovering the serotherapy of tetanus (Behring and Kitasato, 1890) and then an anti-tetanus vaccine that effectively prevents tetanus for a long period of life (WHO, https://www.who.int/news-room/fact-sheets/detail/tetanus). In some countries, the tetanus vaccination schedule may not be accomplished and displacement of individuals to be vaccinated or unfavorable economic conditions may require to restrict vaccination to selective subset of individuals, such as pregnant women to limit the incidence of neonatal tetanus. As tetanus is an intoxication and not an infection, tetanus is not transmissible, and anti-tetanus vaccination does not induce a "herd" effect. If the vaccine is not reinoculated in the second part of life at 10 years intervals, individuals are no longer protected from the risk of developing tetanus following contamination of a necrotic wound with *C. tetani* spores. In most of the world, tetanus is effectively prevented by vaccination reaching the status of "neglected disease." Yet, hundreds of thousands of people dye by tetanus in less developed countries mainly in the fatal form of *tetanus neonatorum*, which affects

Abbreviations: ASE, axonal signaling endosomes; BoNT, botulinum neurotoxin; GFP, green fluorescent protein; H, heavy chain of tetanus neurotoxin; HC, the 50 kDa carboxy-terminal fragment C of tetanus neurotoxin; HCC, the 25 kDa C-terminal part of HC; HCN, the 25 kDa N-terminal part of fragment C of tetanus neurotoxin; In-In, inhibitory interneurons; L, light chain of tetanus neurotoxins; PFT, pore-forming toxin; PSG, poly-sialoganglioside; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein.

neonates (see below) or of maternal tetanus (Njuguna et al. 2020; Thwaites et al. 2015). In addition, it should be considered that this disease is considerably under-reported.

Tetanus has been dealt with in a large number of reviews that have discussed all aspects of the disease, including symptomatology, pathogenesis, immunological aspects of serotherapy and vaccination, the definition of therapeutic protocols, and the structure–function relationship of TeNT. At present, the interest in tetanus has somewhat decreased, although many papers continue to be published, mainly reporting on the epidemiology of tetanus, on unusual new cases in humans and other animals, or on the molecular and cellular biology of TeNT action. Here, we aim at revisiting the symptomatology and the different forms of tetanus in humans in the light of recent acquisitions on the molecular and cellular aspects of TeNT action.

2 | TETANUS

Four forms of tetanus are classified: local tetanus, generalized tetanus, tetanus neonatorum, and cephalic tetanus, as summarized in Table 1.

All these different forms of tetanus derive from an initial necrotic wound contaminated by spores of *C. tetani*. These spores are ubiquitous and enriched in soils containing a high proportion of organic matter in decomposition (including cadavers) as well as in manured and cultivated land because they are also present in the intestinal tract of animals, particularly birds, and in their feces.

Wound localization, its size, and extent of necrotic area together with the number of spores contaminating the wound, the amount of TeNT produced, and its kinetics of distribution in the body are main determinants of the severity of tetanus. The physical status and the age of the patient are other essential determinants for the prognosis of the disease.

Spore contamination of head wounds or of the lesioned inner ear, as in otitis media, are associated with cephalic tetanus; some cases of cephalic tetanus after an eye injury or tonsillectomy have been reported. Traditional medicine treatments or unsterile umbilical cord cut and non-appropriate treatments of the umbilical stump may cause contamination with *C. tetani* spores leading to *tetanus neonatorum* that, together with cephalic tetanus, is associated with short incubation times and high mortality (Thwaites et al., 2015). The use of injectable drugs also exposes to a high risk of developing tetanus (Gonzales et al., 2014).

At variance, a contaminated necrotic wound of limb extremities may cause localized tetanus with muscle rigidity and spasms, limited to the anatomical area around the necrosis. This form of tetanus may then evolve into a generalized tetanus in 2/3 of cases. Other general considerations are not warranted as the development of generalized tetanus may vary significantly with different incubation and onset times. In several cases, the initial lesion was so minor, and tetanus developed so long after, that the first symptoms appeared when the initial wound was no longer visible or even remembered. A review

TABLE 1 The four forms of tetanus

Form of tetanus	Site of wound	Incubation time*	Onset time+	Prognosis
Local	Any part of the body surface, mainly limbs	Few days/weeks. Rigidity and stiffness of the muscles close to the site of TeNT release	It may progress into generalized tetanus in weeks, depending on the amount of TeNT released	Good recovery beginning 2/3 weeks after the development of muscle contractures
Generalized	Any part of the body surface	Few days up to several weeks. Contraction and spasticity of facial and neck muscles then descending to other skeletal muscles	Days/weeks. Generalized spastic paralysis with sudden bursts of contraction of all skeletal muscles. Respiratory deficit and possible later appearance of autonomic dysfunctions	The shorter incubation/onset, the worse is the outcome. Death follows respiratory or heart failure
Neonatal	Umbilical cord	Few days. Spasticity of facial and neck muscles. Inability of sucking	Few days. Similar to generalized tetanus but with more severe symptoms	Negative
Cephalic	Head wounds	Days. Cranial nerve palsy and spasticity of facial and neck muscles	Few days	Similar to that of generalized tetanus

of many cases has led to the definition of severity scores for tetanus that can be used to estimate a prognosis and to define the better therapy to be applied (Ablett, 1956; Cook et al. 2001; Mallick & Winslet, 2004; Phillips, 1967; Thwaites & Farrar, 2003; Thwaites et al. 2006).

The different forms of tetanus have common *initial symptoms* that consist of muscle contractures and spasms derived from unopposed contractures of skeletal muscles, although cephalic tetanus is frequently associated with an initial paralysis of one or more cranial nerves that may delay diagnosis with a consequent increased risk of severe tetanus. Generalized tetanus, which is the most common form of tetanus, usually begins with: (a) stiffness of the jaws that then develops in full contracture of the masseters (*Trismus*, lockjaw) even when the wound is localized far away, (b) contracture of facial and neck muscles (*opisthotonos*) and rigidity of the abdominal and erector spinal muscles, and (c) pharyngeal and laryngeal spasms with dysphagia that prevents swallowing. These symptoms are sufficient to conclude for a diagnosis of tetanus. Their severity may decrease with time until complete recovery. Otherwise, muscle contractures with spasms and convulsions may interest all skeletal muscles of the body and persist until death. Spasmodic contraction of the respiratory muscles causes respiratory deficit that may degenerate into asphyxia, which is the most frequent, eventual, cause of death (Ablett, 1956; Cook et al. 2001; Mallick & Winslet, 2004; Phillips, 1967; Thwaites & Farrar, 2003; Thwaites et al. 2006).

In general, the earlier the symptoms appear, that is, short incubation and onset times (Table 1); the worst is the prognosis. In the past, the outcome of generalized tetanus was frequently death caused by respiratory or cardiac failure. Nowadays, the availability of Intensive Care Units (ICUs) and the definition of protocols of pharmacological intervention have limited the mortality of tetanus to a small percentage of patients (Mallick & Winslet, 2004; Thwaites & Farrar, 2003). However, over activity of the sympathetic nervous system may develop with time in severe cases of tetanus thus complicating the management of the patients even in ICUs. These late autonomic symptoms include fluctuating tachycardia/bradichardia and hypertension/hypotension with considerable sweating; this is accompanied by a relevant increase in the level of noradrenaline and adrenaline in plasma and urines (Hortnagl et al., 1979). These autonomic activities of TeNT are likely to be an indirect result of its inhibition of neurotransmitter release from inhibitory interneurons of spinal cord, brainstem, and thoracic sympathetic ganglia with consequent increased excitability of all spinal efferents, including those impinging on the sympathetic nervous system (Paar and Wellhoner, 1973).

3 | TETANUS NEUROTOXIN (TENT)

3.1 | TeNT producing bacterium

Necrotic wounds of any kind and extension, even minor ones such as those caused by tattooing or circumcision, and abortion performed with primitive customs under non-hygienic conditions, may be contaminated by spores of *C. tetani* (Thwaites et al. 2015). The spores can germinate if appropriate needs of low redox potential, low oxygen tension, pH, and bacterial nutrients are met. Vegetative bacteria multiply without diffusing in the rest of the body and without causing a relevant inflammation. The spores may persist within the wound for several weeks and then germinate if and when favorable conditions develop. This is likely to be a major determinant of the long incubation times (more than a month) not infrequently reported. If the contaminating strain of *C. tetani* harbors the plasmid encoding the gene for TeNT, then the toxin is produced and accumulates in the bacterial cytoplasm until it is released by bacterial autolysis. Toxin synthesis and release may require one to a few days. Two other proteins appear to be relevant for bacterial growth and toxin production: the chromosome-encoded tetanolysin (a pore-forming toxin, PFT) and the plasmid-encoded collagenase (Bruggemann et al. 2003). PFTs are capable of killing cells by osmotic lysis, thus contributing to necrosis. PFTs are particularly effective in killing phagocytic cells, thus preventing bacterial phagocytosis and digestion. The role of tetanus collagenase is unknown, but it could contribute to shaping a niche for the growth of the *C. tetani* colony by hydrolyzing the extracellular matrix and providing amino acid nutrients.

3.2 | TeNT Structure

Tetanus neurotoxin is released as a single polypeptide chain of 150 kDa (1,315 amino acid residues) (Bruggemann et al. 2015). Few variants of the reference strain (Massachusetts E88), very similar among them, are known (Chapeton-Montes et al. 2019; Cohen et al. 2017). Selective proteolysis of an exposed loop, subtended by a disulfide bridge, present at one-third of the 150 kDa polypeptide chain occurs within the necrotic wound by bacterial or tissue proteases. This cleavage generates two polypeptide chains (H, 100 kDa and L, 50 kDa) held together by non-covalent interactions, by a polypeptide belt extending from the H chain and encircling the L chain and by a single interchain disulfide bond, as apparent in the crystallographic structure of TeNT (Masuyer et al. 2017). The integrity of the interchain disulfide bond is essential for neurotoxicity (Schiavo et al. 1990).

As schematically shown in Figure 1a, the toxin is organized in four domains that fulfil different tasks of the multi-steps chain of events leading to tetanus: (a) the N-terminal globular domain L (50 kDa, red) is a metalloprotease whose activity is displayed in the neuronal cytosol; (b) the intermediate HN domain (50 kDa, yellow) is characterized by two parallel long alpha helices and assists the trans-membrane crossing of the L chain (Dong et al. 2019; Masuyer et al. 2017; Pirazzini et al. 2016). The two α-helices are connected by a charged loop (^{767}DKE^{769}) that is essential for TeNT neurotoxicity (Zuverink et al., 2020); (c) the HCN domain (25 kDa, purple) consists of 16 β-strands and 4 α-helices arranged in a jelly roll motif, closely similar to that of carbohydrate binding proteins of the legume lectin family (Deppe et al., 2020); and (d) the HCC domain (25 kDa, green)

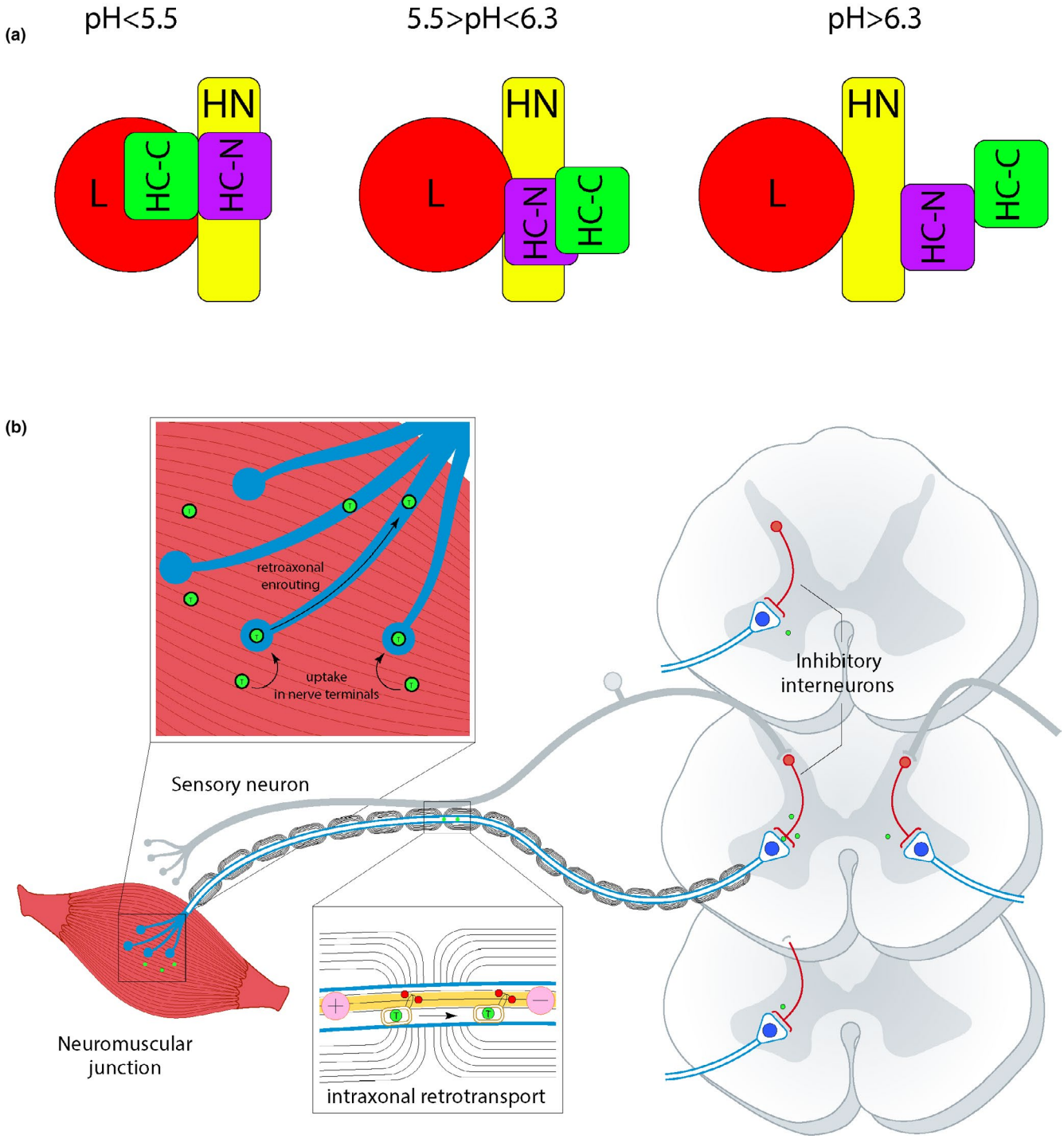

FIGURE 1 (a) Schematic structure of TeNT and of its different conformations adopted at the indicated pH values. (b) The long journey that TeNT undertakes to reach its neuronal targets within the CNS. TeNT (red dots) enters peripheral nerve terminals within endosomes that undergo retroaxonal transport to the perikaryon. TeNT is then released in the external medium and binds to inhibitory interneurons impinging on the peripheral neurons as well as other inhibitory interneurons located at different levels of the spinal cord and of the brain stem

harbors two high affinity binding sites for sugars: one for sialic acid and one for the oligosaccharide portion of polysialogangliosides (PSG) (Binz & Rummel, 2009; Chen et al., 2009; Rummel et al., 2003). In addition, it has one binding site for the protein nidogen (Bercsenyi et al. 2014). These binding interactions are essential for neurotoxicity (Bercsenyi et al. 2013; Dong et al. 2019; Rummel, 2017).

Such a four-domain structure is shared by the botulinum neurotoxins (BoNT) produced by different Clostridia species, but, at variance from TeNT, the BoNTs cause the peripheral neuroparalytic syndrome of botulism characterized by a flaccid paralysis (Johnson & Montecucco, 2008). This is attributed to the BoNT binding both to PSG and to the luminal domain of a synaptic vesicle protein (SV2 for

BoNT serotype A and E or synaptotagmin for BoNT serotype B, D/C, and G), which drives these neurotoxins into the motor axon terminals inside synaptic vesicles with ensuing paralysis (Chen et al., 2009; Dong et al. 2019; Pantano & Montecucco, 2014; Pirazzini et al., 2017b). Masuyer et al. (2017) have shown that TeNT has a remarkable conformational plasticity governed by pH. It adopts the open conformation of botulinum neurotoxins A and B at neutrality, a partially closed one in the pH range 5.5–6.3 and a closed one at pH values <5.5 (Figure 1a). This more compact form is characterized by the HCC and the L domains interacting with each other. This conformational flexibility may be required by TeNT to perform its long molecular journey from the general circulation to the final destination inside the cytosol of inhibitory interneurons of the spinal cord (Masuyer et al., 2017; Montal, 2017).

3.3 | TeNT diffusion in the general circulations and binding to neurons

The permeability of lymphatic and venous capillaries to proteins, particularly in damaged tissues (Egawa et al., 2013; Leak, 1971), suggests that, once released from bacteria, TeNT enters the lymphatic and blood circulations, although the proportion of lymphatic versus venous transport may vary substantially depending on the release site and on the extent of damage of capillaries in the wounded area. Consequently, it can be estimated that TeNT reaches the general circulation in a very short time, that is, minutes. This diffusion process is accompanied by an extensive and rapid dilution of TeNT, likely generating in the first period of time after release of a non-linear concentration gradient from the site of release to the more distant anatomical regions. This consideration is relevant to the development of local tetanus, which is consequent to the higher binding and entry of TeNT in motor neuron terminals around the site of toxin release or injection. Thereafter, TeNT undergoes retroaxonal transport inside motor and sensory neurons and it is released in the spinal cord where it blocks neurotransmitter release from inhibitory interneurons; this in turn impairs the balanced contraction of opposing skeletal muscles located in the anatomical area where TeNT was originally released (Figure 1b). However, it is important to note that the production and release of TeNT may continue for days leading eventually to a general distribution of TeNT in the body fluids with the consequent transition from a local to a generalized form of tetanus. Trismus, which is very frequently the first symptom of tetanus even when the contaminated wound is located far away from the head, may be explained by a higher affinity binding of TeNT to cranial nerve terminals followed by the more rapid delivery to the brain stem and spinal cord owing to their close anatomical connection.

The binding of TeNT to the presynaptic membrane is likely to be the result of an evolutionary process whereby the receptors binding sites in the HCC domain were selected and shaped around the oligosaccharide portions of PSG highly enriched in the presynaptic membrane (Chen et al., 2009; van Heyningen, 1974; Montecucco, 1986; Rummel, 2013; Masuyer et al. 2017). In fact, it should be considered that the hydrophilic oligosaccharide head groups of PSGs project above of the membrane plane thus acting as molecular antennae promoting, together with their number and clustering, the binding of the TeNT while flowing in the inter-synaptic extracellular fluids (Montecucco et al., 2004). In the case of the neuromuscular junction (NMJ), the cholinergic terminal is separated from the muscle by the basal membrane (BM), which is an extracellular matrix bathed by extracellular fluids. BM reversibly binds nidogens (two isoforms), which are glycoproteins that play an essential role in axon guidance during development. TeNT binds both nidogen-1 and nidogen-2 via its HCC domain (Bercsenyi et al. 2014; Surana et al. 2018).

TeNT amino acid sequence is closely similar to that of BoNT type B, and they cleave VAMP (vesicle-associated membrane protein) exactly at the same peptide bond, but cause a spastic and a flaccid paralysis, respectively (Schiavo, Benfenati, et al., 1992; Pantano & Montecucco, 2014). This difference is caused by their action on different anatomical sites depending on different binding specificities to protein receptors. It is possible that their common toxin precursor evolved either to bind the synaptic vesicle protein synaptotagmin, leading to BoNT/B that acts on peripheral neurons, or to bind nidogens that may act as decoy receptors preventing TeNT from binding to synaptic vesicle proteins, and delivering TeNT to PSG-enriched sites of the presynaptic membrane. This is followed by entry into endosomes that are then retroaxonally transported to the perikaryon of α-motor neurons inside the spinal cord. TeNT also binds to the dendrites of sensory and adrenergic neurons, as shown by experiments with radiolabeled toxins, and it is retrotransported to superior cervical ganglia (Erdmann et al. 1975; Habermann & Dimpfel, 1973; Meckler et al., 1990; Stöckel et al. 1975).

3.4 | Retroaxonal transport of TeNT to the spinal cord and brainstem

Internalization of TeNT inside presynaptic peripheral nerve terminals takes place via a particular type of endosomes termed axonal signaling endosomes (ASE) (Schmieg et al., 2014). These endosomes form by an endocytic process controlled by the small GTPase Rab5 followed by their maturation in a process controlled by Rab7, in motor, sensory, and adrenergic neurons (Deinhardt et al. 2006; Surana et al. 2018). ASE carry NGF, neurotrophins, and their receptors, including TrkA and $p75^{NTR}$, to the neuronal perikaryon via a fast retroaxonal transport system whose rate has been estimated around 7 mm/hr in various type of neurons (Sleigh et al. 2017; Stöckel et al. 1975). Contrary to the majority of endosomes, ASE do not have an acidic interior (Lalli & Schiavo, 2002). This feature is very relevant because, otherwise, the low pH would trigger the membrane translocation of the L chain of TeNT into the motor axon before reaching inhibitory interneurons (see below), thus aborting the central action of this metalloprotease (Lalli & Schiavo, 2002; Pirazzini et al. 2016).

The ASE retroaxonal transport is an essential communication system between axon terminals and soma, and any alteration to the proteins (dyneins, adaptors, etc.) involved in such a system leads to

degeneration of motor and sensory neurons (Schmieg et al., 2014; Sleigh et al. 2017; Surana et al. 2018). TeNT and other microbial pathogens (Poliovirus, Bornavirus, rabies virus, etc.) hijack this physiological function to reach the CNS (Charlier et al., 2016; Gluska et al., 2014; Ohka & Nomoto, 2001; Salinas et al., 2010; Taylor & Enquist, 2015). The rate of retroaxonal transport of I^{125}-TeNT inside sympathetic neurons to the superior cervical ganglion of rats was estimated to be about 180 mm/day (Schwab & Thoenen, 1977) and that of the fluorescent protein GFP fused to the HC fragment of TeNT inside the mouse sciatic nerve about 160 mm/day (Sleigh et al. 2017). Comparative data for humans are not available, but, assuming similar figures, the contribution of the intraneuronal centripetal journey of TeNT to the "incubation time" is small in cephalic tetanus, yet significant in the case of local and general tetanus following necrotic wounds of the extremities in adults.

4 | TRANS-SYNAPTIC MOVEMENT OF TENT FROM THE MOTOR NEURON SOMA TO INHIBITORY INTERNEURONS

When the ASE containing TeNT reach the perikaryon of motor neurons localized in the ventral horn of the spinal cord, the toxin is transferred via a trans-synaptic movement to the inhibitory interneurons (In-In) forming synapses with the dendrites and soma of the peripheral motor neurons (Schwab, 1980; Schwab et al. 1979; Schwab & Thoenen, 1977; Stöckel et al. 1975; Stoeckel et al. 1977). This may occur via TeNT-containing ASE fusing with lysosomes followed by release via secretory lysosomal vesicles in the intersynaptic space. In-In targeting within the spinal cord was hypothesized by Sherrington following a comparative analysis of the effects of strychnine and TeNT (Sherrington, 1907). However, In-In were identified by Eccles and collaborators who observed that upon local administration of TeNT within peripheral nerves or directly in the spinal cord (close to the anterior horn), the plurisynaptic Ia, Ib, and In-In inhibition of α-motoneuron was progressively reduced in a few hours after toxin administration (Brooks et al. 1955, 1957). On the other hand, monosynaptic excitation of α-motoneurons by Ia afferents was unaffected and, at the same time, the activation of In-In during polysynaptic α-motoneuron inhibition was also unaffected by TeNT. Altogether, these findings indicated that the reduced inhibition of α-motoneurons does not derive from reduced excitability of inhibitory neurons but rather from a blockade in the release of inhibitory neurotransmitters (glycine or GABA) at their synaptic contacts with α-motoneurons, as subsequently demonstrated (Curtis et al. 1976; Kanda & Takano, 1983).

Schwab and Thoenen conducted extensive work to follow TeNT retroaxonal transport inside motor neurons and destination within the spinal cord. Using electron microscopy and I^{125}-TeNT autoradiography, they provided experimental evidence that after reaching the perikaryon of motor neurons in the ventral horn of the spinal cord via the retrotransport of vesicles, TeNT was transferred via a trans-synaptic movement to the inhibitory interneurons, which form direct synaptic contacts with the dendrites and soma of the peripheral motor neurons (Figure 1) (Schwab et al. 1979; Schwab & Thoenen, 1976). However, it should be noted that the histoautoradiographic experiments were not sensitive enough to exclude the possibility that TeNT was released by motor neuron soma outside the intersynaptic space with In-In or that TeNT could leak out this intersynaptic space to reach other cells that display high affinity binding for TeNT, as outlined in Figure 1b. This effect could contribute to the development of generalized tetanus together with the toxin distributed in the body reaching the different levels of the spinal cord via the different motor neurons.

According to the studies on spinal reflexes control, the TeNT target neurons are the Renshaw cells, as well as Ia and Ib inhibitory interneurons. However, further studies of spinal cord circuits and their role in simple and complex movements (such as locomotion) unraveled new functional parts of inhibitory interneurons input to motor neurons, the existence of other inhibitory interneurons such as the V_0 commissural interneurons, and the sophisticated role of recurrent reciprocal inhibition of inhibitory interneurons (Goulding, 2009; Rybak et al., 2015). These findings paint a very complex system of segmental or multi-segmental spinal cord circuits that control agonist/antagonist motor neuron activity on the ipsilateral body side and the interconnection between the contralateral (left/right) sides (Figure 2). These spinal cord circuits control the generation of segmental locomotory output through central pattern generators (Windhorst, 1996; Ramírez-Jarquín & Tapia, 2018; Shepard, 2004). Pluri-synaptic reflexes, other than exciting or inhibiting homolateral reciprocal muscles, evoke opposite responses in the same reciprocal muscles of the contralateral side. These functional effects on contralateral motoneurons are in fact driven by excitatory and inhibitory collaterals from the homolateral sensory afferents as well from activated homolateral motor neurons (Betley et al., 2009). A clear example of these circuits is the "avoiding reflex," elicited by nociceptor activation. During motor tasks like the removal of limbs from nociceptive stimuli, this reflex activates circuits that generate contralateral motor outputs to maintain body balance. Therefore, it cannot be excluded that TeNT injected/released at the level of a limb muscle affects the contralateral spinal cord or even ascend toward the brain stem, where TeNT is particularly toxic (Rossetto & Montecucco, 2019). Clearly, this matter implicates that the current understanding of the pathophysiology of tetanus is somewhat simplified and calls for ad hoc studies to fully decipher the pathogenic action of TeNT in the spinal cord and brain stem.

5 | THE TENT-INDUCED BLOCKADE OF NEUROTRANSMITTER RELEASE IN INHIBITORY INTERNEURONS

All spinal cord cells that express appropriate receptors can bind TeNT with high affinity; the synaptic vesicle glycoprotein SV2 was recently proposed to act as the central protein receptor of TeNT (Yeh et al., 2010), but this binding does not explain the specificity of

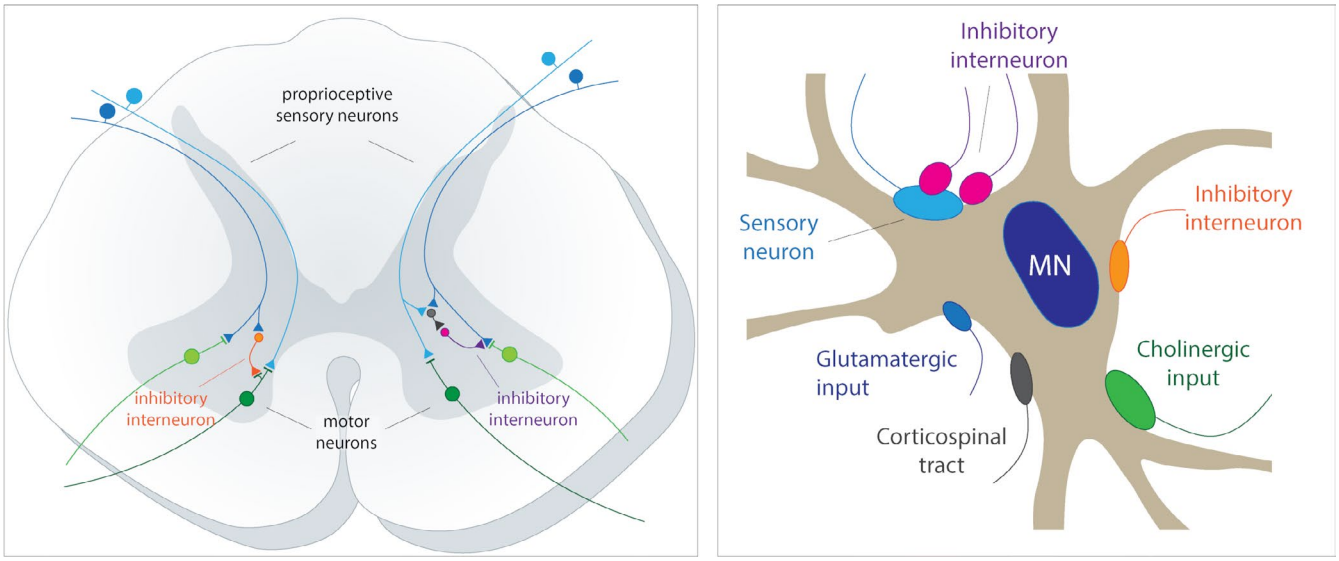

FIGURE 2 Left panel. Schematic view of proprioceptive sensory afferents entering spinal cord myelomere and directly synapsing and exciting muscle motor neuron (light green) and inhibitory interneurons for antagonist muscle motor neuron (dark green). Right panel. Spinal motorneuron excitatory and inhibitory afferents

TeNT for In-In as SV2 is present in all neurons; however, it is possible that the glycosylation of SV2 is different and unique in In-In. TeNT is then endocytosed into synaptic vesicles (Matteoli et al. 1996), wherefrom the L-domain translocates across the membrane in a process driven by the low pH and assisted by the HN domain (Fischer & Montal, 2007a; Pirazzini et al. 2016). During this process, the L chain must remain linked to the HN domain via the interchain disulfide bond in order to lead to the exposure of the L chain on the cytosolic side of the synaptic vesicle membrane (Fischer & Montal, 2007b). Here, it is freed by SS reduction mediated by the NADH-Thioredoxin Reductase-Thioredoxin redox system together with the chaperone protein Hsp90 (Azarnia Tehran et al. 2017; Pirazzini et al. 2013; Pirazzini et al., 2017).

Once released from HN, the L chain displays its Zn^{2+}-dependent protease activity specific for VAMP, which is cleaved at a single site (Gln76-Phe77 in the isoform VAMP2) (Schiavo, Benfenati, et al., 1992). This highly selective biochemical lesion is sufficient to block neurotransmitter release in inhibitory interneurons. TeNT blocks synaptic vesicle exocytosis at synapse but not in isolated axons (Verderio et al., 1999). Of the eight known isoforms of human VAMP, VAMP-1, -2, and -3 were shown to be cleaved by TeNT (Pirazzini *et al.* 2017; Proux-Gillardeaux et al., 2005). The main VAMP isoforms involved in neuroexocytosis are VAMP-1 and VAMP-2, with VAMP-1 being the predominant one in the spinal cord (Jacobsson et al. 1998). Mutations at the TeNT cleavage site in VAMP-1 provide resistance to tetanus in rats and chickens (Patarnello et al. 1993). The Km and Vmax values of the L-chain metalloprotease of TeNT were estimated to be: 4–50 μM and 0.035–0.04 μmol/sec, respectively (Chen & Barbieri, 2008; Sikorra et al. 2008). In *Aplysia californica* cholinergic nerve terminals, 4–10 molecules of TeNT L chain were capable of blocking neurotransmitter release within 20 min at room temperature (Schiavo, Poulain, et al., 1992). This finding indicates that the time required to cleave about 65 copies of VAMP located on each of the about 200 synaptic vesicles present in the presynaptic terminal of a CNS neuron (Ikeda & Bekkers, 2009; Takamori et al., 2006) contributes little to the "time of incubation" of tetanus disease.

As long as the L chain of TeNT remains inside spinal cord neurons, their neuroexocytosis is blocked, paralysis persists, and the tetanized patient may dye. However, the L metalloprotease does not kill the neuron and, with time, it is thermally or chemically inactivated or it may be degraded in proteasomes. Whatever the mode of L inactivation, damaged synaptic vesicle is replaced by novel ones, affected neurons re-establish their neurotransmitter release, and the patient slowly recovers. Clearly, drugs that specifically inhibit or inactivate the TeNT metalloprotease activity inside the neuron, or drugs that accelerate the L-chain inactivation, will shorten the time of recovery and would be most welcome. Given the tetanus present status of neglected diseases, this would require an international funding effort.

6 | CONCLUSIONS

Tetanus, a deadly disease that has taken the lives of millions and millions of people over the centuries, is now well prevented by tetanus toxoid vaccination and by the use of hyperimmune anti-tetanus human antisera. Few cases are recorded in countries where these preventive provisions are available. However, the disease is still taking many lives in less-developed parts of the world. This situation calls for further research efforts to discover drugs that would permit a rapid recovery from tetanus and to develop safer serotherapies employing monoclonal antibodies rather than hyperimmune human antisera. Moreover, the use of TeNT could contribute significantly to studies aimed at learning more about

the inhibitory circuitry involved in balanced skeletal muscles contractions and in lateral/contralateral alternate body movement. In fact, most of the available information on the action of TeNT within the spinal cord were acquired in the period 1955–1975 using electrophysiological and imaging techniques that have been, meanwhile, largely improved in terms of specificity, resolution, and sensitivity. The use of the presently available techniques coupled with TeNT derivatives labelled with novel brilliant fluorescent probes could contribute to identify and map spinal cord and brainstem neuronal circuits involved in neurophysiology and in the pathogenesis of tetanus.

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CONFLICT OF INTEREST

The authors of this manuscript certify that they have no conflict of interest involved in any organization or entity related to the content of the present review.

ORCID

Cesare Montecucco https://orcid.org/0000-0001-7361-5688

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