Endocytosis and retrograde axonal traffic in motor neurons

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Abstract

Spinal cord motor neurons control voluntary movement by relaying messages that arrive from upper brain centres to the innervated muscles. Despite the importance of motor neurons in human health and disease, the precise control of their membrane dynamics and its effect on motor neuron homoeostasis and survival are poorly understood. In particular, the molecular basis of the coordination of specific endocytic events with the axonal retrograde transport pathway is largely unknown. To study these important vesicular trafficking events, we pioneered the use of atoxic fragments of tetanus and botulinum neurotoxins to follow endocytosis and retrograde axonal transport in motor neurons. These neurotoxins bind specifically to pre-synaptic nerve terminals, where they are internalized. Whereas botulinum neurotoxins remain at the neuromuscular junction, tetanus toxin is retrogradely transported along the axon to the cell body, where it is released into the intersynaptic space and is internalized by adjacent inhibitory interneurons. The high neurospecificity and the differential intracellular sorting make tetanus and botulinum neurotoxins ideal tools to study neuronal physiology. In the present review, we discuss recent developments in our understanding of the internalization and trafficking of these molecules in spinal cord motor neurons. Furthermore, we describe the development of a reliable transfection method for motor neurons based on microinjection, which will be extremely useful for dissecting further the molecular basis of membrane dynamics and axonal transport in these cells.

Introduction

Membrane dynamics is a process of paramount importance in all eukaryotic cells. It plays a role in multiple pathways, controlling, among other events, cellular homoeostasis, motility and survival. Due to their specialized functions

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and topology, neurons have developed unique mechanisms for the regulation of their membrane dynamics, which are responsible for neurite outgrowth and remodelling, synaptogenesis and SV (synaptic vesicle) exo-/endocytosis. The precise co-ordination of these processes is ensured by fine-tuned vesicular traffic, leading to the transport of many different ligands and organelles from and to the cell body. Newly synthesized proteins, RNAs and vesicular cargos are delivered to distal neuronal sites via the anterograde transport pathway, whereas retrograde transport is required for the retrieval of organelles and ligands from the cell periphery to the soma [1]. Pathogens and virulence factors, including TeNT (tetanus neurotoxin) and several neurotrophic viruses, induce these transport pathways to enter the central nervous system and to spread to neighbouring cells [2].

Despite the importance of axonal retrograde transport in health and disease, the molecular machinery responsible for this process and its coupling to specific endocytic events at the nerve terminal is poorly characterized. Whereas our understanding of SV dynamics within mammalian synapses has witnessed remarkable progress in the last decade [3], little is known about other endocytic events occurring at the same cellular site. Moreover, the relationship between the rate and co-ordination of SV exo-/endocytosis and other synaptic endocytic events is presently unknown. We sought to fill this gap by using a non-toxic fragment of TeNT (H_C) as a probe to monitor high-affinity binding, internalization and retrograde axonal transport in spinal cord MNs (motor neurons) [4,5].

**TeNT H_C as a probe for neuronal membrane dynamics**

TeNT is expressed by toxigenic strains of *Clostridium tetani*, an anaerobic bacterium, and belongs to the CNT (clostridial neurotoxin) family, which also comprises seven BoNT (botulinum neurotoxin) serotypes. TeNT is the causative agent of tetanus, a life-threatening disease characterized by an involuntary general contraction of skeletal muscles due to the unbalanced activity of the NMJs (neuromuscular junctions). CNTs are structurally organized into heavy and light chains linked by a disulphide bond (Figure 1a) [6]. The light chains are zinc-endopeptidases specific for synaptic members of the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) family, which are core components of the neurotransmitter release apparatus [7]. These proteins [VAMP (vesicle-associated membrane protein)/synaptobrevin, SNAP-25 and syntaxin 1] are necessary for the fusion of SVs with the synaptic plasma membrane; their selective proteolysis accounts fully for the inhibition of neurotransmitter release caused by CNTs *in vivo* [7,8]. As a consequence, CNTs are the tools of choice to study the functions of synaptic SNARE proteins in neurons and secretory cells both *in vitro* and *in vivo* [7,8]. TeNT and BoNT have also been exploited to ascertain additional functions of synaptic SNAREs, such as their role in membrane repair and axonal maintenance [5]. In addition, TeNT is used in experimental models of chronic epilepsy to highlight the effects of repetitive seizures on brain functions [9].

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Alongside these applications, TeNT and BoNT are also ideal tools to study membrane dynamics at the nerve terminal due to their neurospecificity and unique intracellular trafficking features. All CNTs are recruited to the NMJ plasma membrane, where they undergo distinct sorting events. BoNT enters the NMJ and blocks acetylcholine release, causing the flaccid paralysis, which is the clinical trademark of botulism. TeNT is instead recruited to the axonal retrograde transport pathway and reaches the soma of MNs, which is located in the spinal cord [7]. TeNT is then targeted to inhibitory interneurons, where it blocks the release of glycine, thus leading to spastic paralysis. This distinct sorting of TeNT and BoNT is saturable, since at high concentrations BoNT is also transported to the spinal cord, causing central nervous system effects [10].

Figure 1 Scheme of clostridial neurotoxins and their domains. (a) TeNT and BoNT are composed of a heavy (H) and a light (L) chain, held together by a disulphide bridge and other non-covalent interactions. The L chain (in white) is a zinc-endopeptidase specific for SNARE proteins. The H chain is composed of a 50 kDa N-terminal domain (H_N, hatched), which is involved in membrane translocation and a C-terminal portion (H_C) responsible for membrane binding and retrograde transport. This domain is formed by the H_CN (light grey) and H_CC (dark grey) subdomains. H_C fragments are expressed as recombinant proteins tagged at the N-terminus [11]. These tags include a cysteine-rich motif suitable for Alexa-maleimide modification [42]. AA, amino acids. Fluorescent TeNT H_C is used as a probe to monitor axonal retrograde transport in spinal cord MNs (b), here imaged by field emission scanning electron microscopy. Notice the typical large soma, multiple dendritic arborizations and a single long axon with constant diameter. Scale bar, 10 μm.
However, at concentrations relevant for the pathophysiology of tetanus and botulism (picomolar and below), TeNT and BoNT are characterized by a specific targeting to distinct endocytic routes and constitute useful tools for the analysis and identification of these pathways in neurons [5].

The main determinants of the neurospecificity and intracellular targeting of CNTs are encoded by their C-terminal portions, termed HC (Figure 1a) [5]. Isolated HC fragments bind to the functional receptors of TeNT and BoNT, since they counteract the paralysis induced by the parental neurotoxins [11]. HC is composed of two distinct subdomains of almost identical size with different binding specificity. The foremost C-terminal domain of HC (H_CC) contains four distinct carbohydrate-binding regions, which mediate the interaction with surface lipids containing sialic acid, such as the polysialoganglioside GT1b [12,13], and, possibly, with neuronal glycoproteins [14,15]. The structural differences detected in the binding of ganglioside GT1b to the H_CC domain of TeNT and BoNT suggest that this domain might contribute to the specificity of TeNT and BoNT recruitment to the NMJ and their selective trafficking [16,17]. Accordingly, deletions in H_CC cause loss of binding to MNs, the impairment of TeNT to undergo retrograde transport in vivo [18], and a decrease in the neurotoxicity of CNT [15,16]. The N-terminal portion (H_CN) of HC structurally resembles the carbohydrate-binding moiety found in lectins [19]. Despite the fact that no ligand for this portion of the molecule has been reported to date, it has been suggested that H_CN might play a role in a later step of the intoxication process, such as the intracellular sorting of TeNT [5].

Specificity of the binding of clostridial neurotoxins to the neuronal membrane

Multiple receptors participate in the high-affinity interaction of CNTs and their HC fragments to the neuronal membrane. TeNT and BoNT bind to polysialogangliosides [7]. This interaction is biologically important, since CNTs display a reduced activity in neurons in which ganglioside biosynthesis has been impaired [20,21]. However, several considerations, including the limited binding competition among CNTs, suggest that other determinants play a role in the high-affinity interaction of these molecules with the neuronal surface [22]. In addition to acidic phospholipids, specific protein co-receptors have been characterized. BoNT/B and G interact with members of the synaptotagmin family in a GT1b-dependent manner [23–26]. These SV proteins, which are involved in calcium sensing at the synapse, expose their intraluminal domain to the extracellular environment following SV fusion with the plasma membrane [27], potentially allowing CNT binding. These findings suggest that the internalization of at least these two BoNT serotypes may occur via SV endocytosis.

Despite the efforts from different laboratories, less is known regarding the specific receptors for TeNT and the remaining BoNT serotypes. One or more GPI (glycosylphosphatidylinositol)-anchored proteins of 15 kDa have been shown to bind selectively to TeNT and its H_CC fragment, but not BoNT [28,29]. In NGF (nerve growth factor) differentiated PC12 cells, one of these TeNT-interacting factors has been identified as Thy-1 [30], a GPI-anchored
glycoprotein involved in multiple cellular functions, including neurite outgrowth [31]. Although Thy-1 is unlikely to be the main protein receptor for TeNT [5], GPI-anchored proteins play a major role in TeNT binding and uptake, since pre-treating MNs with a phospholipase which cleaves their lipid anchor protects neurons from TeNT intoxication [32]. Altogether, these findings support the hypothesis that one or more still unidentified GPI-anchored glycoproteins act as a physiological TeNT receptor.

GPI-anchored proteins, together with cholesterol, gangliosides and other sphingolipids (but notably not synaptotagmins), are enriched in microdomains of the plasma membrane termed lipid rafts [33,34]. Lipid rafts act as functional platforms for signalling, endocytosis and sorting [34], and might be exploited by pathogens to promote infection or intoxication [35]. The binding of TeNT HC to multiple raft-associated components suggests that the recruitment of TeNT on the neuronal surface is mediated by lipid microdomains. Accordingly, CNTs associate with detergent-resistant glycolipid-enriched membranes in a cholesterol-dependent manner [30,36]. Disruption of lipid microdomains with cholesterol-sequestering drugs abolishes TeNT HC internalization [30] and VAMP/synaptobrevin cleavage [30,32], suggesting that lipid rafts play a central role in TeNT trafficking and represent a common requisite for the recruitment of CNTs to the neuronal plasma membrane. Furthermore, the distinct protein receptors and trafficking of TeNT and BoNT imply that these neurotoxins bind to different lipid rafts with independent intrasynaptic sorting. Strong evidence supporting the presence of pools of membrane rafts undergoing distinct regulation is accumulating rapidly, together with the notion that both the composition and distribution of lipid microdomains are likely to determine their distinct signal outputs and, ultimately, their cellular fate [34].

The high affinity of CNTs, their multiple receptors and the apparent redundancy of some of these molecules in CNT binding to the neuronal membrane have led to the recent proposal that APRs (arrays of pre-synaptic receptors) are involved in this process [22]. APRs are suggested to be dynamic entities sharing several functional features with lipid rafts. Although the initial CNT binding may involve any of their receptor molecules, the polysialoganglioside headgroups, rather than the carbohydrate moieties of glycoproteins, are likely to act as preferred initial binding factors due to their high lateral mobility and high concentration at the NMJ. Polysialogangliosides would act as ‘antennas’ in capturing CNT molecules present in the intersynaptic fluid and allow their clustering in APRs on the pre-synaptic membrane [22]. The presence of different receptor molecules within an APR, each one in several copies, would transform the initial low-affinity reversible membrane interaction [37] to a multivalent high-affinity one, which is virtually irreversible [8,38]. The recruitment of CNTs to APRs is expected to favour the delivery of CNTs to specialized sites for endocytosis and sorting, where these neurotoxins may exploit pre-assembled signalling complexes for their internalization, such as the Trk-dependent neurotrophin signalling cascade, which has been shown to be activated by TeNT HC in cortical neurons [39]. Therefore specific APR signalling might functionally engage a short-range pathway that is responsible for
BoNT localization at the NMJ or, alternatively, couple TeNT endocytosis with the sorting machinery responsible for the long-haul retrograde transport routes to the spinal cord followed by neurotrophins.

Synaptic sorting and axonal transport in MNs

Axonal retrograde transport is essential for neuronal homoeostasis, and its impairment is incompatible with neuronal survival [40]. Despite its importance in neuronal physiology and disease, a limited number of studies have focused on the identification of the machinery responsible for the sorting of endocytic vesicles to the axonal retrograde transport pathway in mammalian neurons. Furthermore, the direct visualization of retrograde transport and its quantitative analysis have been hampered by the paucity of reliable assays in living cells. We have recently established an axonal retrograde transport assay in differentiated spinal cord MNs, which exploits a fluorescent derivative of TeNT H_C as a probe [4]. MNs are isolated from embryonal rat spinal cords (E14) by a purification method that combines metrizamide density centrifugation and magnetic immuno-isolation using antibodies against p75 low-affinity NGF receptors (p75NTR). This procedure exploits both the relative large size of MN somas and the selective early expression of p75NTR in these cells [41]. The same procedure can be adapted to murine MNs by using embryos at E13 (embryonic day 13) and omitting the magnetic purification step [42]. Differentiated MNs [DIV5 (5 days in vitro) onwards] exhibit an extensive dendritic network and a single axon (Figure 1b), which may contain lateral branching.

Differentiated MNs internalize both fluorescently labelled TeNT and TeNT H_C in morphologically identical endocytic structures [4], which display an overlapping speed distribution [5]. These carriers have an average speed that agrees well with the rates observed for TeNT retrograde transport in vitro [8]. Two main groups of organelles are responsible for the retrograde transport of TeNT H_C: round vesicles and tubules. Both types of carriers are characterized by distinct kinetic behaviour; vesicles move discontinuously, alternating frequent pauses with phases of movement, whereas tubules are faster and display a more continuous transport [4]. Both types of carriers are likely to originate from coated invaginations, which appear on the axonal plasma membrane upon incubation of TeNT H_C at 4°C. When neurons were subsequently shifted to 37°C, TeNT H_C was initially found in deep pits and coated vesicles, and was detected later on in uncoated tubulovesicular and round endocytic structures, as seen by electron microscopy [42]. The diameter of vesicular compartments varies between 50 and 100 nm, whereas the average length of tubular structures is 2–3 μm. Analogous coated structures have been observed independently for TeNT internalization in mixed spinal cord cells [43]. However, the nature of the TeNT H_C compartment and its internalization mechanism have been elusive due to, among other factors, the paucity of systems for efficient transfection of fluorescently tagged and dominant-negative mutant proteins in MNs.
Optimization of transfection protocols in spinal cord MNs

We sought to fill this gap by testing different chemical and physical protocols for MN transfection. Lipofectamine 2000 is a cationic lipid-based transfection reagent designed to give high transfection efficiencies coupled to high protein expression levels in different mammalian cell types. We tested a variety of conditions in terms of Lipofectamine 2000 concentration (0.5–1.5%, v/v), amount of DNA (0.5–3 μg), incubation time (2–24 h), expression times (20–120 h) and differentiation state of the transfected MNs (DIV0–DIV4). As a transfection probe, we chose the vector pEGFP-C2, which drives the expression of EGFP (enhanced green fluorescent protein) under the strong human CMV (cytomegalovirus) immediate early promoter. Despite our attempts, we could observe medium–strong cytoplasmic EGFP expression only in a low percentage of MNs: up to 5–10%, if transfected at DIV1, but falling to well below 1% at later differentiation stages. Furthermore, when tested for TeNT HC internalization and axonal retrograde transport [4], cells expressing EGFP did not show many moving TeNT HC carriers. In addition, untransfected cells in the same culture dish, or in control dishes where the exogenous DNA was omitted, displayed only a few slow-moving endosomes, similar to pEGFP-C2 transfected cells, suggesting that Lipofectamine 2000 treatment itself has a deleterious effect on the ability of MNs to sustain an efficient axonal retrograde transport (K. Deinhardt and G. Schiavo, unpublished work). Although the precise explanation of this inhibitory effect is presently unclear, it is possible that the permeability of the MN plasma membrane is altered by the cationic lipid formulation of the transfection reagent, leading to a large and long-term imbalance of the ionic equilibrium and membrane potential of the neurite network, which in turn negatively affects axonal transport.

On the basis of these early findings, we then chose microinjection as the elected protocol to introduce foreign DNA into differentiated MNs. Despite the relative low throughput of this technique, it is generally compatible with viability and functions of fully differentiated neuronal cultures [44], and its efficiency is independent of the maturation stage of the neurons. Upon microinjection of the same EGFP-based vector, MNs were left to recover for 4–72 h and were then tested for TeNT HC internalization and axonal retrograde transport as before. Differentiated MNs expressing high levels of EGFP are able to bind and internalize TeNT HC to the same levels as untransfected cells (Figures 2a and 2b). Moreover, the percentage of still and moving TeNT HC-containing structures in EGFP-expressing MNs is identical with that of control cells (Figure 2e), as well as the speed distribution of retrograde transport carriers (Figure 2f). This last parameter has been calculated by kinetic analysis of time-series images (Figure 2c), as described previously [42]. Therefore microinjection is the method of choice for the expression of high levels of exogenous proteins in differentiated MNs. In addition, microinjection allows the introduction of other cell-impermeant molecules into MNs, such as purified proteins or labelled antibodies.
Molecular motors and fast axonal retrograde transport

Fast retrograde transport of TeNT H<sub>c</sub> in MNs requires both F-actin (filamentous actin) and MTs (microtubules) [42], suggesting that both MT-dependent and microfilament-dependent motors are involved in this process. To impair MT- and F-actin-based motors selectively, MNs were incubated with the dynein inhibitor EHNA [erythro-9-(2-hydroxy-3-nonyl) adenine] [45], the kinesin inhibitor aurintricarboxylic acid [46] and the broad-spectrum myosin inhibitor 2,3-butanedione monoxime [47] following TeNT H<sub>c</sub> internalization. Only the simultaneous treatment of MNs with the three inhibitors stopped TeNT H<sub>c</sub> carriers completely [42], suggesting that multiple classes of molecular motors are involved in fast axonal retrograde transport.

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To overcome the specificity concerns of this pharmacological analysis, the function of dynein and axonal myosins in retrograde transport have been investigated further by using a combination of transfection and genetic approaches. To this end, MNs have been microinjected with p50/dynamitin, a component of the dynein/dynactin complex [48]. Overexpression of p50/dynamitin in mammalian cells disrupts the complex, resulting in dissociation of cytoplasmic dynein from several membrane organelles, including early and late endosomes [49]. MNs expressing p50/dynamitin display an impaired motility of the TeNT H₃ carriers (Figure 2d), with a pronounced increase of still TeNT H₃-positive endosomes (Figure 2e) and a significant shift of the speed-distribution profile towards lower speed values (Figure 2f). Altogether, these findings indicate that cytoplasmic dynein acts as the main motor unit for retrograde transport in differentiated MNs, which is in agreement with the results obtained by overexpressing p50/dynamitin in postnatal MNs [50]. However, recent findings describing a direct link between dynactin and both kinesin II and cytoplasmic dynein [51] complicate the interpretation of the effects of p50/dynamitin overexpression in these cells. This notion is strengthened by the discovery that a point mutation in the MT-binding domain of dynactin causes MN disease in humans [52]. Evidence supporting a direct role for dynein in MN survival has been recently provided by the finding that mutations in dynein heavy chain generate pathological conditions closely resembling amyotrophic lateral sclerosis [53]. Interestingly, fast retrograde transport of TeNT H₃ in MNs isolated from these mice is impaired, confirming a central role of cytoplasmic dynein in this process.

As predicted by the need for F-actin, TeNT transport requires members of the myosin superfamily and, in particular, myosin Va. MNs from myosin-Va-null embryos displayed slower retrograde transport compared with wild-type cells [42]. Therefore the co-ordination of myosin Va and MT-dependent motors is necessary for fast axonal retrograde transport in spinal cord MNs.

Figure 2 p50/dynamitin blocks axonal retrograde transport of TeNT H₃ in differentiated MNs. (a) MNs bind and internalize TeNT H₃–Alexa Fluor 555. (b) Overexpression of EGFP by microinjection of the pEGFP-C2 vector (inset, 26 h post-injection) does not affect TeNT H₃–Alexa Fluor 555 binding and internalization (scale bars, 20 μm) nor its retrograde transport (c). This panel shows time series images of an MN axon (from left to right) containing TeNT H₃-positive carriers (arrowhead, arrow; frames = 9 × 29 μm). In contrast, MNs overexpressing p50/dynamitin are characterized by slower TeNT H₃-positive carriers and a higher number of stationary TeNT H₃-positive vesicles (d) (arrowhead; frames = 7.6 × 29 μm). In both (c) and (d), MN somas were located out of view at the bottom of the image. Intervals between frames are 5 s. (e) Percentage of still and moving TeNT H₃-positive structures in non-injected MNs (empty bars, n = 265) or MNs injected with pEGFP-C2 (grey bars, n = 289) or PCMV-myc encoding p50/dynamitin [49] (solid bars, n = 333). (f) Kinetic analysis of TeNT H₃ carriers in control (○; 71 carriers, 729 single movements), EGFP-expressing (♀; 68 carriers, 674 single movements) and p50/dynamitin microinjected MNs (▲; 56 carriers, 679 single movements). Relative frequencies of speed values for TeNT H₃–Alexa Fluor 555 carriers observed between two consecutive frames (interval = 5 s) are reported. Retrograde movement is conventionally shown as positive.
Physiological role of TeNT H<sub>C</sub> carriers

TeNT H<sub>C</sub> carriers are not acidified during transport, a condition necessary to retain TeNT within their lumen [8]. SV40 (simian virus 40) and *Escherichia coli* are also internalized into neutral compartments that do not belong to the classical endosomal system, which contain glycosphingolipid-rich caveolae [54]. Despite the absence of caveolin in MNs, TeNT could use a distinct endocytic pathway to bypass the classical endosomal–lysosomal system and escape degradation, as suggested by the long half-life of TeNT in spinal neurons [8]. Similarly to TeNT, endogenous ligands might use the same pathway to avoid proteolysis, reaching the neuronal cell body in a biologically active form. TeNT H<sub>C</sub> and NGF share the same retrograde organelles in MNs, a subset of which contains p75<sup>NTR</sup> [4]. NGF is retrogradely transported in MNs and accumulates in their somas without being degraded [55]. Although the endocytic compartment responsible for p75<sup>NTR</sup> trafficking in MNs is not fully characterized [56], recent studies highlighted p75<sup>NTR</sup> association with GT1b [57] and lipid raft components [58]. On this basis, lipid microdomains involved in the retrograde transport of p75<sup>NTR</sup> might play an important role in the trafficking of TeNT and other endogenous and exogenous factors through a specialized endocytic route.

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