The binding fragment of Tetanus Neurotoxin: a probe to study neuronal endocytosis and retrograde transport.

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Abstract
The Clostridial Neurotoxin (CNT) family is composed of Tetanus Neurotoxin (TeNT) and seven serotypes of Botulinum Neurotoxins (BoNT). These toxins specifically target neuronal cells, leading to an irreversible block of neurotransmitter release. CNTs trafficking can be exploited to investigate the organisation of neuronal endocytic pathways, which are still poorly understood.

We have expressed and functionally characterised recombinant carboxy-terminal binding domains (Hc) of TeNT and BoNT/A, /B and /E. These fragments can fully mimic the specific binding and internalisation of the respective holotoxins and provide useful tools for the analysis of neuronal trafficking.

We have used TeNT Hc to study the peculiar intracellular route that distinguishes TeNT from the other CNT family members. In fact, BoNTs act at the presynaptic terminal, whereas TeNT undergoes retrograde transport in the motor neuron and subsequent transcytosis in the adjacent interneuron. Very little is known about the organisation and the dynamics of this special transport route that could be exploited not only by pathogens, but also by physiological ligands for their biological functions.

TeNT Hc was used to characterise a putative TeNT protein receptor and to visualise for the first time axonal retrograde transport in living motor neurons. This experimental system provides a novel assay for the study of retrograde axonal transport and identifies two main types of retrograde carriers requiring both actin microfilaments and microtubules for efficient progression. TeNT Hc compartments lack markers of the classical endocytic pathway and are not acidified during axonal transport. Importantly, TeNT Hc and Nerve Growth Factor (NGF) share retrograde carriers, which are characterised by the presence of the neurotrophin receptor p75NTR. Altogether, these findings reveal the existence of a novel endocytic route to the neuronal cell body that escapes lysosomal targeting and that could be shared by physiological ligands such as NGF.
Acknowledgements

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Many, many thanks to Peter Lacy, Veena Viswanath and all my friends in the U.K, Italy and U.S.A. for their constant support and affection.

This thesis is dedicated to my mother, my husband, my brother and to the memory of my father.
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<td>TeNT H&lt;sub&gt;C&lt;/sub&gt; and NGF share retrograde carriers.</td>
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>ATPase associated with cellular activities</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>ADF</td>
<td>actin-depolymerising factor</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>APP</td>
<td>β-amyloid precursor protein</td>
</tr>
<tr>
<td>APRE</td>
<td>apical recycling endosome</td>
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<tr>
<td>Arf</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>Arp-1</td>
<td>actin-related protein</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BoNT</td>
<td>Botulinum neurotoxin</td>
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<tr>
<td>BPAG</td>
<td>bullous pemphigoid antigen</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BSOCOES</td>
<td>bis[2(succinimidyl carbonyloxy)ethyl] sulphone</td>
</tr>
<tr>
<td>CCD</td>
<td>cooled charge-coupled device</td>
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<tr>
<td>Cer</td>
<td>ceramide</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNT</td>
<td>Clostridial Neurotoxin</td>
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<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DIGs</td>
<td>detergent-insoluble, glycolipid-enriched complexes</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemi-luminescence</td>
</tr>
<tr>
<td>ECV</td>
<td>endosomal carrier vesicle</td>
</tr>
<tr>
<td>EE</td>
<td>early endosome</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<tr>
<td>FESEM</td>
<td>field emission scanning electron microscopy</td>
</tr>
<tr>
<td>FLIM</td>
<td>fluorescence lifetime imaging microscopy</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GalNac</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<tr>
<td>Glc</td>
<td>glucose</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>Hex A</td>
<td>β-N-acetylhexosaminidase A</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>Lat B</td>
<td>latrunculin B</td>
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<tr>
<td>LBPA</td>
<td>lysobisphosphatidic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LE</td>
<td>late endosome</td>
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<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
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<tr>
<td>MCDX</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium with Earle’s salts</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulphonic acid</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>MN</td>
<td>motor neuron</td>
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<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>NeuAc</td>
<td>N-acetylneuraminic acid (sialic acid)</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilament</td>
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<tr>
<td>NGA</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycole</td>
</tr>
<tr>
<td>PI</td>
<td>phosphoinositosides</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>phosphoinositol specific-phospholipase C</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 phosphate kinase</td>
</tr>
<tr>
<td>PIP(4, 5)K</td>
<td>phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PLAP</td>
<td>placental alkaline phosphatase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulphonyl fluoride</td>
</tr>
<tr>
<td>PNGase F</td>
<td>N-Glycosidase F</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PtdIns(3)P</td>
<td>phosphatidylinositol (3) phosphate</td>
</tr>
<tr>
<td>PtdIns(4, 5) P₂</td>
<td>phosphatidylinositol (4,5) biphosphate</td>
</tr>
<tr>
<td>RE</td>
<td>recycling endosome</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMA</td>
<td>spinal muscular atrophy</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosomal asssociated protein of 25 kDa</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>SSV</td>
<td>small synaptic vesicle</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TeNT</td>
<td>Tetanus Neurotoxin</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VAMP</td>
<td>vesicle associated membrane protein</td>
</tr>
<tr>
<td>Vin</td>
<td>vincristine</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus protein G</td>
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Publications


Chapter 1: Introduction
Preface

In the fifth century B.C. Hippocrates described for the first time the symptoms of a sailor affected by a syndrome characterised by hypercontraction of the skeletal muscles. He called this type of paralysis tetanus, (from τετανος, the Greek word for “tension”).

Tetanus was considered a neurological disease until the end of the 19th century, when the potent neurotoxin produced by *Clostridium tetani* was identified as the sole causative agent of the clinical symptoms of tetanus.

The same period also saw the first clinical description of botulism in medical literature, the isolation of *Clostridium botulinum* and its powerful neurotoxin. These fundamental discoveries opened a new research era, which led to the development of tetanus vaccination and the introduction of botulinum neurotoxins as therapeutic agents.

Clostridial Neurotoxins were also destined to open additional fields of investigation: the characterisation of their intracellular activity and their molecular substrates has been crucial for the understanding of membrane fusion during neurotransmitter release and vesicular transport. Moreover, their neurospecific trafficking makes them ideal tools for the investigation of neuronal transport routes, which is the focus of the work presented in this thesis.

This chapter contains a description of the Clostridial Neurotoxin family and their mode of action followed by an introduction to the molecular basis of neuronal endocytic trafficking, including the cytoskeletal components and the motor molecules involved in this process.
1.1 The Clostridial Neurotoxins

The Clostridial Neurotoxin (CNT) family is formed by Tetanus Neurotoxin (TeNT) and seven serotypes of Botulinum neurotoxins (BoNT, defined with letters from A to G). These powerful toxins are produced by bacteria of the genus *Clostridium*, which are widely distributed in the environment, mainly as spores. CNTs bind specifically to neuronal cells, enter their cytosol and ultimately block neurotransmitter release. They are the most toxic substances known, with mouse LD$_{50}$ ranging between 0.1 and 1 ng/kg of body weight. This high toxicity is explained by their absolute neurospecificity and by their enzymatic protease activity (see below) (Herreros et al., 1999).

Tetanus intoxication often leads to death by respiratory or heart failure (Bleck, 1989). This disease was shown to be transmissible and caused by a bacterium which was isolated and named *Clostridium tetani* (Kitasato, 1889). *Clostridium tetani* is a rod-shaped, strictly anaerobic bacterium, which frequently harbours a sub-terminal spore, thus resembling a drumstick ("clostridium" in Latin). Its spores can germinate in the presence of low oxygen, mild acidic pH and abundant nutrients (Popoff, 1995). These requirements are met in anaerobic wounds, where the bacteria can produce a cytoplasmic proteic toxin that is released in the bloodstream after autolysis. The toxin then reaches the neuromuscular junction (NMJ) where it binds to motor neuron (MN) presynaptic terminals, is internalised, retrogradely transported along axons to the cell body located in the ventral horn of the spinal cord (Price et al., 1975; Stöckel et al., 1975). The toxin is then released into the intersynaptic space and is internalised by the adjacent inhibitory interneurons, where it blocks the release of inhibitory neurotransmitters (Bergey et al., 1983; Schiavo et al., 2000; Schwab and Thoenen, 1976). This effect impairs the neuronal circuit that ensures balanced voluntary muscle contraction, causing the typical spastic symptoms that...
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Introduction

characterise tetanus intoxication. These include spasm of the facial muscles (“risus
sardonicus”), trismus (lockjaw), opisthotonus and generalized persistent reflex spasms
triggered by sensory or motor stimuli (Herreros et al., 1999) (Figure 1.1a).

Adult botulism (from the Latin “botulus”, sausage), is an intestinal intoxication caused by
toxigenic strains of C. botulinum (Arnon, 1995), C. barati and C. butyricum (Herreros et
al., 1999). Adult botulism is caused by the ingestion of food contaminated with spores of
C. botulinum and preserved under anaerobic conditions favouring germination,
proliferation and production of the neurotoxin (Smith and Sugiyama, 1988). C. botulinum
spores can also germinate directly in the intestine and release the neurotoxin, but only in
the absence of a normal gut microflora, a condition present in children less than six
months old (infant botulism) or, more rarely, in adults treated with broad-spectrum
antibiotics. Other conditions leading to an altered intestinal anatomy, such as
inflammatory bowel diseases or intestinal surgery can also increase the risk of botulinum
intoxication (Arnon, 1995). Unlike tetanus, botulism very rarely follows wound infection,
due to the very strict requirements for spore germination and vegetative growth, although
wound botulism is increasingly observed in drug users (Hatheway, 1995).

BoNTs are released in complex with other non-toxic proteins that prevent denaturation
and proteolysis in the acidic environment of the stomach lumen. The slightly alkaline
intestinal pH causes the dissociation of the complex and the release of the neurotoxin,
which reaches the bloodstream by transcytosis from the apical to the basolateral side of
intestinal epithelial cells (Maksymowych and Simpson, 1998). BoNTs act at the NMJ
where they block excitatory acetylcholine (ACh) release and hence cause a flaccid
paralysis (Schiavo et al., 2000). Botulism is therefore characterised by a general muscular
weakness, which impairs the normal function of ocular and throat muscles and later
Figure 1.1 Different clinical symptoms of the intoxication caused by TeNT and BoNTs.

(a) Example of spastic paralysis caused by TeNT intoxication. Note the spasm of the facial muscles and the persistent muscle contraction in the arms (from www.cdc.gov).

(b) A case of infant botulism. Note the flaccid paralysis extended to neck and upper body skeletal muscles (from Montecucco, Mol Medicine Today, 1996).
extends to all skeletal muscles (Smith and Sugiyama, 1988) (Figure 1.1 b). In the most severe cases, this generalised flaccid paralysis affects the respiratory and autonomic functions and leads to respiratory failure (Hatheway, 1995; Smith and Sugiyama, 1988). The opposite clinical symptoms of tetanus and botulism, spastic versus flaccid paralysis, result from the distinct trafficking and final sites of action of TeNT and BoNTs rather than from different intracellular activities (Figure 1.2). At pharmacological doses (picomolar and lower) both types of toxins block neurotransmitter release, but TeNT action is specific for central inhibitory synapses, while BoNTs act peripherally at the NMJ (Schiavo et al., 2000).

Tetanus and botulism can be prevented by specific anti-toxin antibodies, since the neurotoxins are the only agents responsible for these diseases. Toxin-neutralising antibodies can be passively acquired with direct injection, or actively induced after administration of a formaldehyde-inactivated toxin (toxoid). Recently, alternative recombinant vaccines based on the non-toxic terminal third of TeNT and BoNT molecules have been established, but their use has been so far limited to restricted groups of individuals (Middlebrook and Brown, 1995).

1.1.1 CNT structure-function relationship

The similar activity of the CNT family members predicts a common structural organisation. CNTs are produced in the bacterial cytosol as a single, inactive polypeptide chain of 150 kDa lacking a leader sequence and are released in the culture medium after bacterial lysis. No proteins are found to be associated with TeNT, whereas BoNTs form multimeric protein complexes with a molecular mass of up to 900 kDa (progenitor toxins) (Popoff and Marvaud, 1999). The single-chain CNT is activated by endogenous or
Figure 1.2 Schematic representation of TeNT and BoNT different trafficking. After entering motor nerve terminals, TeNT (right) is retrogradely transported to the MN cell body, released in the intersynaptic space and internalised by the adjacent inhibitory interneurons of the spinal cord. Only at this level the toxin active chain (red) is translocated into the cytosol where it cleaves the synaptic vesicle protein VAMP, blocking inhibitory neurotransmitter release. In contrast, BoNTs (left) are internalised in endocytic compartments that remain at the NMJ. Upon translocation into the cytosol, the active chain (light blue) cleaves its SNARE targets, ultimately leading to block of excitatory neurotransmission.
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Exogenous proteases which cleave an exposed loop subtended by a disulfide bridge formed by a highly conserved pair of cysteine residues (DasGupta, 1994). The active CNT derived from this proteolytic cleavage consists of a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa) (Figure 1.3 a) which remain associated via non-covalent protein-protein interactions and the conserved interchain disulfide bond, whose integrity is essential for neurotoxicity (de Paiva et al., 1993; Schiavo et al., 1990b). Papain treatment of CNTs at a preferential site in the middle of the H chain generates a carboxy-terminal fragment, called \( H_e \), and a heterodimer formed by the L chain and the amino-terminal portion of the H chain (Helting and Zwisler, 1977).

The recently solved crystal structures of TeNT \( H_e \), BoNT/A and B have provided crucial insights on the mechanism of action of CNTs (Hanson and Stevens, 2000; Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000; Umland et al., 1997). Their structure can be considered a modular assembly of three functional domains involved in the sequential steps mediating CNT toxic action: cell surface binding and internalisation (\( H_C \)), translocation across membranes (\( H_n \)) and final substrate catalytic cleavage (L chain).

These three domains are arranged in a linear fashion with the translocation domain in the middle (Figure 1.3 b). The catalytic domain has a compact structure formed by a mixture of \( \alpha \)-helices and \( \beta \)-sheets. The translocation domain is mainly \( \alpha \)-helical and the binding domain can be clearly divided in two sub-domains. Overall the three functional domains are also structurally distinct, except for a belt belonging to the translocation domain that wraps around the perimeter of the catalytic domain.
Figure 1.3 The three-functional domain structure of CNTs.

(a) Schematic representation of CNTs. The active holotoxins are formed by a 50-kDa light chain (L, blue) linked to a 100-kDa heavy chain (H) via a disulphide bridge, which must be reduced intracellularly for activity. The C-terminal portion of the heavy chain (Hc) is responsible for neurospecific binding, whereas the N-terminus (Hn) is involved in the translocation of the L chain in the cytosol. Structurally, the Hn portion can be subdivided in two 25-kDa domains, HcC (red) and HcN (light green). The L chain retains the zinc-endopeptidase activity responsible for CNT toxic effect. (b) Crystal structure of BoNT/A highlighting the three functional domains: binding (red/light green), translocation (green) and catalytic (blue/light blue). The latter domain is formed by a mixture of β-strands and α-helices and coordinates a zinc atom (yellow dot). The translocation domain (Hn) consists of two long α-helices with a long belt wrapped around the L chain. The Hc is clearly divided in two structural domains with similar size, HcN and HcC. The HcN is a lectin-like domain, with two seven-stranded β-sheets, whereas the HcC adopts a modified β-trefoil fold.
1.1.1.1 Structure of the binding domain (Hc)

Many studies have shown that Hc is responsible for the neurospecific binding and internalisation of CNTs (reviewed in Halpern and Neale, 1995; Schiavo et al., 2000). Based on structural analysis, Hc is composed of two distinct sub-domains (HcN and HcC), rich in β-sheets and of almost identical size (Figure 1.4). The amino-terminal sub-domain (HcN) consists of two 7-stranded antiparallel β-sheets sandwiched together in a jelly roll motif (Lacy et al., 1998; Umland et al., 1997). A similar structure is present in the carbohydrate-binding moiety of plant lectins. Strikingly, the proteins that are structurally more similar to CNT HcN are all known to interact with sugars (e.g. sialidase, lectins). Notably among these are cryia and insecticidal δ-endotoxin, which act by binding glycoproteins and creating leakage channels (Li et al., 1991). An α-helical segment connects the HcN with the HcC. The latter domain adopts a modified β-trefoil fold with a six-stranded β-barrel and a β-hairpin triplet capping the base of the domain. The β-trefoil motif is present in many proteins involved in recognition and binding functions, such as trypsin inhibitors. Interestingly, the whole CNT binding domain tilts away from the rest of the molecule; as a consequence all its surface loops are exposed and available for interaction with the cell surface. The highly conserved sequence of HcN predicts that this lectin-like domain could adopt a similar structure in TeNT and BoNTs. However, the sequence similarity drastically weakens in the HcC domain. Only three residues in the region between strands β19 and β29 of the TeNT Hc structure are conserved throughout the CNT family, but the homology increases again in the residues nearest the carboxy-terminus (Swaminathan and Eswaramoorthy, 2000). Despite the overall low homology, CNT HcCs can still share a common structure due to the presence of 18 hydrophobic buried residues crucial for maintaining the β-trefoil fold. Such a common β-trefoil
Figure 1.4 Crystal structure of the $H_c$ domain of TeNT. The $H_c$ can be structurally divided into two sub-domains. The N-terminal sub-domain ($H_{cN}$, *green*) is a lectin-like domain, formed by two 7-stranded antiparallel $\beta$-sheets sandwiched together in a jelly roll motif. The C-terminal sub-domain ($H_{cC}$, *red*) displays a $\beta$-trefoil motif, which is shared by other proteins involved in recognition and binding.
structure could act as a scaffold for the variable loop regions corresponding to the CNT divergent sequences (Swaminathan and Eswaramoorthy, 2000). At least for TeNT, the HcC appears to be a major determinant for neurospecific binding. Deletion of the last 10 residues from the carboxy-terminus abolishes the surface interaction with spinal cord neurons, whereas removal of the HcN does not appreciably reduce membrane binding (Halpern and Loftus, 1993).

1.1.1.2 Structure of the translocation domain (H\textsubscript{f})

After receptor-mediated internalisation in endocytic vesicles, the catalytic domain of CNTs has to reach the cytosol to modify its target. Several studies have shown that the H\textsubscript{f} domain is responsible for the CNT pore-forming activity (reviewed in Schiavo et al., 2000). The endosomal acidic pH could trigger a conformational change in the amino-terminal portion of the H chain, allowing the formation of a pore in the membrane that would favour the translocation of the catalytic domain into the cytosol. However, the exact mechanism of this process remains one of the less understood aspects of CNT intoxication. The translocation domain consists of two long \(\alpha\)-helical regions, each about 105 Å long forming coiled coils (Lacy et al., 1998) (Figure 1.3 b). Similar helical bundles have been found in other toxins, such as colicin Ia, or the nucleotide exchange factor GrpE and some viral proteins (Schiavo et al., 2000). A search for putative membrane spanning regions in the translocation domain identified two areas predicted to adopt an \(\alpha\)-helical conformation in two CNTs (residues 637-659 for BoNT/B and 659-681 for BoNT/A). This BoNT/A segment was also found to increase permeability of lipid bilayers, making it a candidate pore-forming area (Oblatt-Montal et al., 1995). However, in the crystal structures of both BoNT/A and B these regions are not helical, but assume a strand-like
conformation (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000). This finding suggests that these segments could be very flexible and may therefore be involved in a pH-dependent conformational change, causing the exposure of previously hidden residues. Validation of this hypothesis could be provided by the still awaited comparison of CNT crystal structures at low and neutral pH. Finally, a very peculiar feature present in CNT H₄ is a long loop that wraps around the catalytic domain. Interestingly, this “belt” region covers the active site in BoNT/A, but not in BoNT/B (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000). The shorter length of this particular belt in BoNT/B and E compared to A could be a helpful feature in the design of selective inhibitors for certain BoNT serotypes.

1.1.1.3 Structure of the catalytic domain

The CNT catalytic domain is characterised by the presence of the HEXXH zinc-binding motif of zinc-endopeptidases (Herreros et al., 1999). Accordingly, the CNT proteolytic activity is zinc-dependent and heavy metal chelators generate inactive apo-neurotoxins by removing the bound zinc atom (Schiavo et al., 1992b; Schiavo et al., 1992c; Wright et al., 1992). The eight CNTs cleave three targets of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) family (Jahn and Sudhof, 1999) (Figure 1.5). TeNT and BoNT/B, /D /F and /G cleave vesicle-associated membrane protein (VAMP); BoNT/A and /E cleave synaptosomal-associated membrane protein of 25 kDa (SNAP-25) and BoNT/C cleaves both syntaxin and SNAP-25 (see below, paragraph 1.1.5).

Based on the crystal structures of BoNT/A and BoNT/B, the CNT catalytic domain appears to adopt a globular fold consisting of a mixture of α-helices and β-sheets and strands (Hanson and Stevens, 2000; Lacy et al., 1998; Swaminathan and Eswaramoorthy,
Figure 1.5 Schematic structure of the SNARE proteins cleaved by CNTs. VAMP is a type-II membrane protein anchored to SSVs by a single transmembrane domain (dark grey). VAMP is characterised by a short intraluminal domain, a conserved 60 residue-long cytosolic portion able to form coiled coils and a poorly conserved N terminus. SNAP-25 is bound to the presynaptic membrane by several palmitoylated cysteine residues located in the middle of the molecule. Syntaxin is another type-II membrane protein, mainly found on the neuronal plasma membrane. The N-terminal portion is exposed to the cytosol and is followed by a transmembrane domain and few extracellular residues. The arrows indicate the sites of CNT cleavage. The domains participating in the formation of the SNARE complex are also shown (yellow boxes).
The active site zinc is buried inside a cavity with a very high negative electrostatic potential. As previously suggested by chemical and mutagenesis experiments, the zinc atom in BoNT/A is directly coordinated by His 222 and His 226 present in the HEXXH motif. The Glu 223 coordinates a water molecule as the third ligand, while Glu 261 is the fourth zinc ligand (Hanson and Stevens, 2000). Similarly, for BoNT/B the metal atom is coordinated by His 229, His 233 and Glu 267 (Swaminathan and Eswaramoorthy, 2000). Overall, 17 of the 22 residues within 8 Å of the zinc are identical in BoNT/A and BoNT/B. As the distance increases, more differences start to emerge, suggesting that the substrate specificity between the two toxins may not be due to the residues immediately adjacent to the zinc site, but may be the consequence of a long range effect, near the mouth of the cavity. In BoNT/B three sets of loops in the light chain undergo a conformational change upon separation from the heavy chain or upon VAMP binding. The movement of these loops therefore seems to be crucial for the catalytic activity, and most likely similar conformational changes could also be valid for BoNT/A upon separation of the heavy and light chains (Singh, 2000).

The proposed catalytic mechanism for CNTs is very similar to other metalloendopeptidases of the zincin and metzincin superfamilies (Herreros et al., 1999). However, the structural similarity is only confined to the α-helix containing the HEXXH motif. For this reason, CNTs can be considered a distinct group of metalloproteases with a unique modular structure designed to perform the 4 steps of the intoxication process: a) binding, b) internalisation, c) membrane translocation and d) intracellular cleavage of specific substrates (Schiavo et al., 2000) (Figure 1.6).
Figure 1.6 Schematic representation of the CNT intoxication process. CNTs bind to the neuronal membrane through interaction of their binding domain (B, blue) with one or more receptors. The neurotoxins are then internalised in an endocytic compartment. Acidification of the vesicular lumen favours the translocation of the neurotoxin light chain (A, red) into the cytosol. The active light chain is then able to cleave its target, impairing neurotransmitter release.
1.1.2 Neurospecific binding

After diffusion in the bloodstream, TeNT and BoNTs reach the presynaptic membrane of cholinergic terminals where they bind. TeNT may also bind to sympathetic and adrenergic fibres (reviewed in Rossetto et al., 2001). Numerous studies have examined CNT binding using both morphological and biochemical techniques in different systems including primary neuronal cultures, cell lines and various membrane preparations (reviewed in Halpern and Neale, 1995; Schiavo et al., 2000). These reports have shown that TeNT and BoNT bind to membrane receptors and are subsequently internalised. Morphological studies have visualized the binding of BoNT/A and /B to the unmyelinated area of motor nerve terminals (Black and Dolly, 1986). However, the identification of specific, high-affinity CNT receptors still remains unresolved. Numerous studies have shown that CNT bind to gangliosides found in the neuronal plasma membrane (reviewed in Halpern and Neale, 1995; Montecucco, 1986).

Gangliosides consist of sialic acid-containing oligosaccharides linked to ceramide (Figure 1.7). The prototype of these lipids is GM₁, which has a single sialic acid attached to the internal galactose of the oligosaccharide backbone (Galβ3GalNAcβ4-(NeuAcα3)Galβ4GlcβCer). Polysialogangliosides contain multiple sialic acid residues linked to either of the galactose residues. Accordingly GD₁b is characterised by a double sialic group linked to the internal galactose, whereas GT₁b and GQ₁b also have one or two sialic acid molecules connected to the terminal galactose, respectively.

CNTs show maximal binding to members of the G₁b series (GT₁b, GD₁b, GQ₁b) (Halpern and Neale, 1995). Pretreatment of NMJ preparations with G₁b gangliosides prevents the BoNT-dependent block of neurotransmitter release and decreases TeNT retrograde transport (Stöckel et al., 1977). However, the removal of sialic acid in neuronal
Figure 1.7 Scheme of ganglioside structure.
Gangliosides consist of a carbohydrate backbone linked to ceramide formed by sphingosine and a fatty acid (top panel). One or more molecules of N-acetylneuraminic acid (NANA, or sialic acid) are bound to the galactose (Gal) of the sugar chain. Shown here is GT\textsubscript{1b}, the ganglioside with highest affinity for TeNT. Different gangliosides are characterised by the number and the position of sialic acid (bottom panel).
Glc, glucose; GalNAc, N-acetylgalactosamine.
membranes with neuraminidase decreases, but does not abolish CNT binding (Bigalke et al., 1986). Mouse spinal cord cultures treated with fumonisin B1, an inhibitor of ganglioside synthesis, do not bind TeNT and are not sensitive to the toxin action. Addition of exogenous gangliosides to fumonisin-treated cultures restores the ability of TeNT to bind to neuronal membranes and to block neurotransmitter release, suggesting that gangliosides are a necessary component of the receptor mechanism for TeNT (Williamson et al., 1999). However, several lines of evidence argue against a model proposing polysialogangliosides as the sole CNT receptors. First, many neuronal cells contain gangliosides to which TeNT and BoNTs bind, but the two types of neurotoxins act preferentially on certain neuronal subtypes in vivo (Halpern and Neale, 1995); second, CNT binding is trypsin sensitive and many studies with cultured cells and brain homogenates have shown the involvement of a protein component in toxin binding (Parton et al., 1988; Pierce et al., 1986; Schiavo et al., 1991; Yavin and Nathan, 1986).

Finally, two classes of CNT binding sites with different affinities appear to be present on the surface of CNT-sensitive cells (Montecucco, 1986). Based on these observations, Montecucco proposed a “double receptor” model, in which polysialogangliosides could act as low-affinity receptors. The membrane-bound ganglioside-toxin complex could then move laterally to reach and bind a high-affinity protein receptor. According to this model, polysialogangliosides, which are very abundant on the neuronal surface, would form an effective system to “capture” the toxin and facilitate its interaction with the specific protein receptor, present in low amounts at the synapse (Montecucco, 1986).

The organisation of CNT Hc in a lectin-like domain and a protein-interacting β-trefoil scaffold suggests that CNTs might have multiple interactions with both protein and sugar components on the cell membrane. The recent crystal structures of TeNT Hc with an
analogue of GT\textsubscript{1b} or soaked with different carbohydrates provide some insight into the possible node of ganglioside binding (Emsley et al., 2000; Fotinou et al., 2001) (Figure 1.8). The overall structure of such complexed H\textsubscript{C} is virtually identical to that of apo H\textsubscript{C}, suggesting a "lock and key" type of binding between TeNT and gangliosides, an observation valid also for BoNT/B bound to sialyllactose (Swaminathan and Eswaramoorthy, 2000). Emsley and collaborators determined the structures of TeNT H\textsubscript{C} crystals soaked in solutions of lactose, galactose, N-acetylgalactosamine (NGA), and sialic acid, the building blocks of polysialogangliosides (Emsley et al., 2000). The main conclusion from these studies is that TeNT appears to have multiple carbohydrate-binding sites present in the H\textsubscript{C}C domain. The lactose-binding site is located close to His 1293, which has been identified from photoaffinity labelling as a component of a ganglioside-binding pocket (Shapiro et al., 1997). Another broad surface (Asp 1147-Asn 1226) seems to be able to accommodate sialic acid and NGA at the same time, while a single galactose-binding area is present in a depression on the protein surface created by a loop from residues 1180 to 1196. A GT\textsubscript{1b} synthetic analogue is also able to interact with two distinct sites on TeNT H\textsubscript{C}: the Gal4-GalNAc3 site is a groove formed by Trp 1289, His 1271, and Tyr 1290, whereas the disialic site consists of residues Asp 1147, Arg 1226, Asn 1216, Asp 1214 and Tyr 1229 (Fotinou et al., 2001; Sutton et al., 2001). These structural data suggest that a single ganglioside could bind simultaneously to more than one TeNT molecule, therefore achieving a cross-linking effect on the neuronal surface. This could facilitate the recruitment of the toxin to sites enriched in high affinity protein receptors possibly important for the subsequent internalisation process.

In agreement with the double receptor model, BoNT/B interacts with the intravesicular domain of the synaptic proteins synaptotagmin I and II in the presence of
Figure 1.8 Structural regions involved in TeNT Hc binding to different carbohydrates.
TeNT Hc has multiple carbohydrate-binding sites located in the C-terminal portion. Shown here are the lactose-binding pocket, located close to His 1293 and Tyr 1290, a second large pocket (Asn 1147 - Asn 1226), able to simultaneously interact with both sialic acid and N-acetylgalactosamine (NGA), and a galactose-binding region (Tyr 1180 - Ile 1196). The positions and orientations of the carbohydrates point to the possible simultaneous interaction of TeNT Hc with more ganglioside molecules.
polysialogangliosides (Nishiki et al., 1994; Nishiki et al., 1996a). This result has been extended to BoNT/A and /E (Li and Singh, 1998), suggesting that synaptotagmins might act as receptors for all BoNTs. However, this is in contrast with competition experiments demonstrating that different serotypes have different receptors (Evans et al., 1986; Habermann and Dreyer, 1986). It is possible that BoNTs could interact with distinct synaptotagmin isoforms, but the exact role of these proteins in toxin binding and uptake still needs to be defined.

BoNTs act only at the NMJ, while TeNT has to reach the inhibitory interneurons of the spinal cord to perform its activity (Halpern and Neale, 1995). This different trafficking suggests that these toxins might interact with different receptors. In the case of TeNT, the different toxicity reported at peripheral and central levels indicates that binding to the NMJ and to central synapses in the spinal cord might involve distinct receptors that remain to be discovered.

1.1.3 Endocytosis

After binding to the neuronal membrane, CNTs are internalised in vesicular compartments in a temperature- and energy-dependent process (Dolly et al., 1984; Staub et al., 1986). Currently, we still lack a consistent view of the internalisation pathway followed by CNTs, probably due to the variety of cellular systems used by different investigators. The uptake of TeNT has been examined in spinal cord cultures, where surface-bound TeNT is internalised in vesicular structures (Critchley et al., 1985). In these spinal neurons, gold-labelled TeNT was found associated with coated pits and coated vesicles at early internalisation time points, and later in tubular and vesicular uncoated structures and multivesicular bodies (MVBs) in cell somas and axons (Parton et al., 1987). In contrast,
uncoated structures seemed to mediate TeNT entry in ganglioside-treated BALB/c 3T3 cells, with the toxin-gold complex found at later time points in lysosomes and MVBs (Parton et al., 1988).

Matteoli and collaborators detected colocalisation between TeNT and SSV markers following depolarisation in differentiated hippocampal neurons (Matteoli et al., 1996). Neuroexocytosis is tightly coupled with recycling (Cremona and De Camilli, 1997), and it had been previously shown that nerve stimulation facilitates CNT intoxication (Herreros et al., 1999). Based on these observations, it was proposed that TeNT endocytosis might occur through small synaptic vesicle (SSV) recycling. However, TeNT is inactive at the NMJ at 18°C, even in the presence of high-frequency stimulation, while it is fully active at 25°C (Schmitt et al., 1981). Moreover, TeNT still maintains a full ability to intoxicate NMJ treated with BoNT/A, where neurotransmitter release is completely blocked (Habermann and Erdmann, 1978). These results therefore suggest that the mechanism of TeNT internalisation at the NMJ could differ from that seen at central level.

Hippocampal neurons are also susceptible to intoxication with high doses of BoNTs, and BoNT/B and /F seem to use an endocytic route distinct from SSV recycling (Verderio et al., 1999). However, further work is still needed for the full characterisation of the neuronal endocytic pathways followed by CNTs.

1.1.4 Translocation into the cytosol

Whatever the nature of the compartment containing the internalised CNTs, the light chain has to cross the hydrophobic barrier of the lipid bilayer and reach the cytosol to cleave its target. The different trafficking of TeNT and BoNTs at the NMJ indicates that translocation and internalisation represent distinct steps triggered by different mechanisms.
Compelling evidence shows that TeNT and BoNTs have to be exposed to a low pH step for nerve intoxication to occur (Adler et al., 1994; Matteoli et al., 1996; Simpson, 1982; Simpson, 1983; Simpson et al., 1994; Williamson and Neale, 1994). Since a non-acid-treated light chain still blocks exocytosis when applied intracellularly, it is plausible to think that exposure to low pH is necessary for membrane translocation, as previously demonstrated for other bacterial protein toxins (Montecucco et al., 1994). Low pH causes a structural change in CNTs from a water-soluble, "neutral" to an "acid" conformation, characterised by the exposure of hydrophobic regions. This process enables the penetration of both heavy and light chains into the hydrophobic core of the lipid bilayer (Boquet and Duflot, 1982; Boquet et al., 1984; Cabiaux et al., 1985; Menestrina et al., 1989; Montecucco et al., 1989; Schiavo et al., 1990a). In planar lipid bilayers CNTs form high-conductance ion channels (Beise et al., 1994), formed by oligomerisation of the Hₙ domain. Interestingly, a short amphipathic region flanking the Hₙ long α-helices is able to form channels with properties similar to the channels obtained with intact neurotoxin. It was therefore suggested that the channel may be formed by a toxin tetramer that brings four of these amphipathic segments together with their hydrophilic residues oriented towards the lumen of the pore (Montal et al., 1992; Oblatt-Montal et al., 1995). This model is consistent with the three-dimensional reconstruction of the channel formed by BoNT/B in phospholipid bilayers (Schmid et al., 1993). However, the precise mechanism of the translocation step and the exact nature of the channel are still debated. A possible model envisages the passage of the light chain through a channel open laterally to lipids, more than through a completely proteinaceous pore (Montecucco et al., 1994). The heavy and light chains are supposed to change conformation at low pH in a concerted fashion, with the exposure of their hydrophobic
surfaces allowing contact with the hydrophobic core of the lipid bilayer. The heavy chain would form a transmembrane hydrophilic cleft enabling the passage of a partially unfolded light chain, with its hydrophobic residues facing the membrane lipids. The neutral cytosolic pH could then facilitate light chain refolding, involving the possible participation of chaperones and the reacquisition of the zinc ion previously released by the histidines protonated at low pH. The process also includes the reduction of the disulfide bond bridging the H and L chain, a necessary step to cause neurotoxicity (Schiavo et al., 1990b). At present, it is still unclear when and where the reduction of the interchain disulfide bond occurs. After the successful translocation of the L chain in the cytosol, the hydrophilic cleft of the H chain would tighten up to reduce the interaction with the hydrophobic core of the vesicle membrane. This process is thought to leave a channel in the membrane consisting of two rigid protein walls and a flexible lipid region. This particular "pore" would be responsible for the ion-conducting properties of CNTs and would be a consequence of the translocation process, more than its prerequisite.

1.1.5 Intracellular proteolytic activity: SNARE cleavage

The nature of CNT catalytic activity was discovered following the observation they all share the zinc-binding motif of zinc-endopeptidases (HEXXH) (Kurazono et al., 1992; Schiavo et al., 1992b; Schiavo et al., 1992c; Wright et al., 1992). Only three CNT targets, belonging to the SNARE family (Bock and Scheller, 1997; Söllner et al., 1993) have been identified. TeNT and BoNT/B, /D, /F and /G cleave VAMP at different sites (Schiavo et al., 1992a; Schiavo et al., 1994; Schiavo et al., 1993a; Schiavo et al., 1993c; Yamasaki et al., 1994a; Yamasaki et al., 1994b; Yamasaki et al., 1994c). Interestingly, TeNT and BoNT/B act on the same VAMP site (Gln76-Phe77) and yet they cause spastic and flaccid
paralysis, respectively (Schiavo et al., 1992a), conclusively demonstrating that the
different clinical symptoms of tetanus and botulism are due to distinct sites of intoxication
rather than a different mechanism of action.

BoNT/A and /E cut two distinct peptide bonds within SNAP-25, whereas BoNT/C cleaves
both syntaxin and SNAP-25 (Binz et al., 1994; Blasi et al., 1993; Foran et al., 1996; Osen
Sand et al., 1996; Schiavo et al., 1993a; Schiavo et al., 1993b; Schiavo et al., 1995;
Vaidyanathan et al., 1999; Williamson et al., 1996).

SNARE proteins have been implicated as crucial components in most, if not all,
intracellular membrane trafficking events studied so far (reviewed in Chen and Scheller,
2001; Jahn and Sudhof, 1999). In eukaryotic cells, molecules need to be delivered to their
correct intracellular destinations without compromising the structural integrity of cellular
compartments. To achieve this, transport vesicles bud from a donor organelle and then
dock and fuse with a target membrane. SNAREs were originally divided into v-SNAREs
and t-SNAREs based on their vesicle or target membrane localisation (Bock and Scheller,
1997; Sollner et al., 1993). According to the “SNARE hypothesis” (Rothman, 1994), each
type of vesicle has a unique v-SNARE that pairs up with distinct cognate t-SNAREs at the
appropriate target membrane, enabling membrane fusion. More than a hundred SNARE
proteins from diverse organisms have been discovered. Sequence analyses revealed that
all SNAREs share a homologous domain of ~60 aminoacids that is referred to as the
SNARE motif (Jahn and Sudhof, 1999), important for the association into core complexes
involved in membrane fusion (see below). SNAREs are currently classified into Q- and R-
SNAREs, depending on the presence of either a glutamine or an arginine in a central
position of the SNARE motif (Jahn and Sudhof, 1999).
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The known mammalian SNAREs can be distinguished as members of the syntaxin, VAMP or SNAP-25 families. Both syntaxin and VAMP are anchored to the membrane by a carboxy-terminal transmembrane domain, whereas SNAP-25 is peripherally attached to the membrane by palmitoylation of four cysteine residues in the central region of the protein (Jahn and Sudhof, 1999) (Figure 1.5). Biochemical studies have shown that the soluble coiled-coil forming domains of recombinant syntaxin, SNAP-25 and VAMP form an extremely stable complex (Figure 1.5) (Fasshauer et al., 1998; Poirier et al., 1998a). The crystal structure of the SNARE complex shows that one coil of syntaxin and VAMP, and two coils of SNAP-25 intertwine to form a four-stranded coiled-coil structure (Sutton et al., 1998), which induces the juxtaposition of a vesicle to the target membrane and is involved in their fusion (Figure 1.9). Weber et al. demonstrated that the assembly of such “SNAREpins” between artificial lipid bilayers triggers spontaneous fusion (Weber et al., 1998). As would be expected from a very stable complex, ATP is needed to dissociate it into monomeric components. Disassembly is carried out by two types of proteins, the ATPase NSF and members of the SNAP adaptor protein family, which were initially discovered as essential factors in a Golgi transport assay (Clary et al., 1990; Malhotra et al., 1988).

Recent work performed with yeast and mammalian SNAREs anchored to liposomes has reinforced the concept that the specificity of membrane fusion events relies on a precise pattern of v-SNARE-t-SNARE interactions (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000). Most of the SNARE proteins are found in specific cellular compartments (Jahn and Sudhof, 1999; Scales et al., 2000), suggesting that they have selective functional involvement in distinct intracellular trafficking steps. Although the specificity of intracellular membrane fusion is associated with the physico-chemical
Figure 1.9 Crystal structure of the SNARE complex.
Model of the SNARE complex involved in the fusion of SSV with the presynaptic plasma membrane. The coiled-coil segment in the cytosolic portion of the SSV protein VAMP (blue) interacts with one coil of syntaxin (red) and two coils of SNAP-25 (green) to form a four-helical bundle complex. The loop between the two coiled domains of SNAP-25 is shown as an unstructured polypeptide chain. Formation of this SNARE complex plays a crucial role in joining the SSV and presynaptic membranes, a process impaired by CNT-mediated cleavage (black arrows).
properties of SNAREs, a finer spatial and temporal control of SNARE-mediated fusion is provided by additional regulatory systems, such as Rab GTPases (Zerial and McBride, 2001) (see also below, paragraph 1.2.4) and other proteins helping vesicle tethering (Pelham, 2001).

The molecular basis of CNT specificity for their substrates is only partially known. Sequence comparison of the different SNAREs has revealed the presence of a nine-residue-long motif, consisting of three carboxylate residues alternating with hydrophobic and hydrophilic residues (Rossetto et al., 1994). This motif is present in two copies in VAMP and syntaxin and in four copies in SNAP-25 and seems necessary for the specific binding of CNTs, since only protein segments including at least one motif at the correct distance from the target site are cleaved by neurotoxins.

1.1.6 The CNT substrates

1.1.6.1 SNAP-25

The t-SNARE SNAP-25 is a major palmitoylated protein in the CNS. It is localised in the membrane through the palmitoylation of cysteine residues present in the middle of the polypeptide chain (Figure 1.5) (Hess et al., 1992; Veit et al., 1996). It is highly conserved from yeast to humans, and forms the core of the neuroexocytotic apparatus with the other t-SNARE syntaxin and with the v-SNARE VAMP (Söllner et al., 1993). Furthermore, it forms a stoichiometric complex with the Ca\(^{2+}\) sensor synaptotagmin present on SSVs, and this interaction is thought to be crucial for the Ca\(^{2+}\)-dependent phase of neurotransmitter release (Banerjee et al., 1996; Schiavo et al., 1997). SNAP-25 has also been demonstrated to interact in a Ca\(^{2+}\)-dependent manner with Hrs-2, an ATPase having a regulatory effect on neuroexocytosis (Bean et al., 1997). SNAP-25 is necessary for axonal growth during
neuronal development and nerve terminal plasticity in the mature nervous system (Geddes et al., 1990; Osen Sand et al., 1993). The SNAP-25 A splice variant is mainly present in the embryo, whereas SNAP-25 B starts to be expressed at birth in the nervous and endocrine system (Bark and Wilson, 1994). Another isoform, SNAP-23, is expressed only outside the nervous system (Mollinedo and Lazo, 1997; Ravichandran et al., 1996). Interestingly, SNAP-23 can substitute SNAP-25 in some regulated secretory pathways, but it is insensitive to cleavage by BoNT/E (Sadoul et al., 1997). Finally, SNAP-29, a longer isoform of SNAP-25 with a conserved BoNT/E cleavage site has been found in multiple membrane compartments (Steegmaier et al., 1998).

1.1.6.2 Syntaxin

The syntaxin family includes more than 20 syntaxin isoforms in mammals, and many homologs in yeast, insects and plants (Bennett et al., 1993; Bock and Scheller, 1997). Syntaxin is a type II membrane protein localised mainly on the neuronal surface (Figure 1.5). This protein has the amino-terminal portion exposed to the cytosol followed by a transmembrane region and only a few extracellular residues. The amino-terminal domain consisting of three long \( \alpha \)-helices is likely to be involved in protein-protein interaction (Fernandez et al., 1998), whereas the central region participates in the formation of the SNARE complex (Poirier et al., 1998b; Sutton et al., 1998). This last process is controlled by the interaction of syntaxin with Munc-18 and other accessory proteins and it is regulated by protein kinase C (Fujita et al., 1996; Hirling and Scheller, 1996; Südhof, 1995).

Syntaxin is also found in association with several types of \( \text{Ca}^{2+} \) channels at active zones (Atlas, 2001). It interacts with some synaptotagmin isoforms in a \( \text{Ca}^{2+} \)-dependent fashion.
(Südhof, 1995), and undergoes with SNAP-25 a recycling process in organelles undistinguishable from SSVs (Walch-Solimena et al., 1995). Syntaxins are important for neuronal development but also for survival, since BoNT/C, unlike the other CNTs, has degenerative effects on neurons (Kurokawa et al., 1987; Osen Sand et al., 1996; Williamson and Neale, 1998). Finally, syntaxins seem to be implicated in synaptic plasticity, since they are differently expressed during long-term potentiation, probably regulating Ca\(^{2+}\) entry via selective interaction with specific Ca\(^{2+}\) channels (Herreros et al., 1999).

1.1.6.3 VAMP

VAMP, also known as synaptobrevin, is the v-SNARE prototype and is found in SSVs, dense core granules and synaptic-like microvesicles (Südhof, 1995). Ten VAMP isoforms have been identified so far (Advani et al., 1998; Bock and Scheller, 1997; Galli et al., 1998; Wong et al., 1998; Zeng et al., 1998) and are present in all vertebrate tissues, although their abundance and distribution differ (Rossetto et al., 1996; Trimble, 1993). VAMP can be divided into four different domains (Figure 1.5). The cytosolic portion contains a proline-rich and isoform-specific amino-terminal segment and a conserved coiled-coil region participating in SNARE complex formation. This portion also contains sites of phosphorylation for calmodulin-dependent protein kinase and casein kinase (Nielander et al., 1995). A single transmembrane domain anchors VAMP to the SSV and is followed by a poorly conserved intravesicular tail of variable length in different species. On the SSV, the availability of VAMP for the formation of the SNARE complex is regulated by its association with synaptophysin, a major component of the SSV
membrane, and with subunits of the V-ATPase (Calakos and Scheller, 1994; Edelmann et al., 1995; Galli et al., 1996; Washbourne et al., 1995).

So far, three VAMP isoforms have been extensively studied: VAMP-1, VAMP-2 and the non-neuronal cellubrevin (Baumert et al., 1989; McMahon et al., 1993; Trimble et al., 1988). Each of these isoforms shows a distinct pattern of interactions with specific factors, as shown for example by the isolation of BAP31, a sorting protein that controls the trafficking of VAMP-1 and cellubrevin, but not of VAMP-2 (Annaert et al., 1997). The latter isoform appears to have a role in SSV biogenesis, as shown by the study of its interaction with the adaptor complex AP3. In fact, CNT-mediated cleavage of VAMP-2 from endosomes blocks SSV formation in vitro (Salem et al., 1998).

1.1.7 Therapeutic uses of CNTs

1.1.7.1 BoNTs

The large amounts of data collected on BoNTs action at the synapse and the improved techniques of biochemical purification of these neurotoxins have laid the foundations for the clinical use of BoNTs as therapeutic agents in pathologies characterised by hyperfunction of cholinergic terminals (Herreros et al., 1999). The first pioneering therapeutic administration of BoNTs were performed in monkeys to correct experimental strabismus (Scott, 1989). Following this landmark work, a large number of studies have clearly indicated BoNTs as safe therapeutic agents in a variety of human pathologies (Rossetto et al., 2001). Injections of low doses of BoNTs into the hyperactive muscle lead to suppression of the symptoms lasting for a few months. BoNT is currently the best available treatment for certain types of strabismus and blepharospasm. Other pathological conditions that benefit from BoNTs administration include dystonias, hemifacial spasm,
achalasia and torticollis. Furthermore, BoNTs have recently been established as successful cosmetic agents to reduce facial wrinkles (Herreros et al., 1999).

The most widely used serotype is BoNT/A, but other types are also under clinical trials (Eleopra et al., 1998). There are very few side effects linked to BoNT/A administration, one of the most common resulting in immunisation induced by high-dose and long-term treatments. In this case, other BoNT serotypes and BoNT/C appear to be the best choice, since BoNT/B, E and F induce only short-term beneficial effects (Eleopra et al., 1997). Finally, other possible applications of BoNTs include those cases where only a very short treatment is required, such as the reduction of muscle tension in complex bone fractures (Rossetto et al., 2001). The choice of the BoNT serotype could therefore be adapted to the pathological condition and the desired duration of effect.

1.1.7.2 TeNT

TeNT is used in animal models to induce experimental epilepsy and neuronal degeneration (Herreros et al., 1999), but is not currently used for therapeutic purposes. However, the ability of the non-toxic TeNT \( H_c \) to undergo retrograde axonal and trans-synaptic transport has stimulated some interesting research on the use of this toxin fragment for the delivery of biological agents to the nervous system. A TeNT \( H_c \)-enzyme conjugate was able to repair a lysosomal enzyme defect in cultured neuronal cells. Lysosomal storage diseases causing neurodegeneration can arise from genetic defect in lysosomal enzymes, such as \( \beta-N \)-acetylgalactosaminidase A (Hex A). When added to the culture medium, HexA chemically conjugated with TeNT \( H_c \) could replace this enzyme in cerebral cortical neurons derived from a feline model of human GM2 gangliosidosis (Tay-Sachs and Sadhoff diseases), abolishing the abnormal accumulation of GM2 in lysosomes.
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(Dobrenis et al., 1992). Other studies (Figueiredo et al., 1997; Francis et al., 1995) have reported the targeted delivery of the free radical scavenging enzyme superoxide dismutase (SOD) to neuronal cells using a SOD-TeNT Hc hybrid protein. This application could be exploited for the treatment of a variety of neurological disorders associated with free radical toxicity (e.g. stroke, trauma). In another interesting work, Coen et al. investigated the potential use of TeNT as an in vivo neurotropic carrier by showing that a hybrid lacZ-TeNT Hc fusion protein injected intramuscularly in mice could undergo retrograde transsynaptic transport and, at the same time, retain the β-galactosidase enzymatic activity (Coen et al., 1997). Although further experimental work is needed, these results suggest that TeNT Hc could be a useful tool for the specific targeting of therapeutic or diagnostic molecules in neuronal cells.
1.2 Endocytic pathways

1.2.1 Clathrin-mediated endocytosis

Eukaryotic cells use endocytosis for a variety of purposes, including nutrient uptake, SSV recycling, remodeling of the plasma membrane, regulation of cell-surface expression of signalling receptors, and the establishment of cellular polarity. It is therefore not surprising that cells employ differently regulated endocytic pathways, according to the cargoes and their final destinations (Figure 1.10). Clathrin-mediated endocytosis is so far the best-characterised endocytic process (Marsh and McMahon, 1999). However, recent work has highlighted the existence of other types of endocytic pathways, such as caveolar uptake, various clathrin-independent mechanisms and macropinocytosis, which still remain largely unknown (Nichols and Lippincott-Schwartz, 2001).

Clathrin-mediated endocytosis is used to internalise a variety of ligands, such as growth-factors, antigens and recycling receptors and is involved in two crucial transport steps: endocytosis from the plasma membrane to early endosomes and transport from the trans-Golgi network (TGN) to endosomes. In addition, it can also function in budding from endosomes, immature secretory granules and other sites in the cell (Kirchhausen, 2000; Mellman, 1996).

In neurons the best-studied example of clathrin-mediated endocytosis is recycling of SSVs after exocytosis (Murthy and Stevens, 1998; Slepnev and De Camilli, 2000). The process is characterised by the progressive assembly of a clathrin scaffold which provides a mechanical means to deform the membrane into a “coated pit” (Marsh and McMahon, 1999) (Figure 1.11). A similar mechanism is also used for transferrin internalisation which is confined to the somatodendritic domain (Mundigl et al., 1993; Parton et al., 1992). In this first phase, cargoes and cargo receptors are concentrated by a still poorly understood...
Figure 1.10 Schematic representation of structures involved in endocytic pathways.
The "classical" coated pathway (1) requires the participation of the clathrin coat and the dynamin-dependent fission of the budding endocytic vesicle. However, endocytosis can occur also through caveolae, uncoated flask-shaped invaginations characterised by the presence of caveolins (2). These endocytic structures may also require dynamin for the fission step. Clathrin- and caveolae-independent pathways have also started to emerge (3). They may possibly involve the participation of lipid rafts, but the molecular components involved are still unknown. Macropinocytosis (4) consists of fluid internalisation in large endocytic vesicles formed after the closure of lamellipodia. The process involves the participation of the actin cytoskeleton and PI3 kinase. (Adapted from Sandvig, EMBO J, 2000).
mechanism into the budding vesicle, which then matures and eventually pinches off from the plasma membrane. A subsequent energy-dependent uncoating step follows, giving rise to a vesicle ready to continue its trafficking in the cytoplasm or, in the case of a SSV, to be refilled with neurotransmitter and re-enter the exocytic cycle (Brodin et al., 2000).

The unit elements of the clathrin lattice are three-legged triskelia, comprising three heavy and three light chains that form stable oligomeric complexes (Figure 1.12). The clathrin coat is assembled on the cytoplasmic face of the plasma membrane by the recruitment of the adaptor complex AP-2, a heterotetramer consisted of two large subunits, α and β2 (each of ~ 100 kDa), and two smaller chains, μ2 (50 kDa) and σ2 (20 kDa). The AP-2 complex not only links clathrin to the membrane, but also coordinates coat assembly with the selection of protein or lipid cargoes, concentrating them in the emerging bud (Kirchhausen, 2000). Indeed, the AP-2 core region binds both to membrane proteins, such as synaptotagmin and proteins containing endocytic motifs, and to membrane lipids, such as phosphoinositides (PIs) (Bonifacino and Dell'Angelica, 1999; Gaidarov and Keen, 1999; Slepnev and De Camilli, 2000; von Poser et al., 2000). The binding of AP-2 to clathrin is instead mediated primarily by the β2 subunit at the level of the “ear” domain and in the hinge region connecting the ear to the trunk domain (Robinson and Bonifacino, 2001; Shih et al., 1995). The coat also contains an additional protein named AP180 in neurons and CALM in other cells which is able to interact with clathrin, the AP-2 ear domain and membrane PIs, thereby assisting coat assembly and possibly controlling vesicle size (Ford et al., 2001; Tebar et al., 1999; Zhang et al., 1998). A plethora of accessory factors assisting the formation of clathrin-coated vesicles have been identified (Slepnev and De Camilli, 2000; Takei and Haucke, 2001) (Figure 1.12). These factors help coordinate coat formation with biochemical changes in the lipid bilayer and local
Figure 1.11 Schematic representation of clathrin-dependent endocytosis of SSV.
The AP-2 adaptor protein acts as a bridge between membrane proteins and lipids and
the clathrin coat, which enables the formation of a "coated pit" enriched in lipid or
protein cargoes. The process can be assisted by AP180. The actin cytoskeleton and a
vast array of accessory factors is involved in the maturation of the emerging bud, which
undergoes a dynamin-dependent fission step. The final uncoating process requires
synaptojanin-dependent PtdIns(4,5)P$_2$ (PIP$_2$) hydrolysis and other accessory factors
such as hsc70 and auxilin.
(Adapted from Takei, Trends Cell Biol, 2001).
Figure 1.12 Components of the endocytic machinery.
Schematic representation of coat components, membrane factors and accessory proteins involved in clathrin-mediated endocytosis. Described on the right are their putative functions.

C2, protein kinase C homology 2 domain; DPW, Asp-Pro-Trp; SH3, Src-homology 3; EH, eps15 homology domain; ENTH, epsin amino-terminal homology; GED, GTPase-enhancing domain; J, DnaJ domain; LPA-Atase, lysophosphatidic acid acyl transferase; NPF, Asn-Pro-Phe; PH, pleckstrin homology domain; PRD, proline-rich domain; Sac1, suppressor of actin 1; SBD, substrate-binding domain.

(Adapted from Takei, Trends Cell Biol, 2001).
modification of the actin cytoskeleton and contribute to integrate intracellular signalling pathways with endocytosis. During maturation of the emerging vesicle, membrane curvature increases to form a deeply invaginated pit that finally detaches. The fission step requires the action of the GTPase dynamin (Marks et al., 2001). Although its mechanism of action is still not completely understood, dynamin appears to interact with its binding partners amphiphysin and endophilin to form oligomeric rings around the stalks of coated pits (Hinshaw, 2000; Hinshaw and Schmid, 1995). This finding has also been supported by the use of \textit{in vitro} assays where the ability of these different proteins to tubulate liposomes can be tested (Farsad et al., 2001; Takei et al., 1998; Takei et al., 1999). The emerging view considers dynamin as both a classical GTPase and a mechanochemical enzyme. The GTPase activity of dynamin, whether or not necessary for fission (Sever et al., 1999), is important for its function and is regulated by its binding partners, its own state of oligomerisation and by PIs (Slepnev and De Camilli, 2000). In this regard, phosphatidylinositol-4, 5-biphosphate (PtdIns(4, 5)P$_2$) is certainly one of the major players in promoting clathrin coat assembly, regulating dynamin function and, at the same time, also nucleating actin filaments (Osborne et al., 2001; Rohatgi et al., 1999; Takei and Haucke, 2001). Mice deficient in synaptojanin, a PI phosphatase able to dephosphorylate PtdIns(4, 5)P$_2$ to phosphatidylinositol (PtdIns), accumulate clathrin-coated vesicles and PtdIns(4, 5)P$_2$ at the synapse, suggesting that hydrolysis of this PI could be involved in the uncoating step (Cremona et al., 1999). By interacting with the three main factors involved in fission (dynamin, endophilin and amphiphysin), synaptojanin seems therefore to couple vesicle budding to the uncoating reaction (Slepnev and De Camilli, 2000). This last step is also assisted and regulated by other accessory proteins, such as hsc70, a member of the DnaK family heat-shock proteins. The relatively low ATPase activity of this molecular
chaperone can be substantially enhanced by auxilin, another accessory factor, highly abundant at nerve terminals that helps in the targeting of hsc70 to the coat (Slepnev and De Camilli, 2000; Ungewickell et al., 1995). However, other components of the “uncoating” machinery await further characterisation. One of these is the recently discovered human homolog of Drosophila stoned B (hStnB/stonin 2), which also seems to be involved in other endocytic steps, such as cargo selection and modulation of coat assembly (Martina et al., 2001).

1.2.2 Macropinocytosis
An increasing amount of work in different cell types has highlighted the presence of other clathrin-independent endocytic routes (Figure 1.10). Historically, phagocytosis and macropinocytosis are the oldest known uncoated pathways. Phagocytosis is primarily performed by professional phagocytes like neutrophils, macrophages and dendritic cells, and generally leads to the uptake of particles (e.g. microbes) with a diameter longer than 0.5 μm, whereas macropinocytosis is generally associated with ruffling membranes and not often found in unstimulated, resting cells (Cardelli, 2001). This clathrin-independent process results in fluid internalisation through large, irregular endocytic vesicles more than 1 μm in diameter, formed by the closure of lamellipodia. The molecular mechanism regulating macropinocytosis and also the subsequent recycling of the macropinosome membrane components are still under investigation. Studies in the amoeba Dictyostelium have demonstrated a requirement of phosphatidylinositol 3-phosphate kinase (PI3K) and actin polymerisation for macropinocytosis to occur (Cardelli, 2001), whereas recent work in HeLa cells has demonstrated the involvement of the GTPase ADP-ribosylation factor (Arf) 6, in the control of macropinosome dynamics (Brown et al., 2001). By activating
phosphatidylinositol 4-phosphate 5-kinase (PIP\(4, 5)\)K, Arf 6 seems to stimulate local production of PtdIns(4, 5)\(\text{P}_2\), pointing to actin-based cytoskeleton components as possible downstream effectors in the formation of macropinosomes (Honda et al., 1999). Whether a regulated recycling of micropinosome components could then play a role in generating heterogeneity in the plasma membrane during processes such as cell activation and motility still remains to be fully understood.

1.2.3 Endocytosis through caveolae and lipid “rafts”

Caveolae, the “little caves” first identified in epithelial cells, have recently emerged as the site of important dynamic regulatory events at the plasma membrane (Figure 1.10). These flask-shaped, non-coated plasma membrane invaginations appear to serve different functions, such as transcytosis, potocytosis (large-scale uptake of fluids and small molecules), polarised trafficking of proteins, cholesterol transport, and regulation of signal transduction (reviewed in Anderson et al., 1992; Kurzchalia and Parton, 1999). Biochemically, caveolae are cholesterol- and sphingolipid-enriched microdomains created and maintained by caveolins, members of a family of cholesterol-binding proteins. Caveolins are characterised by a hairpin-like topology, with an intermembrane loop and an amino-terminal cytoplasmic domain involved in interactions with a number of signalling proteins (Figure 1.13 c) (reviewed in Okamoto et al., 1998). There are three members of the caveolin family; caveolin-1 and -2 are present in a variety of cell types and are abundant especially in endothelial cells, whereas caveolin-3 has been detected only in skeletal and cardiac muscle and astrocytes (Ikonen and Parton, 2000; Nishiyama et al., 1999). Caveolin was thought to be absent from the nervous system, but recent reports have shown the presence of caveolin-1 and -2 in neurons (Galbiati et al., 1998), and have
associated them with the formation of protein complexes during induction of synaptic potentiation (Braun and Madison, 2000). However, a full understanding of caveolin function in neurons awaits further investigation. The discovery that caveolae contain one key element of the machinery participating in vesicle biogenesis, the GTPase dynamin, suggests that they can be involved in membrane internalisation (Henley et al., 1998; Oh et al., 1998). Dynamin and GTP can cause fission of caveolae from plasma membrane in vitro (Oh et al., 1998), but the extent of caveolar uptake in non-endothelial cells still has to be determined. Interestingly, a recent study based on the analysis of Simian Virus 40 trafficking in fibroblast-like cells has suggested the existence of a caveolae-dependent endocytic pathway employing a compartment, the “caveosome”, distinct from the classical transferrin-labelled endosomes (Pelkmans et al., 2001). The existence of similar uncoated pathways representing an alternative to the classical clathrin-mediated endocytosis in different cell types remains an active field of research.

Caveolae represent a particularly stable subset of the so-called “lipid rafts”, dynamic assemblies of cholesterol and sphingolipids in the external leaflet of the membrane bilayer (Simons and Ikonen, 1997). The principle governing the formation of these membrane “microdomains” is based on the preferential interaction of the cholesterol steroid ring with the fully saturated acyl chains of sphingolipids rather than with the “kinked” chains of unsaturated glycerophospholipids (Figure 1.13 b), resulting in the generation of distinct liquid-ordered phases in the lipid bilayer dispersed in a liquid-disordered matrix of glycerolipids. The initial raft hypothesis was based on the discovery in polarised epithelial cells that in the biosynthetic pathway glycosphingolipids formed clusters within the exoplasmic leaflet of the Golgi membrane (Simons and van Meer, 1988). These microdomains, resistant to non-ionic detergent solubilisation, were considered to represent
Figure 1.13 Schematic representation of lipid "rafts" and caveolae in the plasma membrane.

Rafts (pink-shaded regions) segregate from the other regions of the lipid bilayer (blue) due to their enrichment in cholesterol (b, grey), sphingomyelin and glycosphingolipids (b, red) in the exoplasmic leaflet. Glycerolipids like phosphatidylserine and phosphatidylethanolamine (b, green) are found in the cytoplasmic leaflet. Proteins can associate to rafts through a GPI anchor (a, red) or through their transmembrane domain, like the influenza virus haemagglutinin (HA) (a, yellow). Caveolae represent a particularly stable example of lipid rafts, formed by self-associating caveolin molecules (c, grey) with a hairpin-like conformation.
(Adapted from Simons, Nature, 1997)
sorting centres for proteins destined for their polarised delivery to the plasma membrane. Because of their high lipid content, these detergent-insoluble, glycolipid-enriched complexes (DIGs) partition to low-density during gradient centrifugation (Simons and Toomre, 2000). Such isolation of DIGs has now become a common tool to determine whether membrane proteins of interest are included or excluded from the lipid microdomains. High “raft affinity” is generally due to the presence of lipid anchors or hydrophobic modifications that can promote the interaction with the lipid saturated environment (Figure 1.13 a). Proteins particularly enriched in lipid rafts include glycosylphosphatidylinositol (GPI)-anchored proteins, doubly acylated proteins such as Src-family kinases, and cholesterol-linked and palmitoylated proteins, such as Hedgehog (reviewed in Ikonen, 2001; Simons and Toomre, 2000). Experimental evidence has involved rafts in the delivery of proteins to the plasma membrane of different cell types (reviewed in Ikonen, 2001), including hippocampal neurons (Ledesma et al., 1999). In addition, rafts are also found in early endocytic organelles (Ikonen, 2001; Mukherjee et al., 1998), and it is becoming increasingly clear that these microdomains play an important role not only in the early phase of endocytosis, but also in the following sorting steps. Some clues have emerged from studies on the trafficking of GPI-anchored proteins, which are endocytosed independently of clathrin and cycle from the plasma membrane to the Golgi through a pathway that would normally serve to regulate lipid raft distribution within cells (Nichols et al., 2001). Moreover, association with cholesterol-rich microdomains appears to be responsible for the endocytic retention of GPI-anchored proteins in recycling compartment (Chatterjee et al., 2001).

Lipid rafts also seem to perform an important role on the plasma membrane, acting as concentration platforms for individual receptors activated by clustering due to ligand
binding or, artificially, by antibody-mediated crosslinking (Figure 1.14). Lateral cross-linking of raft components has been shown to create stabilised raft patches that may represent functional signalling centres (Harder et al., 1998). Binding to rafts could help recruit signalling components to a new micro-environment, where for example phosphorylation states can be modified, resulting in downstream signalling. Several examples of signalling processes involving rafts have been reported, and these include the immunoglobulin (Ig) E-triggered cascade during the allergic immune response, and, in neurons, glial cell-line derived neurotrophic factor (GDNF) signalling (reviewed in Simons and Toomre, 2000) (Figure 1.15). Binding of GDNF to the GPI-anchored GFRα1 coreceptor localised in rafts can recruit the receptor tyrosine kinase c-Ret to the lipid microdomain, thus activating a signalling pathway involving FRS2 and Src-family kinases (Tansey et al., 2000).

Interestingly, a number of pathogens and toxins seem to exploit rafts for internalisation in a variety of different cells (Fivaz et al., 1999). One of the best-studied examples is cholera toxin. This toxin binds through its B subunit to the ganglioside GM1, found both in lipid rafts and clathrin-coated pits (Lencer et al., 1999; Tran et al., 1987). Notably, constitutive clathrin-independent endocytosis of cholera toxin B subunit occurs in cells lacking caveolin and there is evidence that only non-clathrin pathways lead to the Golgi apparatus, a mandatory target for cholera toxin activity (Orlandi and Fishman, 1998). The emerging picture suggests the existence of multiple endocytic pathways, clathrin-, caveolae-, or simply raft-dependent that act both at the plasma membrane and at subsequent sorting steps, but the levels of overlap among these routes still have to be fully investigated.
Raft regions are present in the plasma membrane (pink) and in caveolae, invaginations stabilised by caveolins (black spikes). Rafts outside caveolae are dynamic and smaller than caveolar regions. Raft markers (white) preferentially associate with membrane lipid microdomains within and outside caveolae (top). Antibody- or ligand-mediated clustering leads to the formation of raft patches enriched in raft components. The raft patches can fuse to form a stabilised large raft (bottom left). Patched components can also move to caveolae (bottom right).

(Adapted from Harder, Curr Opin Cell Biol, 1997)
Figure 1.15 GDNF signalling through lipid rafts.
GDNF (purple) binding to the GPI-anchored GDNF receptor (GFRα1, blue) leads to recruitment of the transmembrane signalling receptor tyrosine kinase c-Ret (orange) from non-lipid raft compartments to lipid microdomains. This leads to activation of the Src-family of kinases (red) and FRS2 (yellow). Prevention of c-Ret recruitment to rafts using transmembrane-anchored GFRα1 substantially decreases GDNF-stimulated intracellular effects.
(Adapted from Saarma, Trends Neurosci, 2001).
1.2.4 Rab proteins: coordinators of intracellular trafficking

The endocytic system of higher eukaryotic cells relies on a very complex and dynamic network of organelles each characterised by specific membrane domains and protein components. Typically, endocytosed molecules enter early endosomes (EEs), which are important sorting stations. Internalised proteins can be directed to a recycling endosome (RE) to return back to the plasma membrane (e.g. transferrin receptor), whereas those destined to degradation are routed to MVBs, late endosomes (LEs), and finally to lysosomes (reviewed in Gruenberg, 2001). The boundaries of all these compartments are often quite elusive, due to dynamic interactions, exchange of contents and the distribution of regulatory components throughout several types of endocytic organelles. In order to maintain organelle identity and ensure the correct trafficking of cargoes, the cell preserves the specificity of membrane fusion between compartments through topological pairing of cognate SNAREs (Parlati et al., 2000). However, SNAREs inevitably transit throughout different compartments during trafficking. An additional level of spatial and temporal regulation of the fusion event is therefore required. Rab GTPases together with their effectors serve as a regulatory system in the endocytic pathway not only by coordinating SNARE-dependent membrane fusion, but also by determining compartmental organisation of the organelles with their presence in distinct functional domains (reviewed in Zerial and McBride, 2001). Rab GTPases act as molecular switches, oscillating between a GTP- and a GDP-bound state. In the activated GTP state, Rab proteins can bind to a plethora of different cytoplasmic effectors, whose activities vary according to the type of organelle and the transport step involved. Certain combinations of Rab proteins and effectors seem to be compartment-specific, and this finding has helped to establish them as markers of defined endocytic routes (Figure 1.16). For example, Rab 5 regulates clathrin-mediated
transport from the plasma membrane to the EE, as well as homotypic EE fusion (Bucci et al., 1992). The Rab 5 effector EEA1 acts as a tethering/docking factor of EEs, where it is exclusively found (Christoforidis et al., 1999a; Zerial and McBride, 2001). EEA1 contributes to the spatial organisation of vesicle trafficking with its ability to interact with syntaxin 6, implicated in TGN to endosome transport (Parlati et al., 2000), and syntaxin 13, required for endosome fusion and recycling (McBride et al., 1999; Prekeris et al., 1998). As for the initial endocytic steps, PIs are also involved in the organisation of early endosomal membranes. Indeed, PI3Ks are Rab 5 effectors (Christoforidis et al., 1999b), leading to the generation of PtdIns(3)P, which is enriched in EEs (Gruenberg, 2001). The presence of this lipid in turn allows the binding of other Rab 5 effectors through their FYVE or PHOX domains, (Simonsen and Stenmark, 2001; Simonsen et al., 2001) ultimately leading in the regulation of docking/fusion and SNARE targeting.

A recent study highlighted the compartmentalisation of different GTPases within the endosomal membrane and revealed three main populations of endosomes: one containing only Rab 5, another Rab 4 and Rab 5, and a third Rab 4 and Rab 11. The first two groups would represent a type of early/sorting endosome, whereas the latter would correspond to a RE. The distribution of Rabs in distinct domains of the same endosomal compartment could be translated into different effector platforms contributing to the sorting process (Sonnichsen et al., 2000).

After leaving EEs, recycling molecules are found in distinct tubular structures, the REs. These compartments are less acidic than early endosomes and usually show a perinuclear distribution (reviewed in Gruenberg, 2001). Rab 11 was initially demonstrated to be important for transferrin transport in non-polarised cells (Ullrich et al., 1996). In addition, this protein seems to regulate the return of recycling receptors on the plasma membrane
Figure 1.16 Rab-regulated endocytic pathways in non-polarised cells. Receptors and other molecules internalised through clathrin-coated vesicles (CCV) reach the early endosome (EE), where sorting occurs. Some receptors are recycled back to the plasma membrane, at least in part through recycling endosomes (RE). Molecules destined to degradation are instead delivered from early endosomes to endosomal carrier vesicles (ECV), late endosomes (LE) and finally to lysosomes (L). See text for description. (Adapted from Somsel Rodman, J Cell Sci, 2000).
and to participate in membrane transport exiting the Golgi (reviewed in Somsel Rodman and Wandinger-Ness, 2000). Rab 11 therefore stands out as a factor ensuring cross-talk between the endocytic and exocytic pathways, and is probably only one of many still to be identified. Recycling to the plasma membrane does not appear to be a simple step, since at least a fast and a slow route can be distinguished (Hao and Maxfield, 2000; Sheff et al., 1999), and proteins can return to the plasma membrane also after direct sorting from EEs (Gruenberg, 2001).

Molecules destined for degradation are delivered from EEs to “endosomal carrier vesicles” (ECVs) or MVBs. The pathway then continues with transport to LEs and finally to lysosomes (Figure 1.16). A dominant negative Rab 7 mutant has been shown to strongly inhibit transport from EEs to LEs, suggesting the requirement of this GTPase in the degradative pathway (Feng et al., 1995). The progression from ECV/MVB to LEs is still obscure and the Rab proteins involved remain to be identified, although it seems clear that EEs, REs and ECV/MVBs have a distinct biochemical composition. ECV/MVB are characterised by multiple membrane invaginations enriched in PtdIns(3)P (Gillooly et al., 2000). In contrast, LEs lack this lipid and have lysobisphosphatidic acid (LBPA) in their internal membranes (Kobayashi et al., 1998), whereas the highly glycosylated protein LAMP1 is localised on their limiting membrane (Gruenberg, 2001). As revealed by in vitro liposome assays, LBPA could function by presenting lipids and proteins that need to be degraded to the hydrolytic machinery (Wilkening et al., 1998). LBPA membranes are also important for transport through late endosomes of molecules to be redistributed to lysosomes, such as the mannose 6-phosphate receptor that ensures the delivery of lysosomal hydrolases from TGN to lysosomes (Kobayashi et al., 1998). Rab 9 has been shown to regulate the transport of the receptor back to the TGN (Lombardi et al., 1993;
Somsel Rodman and Wandinger-Ness, 2000). In addition, LBPA-containing membranes could serve as a collecting and distribution device for low-density lipoprotein (LDL)-derived cholesterol, since anti-LBPA antibodies cause cholesterol accumulation in late endosomes, similar to that seen in the cholesterol-storage disorder Niemann-Pick type C (Kobayashi et al., 1999). LEs and lysosomes are difficult to distinguish, since they both contain lysosomal enzymes, have an acidic pH (5.5), and have similar proteins on their outer membrane (Gruenberg, 2001). Moreover, the two compartments can interact dynamically, forming hybrid organelles (Mullock et al., 1998).

The complexity in the organisation of endocytic intermediates is further increased by the role of lipid microdomains in endosomal trafficking. Recent evidence points to the existence of endocytic cholesterol-sensitive pathways for the transport of plasma membrane lipids and raft markers. After clathrin-independent internalisation, molecules could be delivered to the “classical” EE/RE system and subsequently targeted to the Golgi, as observed with the Shiga toxin B subunit (Falguieres et al., 2001). Other components enriched in rafts, such as GPI-anchored proteins, can be delivered to peripheral tubular-vesicular endosomes devoid of classical endocytic markers and then converge in the RE, where they are retained by a mechanism involving the GPI anchor (Chatterjee et al., 2001). However, other studies have identified clathrin-independent pathways targeted to the Golgi which seem to bypass the classical endocytic system altogether (Nichols et al., 2001; Puri et al., 2001), although the identity of the traffic intermediates still remains an interesting enigma to solve.
1.2.5 Endocytosis in polarised cells: the epithelial and neuronal models

Polarised cells have endocytic pathways characterised by common as well as distinct features compared to non-polarised cells. Epithelial cells, such as Madin Darby canine kidney (MDCK) cells, are organised in apical and basolateral membrane domains separated by tight junctions (Figure 1.17) (Mellman, 1996). A similar situation is found in neurons, where the somatodendritic region has different properties from the axonal compartment (Winckler and Mellman, 1999). In MDCK cells, endocytosis occurs at both apical and basolateral sites, where Rab5-positive EEs can be detected. Apical and basolateral molecules can then be recycled to their respective membrane domains, transcytosed, or delivered to LEs (Figure 1.17) (Gibson et al., 1998; Somsel Rodman and Wandinger-Ness, 2000). The apical recycling endosome (APRE) stands out as a specialised organelle, similar to REs in non-polarised cells (Apodaca et al., 1994; Odorizzi et al., 1996). APRE controls recycling and transcytosis and represents a major hub of postendocytic membrane traffic, shared by the apical and basolateral domains. Recently, Rab 11 and two epithelia-specific Rab proteins, Rab 17 and Rab 25, have been identified as traffic regulators in the APRE (Wang et al., 2000; Zacchi et al., 1998), but the details of their coordinating action are still unclear.

In neurons, much effort has focussed on the exo-endocytic steps underlying the SSV cycle (Südhof, 1995). However, little is known about the general organisation of the endosomal system (Figure 1.18). The fungal metabolite brefeldin A (BFA) has been largely used in the study of membrane trafficking. This drug inhibits the GDP-GTP exchange on ARF, thus preventing binding of ARF and cytosolic coat proteins to donor membranes (Peyroche et al., 1999). The result is a block of membrane export from the ER and a redistribution of Golgi membrane components to the ER. Moreover, the TGN mixes with
Figure 1.17 Endocytosis and transcytosis in polarised epithelial cells.
Molecules are internalised from either the apical or the basolateral plasma membrane in apical (AEE) and basolateral (BEE) early endosomes, respectively. Similarly to non-polarised cells, molecules destined for degradation are delivered to the late endosome and lysosome. Apical and basolateral molecules can also be directly recycled to their respective membrane domains, or be transcytosed after transport through the apical recycling endosome (APRE), a specialised organelle shared by the apical and basolateral pathways. See also text.
(Adapted from Somsel Rodman, J Cell Sci, 2000).
the recycling endosomal system which undergoes rapid tubulation also observed in other organelles, such as endosomes and lysosomes (Sata et al., 1999). In neurons, BFA seems to selectively disrupt endocytic trafficking in the somatodendritic compartment, suggesting that the endocytic systems in dendrites and axons could be functionally different (Mundigl et al., 1993). Moreover, transferrin is internalised through its receptor only in the somatodendritic domain, which could then be considered analogous to the basolateral domain of polarised epithelial cells, where the receptor is located (Gibson et al., 1998; Mundigl et al., 1993). The dendrites of cultured hippocampal neurons contain extensive networks of tubular EEs and MVB-like structures, possibly involved in active recycling or sorting to the degradative pathway (Parton et al., 1992). In these cells Rab 5 shows a functional association with both somatodendritic and axonal EEs (de Hoop et al., 1994). However, these two compartments appear to be differentially regulated, since the Rab 5 effector EEA1 is only found in the cell body, in line with its presence only on basolateral sorting endosomes in polarised epithelial cells (Wilson et al., 2000).

Recent studies have also highlighted a high level of complexity in neuronal recycling routes. For example, syntaxin 13, primarily found in tubular EEs and REs in non-polarised cells, is present in round stationary and mobile tubular structures along axons and dendrites in hippocampal neurons (Prekeris et al., 1999), whereas transferrin can follow a transcytotic pathway, being internalised in the cell body and released in the axon (Hemar et al., 1997).

Nearly all axonal endocytosis seems to occur at growth cones or presynaptic sites, and endocytic organelles that exit these regions are transported unidirectionally back towards the soma (Overly and Hollenbeck, 1996). Analysis of the β-amyloid precursor protein (APP) transport in central neurons suggests the existence of an endocytic pathway
Figure 1.18 Schematic model of endocytic trafficking in neurons.

Internalisation via coated pits occurs at presynaptic sites (1) and in the somatodendritic domain. In the nerve terminal, endocytosed cargoes are sorted in the early endosome. They can recycle back to the plasma membrane (2) or enter MVB/ECV and be directed to a retrograde axonal route (3). Sorting could also occur during transport to the cell body. Acidification steps have been found to occur in a region 50-150 μm from the axonal terminal and in axonal branch points (4, red arrows). Most endocytic organelles are acidified by the time they reach the proximal axon.

Ligands internalised from dendritic membranes can follow the degradative pathway to reach lysosomes (5), or return to the plasma membrane through recycling endosomes (6). In hippocampal neurons, ligands like transferrin can also undergo transcytosis, being internalised in dendrites and released in the axonal domain (7). Some of the proteins found to participate in neuronal endocytic pathways are also indicated.

ECV, endosomal carrier vesicle; EE, early endosome; G, Golgi; LE, late endosome; LY, lysosome; MVB, multivesicular body; N, nucleus; RE, recycling endosome.
envisaging internalisation through SSV recycling followed by sorting into retrograde axonal carriers directed to the cell body (Marquez-Sterling et al., 1997). The retrograde compartments could be MVBs, possibly functioning as intermediates from EEs at the presynaptic membrane to LEs located in the cell body (Hollenbeck, 1993b; Parton et al., 1992). However, several reports have shown that late-endocytic and lysosomal organelles are not restricted solely to the somatodendritic domain and that acidification of membrane carriers occurs at distal axons and at axonal branching points (Overly and Hollenbeck, 1996). In addition, time-lapse observations have suggested that sorting might occur along retrograde transport routes (Prekeris et al., 1999). A full understanding of neuronal endocytic pathways will require the identification of the molecular components responsible for what seems to be a complex network of trafficking routes in both the somatodendritic and axonal compartments.
1.3 The neuronal cytoskeleton

Neurons are characterised by complex and diverse shapes that are at the basis of their signalling properties and their pattern of synaptic connections. They are highly polarised cells, usually with multiple dendrites and a long axon. This latter process can be extremely long, such as in MNs, where it can extend up to several meters in large mammals and can have volumes that are many orders of magnitude larger than the cell body. Moreover, axons and dendrites display an amazing variety of branching patterns that are reproduced according to the neuronal cell type and function. The maintenance of such complex shapes poses a challenge to the cytoskeleton components, which are essential not only for the development and support of neuronal processes, but also for their plastic remodelling in response to electrical activity or injury (Burgoyne, 1991). This requires the ability to form both very stable and highly dynamic structures, an intrinsic property found in the cytoskeletal components (see below). In addition, the cytoskeleton provides the fundamental system ensuring the transport and the correct targeting of organelles, vesicles and molecules to axons and dendrites, thereby playing a vital role in neuronal physiology.

Elegant electron microscopy (EM) observations have provided an opportunity to examine the organisation of the neuronal cytoskeleton (Hirokawa, 1982). The axonal and dendritic processes contain tightly-packed long filaments, whereas a variety of organelles appear embedded in the cytoskeletal matrix. In neurons, three main cytoskeletal components can be distinguished: microtubules, with a diameter of 20-25 nm, neurofilaments, the neuronal version of intermediate filaments, whose diameter is 10 nm and actin microfilaments with a diameter of about 5-8 nm. Microtubules and neurofilaments are generally present throughout the neuronal cytoplasm, whereas actin microfilaments appear to be
concentrated in a cortical network under the axonal plasma membrane and are particularly abundant in growth cones (Bradke and Dotti, 1999).

1.3.1 Microtubules

Microtubules (MTs) are polymers consisting of tubulin, a heterodimer of two globular proteins of approximately 50 kDa each, called α- and β- tubulin (Nogales et al., 1999). Tubulin dimers are arranged longitudinally to form protofilaments, 12-15 of which are joined through lateral association to form a hollow cylinder. The alignment of tubulin dimers within the MT leaves α-tubulins exposed at one end and β-tubulins exposed at the other, which gives MTs intrinsic structural polarity (Nogales et al., 1999). MTs are dynamic, since they can grow or shrink by addition or loss of the tubulin dimers (Desai and Mitchison, 1997). As a consequence of their intrinsic polarity, MTs also display a kinetic polarity as the rate of polymerisation is different at the two ends; the faster growing end is the plus end (β-tubulins exposed), and the slower growing end is the minus end (α-tubulins exposed) (Figure 1.19). The ends of MTs switch stochastically between periods of slow growth and rapid shrinkage; the transition from growth to shrinkage is called “catastrophe”, whereas the return to growth is called “rescue”. The frequent interconversion between these two states in a population of MTs is commonly referred to as “dynamic instability”, and is due to the GTPase activity of β-tubulin (Erickson and O'Brien, 1992). The first step in MT growth consists of the addition of GTP-tubulin on to the plus end. Incorporation of tubulin into a MT stimulates GTP hydrolysis (Stewart et al., 1990) (Vandecandelaere et al., 1999). EM studies suggest that nucleotide hydrolysis causes a structural change in the tubulin subunit from a “straight” (GTP-bound) to a “curved” (GDP-bound) conformation, resulting in decreased affinity for the neighbouring
subunits and dissociation from the polymer (Hyman et al., 1995; Muller-Reichert et al., 1998). Since GTP hydrolysis does not occur instantaneously after tubulin addition, MTs in the growth phase are thought to maintain a stabilising “cap” of GTP-tubulins that prevents the unstable GDP-tubulin within the lattice from adopting its preferred curved conformation (Caplow and Shanks, 1996; Drechsel and Kirschner, 1994). The energy of hydrolysis is therefore stored in the lattice in the form of strain (Caplow et al., 1994; Mickey and Howard, 1995). However, the rate of GTP hydrolysis is close enough to the rate of addition, so that sometimes hydrolysis catches up, exposing GDP-tubulins at the MT plus end. This results in the formation of curved protofilament peels, which are characteristic of depolymerising ends (Mandekow et al., 1991; Tran et al., 1997). Finally, the release of free energy that accompanies relaxation of the strained GDP-tubulin promotes a rapid MT “catastrophe” (Caplow et al., 1994). Support for this model comes from the finding that tubulin polymerised with a slowly-hydrolysable GTP analogue forms stable MTs lacking dynamic instability (Caplow et al., 1994; Hyman et al., 1992). Precise regulation of MT dynamics is crucial for cellular function and is achieved through an array of additional stabilising factors, such as MT-associated proteins (MAPs) (Mandekow and Mandekow, 1995), and the recently discovered destabilising proteins Op18 and XKCM1 (McNally, 1999). Phosphorylation regulates the activity of many of these factors, thus linking MT dynamics to intracellular and extracellular signalling (Walczak, 2000).

In the neuronal cell body MTs are nucleated from the centrosome which is apparently randomly located and with only a few MTs attached at any one time (Yu et al., 1993). The mechanism for MT release from centrosomes is not completely understood, but is thought to involve the MT-severing protein, katanin (McNally and Vale, 1993). MTs are then
Figure 1.19 Dynamic properties microtubule polymers.

Microtubules are polymers formed by the tubulin, a heterodimer of α- and β-subunits. Polymerisation occurs by the addition of GTP-tubulin (yellow) to the plus end. The incorporated monomer hydrolyses GTP to GDP (orange). If the rate of hydrolysis increases, GDP-tubulin is exposed at the plus end, leading to fast microtubule depolymerisation (catastrophe), unless addition of GTP-tubulin recaps the plus end (rescue). The dissociated GDP-tubulin monomers can rapidly exchange their nucleotides for GTP to continue the polymerisation cycle.
mobilised and directed towards neuronal processes, but the organisation of this delivery process is still debated. In axons, MTs are uniformly oriented with the plus ends pointing towards the terminal and minus ends toward the cell bodies (Burton and Paige, 1981; Heidemann et al., 1981). In contrast, the orientation in the dendrites is much more complex, since these processes contain MTs with mixed polarity with respect to the cell soma (Burton, 1988). In hippocampal neurons, however, MTs with mixed polarity in the proximal dendrites were found to switch to a uniform polarity in the distal dendritic regions, with the plus ends pointing away from the cell body just as in axons (Baas et al., 1988). Controlling the polarity of MTs in dendrites and axons may therefore contribute to achieve correct targeting of organelles in these processes. Interestingly, MTs in neurons are significantly more stable than in other cell types (Baas et al., 1994; Morris and Lasek, 1982; Webb and Wilson, 1980). This property could be fundamental for the ability to extend processes and to transport molecules over long distances (Hirokawa, 1994; Mandelkow and Mandelkow, 1995).

1.3.2 Actin microfilaments

Similarly to MTs, actin also forms highly dynamic filaments. Actin is a 43-kDa protein able to self-assemble into linear polymeric microfilaments (F-actin) arranged in a double helical conformation. Actin monomers (G-actin) bind to either ATP or ADP and the formation of the filaments is accompanied by nucleotide hydrolysis. The principles regulating the assembly of actin are similar to those observed for MTs, but different association and dissociation rate constants as well as ATP hydrolysis rate confer a distinct dynamic behaviour to actin microfilaments (Pantaloni et al., 2001). These structures are also polar, since addition of ATP-actin occurs at the plus end (or “barbed” end) and
dissociation at the minus or "pointed end", after ATP hydrolysis within the incorporated subunits and release of $P_i$ into solution (Figure 1.20). The monomers in solution can then exchange ADP for ATP and re-enter the cycle. At steady state, such a "treadmilling" process is at equilibrium, with the net assembly of subunits at the plus end equaling the net disassembly at the minus end. Filament length and number are relatively constant. In vivo, regulatory factors control the treadmilling process and the availability of ATP-actin to finely tune actin-based motility in response to environmental or intracellular signals (Pantaloni et al., 2001). For example, actin-depolymerising factor (ADF)/cofilin accelerates pointed-end depolymerisation, which is the rate-limiting step in the treadmilling ATPase cycle (Carlier et al., 1997). As a consequence, a higher steady-state concentration of monomeric ATP-actin is established, which supports faster barbed-end growth and increases the rate of actin-based motility. Another protein, profilin, specifically binds ATP-actin monomers and facilitates their addition exclusively at barbed ends (Pantaloni and Carlier, 1993). The joined activity of profilin and ADF/cofilin can have a synergistic effect, resulting in an enhancement of treadmilling processivity (Didry et al., 1998). Genetic and biochemical studies have identified several other actin-binding proteins important for the regulation of motility, like capping proteins, or the WASP-Arp2/3-nucleating complex, which allows F-actin branching and connects actin to a variety of signaling pathways (reviewed in Machesky and Insall, 1999). In neurons, actin-based motility is crucial for a variety of morphogenetic processes, ranging from growth-cone movement and the establishment of neuronal polarity during development (Bradke and Dotti, 2000), to plastic remodeling of dendritic spines in maturity (Matus, 2000). Recent studies have shed light on the role of the actin cytoskeleton in the endocytosis of SSVs during the recycling process (Qualmann et al., 2000; Slepnev and De Camilli,
Figure 1.20 Dynamic properties of actin microfilaments. 
Actin microfilaments are polymers formed by actin subunits able to bind either ATP or ADP. ATP-bound actin monomers add onto the filament at the plus (or "barbed") end. After nucleotide hydrolysis, ADP-actin readily dissociates from the minus (or "pointed") end of the filament. Individual actin subunits therefore "treadmill" through the polymer, a phenomenon that can be used to produce mechanical force.
2000). In addition, it is becoming increasingly clear that organelle transport in neurons can also rely on F-actin. Vesicles from squid axoplasm can be transported along actin filaments *in vitro* (Kuznetsov et al., 1992), mitochondria move bidirectionally along F-actin in axons (Morris and Hollenbeck, 1995), and actin-based motors seem to be involved in the movements of vesicles in regions poor of MTs, such as growth cones or synaptic terminals (Bridgman, 1999). However, the molecular mechanisms of how such actin-based transport is achieved and regulated are still unclear.

### 1.3.3 Neurofilaments

Neurofilaments (NFs) have an intermediate size between actin microfilaments and MTs and fall into the class of cytoskeletal components known as intermediate filaments which also includes keratins, vimentins, glial fibrillary acidic protein (GFAP) and nuclear lamins. They are composed of three polypeptide subunits: NF-H (high; 112 kDa), NF-M (middle; 102 kDa), and NF-L (low; 68 kDa) (Perrone Capano et al., 2001). These proteins contain a central α-helical region able to form coiled-coil dimers. The dimers then form tetramers that assemble to form NFs without the need for nucleotide hydrolysis. However, the mechanism of NF formation is still not completely understood. These structures have been considered historically as mechanical reinforcers, but their complex composition suggests that they are more than simple mechanical scaffolds in neurons. The architectural organisation of the NF network in axons is characterised by longitudinally oriented and regularly spaced filaments with unique “side arms” formed by NF-M and NF-H tail domains. These projections, as well as other sites of the NF proteins, can be phosphorylated by a variety of kinases and in response to physiological stimuli, such as depolarisation (Nixon, 1998). The result is a control of the interaction of NF with other
cytoskeletal components and a modulation of filament spacing, ultimately affecting axonal calibre. Such modification can have functional significance, since axonal diameter is a major determinant of the conduction velocity of nerve impulses along axons (Perrone Capano et al., 2001). NFs are the most abundant cytoskeletal components in large neurons and myelinated axons, particularly those belonging to large motor and sensory neurons. It is therefore not surprising that aberrant accumulation of these filaments, as observed in neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Julien, 1999) has deleterious effects on both axonal transport and radial axonal growth.

1.3.4 Interaction between neuronal cytoskeletal elements

The neuronal cytoskeletal components described above do not represent independent systems, but are interconnected in a complex network supporting the whole neuronal cell. EM studies in squid axoplasm have shown a large number of long actin filaments joining together and running co-axially with MT bundles in the giant axon (Bearer and Reese, 1999), suggesting a physical association between these two components. General integrators of the cytoskeleton that have recently received much attention are plakins, a family of large coiled-coil dimeric proteins, of which desmoplakin, bullous pemphigoid antigen 1e (BPAG1e) and plectin are the prototypes (Klymkowsky, 1999). Two splice forms of the BPAG1 gene are able to interconnect NFs with the axonal actin cytoskeleton, whereas another isoform specifically localises to MTs, enhancing their stability to cold and to depolymerising agents (Yang et al., 1999). BPAG plakins therefore appear to be integrators of all three cytoskeletal networks, a feature that in neurons could be
responsible for the high degree of MT stabilisation, ultimately allowing axonal transport over long distances.

1.3.5 Drugs affecting the cytoskeleton

1.3.5.1 MT-disrupting agents

Three main classes of tubulin-binding drugs, colchicine analogues, vincas, and taxanes have been identified according to the site of protein interaction. Colchicine inhibits MT polymerisation, possibly by binding to the cleft between the α- and β-monomers, facing the lumen of the MT. The widely used drug nocodazole also has a similar mechanism of action (Downing, 2000). Vinca alkaloids (such as vinblastine and vincristine) stop MT dynamics by binding to the surface of the β-tubulin exposed at the plus-end, thus inhibiting elongation (Downing, 2000). At high concentration, protofilaments are seen to form spirals, leading to the accumulation of paracrystalline aggregates (Weber et al., 1975). Taxol and related taxanes instead bind to a pocket in the tubulin structure lined by several hydrophobic residues and located in the middle of the β-monomer. The final effect of these drugs is to stabilise MTs by inducing GDP-tubulin to adopt an elongated conformation reminiscent of the GTP-bound form, thus counteracting the protofilament tendency to curl (Arnal and Wade, 1995).

1.3.5.2 F-actin-disrupting agents

The most common actin-disrupting drugs are the fungal metabolites cytochalasins, and latrunculins, marine macrolides isolated from the Red Sea sponge Latrunculia magnifica. Several types of cytochalasins (A-J) have been identified, varying in potency and effects. They generally act by inhibiting actin polymerisation through the block of monomer
addition at the plus-end. In addition, they possess some actin filament-severing activity (Cooper, 1987).

In contrast, latrunculins disrupt F-actin organisation by sequestering G-actin monomers through the formation of a 1:1 complex (Spector et al., 1989). Other drugs affecting the actin cytoskeleton are jasplakinolide, a cell-permeable compound that stabilises F-actin and reorganises actin filaments into a tight cortical layer adjacent to the plasma membrane (Bubb et al., 2000), and swinholide A, also isolated from a marine sponge, *Theonella swinhoei*. This natural compound has a mechanism of action similar to latrunculins, since it is able to interact with G-actin monomers, sequestering them from the treadmilling process. In addition, it also displays a filament-severing activity leading to an increase in exposed filament ends and a subsequent acceleration of F-actin depolymerisation (Bubb et al., 1995).
1.4 Mechanisms of neuronal transport

The distinct biochemical composition of the neuronal compartments (soma, dendrites, axon) is maintained by the continuous and selective transport of material from the site of synthesis to its final destination. This process requires a highly efficient machinery able to ensure transport of molecules over long distances.

Much work has focussed on the analysis of axonal transport and has mainly relied on nerve ligation/accumulation studies, live observation of organelle transport in living neurons or in vitro models of extruded axoplasm (reviewed in Goldstein and Yang, 2000; Vallee, 1991). Anterograde axonal transport from the cell body towards the nerve terminal ensures delivery of newly synthesized proteins, organelles and preformed synaptic boutons to their final destinations (Ahmari et al., 2000). Retrograde axonal transport instead moves materials such as organelles, lysosomes, endocytic vesicles and neurotrophins from the synapse to the cell body (Goldstein and Yang, 2000; Vallee, 1991; Whitmarsh and Davis, 2001). In addition, pathogens, such as Herpes simplex virus (Bearer et al., 2000) and TeNT (Herreros et al., 1999) must be retrogradely transported to perform their biological action.

Anterograde axonal transport is divided into three major classes, a fast component and two slow components, A and B. Fast anterograde (0.1-5 μm/s) transport is used to move mostly vesicles and organelles, and possibly secreted molecules, such as neurotrophins, to the synapse. Slow component A is employed for the transport of MTs and NF proteins; slow component B includes the transport of microfilaments and other cytoplasmic proteins, such as clathrin, spectrin, and metabolic enzymes (Vallee, 1991). The speed range for these slow components is between 1-50 nm/s.
Like axons, dendrites also require an efficient delivery system for soluble proteins, various organelles and vesicles, cytoskeletal elements, and even components of the translation machinery (Tiedge and Brosius, 1996). However, the mechanisms and motor proteins involved in dendritic transport await elucidation.

Much of neuronal traffic is generated by MT-based transport (Goldstein and Yang, 2000). This system is based on motor proteins that use the chemical energy derived from ATP hydrolysis to transport a variety of neuronal cargoes along polar MT “tracks” (Figure 1.21). However, new evidence is also highlighting important roles of actin-dependent motors in neuronal transport and strengthening the concept of a functional interrelationship between MT- and actin-based motility systems (Bridgman, 1999; Kamal and Goldstein, 2000).

1.4.1 MT-based motors

1.4.1.1 Kinesins

MT-dependent motors include the large family of kinesins and dyneins. Apart from a few exceptions, kinesins are generally responsible for fast anterograde transport directed towards the MT plus-end. In contrast, dynein is a minus-end directed motor, currently considered to be the main protein responsible for axonal retrograde transport.

Kinesins belong to a superfamily of MT-associated ATPases that perform different functions in living cells (Goldstein and Philp, 1999; Hirokawa, 1998). These motor proteins hydrolyse ATP using a conserved catalytic core containing both MT- and nucleotide-binding sites and displaying MT-stimulated ATPase activity (Vale et al., 2000). Kinesins are divided in three groups – Kin N, Kin C and Kin I, according to the relative position of the catalytic core within the entire polypeptide chain (Figure 1.22) (Vale and
Figure 1.21 The basic organisation of microtubule- and actin-based molecular motors.
Schematic organisation of the microtubule-dependent conventional kinesin (left) and cytoplasmic dynein (right) and of the actin-dependent myosin (centre). All three motors consist of a dimer of two heavy chains (red), a stalk (light green) and several associated polypeptides (yellow). The heavy chains, also termed "heads", are the motor domains with nucleotide and track binding sites. In dynein, the microtubule-binding sites are located at the tip of the "antennae" protruding from the large globular motor domains.
(Adapted from Woehlke, Nat Rev Mol Cell Biol, 2000).
Figure 1.22 Scheme of conventional and minus-end directed kinesins.
The different position of the catalytic core, neck, stalk and tail regions of the plus-end
directed conventional kinesin and the minus-end directed ncd. The catalytic core
contains the ATP- and microtubule-binding sites, whereas the neck regions translate the
change in nucleotide-binding state into production of the power stroke required for
movement.
(Adapted from Sablin, Curr Opin Cell Biol, 2000).
Fletterick, 1997). All Kin N proteins identified so far act as plus-end directed motors, whereas all Kin C members move towards the MT minus-end (Vale et al., 2000). The function of Kin I proteins still has to be conclusively defined, since some work has suggested they possess MT motor activity (Aizawa et al., 1992; Noda et al., 1995), whereas other reports have indicated a possible role in the regulation of MT dynamics (Desai et al., 1999).

The recently described crystal structures of plus-end-directed conventional kinesin and minus-end-directed non-claret disjunctional (ncd) kinesin have shed light on the structural and functional organisation of these motors (Kozielski et al., 1997; Sablin et al., 1998). Kinesins usually consist of a dimer of two heavy chains and associated polypeptides, consisting of two light chains in most of mammalian kinesins. Overall, four domains can be distinguished: the catalytic and motor core, the “neck”, the stalk and the tail. The catalytic core is formed by two motor “heads” able to hydrolyse ATP. Adjacent to the catalytic core is the “neck” region, which has been shown to be primarily responsible for determining direction (Case et al., 1997; Endow and Waligora, 1998; Henningsen and Schliwa, 1997). According to the current hypothesis, the kinesin catalytic domain would function as an “allosteric converter”, transducing information about its MT- and nucleotide-binding state to the neck region. The necks would then respond with a conformational change, producing a “power stroke” able to generate movement. Kinesin is a processive motor, i.e. it moves along MTs in “steps” that can be repeated several times without detaching from the MT. A precise coordination of the two motor domains is required to achieve such processivity (Woehlke and Schliwa, 2000) (Figure 1.23). In solution, a kinesin dimer contains one ADP per motor head. After binding to the MT, one head releases ADP and can detach only if it binds and hydrolyses another ATP molecule.
Figure 1.23 Model of kinesin progression on the microtubule.

In solution each of the two kinesin heads binds one ADP. One head binds to the microtubule, loses ADP and can detach only upon binding of ATP. ATP hydrolysis produces the "power stroke" that allows interaction of the other ADP-bound head to the microtubule. Processivity is therefore ensured by the alternate binding of one motor head at the time onto the microtubule.

(Adapted from Woehlke, Nat Rev Mol Cell Biol, 2000).
During this nucleotide hydrolysis step, the second head is allowed to bind to the MT, and the hydrolysis cycle continues, accompanied by the “power stroke” produced by the neck region. Such a coordinated “hand over hand” mechanism would ensure processive motion, that for conventional kinesin is translated in a succession of rapid 8 nm steps (Coy et al., 1999; Svoboda et al., 1993; Visscher et al., 1999). However, minus-end-directed kinesins do not seem to be as processive as plus-end directed ones, but the exact reason for this discrepancy remains undetermined (Foster and Gilbert, 2000; Mackey and Gilbert, 2000).

The enzymatic activity and cellular function of kinesins are regulated at different levels, including phosphorylation, interaction with small GTPases, and, not least, cargo binding and intramolecular folding (Muresan, 2000). It has been suggested that kinesin light chains inhibit binding of the heavy chains to MTs (Verhey et al., 1998), possibly in a phosphorylation-dependent fashion (Hollenbeck, 1993a; Matthies et al., 1993). Interaction of the tail domain with the cargo would produce a conformational change that relieves inhibition of the motor domain, allowing movement to start. This model would also explain how excessive movements of unloaded motor molecules are prevented. However, the basic mechanisms of cargo recognition and motor activation are still under investigation. The number of different cargoes in neurons certainly exceeds the number of axonal kinesins. As a consequence one type of kinesin is likely to carry several cargoes not only in axons, but also in dendrites (Muresan, 2000). On the other hand, there are examples of kinesin selectively localised in neuronal domains, such as the plus-end-directed KIF21B, which is enriched in dendrites (Marszalek et al., 1999). Several cargoes could also act as kinesin receptors. Examples are fodrin (Takeda et al., 2000), the adaptor complex AP1 (Nakagawa et al., 2000), the small GTPase Rab6 (Echard et al., 1998), vesicle associated myosin V (Huang et al., 1999b), APP (Kamal et al., 2000) and JIP1,
JIP2 (Verhey et al., 2001) and JIP3 (Bowman et al., 2000), scaffolding proteins in JNK/MAPK signalling.

Kin C family members represent a peculiar class of minus-end-directed kinesins. Most of them have functional roles in mitosis and meiosis, like Drosophila ncd. However, three members of this family have been identified in mouse brain, KIFC1, KIFC2 and KIFC3. The homodimeric kinesin KIFC2 seems to be involved in intracellular transport processes, since it was found associated with MVB-like organelles in soma and dendrites (Saito et al., 1997) and some axonal vesicular structures (Hanlon et al., 1997). KIFC2 may therefore contribute to axonal retrograde transport, together with the minus-end-directed dynein (see below). The role of KIFC1 has not been determined yet, whereas KIFC3 has been localised to synapses of retinal photoreceptor cells, suggesting a role at the ribbon synapse. However, knockout mice for KIFC3 develop normally, suggesting a dispensable function for this widely expressed kinesin (Goldstein and Yang, 2000).

1.4.1.2 Dynein

Cytoplasmic dynein was originally purified from neuronal tissue and characterised as a MT-dependent motor (Vallee et al., 1988; Yoshida et al., 1990). Dynein is a big multisubunit complex (1.2 MDa) formed by two heavy chains, which fold to form the two large motor heads and the stalks and are responsible for ATPase activity and force production (Hirokawa, 1998) (Figure 1.24). Each dynein motor unit contains six AAA (ATPases associated with cellular activities) domains, at least four of which are potentially able to interact with ATP. However, the actual number of nucleotide-binding sites is still unknown (King, 2000). In striking contrast to kinesin, the MT-binding domain is segregated from the motor head, being located at the tip of an antiparallel coiled-coil
Figure 1.24 The dynein-dynactin complex.
Dynein is linked to cargo membranes indirectly through the dynactin complex, formed by 11 different subunits. The p150Glued dimer acts as a bridge between microtubules, dynein, and Arp-1. The latter protein interacts with F-actin and spectrin present on cargo organelles, thus playing an important role in the vesicle-motor connection.
(Adapted from Hirokawa, Science, 1998).
structure which forms an "antenna" protruding from the large globular motor domain (Vallee and Gee, 1998). A set of associated polypeptides consisting of intermediate, light-intermediate, and light chains together with the dynactin complex (Hirokawa, 1998) completes the structural organisation of dynein. In particular, dynactin is now believed to be required for most, if not all, of dynein-mediated cellular activities, which include spindle assembly, vesicle targeting to the Golgi, and retrograde axonal transport (Hirokawa et al., 1990; Karki and Holzbaur, 1999; Lye et al., 1987; Paschal et al., 1987; Schnapp and Reese, 1989; Schroer et al., 1989). The dynactin complex consists of 11 different subunits (Figure 1.24), comprising a dimer of p150Glued, 4 or 5 50-kDa dynamitin subunits, and 8 or 9 actin-related proteins (Arp-1) molecules (Hirokawa, 1998). The dynactin complex not only acts as a binding platform for the association of dynein with its cargo, but also increases motor processivity. In extruded squid axoplasm, disruption of dynein-dynactin complex prevents dynein binding to vesicles, thus stopping MT-based transport (Waterman-Storer et al., 1997). p150Glued seems to act as an important linker between dynein, cargo and MTs. In fact, this protein is able to interact not only with one of the dynein intermediate chains (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), but also with MTs and Arp-1 (Waterman-Storer et al., 1995). Functionally, Arp-1 may serve as a structural element of a vesicle tethering complex, possibly through its interaction with spectrin present on axonal vesicles (Muresan et al., 2001). Interestingly, Arp-1 is able to interact more with F-actin than with globular actin in vitro (Melki et al., 1993). Furthermore, it has been shown that one molecule of actin is associated with each dynactin complex (Schafer et al., 1994). Taken together, these data suggest a direct interaction of dynactin with both MT and actin networks during dynein-mediated transport.
A clear model of dynein-mediated motility is still lacking, mainly because of the unresolved issue of how energy released by ATP hydrolysis within the motor head is used to produce force at the MT-binding stalk tip. As dynein dissociates during its mechano-chemical cycle, the dynactin complex could increase motor processivity by transiently tethering the cargo organelle to MTs through the long and flexible linker p150Glued. This system could therefore prevent diffusional loss of cargo from the cytoskeletal substrate and favour the rebinding of dynein to continue translocation (King and Schroer, 2000). It seems plausible that the regulation of the dynactin-MT interaction could be achieved through post-translational modifications, such as phosphorylation, although conclusive evidence for this hypothesis is still lacking.

1.4.2 Actin-based motors: myosins

The actin-based myosin motors form a superfamily of at least 18 known classes (Hodge and Cope, 2000). The general structural organisation of myosins is similar to the one described for MT-based motors, kinesin and dynein (Figure 1.21). The heavy chain includes the catalytic core able to hydrolyse ATP and a "converter" domain, which translates the structural changes initiated in the core into a swing of an adjacent lever region containing the binding sites for a variable number of myosin light chains (Mermall et al., 1998; Spudich, 2001). The structure is completed by a carboxy-terminal tail, containing both coiled-coil regions and domains aimed at targeting the motors to cargoes (Hasson and Mooseker, 1997). Five non-muscle, or "unconventional" myosin classes (I, III, V, VI, and VIIa) have been identified in the nervous system (Hasson and Mooseker, 1997), whereas "conventional" myosin, (myosin II), important in muscle contraction, is
also likely to participate in the F-actin flow at the basis of growth cone dynamics (Suter and Forscher, 1998).

Myosins were usually thought to move towards the plus-end of actin microfilaments until myosin VI was shown to move in the opposite direction (Wells et al., 1999). Moreover, analysis of chimeric myosins demonstrated that the direction of motility is determined by the motor core domain, opening the possibility that other minus-end-directed myosins exist (Homma et al., 2001).

Recent reports have highlighted a possible role of the actin cytoskeleton and myosin motors in axonal transport. Axoplasmic organelles in the squid giant axon move on both MTs and actin microfilaments, and appear to be associated with myosin motors (Bearer et al., 1993; Kuznetsov et al., 1992). Moreover, mitochondria have been shown to move on actin microfilaments in axons of vertebrate neurons, possibly through myosin motors (Morris and Hollenbeck, 1995). Myosin I and V exhibit a punctate staining in different neuronal domains, suggesting their association with vesicular structures (Evans et al., 1997; Lewis and Bridgman, 1996). A recent study showed that myosin Va-associated organelles move bidirectionally in living neurons and proposed that this motor could help the distribution of vesicles in the absence of MTs through local short-range movements (Bridgman, 1999). Indeed, the recent demonstration that myosin V is a processive motor supports the idea that this protein could play an important role in organelle transport (Mehta et al., 1999). The presence of a putative "lever arm" three times longer than myosin II allows myosin V to take larger steps, corresponding to 36 nm, the helical repeat of the actin microfilament (Spudich, 2001; Walker et al., 2000). EM studies combined with biophysical experiments have proposed a possible model of myosin V stepping on actin filaments, where ATP binding allows the trailing motor head to detach from actin
and take a large 72 nm step (Spudich, 2001). However, many aspects of myosin motility, including how bidirectional motility can be achieved in axonal organelles, still have to be clarified.
1.5 Objectives

CNTs specifically interact with the neuronal plasma membrane and exploit neuronal trafficking routes to reach the substrates of their catalytic activity. For this reason they represent an interesting model for the study of neuronal endocytic pathways, which are still largely uncharacterised. The \( H_c \) fragment, a structurally distinct domain in the CNT molecule, is responsible for the interaction of these neurotoxins with their acceptors on the neuronal surface. These features make \( H_c \) a suitable, non-toxic tool for the characterisation of unknown CNT receptors and, in general, for the study of neuronal endocytosis and trafficking. This important goal could be facilitated by the expression of recombinant CNT \( H_c \) fragments, a strategy so far applied only for TeNT \( H_c \) in a limited number of studies. The first part of chapter 3 describes the production and functional characterisation of recombinant TeNT, BoNT/A, /B and /E \( H_c \) fragments appropriately designed for their use in biochemical and cell biological assays aimed at the study of CNT neurospecific trafficking.

The \( H_c \) fragment represents an ideal probe for the characterisation of the peculiar intracellular route that distinguishes TeNT from other CNTs. In fact, BoNTs act at the NMJ, whereas TeNT has to undergo retrograde transport in the MN and transcytosis in the adjacent interneuron to block neurotransmission. Very little is known about the organisation and the dynamics of this special neuronal transport route that could be used not only by pathogens, but also by physiological ligands for their biological functions. Although some types of polygangliosides are commonly recognised as TeNT receptors, experimental evidence has indicated the participation of one or more proteins in
neurotoxin binding. The second part of chapter 3 describes the characterisation of a putative protein receptor for TeNT using its recombinant H\textsubscript{C} fragment.

The final section of chapter 3 focuses on the analysis of TeNT H\textsubscript{C} neuronal trafficking. We established a novel retrograde transport assay in living MNs using a fluorescent version of TeNT H\textsubscript{C}. The results described provide important clues on the organisation of a trafficking pathway in a still much unexplored neuronal cell type.
Chapter 2: Materials and Methods
2.1 Materials

2.1.1 Chemicals

Chemicals used were all of analytical grade or higher and were purchased from the following companies unless otherwise stated: Sigma, Fluka, BDH, Calbiochem, Amersham Pharmacia Biotech. Molecular biology reagents were from Qiagen, Promega and Clontech unless otherwise stated. All radiochemicals were purchased from Amersham Pharmacia Biotech. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from DAKO. Fluorescent conjugated secondary antibodies, Alexa™ Fluor 488, 546, 594 maleimides, Alexa™488 phalloidin, Texas Red sulfonyl chloride, Texas Red-dextran (molecular weight 3000), Texas Red-transferrin, Lysotracker Red DND-99, Lysotracker Green DND-26 were obtained from Molecular Probes.

Phosphate buffered saline (PBS), Hanks’ buffer, Dulbecco’s Modified Eagle’s Medium (DMEM), minimum essential medium with Earle’s salts (MEM) and bacterial growth media (2YT and LB) were all supplied by ICRF laboratory services.

2.1.2 Antibodies

The following antibodies were used in this work:

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2.1.3 Constructs and recombinant proteins

TeNT Hc (residues 855-1314; accession number X04436) in the vector pTTQ8 was a gift from Dr. J. Halpem (FDA, Bethesda, Maryland, U.S.A.) (Halpern et al., 1990).

Recombinant TeNT Hc mutants were obtained from Dr. N. Fairweather (Imperial College, London, U.K.).

2.1.4 C. botulinum genomic DNA

Genomic DNA from C. botulinum serotypes A, B and E was obtained from Dr. M. R. Popoff (Institute Pasteur, Paris, France).
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2.1.5 Purified CNTs
TeNT, BoNT/A, BoNT/B and BoNT/E were obtained from Drs. O. Rossetto and C. Montecucco (University of Padova, Italy).

2.1.6 Cell lines
A rat pheochromocytoma (PC12) subclone shown to bind TeNT with high affinity (Sandberg et al., 1989) was obtained from Dr. T.B. Rogers (University of Maryland, USA).

2.2 Methods
2.2.1 Electrophoresis and Western blotting
One dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) enables the separation of proteins based on their relative molecular mass and differential mobility through the acrylamide gel matrix. Separating gels were usually a single percentage but where specified, linear gradient gels were prepared. Proteins were visualised by Coomassie Blue staining or Western blotting.

2.2.1.1 Coomassie Blue staining
Gels were fixed and proteins stained using a solution of 0.2% Coomassie Blue in 45% methanol, 10% acetic acid for 30 min. Destaining was carried out using 10% methanol, 7% acetic acid.
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2.2.1.2 Western blotting

For Western blotting, proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell) at 100 V for 1 h in 200 mM glycine, 25 mM Tris base, 10% methanol. Transferred proteins were visualised by staining with 0.2% Ponceau S, 3% trichloroacetic acid. Membranes were blocked by incubation for 1 h at room temperature with 5% dried skimmed milk (Marvel) or 5% blocking reagent (Amersham Pharmacia Biotech) in 135 mM NaCl, 100 mM Tris-HCl, pH 7.6, 0.05% Tween-20 (Tris-buffered saline with Tween-20, TBST ). Primary and secondary HRP-conjugated antibodies were applied in fresh blocking solution. Washes were performed with TBST and antibodies were visualised using Enhanced Chemi-Luminescence detection (ECL, Amersham Pharmacia Biotech).

2.2.2 Generation of recombinant proteins

2.2.2.1 Preparation of the pGEX-4T3-VSV-G and pGEX-4T3-HA vectors

Oligonucleotides with the sequence 5' -GATCTTACCAGTAGATAGAAGAGACAG-GCTGGGAAGCGTGCTCATCTGTGTCATATG-3' and 5'-AATTTATTGAACAGATTCGACGACGCTTTCCCAGCTCTGTCCCATCTCTATATCGGTGTAA-3' were phosphorylated with T4 polynucleotide kinase (New England Biolabs) for 30 min at 37°C and then annealed at a final concentration of 1 μM in 40 mM Tris-HCl, pH 7.5; 20 mM MgCl₂, 50 mM NaCl. The double strand linker was then ligated into the BamHI/EcoRI site of pGEX-4T3 (Amersham Pharmacia Biotech). The resulting vector (pGEX-4T3-VSV-G) encodes for glutathione S-transferase (GST), which is fused at the carboxy terminus, downstream of the thrombin cleavage site, to the sequence YTDIEMNRLGK, corresponding to the vesicular stomatitis virus protein G epitope (VSV-G) (Gallione and Rose, 1985; Soldati and Perriard, 1991). This epitope tag is followed by the
phosphorylation motif recognised by protein kinase A (RRASV) (Kaelin et al., 1992), which allows radiolabelling of the fusion protein of interest. The same procedure was followed to prepare the vector pGEX-4T3-HA in which the sequence YPYDVPDYA corresponding to the influenza virus Hemagglutinin epitope (HA) replaces the VSV-G epitope. In this case, the oligonucleotides

5’GATCTTACCCCTACGACGTCCTACGATCCCGTCGTGCATCTGTTCATATGCCATGG-3’ and 5’AATTCCATGGCATATGAACAGATGCACGACGGGATCCGGTGGTCGGGCACGTCGATGGGTAA-3’ were inserted in the BamHI/EcoRI site of pGEX-4T3 following the procedure described above.

2.2.2.2 PCR amplification and cloning of TeNT, BoNT/A, BoNT/B and BoNT/E Hce

Polymerase Chain Reaction (PCR) was performed using as a template crude genomic DNA from selected toxigenic C. botulinum strains (Fach et al., 1995). The strain P64 which is related to strain 62A (Binz et al., 1990) was used for BoNT/A, NCTC 11219 (Whelan et al., 1992) for BoNT/E and the proteolytic strain B600 for BoNT/B. The following primers were used: for BoNT/A, residues 845-1295, 5’-CCATGGAGTACAGATATACCTTTTTCAGCTTTTC-3’ and 5’GTCGACTTCAGTGGCCTTTCTCCCCATC-3’ (GenBank accession number M30196); for BoNT/E, residues 820-1252, 5’-CCATGGAAATAATAGTTATCTTTTAAGCTTTCTTC-3’ and 5’-GTCGACTTTATTTTTCTTGCCATCCATGTTTCTCAG-3’ (accession number X62683); for BoNT/B, residues 832-1290, 5’-CCATGGAAAAACATTATGCCGTGTGATCTTTCAA-3’ and 5’GTCGACTTTATTCAGTCCACCCTCATCTTTTAG-3’. The resulting BoNT/B Hc fragment constitutes a new BoNT/B variant and its sequence is now available with the accession number AJ242628. Following PCR amplification, DNA
inserts were sub-cloned into pCR2.1 (Invitrogen), excised with NdeI/XhoI (BoNT/A) and NcoI/SalI (BoNT/B and E) and then ligated into pGEX-4T3-HA (for BoNT/B and E) or pGEX-4T3-VSV-G (BoNT/A). TeNT Hc (residues 855-1314; accession number X04436), already available in the pTTQ8 vector (Amersham Pharmacia Biotech) (Halpem et al., 1990), was excised with SalI/PstI, blunt-ended with T4 DNA polymerase (New England Biolabs) following manufacturer recommendations and ligated in the SmaI site of pGEX-4T3-VSV-G. The sequences of all CNT Hc inserts were checked by automated sequencing of both DNA strands.

2.2.2.3 Expression and purification of recombinant TeNT and BoNT HcS

pGEX-4T3-VSV-G and pGEX-4T3-HA vectors containing the appropriate Hc insert were used to transform TG1 or BL21(DE3)pLys strains (Stratagene) and protein expression was induced by incubation for 4 h at 30°C in the presence of 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) (Guan and Dixon, 1991). For BoNT/B, TOPP3 cells (Stratagene) were used without induction by IPTG. Bacterial suspensions were then pelleted, resuspended on ice with in cold phosphate-buffered saline (PBS), 0.05% Tween 20, 2mM EDTA, 0.1% β-mercaptoethanol, 4 μg/ml pepstatin, 1 mM benzamidine, 1% Pefabloc (Roche Molecular Biochemicals). After two rounds of French press (900 psi), the lysate was ultracentrifuged at 40,000 rpm for 20 min. The clear supernatant containing the recombinant GST-fusion protein was then adsorbed on glutathione-agarose beads (Sigma) and sequentially incubated with 50 mM Tris-HCl pH 7.4, 2 mM ATP, 10 mM MgSO4 for 10 min at 37°C followed by PBS containing 0.5 M NaCl and 0.05 % Tween 20. The tagged Hc fragments were released by incubation with thrombin (Guan and Dixon, 1991) in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% β-mercaptoethanol for 30 min at room
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temperature. A last elution step with 0.5 M NaCl in the same buffer was also performed. The eluted proteins were usually concentrated with dialysis against 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 15 % polyethylene glycol (PEG) 20,000, 0.1 mM dithiothreitol (DTT) and subsequently dialysed against 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 mM DTT and, after freezing in liquid nitrogen, stored at -80°C. When necessary, the eluted proteins were purified by metal-chelating chromatography using HiTrap columns (Amersham Pharmacia Biotech) (Rossetto et al., 1992). 1 ml HiTrap column was preloaded with 5 ml of 100 mM ZnSO₄, followed by 10 ml of distilled water and then equilibrated with 50 mM HEPES-NaOH, pH 7.4, 150 mM NaCl (buffer A). After loading and extensive washing with buffer A, the Hc fragments were eluted with a linear gradient of imidazole in buffer A (0-25 mM). Pooled fractions were dialysed against 20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 10 % glycerol, 0.1 mM DTT and, after freezing in liquid nitrogen, stored at -80°C.

2.2.2.4 PCR amplification and cloning of TeNT HcC and HcN

TeNT HcN (residues 856 to 1110) and HcC (residues 1111 to 1315) specific DNAs were amplified by standard PCR from the pGEX-4T3-VSV-G-TeNT Hc plasmid using the following primers: 5' CATATGTCACACACACCATCCTTTCTTCTTCT-3' and 5' GTC GACTTATAATATAACATTGTGTCATAA-3' for the HcN sub-domain and 5' CATATGTCATATAACCTTTTATATAGACACTTC-3' and 5' GTCGACTTAAATCATTTG TCCATCTTATC-3' for the HcC sub-domain.

The fragments were then inserted into the PCR-Script cloning vector (Stratagene) and subcloned into pGEX-4T3-VSV-G in the Ndel/SalI site following to the VSV-G epitope
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and the consensus sequence for protein kinase A phosphorylation. The sequences of the inserts were checked by automated sequencing.

2.2.2.5 Expression and purification of recombinant TeNT \( H_C \) and \( H_N \)

Vectors containing the two sub-domains were transformed into BL21(DE3)pLys (Stratagene, USA). GST-fusion proteins were expressed for 4 h at 30°C in the presence of 0.4 mM IPTG. Bacterial suspensions were lysed through French press and adsorbed on glutathione-agarose beads as described above. After thrombin cleavage, the proteins were concentrated snap-frozen in liquid nitrogen and stored at -80°C. In some cases, the sub-domains were further purified on MonoQ anion exchange column (Amersham-Pharmacia Biotech, UK).

2.2.2.6 Preparation of recombinant Cys-TeNT \( H_C \)

Phosphorylated oligonucleotides 5'-GAT CCG CAG AGG CAG CAG CAC GAG AGG CTT GTT GTC GAG AGT GTT GTG CAC GAG AGG CAG CAG CAC GAG CG-3' and 5'-AAT TCG CTC GTG CTG CTG CCT CTC GTG CAC A AC ACT CTC GAC A AC A AG CCT CTC GTG CTG CTG CCT CTG CG-3' were annealed and then ligated into the BamHI/EcoRI site of pGEX-4T3 (Amersham Pharmacia Biotech, Bucks, UK), resulting in the pGEX-4T3-Cys vector. TeNT \( H_C \) was excised from the pGEX-4T3-VSV-G vector with SalI/EcoRI and ligated into the SalI/EcoRI site of pGEX-4T3-Cys. The resulting plasmid (pGEX-4T3-Cys-TeNT \( H_C \)) encodes for GST fused at the carboxy terminus, downstream of the thrombin cleavage site, to the peptide A EAAAR EACCR ECCAR EAAAR and to TeNT \( H_C \). The underlined domains are predicted to form \( \alpha \)-helices with the thiol groups of the cysteines properly oriented for labelling with
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Fluorescein arsenical helix binder (FLASH) (Griffin et al., 1998). The tagged TeNT H_c fragment was expressed and purified following the standard procedure described above for the other recombinant TeNT fragments, concentrated through dialysis against 10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 15% PEG 20,000, 5 mM β-mercaptoethanol and dialysed overnight against 20 mM HEPES-NaOH pH 7.4; 100 mM NaCl, 10% glycerol, 0.1 mM β-mercaptoethanol.

2.2.2.7 Preparation of recombinant Cys-TeNT H_c and H_c

TeNT H_cN (residues 856 to 1110) and H_cC (residues 1111 to 1315) specific DNAs were amplified by standard PCR from the pGEX-4T3-Cys-TeNT H_c plasmid using the following primers: 5'-GGG CTG GCA AGC CAC GTT TGG TG-3' and 5'-GTC GAC TTA ATA ACT TGT GTA TAA TTT TTC-3' for H_cN and 5'-GAA TTC TAT AAC CTT TTT AAG AGA CTT C-3' and 5'-GTC GAC TTA ATC ATT TGT CCA TCC TTC ATC-3' for H_cC.

The amplified sub-domains were then inserted into pBluescript (Stratagene) and sub-cloned into the Sall/EcoRI sites of pGEX-4T3-Cys. Cys-TeNT H_cN and H_cC were expressed and purified using the same procedure followed for Cys-TeNT H_c.

2.2.3 Cell culture

2.2.3.1 PC12 cells

PC12 cells were cultured in DMEM containing 7.5% foetal calf serum, 7.5% horse serum (Sigma, UK). Cells were grown at 37°C in a humidified atmosphere containing 10% CO_2 on plastic dishes precoated with collagen extracted from rat tails. Cells were seeded in 12-well plates (Costar-Corning, USA) at a density of 25,000 cells/well. After 24 h, the
medium was supplemented with 75 ng/ml of nerve growth factor (NGF) 7S; Alexis, USA). Cells were used after 7-8 days of NGF treatment.

2.2.3.2 Primary culture of mouse spinal cord cells

Whole spinal cords from E13 mice were isolated in 77 mM HEPES-NaOH, pH 7.3, 4.2 mM NaHCO₃, 5.4 mM KCl, 136.8 mM NaCl, 68 mM sucrose, 32 mM glucose (dissection buffer), minced and dissociated in 0.25% trypsin in dissection medium at 37°C for 30 min (Fitzgerald, 1989). After pelleting, spinal cord fragments were repeatedly triturated in MEM containing 32 mM glucose, 0.04 mg/ml Dnase, 4 mM L-glutamine, 10% foetal calf serum, 10% heat inactivated horse serum (plating medium). Cells suspended in plating medium were seeded in 12 well plates (Corning Costar) or on glass coverslips coated with a mixture of polyornithine and collagen (Fitzgerald, 1989; Williamson et al., 1996) at a density of 200,000 cells/coverslip or 500,000/well. 24 h after plating, the medium was substituted with MEM containing 32 mM glucose, B27 supplement (Gibco-BRL), 5% heat-inactivated horse serum (5% v/v) and 2 mM L-glutamine (2 mM). Cultures were grown in a humidified 10% CO₂ incubator at 37°C. Five days after plating, 35 µg/ml of uridine and 15 µg/ml of 5'-fluoro-2'-deoxyuridine were added to the medium for 96 h to block cell proliferation. Medium was changed every 3-4 days. Experiments were usually performed with cells kept in culture for 2 weeks.

2.2.3.3 Primary culture of rat motor neurons

Rat spinal cord MNs were purified from E14 rat embryos (Arce et al., 1999; Henderson et al., 1995). Briefly, ventral spinal cords were dissected, dissociated with 0.025% trypsin (GIBCO-BRL, USA) for 10 min at 37 °C and triturated. Cells were centrifuged through a
4% BSA cushion, resuspended and recentrifuged through a 6.5% metrizamide cushion prepared in L-15 medium (Gibco-BRL). The cells found at the metrizamide interphase (1 ml) represented the purest MN fraction (approximately 90%, based on the immunostaining with the MN markers Islet 1/2 (Ericson et al., 1992)) (Figure 3.9). Cells were immediately plated on 35 mm glass bottom microwell dishes (MatTek Corporation) at a density of 25,000/dish and used for live imaging experiments after one week in culture.

The pelleted cells and the supernatant were collected through a bovine serum albumin (BSA) cushion and resuspended in 50 µl of PBS with 0.5% BSA (PBS-BSA) plus 50 µl of mouse anti-rat p75NTR hybridoma supernatant (Chandler et al., 1984), which recognises the neurotrophin receptor p75NTR specifically expressed by rat MNs at E14 (Yan and Johnson, 1988). Cells were incubated with the p75NTR hybridoma supernatant at 12°C for 15 min, washed with PBS-BSA and centrifuged through a BSA cushion. The pellet was resuspended in 80 µl of PBS-BSA and incubated with 20 µl of goat anti-mouse immunoglobulin G (IgG) magnetic microbeads (Miltenyi Biotec) for 15 min at 12°C. After another centrifugation through a BSA cushion cells were resuspended in 500 µl of PBS-BSA and purified by magnetic cell sorting using a Mini Macs magnet and a large cell separation column (Miltenyi Biotec). Purified MNs were seeded on glass coverslips coated with polyornithine and laminin at a density of 40,000 cells/coverslip for immunocytochemistry. For crosslinking experiments MNs were seeded on 12-well plates at a density of 125,000 cells/well. Cultures were maintained in a humidified 7.5% CO₂ incubator at 37°C in Neurobasal medium (Gibco-BRL) containing B27 supplement, 2% horse serum, 0.5 mM L-glutamine, 25 µM β-mercaptoethanol, 10 ng/ml rat ciliary neurotrophic factor and 100 pg/ml rat glial cell line-derived neurotrophic factor (both from
R&D Systems). L-glutamate (25 μM) was added to the medium for the first four days in culture. Cells could be usually maintained in culture for a maximum of 2 weeks.

2.2.4 Cell treatments

2.2.4.1 CNT binding to mouse spinal cord cells

Mouse spinal cord cultures were cooled to 4°C, washed twice with 0.1% BSA in Hanks’ buffer (20 mM HEPES-NaOH, pH 7.4, 0.44 mM KH$_2$PO$_4$, 0.42 mM Na$_2$HPO$_4$, 5.36 mM KCl, 136 mM NaCl, 0.81 mM MgSO$_4$, 1.26 mM CaCl$_2$, 6.1 mM glucose) and incubated with recombinant TeNT $H_C$, native TeNT, BoNT/B $H_C$ (80 nM), BoNT/A $H_C$ or BoNT/E $H_C$ (200 nM) for 1 h at 4°C. In selected samples, native TeNT (20 μM) was pre-incubated at 4°C for 15 min before addition of the recombinant $H_C$.

2.2.4.2 Binding of recombinant CNT $H_C$s, wild-type and mutant TeNT $H_C$ fragments to PC12 cells and rat MNs

Cells on coverslips were cooled to 4°C for 15 min and then washed twice with 0.2% BSA in Hanks’ buffer before incubation with CNT fragments at a concentration ranging between 40 and 100 nM in Hanks’ buffer containing 0.2% BSA for 1 h at 4°C. After washing twice with Hanks’ buffer, cells were fixed and processed for immunocytochemistry as described below using a monoclonal anti-VSV-G (for TeNT and BoNT/A $H_C$) (1:100), anti-HA (for BoNT/B and BoNT/E $H_C$) (1:50), or polyclonal anti-TeNT $H_C$ antibodies (wild type and mutant TeNT $H_C$ fragments) (1:500).
2.2.4.3 Internalisation of VSV-G-tagged TeNT H\text{c}, H\text{c}N and H\text{c}C in MNs

MNs were washed twice and incubated for 1 h at 37°C with the H\text{c} or the sub-domains (40 nM) in serum-free culture medium. After fixation and permeabilisation, cells were blocked and immunostained using an anti-VSV-G antibody. Controls were performed by omitting the H\text{c} or its sub-domains in the incubation buffer.

2.2.4.4 Immunocytochemistry

PC12 cells were fixed in 3.7% paraformaldehyde in PBS, while spinal cord cells and MNs were fixed in 4% paraformaldehyde, 20% sucrose in PBS for 15 min at room temperature. After rinsing twice with PBS, cells were incubated for 20 min with 50 mM NH\text{4}Cl in PBS, washed and then blocked with 2% BSA, 0.25% porcine skin gelatin, 0.2% glycine, 15% foetal calf serum in PBS (blocking buffer) for 30 min to 1 h. Cells were then generally incubated for 1 h with monoclonal or polyclonal primary antibodies diluted in PBS containing 1% BSA, 0.25% porcine skin gelatin (antibody buffer). After rinsing, cells were incubated for 25 min with fluorescent anti-mouse or anti-rabbit secondary antibodies diluted 1:200 in antibody buffer. When necessary, cells were permeabilised using 0.1-0.2% Triton X-100 in blocking buffer. Coverslips were mounted on slides with Mowiol 4-88 (Harco) and stored at 4°C.

2.2.4.5 SNARE analysis

Spinal cord cultures were rinsed twice with MEM and incubated for 20 h at 37°C in serum-free culture medium with different CNTs (TeNT and BoNT/A 100 pM, BoNT/E 500 pM, BoNT/B 2.5 nM). Some samples were pre-incubated with the recombinant H\text{c} (1 nM, 10 nM, 100 nM and 1 \mu M) for 1 h at 37°C before adding the corresponding CNT.
SNARE detection was carried out by immunostaining with a polyclonal antibody recognising the carboxy-terminus of SNAP 25 (1:200) (Osen Sand et al., 1993) or with a monoclonal anti-VAMP-2 (1:100) (Edelmann et al., 1995). For Western blot analysis, spinal cord cells were washed twice with PBS and then scraped in the same buffer. Proteins were recovered by precipitation with 6.5% trichloroacetic acid (TCA) and analysed by SDS-PAGE containing urea (Söllner et al., 1993). Western blotting was performed by using anti-VAMP-2 antibody (1:200) or anti-SNAP-25 polyclonal antibodies (1:200) recognising the full-length or the carboxy terminus of the protein (Edelmann et al., 1995), followed by ECL detection. For quantitation, protein recovery was normalised using syntaxin 1 immunoreactivity as internal standard.

2.2.5 In vitro binding to gangliosides

Hc binding to purified polysialogangliosides was monitored by dot-blot assay (Thomas et al., 1999) by using 0.5 µg of phosphatidylcholine (PC) (Avanti) or purified gangliosides G\textsubscript{M1}, G\textsubscript{D1b}, G\textsubscript{T1b} and G\textsubscript{Q1b} adsorbed on nitrocellulose. After blocking with 5% dried skimmed milk in PBS, Hc fragments (80 nM) were diluted in 20 mM Tris-acetate, pH 6.0, 5% milk and incubated for 2 h at room temperature with the nitrocellulose. CNT binding was detected with anti-VSV-G (1:1,000) and anti-HA (1:200) antibodies, followed by an anti-mouse HRP-conjugated IgG and ECL.

2.2.6 Mouse phrenic nerve-hemidiaphragm assay

These experiments were carried out in collaboration with Dr. O. Rossetto, University of Padova, Italy. Mouse phrenic nerve-hemidiaphragms were dissected from animals weighing about 20 g, mounted in 10 ml of oxygenated (95% CO\textsubscript{2} - 5% O\textsubscript{2}) Krebs-Ringer...
solution (Gibco-BRL) containing 11 mM glucose, pH 7.4 and kept at 37°C. The phrenic nerve was stimulated via two ring platinum electrodes by supramaximal stimuli of 3-6 V amplitude and 0.1 ms pulse duration with a frequency of 0.1 Hz. Isometric muscle contraction was monitored via a displacement force transducer connected to a recorder. In control experiments (without any added toxin), the amplitude of muscle contraction under stimulation was constant for at least 8 h. CNTs were added to the bath at a concentration of 10 nM for TeNT and 0.2 nM for BoNTs and incubated with toxin in physiological medium at 25°C for 60 min without nerve stimulation (Simpson, 1980). At the end of incubation, tissues were washed and transferred to 37°C in a bath without CNTs. Nerve stimulation was applied until a reduction of 90% of the initial muscle twitch was observed. In the competition assays, mouse phrenic nerve-hemidiaphragms were incubated with each H_c fragment (H_c:CNT ratio 100:1 for TeNT and 1,300:1 for BoNTs) for 15 min at 25°C followed by a co-incubation with the parental CNT at the same concentration of the control for 60 min at 25°C without nerve stimulation. After incubation, tissues were washed and paralysis times were monitored. Results were expressed as the time necessary to obtain a 50% reduction of the initial muscle response following nerve stimulation. Data are the average of 3 experiments.

2.2.7 Radiolabelling

All recombinant proteins expressed in the pGEX-4T3-VSV-G vector (TeNT H_c and the H_cN and H_cC) contain the consensus sequence for protein kinase A phosphorylation (Kaelin et al., 1992). For radiolabelling, 5 μg of protein were incubated (5 min, 30°C) with 100 μCi of [32P]-γ-ATP (10 mCi/ml, 3000 Ci/mmol) in 50 mM 2-(N-morpholino)ethanesulphonic acid (MES) NaOH, pH 6.9, 10 mM MgCl₂, 1 mM DTT, 0.5
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5 mM EDTA, 1 mg/ml ovalbumin together with 0.4 mU of the catalytic subunit of protein kinase A (4 mU/µl; Roche Boehringer-Mannheim) diluted in 5 mM MES NaOH pH 6.9, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mg/ml ovalbumin. Free [³²P]-γ-ATP was separated from the labelled-protein on PD-10 columns (Amersham-Pharmacia Biotech) equilibrated with 0.1% ovalbumin in PBS. The fraction containing the maximal amount of radiolabelled protein was stored at -80°C. Specific activity was on average 2000 Ci/mmol.

2.2.8 Crosslinking experiments

These experiments were carried out in collaboration with Dr. J. Herreros. Cells (PC12, spinal cord cells or MNs) were cooled on ice, washed with ice-cold Hanks' buffer and incubated with [³²P]-TeNT Hc, [³²P]-TeNT HcN or [³²P]-TeNT HcC sub-domains (all 200-300 pM) in 0.2% BSA-Hanks' for 2 h on ice. In competition assays, cells were preincubated for 20 min with 2 µM cold proteins, before adding the radiolabelled protein of interest. After binding, cells were washed with Hanks' and incubated for 10 min at 4°C with 0.22 mM of bis[2(succinimidylxycarbonyloxy)ethyl]sulphone (BSOCOES; PerBio-Science) in Hanks' buffer prepared just before use. The reaction was stopped by removing the crosslinker and adding 20 mM glycine in the same buffer. Cells were then solubilised in 4% octyl-β-D-glucopyranoside (Roche Boehringer-Mannheim) in Hanks'supplemented with the following protease inhibitors: 1 mM iodoacetamide, 100 µM phenylmethanesulphonyl fluoride (PMSF), 1 mM benzamidine, 1 µg/ml aprotinin and 1 µg/ml leupeptin. After 5 min, detergent was diluted up to 0.8% and proteins were precipitated with 6.5% trichloracetic acid. Samples were prepared for SDS-PAGE and analysed in 6-12% or 12-18% acrylamide gradient gels (Laemmli, 1970), followed by autoradiography. Quantitative measurements were performed using PhosphorImager.
(Molecular Dynamics). Background readings for each lane were subtracted to calculate both the total $[^{32}\text{P}]-\text{TeNT H}_c$ binding and the amount of the 65-kDa crosslinking band.

For phosphoinositol-phospholipase C (PI-PLC) treatment, cells were washed in serum-free medium, pretreated (1 h, 37°C) with different amounts of PI-PLC and extensively washed. Cells were cooled on ice, washed with Hanks’ buffer (Herreros et al., 2000a) and incubated (1 h, 4°C) with 300 pM $[^{32}\text{P}]-\text{TeNT H}_c$ in Hanks’ buffer containing 0.2% BSA. After binding, cells were washed and crosslinked (10 min, 4°C) with 0.22 mM of BSOCOES in Hanks’ buffer. The reaction was stopped and cells were solubilised as described above. Proteins were analysed in 6-12% acrylamide gradient gels followed by autoradiography.

2.2.9 Retrograde transport assay in living MNs

2.2.9.1 Fluorescent labelling of proteins

Prior to labelling with FLASH, Cys-TeNT H$_c$ (1 n mole) was reduced with 12.5 mM β-mercaptoethanol for 30 min at 37°C. The labelling reaction was performed using 5 n moles of FLASH-EDT$_2$ (Aurora Biosciences) and carried out for 90 min at room temperature in the dark. Labelling of Cys-TeNT H$_c$, Cys-H$_c$N or Cys-H$_c$C with the Alexa maleimides was achieved by overnight incubation at 4°C using 20 moles of dye per mole of protein after dialysing the recombinant proteins (15 n moles) against degassed 20 mM HEPES-NaOH, pH 7.4, 100 mM NaCl. The labelling reaction was stopped with an excess of glutathione in HEPES buffer. Free fluorescent dye was removed on PD-10 desalting columns equilibrated with PBS. Typically 15 fractions (200 µl each) were collected in PBS and the peak of fluorescent protein corresponded to fractions 7-9. A sample of each collected fraction was loaded on a 15% SDS-PAGE. Fractions containing labelled proteins
which appeared as fluorescent bands under UV light were pooled and stored at 4°C. The extent of labelling was, on average, 1.8 moles of dye per mole of protein.

For TeNT labelling, 600 µg of TeNT were diluted in 100 mM NaHCO$_3$, pH 8.8; 150 mM NaCl and incubated for 1 h at 4°C with 600 µg of Texas Red sulfonyl chloride dissolved in dimethyl formamide under stirring in the dark. The labelled toxin was then purified on a PD-10 column equilibrated in PBS. The labelling ratio was 2.2 moles of dye per mole of protein.

For NGF labelling, 100 µg of lyophilized mNGF 7S (Alomone Labs) were reconstituted in 500 mM NaHCO$_3$, pH 8.0 and incubated for 1 h at room temperature in the dark with 100 µg of Texas Red sulfonyl chloride. The labelled NGF was then purified from the free dye on a PD-10 column previously equilibrated with 0.2% acetic acid (Sandow et al., 2000). Texas Red-NGF contained about 4 moles of dye per mole of protein and could differentiate PC12 cells to a neuronal phenotype similarly to unlabeled NGF (not shown).

For BSA labelling, 700 µg of embryo-tested BSA were dissolved in 100 mM NaHCO$_3$, pH 8.8, 150 mM NaCl and incubated for 1 h at 4°C in the dark with 500 µg of Texas Red sulfonyl chloride as described above. The labeled BSA was then purified on a PD-10 column equilibrated with PBS. The degree of labelling was about 2 moles of dye per mole of protein.

### 2.2.9.2 Time-lapse imaging

After 1 week in culture, rat MNs were incubated with 30-40 nM fluorescent TeNT, TeNT H$_C$, H$_C$N or H$_C$C in Neurobasal medium at 37°C for 15 min. Alternatively, cells were cooled and incubated with fluorescent TeNT H$_C$ for 15 min at 4°C. Cells were washed three times with DMEM, warmed to 37°C and imaged in warm DMEM (without phenol
red, riboflavin, folic acid, penicillin and streptomycin) and supplemented with 30 mM HEPES-NaOH, pH 7.3. Cells were placed in a humidified steel chamber maintained at a constant temperature of 37°C. For time-lapse low-light microscopy, fluorescent images were acquired every 1-5 seconds with a Nikon Diaphot 200 inverted microscope equipped with a Nikon X100, 1.3 NA Plan Fluor oil-immersion objective using standard Nikon FITC B-2A or Texas Red G-2A filters and a Hamamatsu C4742-95 Orca cooled charge-coupled device (CCD) camera (Hamamatsu Photonic Systems) controlled by the Kinetic Acquisition Manager 2000 software (Kinetic Imaging Limited). For two-color video microscopy, a double dichroic filter for imaging of fluorescein and Texas Red was used (Filter Set XF53, Omega Optical). Exposure times were between 300 and 500 ms. For time-lapse confocal microscopy, fluorescent images were acquired with continuous scanning using a Zeiss LSM 510 confocal microscope equipped with a Zeiss X63, 1.40 NA Differential Interference Contrast (DIC) Plan-Apochromat oil-immersion objective. A region of interest was chosen and simultaneously excited using the 488 nm and 543 nm wavelengths of a krypton-argon and helium-neon lasers, respectively. Images were collected by averaging twice at a single focal plane and the gain/offset parameters were adjusted in order to prevent bleed-through between the green and red channels. Cells were constantly kept at 37°C during the whole observation. Time series images were then processed by applying a 3X3 low pass filter option of the LSM 510 software.

2.2.93 Competition experiments

MNs were cooled to 4°C, washed twice with 0.2% BSA in Hanks’ buffer and incubated with 30 nM TeNT H_c-Alexa488 for 20 min at 4°C. In selected samples, a 100 fold excess of recombinant VSV-G-tagged TeNT H_c was pre-incubated at 4°C for 15 min before
addition of the fluorescent TeNT $H_C$. Cells were then washed three times in Hanks’ buffer and fixed in 4% paraformaldehyde, 20% sucrose in PBS for 15 min at room temperature. After washing, cells were then mounted with Mowiol 4-88 (Harco) and stored at 4°C. Alternatively, cells were pre-incubated at 37°C for 10 min with a 100 fold excess of TeNT (4 μM) before adding the fluorescent TeNT $H_C$ (40 nM) for 15 min at 37°C. Cells were then washed and imaged with low-light microscopy as described above.

In another set of experiments, MNs were preincubated with a 100 fold excess (4 μM) of mutant TeNT $H_C$ fragments for 20 min at 37°C before adding 40 nM of TeNT $H_C$-Alexa488 for 15 min at 37°C. Cells were then washed and imaged.

2.2.9.4 Drug treatments

Drug stock solutions were prepared: 1 mM latrunculin B (Lat B) and 5 mg/ml nocodazole in dimethyl sulphoxide (DMSO) and 2 mM vincristine (Vin) in ethanol. For the effect on TeNT $H_C$ endocytosis, MNs were pretreated with 0.5 μM Lat B for 25 min at 37°C, cooled to 4°C and incubated with 40 nM fluorescent TeNT $H_C$. Cells were then washed and the medium replaced with warm DMEM buffered with 30 mM HEPES-NaOH, pH 7.3 containing Lat B during the whole observation. For the effect on transport, 0.5 μM Lat B, 10 μg/ml nocodazole, or 5 μM Vin were added to the cells about 20 min after removing the fluorescent TeNT $H_C$, when retrograde vesicles were visible. Imaging was resumed at least 30 min after Lat B or nocodazole addition or 40 min after Vin addition. In parallel control cells, an equal amount of DMSO or ethanol (final concentration 0.2%) was added and imaging was performed at similar time points to the drug-treated samples.
2.2.9.5 Double labelling experiments

MNs were incubated with 40 nM TeNT Hc-Alexa488 and 0.2 mg/ml Texas Red-dextran for 30 min at 37°C. Alternatively, cells were incubated with 40 nM TeNT Hc-Alexa488 and 50 nM Lysotracker Red DND-99 for 20 min at 37°C. In other cases, cells were incubated with 0.9 mg/ml Texas Red dextran overnight at 37°C. Lysotracker Green DND-26 (80 nM) was then added for 30 min. Cells were then washed three times and imaged with low-light or confocal microscopy.

In another set of experiments, MNs were cooled at 4°C and incubated with 40 nM TeNT Hc-Alexa488 and 1.75 µM Texas Red-BSA or 40 nM Texas Red-NGF for 20 min at 4°C. Cells were then washed three times, warmed to 37°C and imaged.

For the experiments with TeNT Hc and transferrin, MNs were washed with warm Neurobasal medium and incubated with 40 nM TeNT Hc-Alexa488 and 80 µg/ml Texas Red-transferrin for 45 min at 37°C, washed and imaged with low-light or confocal microscopy. In some experiments, cells were treated with 20 µg/ml BFA immediately after the incubation with the proteins. The drug was kept in the medium throughout the observation time.

In other experiments, MNs were incubated with 40 nM TeNT Hc-Alexa488 and 40 nM TeNT-Texas Red for 15 min at 37°C, washed and imaged with low-light microscopy.

2.2.9.6 Tracking and data quantification

The Motion Analysis software (Kinetic Imaging) was used for manual vesicle tracking. Stacks of time-lapse sequences were analyzed for movement of fluorescent structures. Only moving vesicles that could be tracked for at least 3 time points were considered. Tracking was stopped when the fluorescent vesicles went out of focus or could not be
distinguished from other overlapping vesicles. The distance covered by a carrier between two consecutive frames or between the initial and the final tracking points was used to determine speed and average speed, respectively. Statistical analysis was performed using SigmaPlot 2000 (v 6.0; SPSS Science). Curve fitting was obtained by using KaleidaGraph (v 3.5; Synergy Software) and Origin (v 4.0; Microcal Software).

8-bit images from time-lapse experiments were assembled into movies with the Kinetic Imaging software using the Microsoft Video 1, 100% quality compression algorithm. Single frame-images were processed for presentation in Adobe Photoshop®5.5.

2.2.10 Electron Microscopy

These experiments were carried out in collaboration with Steve Gschmeissner (ICRF Electron Microscopy Unit). Cells were cooled to 4°C, washed twice with 0.2% BSA in Hanks’ buffer and incubated with 40 nM gold-conjugated TeNT Hc (Aurion, Wageningen, The Netherlands). After washing three times with 0.2% BSA in Hanks’ buffer, cells were either fixed immediately or warmed to 37°C for 5 or 20 min before washing and fixing for 1 h with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (Sorensen’s buffer). Cells were then washed and stored at 4°C in the same buffer before being processed for EM. Alternatively, cells were incubated at 4°C with 40 nM VSV-G-tagged TeNT Hc for 20 min, washed three times with 0.2% BSA in Hanks’ buffer and blocked with 2% BSA, 0.25% porcine skin gelatin, 0.2% glycine, 15% FCS in PBS for 15 min at 4°C. Cells were then washed twice in Hanks’ buffer and incubated with mouse anti VSV-G antibody (1:80) diluted in in PBS containing 1% BSA, 0.25% porcine skin gelatin for 30 min at 4°C. After rinsing three times in Hanks’ buffer, cells were incubated with 10 nm gold-conjugated goat anti-mouse IgG (1:100; British BioCell International) for 25 min at
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4°C, washed six times with Hanks’ buffer and immediately fixed or warmed to 37°C for 5 or 20 min before fixation with glutaraldehyde as above. After washing with Sorensen’s buffer, the cells were then postfixed with 1% osmium tetroxide for 30 min, washed several times, dehydrated in ascending ethanol, infiltrated and embedded in araldite over 2 days. Thin sections were stained with methanolic uranyl acetate and lead citrate for straight morphology examination or aqueous uranyl acetate for immunogold samples. Sections were then observed with a JEOL 1010 transmission electron microscope (JEOL Inc.).

2.2.11 Field Emission Scanning Electron Microscopy (FESEM)

MN were washed twice with 0.2% BSA and incubated at 4°C with 40 nM VSV-G-tagged TeNT H_c for 20 min. Cells were then blocked as described above and incubated with polyclonal anti-VSV-G and monoclonal anti-Thy-1 antibodies for 45 min at 4°C. After washing with Hanks’ buffer, cells were incubated with 10 nm gold-conjugated goat anti-mouse IgG and 5 nm gold-conjugated protein A (1:100; British BioCell International) for 25 min at 4°C. After washing several times, samples were finally fixed with 4% paraformaldehyde in Sorensen’s buffer for 1 h at room temperature.

Coating for FESEM was performed with a high-resolution beam coater model 681 (Katan) operated at 10kV for 3 min at 5x10^-3 torr. Samples were observed with a JEOL FESEM 6700F equipped with a solid state back-scattered electron (BE) detector (model SM74070). Images were taken at 20kV with a probe current of 20 µA. Secondary emission and back-scattered electron images were then pseudocoloured and digitally overlapped.
2.3 Supplemental Material

2.3.1 Description of videos

Video 1. Example of TeNT Hc trafficking in MNs. Cells were incubated with TeNT Hc-Alexa488 for 15 min at 37°C, washed and imaged with low light time-lapse microscopy. TeNT Hc enters endocytic structures of different shape in the MN axon, dendrites and cell body. Note the retrogradely-biased transport in the axon, the bi-directional movement of tubular structures along dendrites and the dynamics of vesicles in the cell body. Intervals between frames are 5 s. The movie consists of 40 consecutive frames played at 5 frames/s. The image is 64 μm x 85 μm. See also Figure 3.21 a-g.

Video 2. TeNT Hc retrograde transport along the axon of a MN treated only with vehicle containing DMSO. The cell body is located at the top. Frames were taken every 5 s. Shown here is a movie consisting of 31 frames played at 5 frames/s. The image is 15 μm x 43 μm. See also Figure 3.34 a.

Video 3. Fluorescent TeNT is also retrogradely transported in living MNs. Cells were incubated with 40 nM TeNT-Texas Red for 15 min at 37°C, washed and imaged by low-light microscopy. The cell body is located at the bottom. Frames were taken every 5 s. Shown here is a movie consisting of 46 frames played at 5 frames/s. The image is 5 μm x 30 μm. See also Figure 3.28.

Video 4. Fluorescent TeNT and its Hc domain are transported in the same retrograde carriers in living MNs. Cells were incubated with 40 nM TeNT-Texas Red and 40 nM...
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TeNT Hc-Alexa488 for 15 min at 37°C, washed and imaged by double-colour low-light microscopy. Frames were taken every 5 s. Shown here is a movie consisting of 35 frames played at 5 frames/s. The image is 65 μm x 45 μm. See also Figure 3.30.

Video 5. Fluorescent TeNT HcC is internalised and retrogradely transported in living MNs. Cells were incubated with 40 nM TeNT HcC-Alexa488 for 15 min at 37°C, washed and imaged by low-light microscopy. Frames were taken every 5 s. Shown here is a movie consisting of 80 frames played at 5 frames/s. The image is 64 μm x 85 μm. See also Figure 3.31 f.

Video 6. Fluorescent TeNT HcN gives a faint vesicular staining restricted to the somatodendritic domain of living MNs. Cells were incubated with 40 nM TeNT HcN-Alexa488 for 15 min at 37°C, washed and imaged by low-light microscopy. Frames were taken every 5 s. Shown here is a movie consisting of 24 frames played at 5 frames/s. The image is 64 μm x 85 μm. See also Figure 3.31 g-h.

Video 7. Effect of F-actin disruption on TeNT Hc axonal retrograde transport. Treatment of cells with 0.5 μM Lat B for 20 min causes the vast majority of TeNT Hc endocytic structures to stop or oscillate. The cell body is located at the bottom. Frames were taken every 5 s. Shown here is a movie consisting of 31 frames played at 5 frames/s. The image is 11 μm x 38 μm. See also Figure 3.34 b.

Video 8. Effect of F-actin disruption on TeNT Hc trafficking in MN cell bodies. Treatment with 0.5 μM Lat B for 40 min perturbs the dynamics of TeNT Hc organelles
both in the cell body and in dendrites. Frames were taken every 5 s. Shown here is a movie consisting of 40 frames played at 5 frames/s. The image is 64 μm x 85 μm.

**Video 9.** Effect of microtubule depolymerisation on TeNT Hc axonal retrograde transport. Treatment of cells with 5 μM Vin for 50 min causes the vast majority of TeNT Hc carriers to stop or oscillate. The cell body is located at the top. Frames were taken every 5 s. Shown here is a movie consisting of 32 frames played at 5 frames/s. The image is 9 μm x 35 μm. See also Figure 3.37 a.

**Video 10.** Effect of microtubule disruption on TeNT Hc somatodendritic trafficking. Treatment with 5 μM Vin for 50 min stops the trafficking of TeNT Hc endocytic structures in both cell body and dendrites. Frames were taken every 5 s. Shown here is a movie consisting of 36 frames played at 5 frames/s. The image is 64 μm x 85 μm.

**Video 11.** TeNT Hc carriers do not colocalise with lysosomes or acidic organelles. Rat MNs were incubated with 40 nM TeNT Hc-Alexa488 (green) and 50 nM Lysotracker DND-99 (red) for 20 min at 37°C. Cells were washed and imaged with double-colour low-light microscopy. The cell body is located to the right. Frames were taken every 5 s. Shown here is a movie consisting of 31 frames played at 5 frames/s. The image is 57 μm x 19 μm. See also Figure 3.47 a-c.

**Video 12.** TeNT Hc carriers colocalise with Texas Red-NGF labelled compartments. MNs were incubated with 40 nM TeNT Hc-Alexa488 and 40 nM Texas Red-NGF for 30 min at 37°C. Cells were then washed and imaged by confocal microscopy. The cell body is
located to the right. Frames were taken every 0.24 s. Shown here is a movie consisting of 159 frames played at 10 frames/s. The image is 47 μm x 7 μm. See also Figure 3.50 a-c.
Chapter 3: Results
3.1 Functional characterisation of recombinant CNT H_c fragments

3.1.1 Expression and characterisation of recombinant CNT H_c fragments

A large amount of work has demonstrated that the H_c portion is responsible for the specific binding of CNTs to neuronal membranes (reviewed in Halpern and Neale, 1995; Herreros et al., 1999; Schiavo et al., 2000). We sought to express this toxin domain in a recombinant, epitope-tagged form and use it for the analysis of CNT binding and trafficking in neuronal cells. Sequence alignments and the recently solved crystal structures of TeNT H_c, BoNT/A and /B (Knapp et al., 1998; Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000; Umland et al., 1997) highlight the similar organisation of CNT H_c fragments and predict that both the folding and the binding activity of the H_c domains are independent of the rest of the neurotoxin molecule. To test this hypothesis, we expressed and purified GST-fusion proteins containing CNT H_c fragments in E. coli (Guan and Dixon, 1991). We concentrated our efforts on the expression and functional characterisation of the H_c fragment of TeNT and of BoNT/A, /B and /E, the three BoNT serotypes most commonly involved in human botulism.

We adopted a PCR approach to amplify BoNT H_c-s using crude genomic DNA from selected toxigenic C. botulinum strains as a template and primers chosen on the basis of CNT sequence alignment. TeNT H_c was already available in the laboratory.

PCR amplification with BoNT/A and E specific primers generated fragments corresponding to the published sequences, whereas with BoNT/B primers the same procedure allowed the isolation of a new BoNT/B variant with 91% identity to M81186 and Y13630 (strain Danish, ATCC 43757, proteolytic) and 88% identity to X71343 (strain Eklund, ATCC25765, non-proteolytic) at the amino acid level (Figure 3.1). The neurotoxin corresponding to this new variant is fully toxic and is immunologically
Figure 3.1 Sequence alignment of CNT H₃ fragments.
Shown in blue is the new BoNT/B strain isolated in our study, followed by the already known proteolytic (Pr) and non-proteolytic (NP) BoNT/B strains and the other CNT H₃s used in our experiments. Highlighted in yellow are the identical residues (asterisks), whereas similar aminoacids are shown in green (double dots). Differing residues are highlighted in red.
indistinguishable from the classical B serotype (M.R. Popoff, personal communication). The sequence of this BoNT/B variant has been deposited in GeneBank with accession number AJ242628.

The amplified CNT $H_c$s were cloned in modified versions of the expression vector pGEX, encoding GST followed by a thrombin cleavage site and either the VSV-G (pGEX-4T3-VSV-G) or the HA (pGEX-4T3-HA) epitopes. The epitope tag is also followed by the protein kinase A consensus motif (Kaelin et al., 1992), allowing radioactive labelling of the expressed fragments for crosslinking experiments (see below) (Figure 3.2 A). This avoids direct chemical modification of the residues within the $H_c$ and minimises the possibility of loss of biological activity. In the case of TeNT, BoNT/A and E, this procedure allowed the isolation of recombinant proteins with an apparent molecular weight ranging from 44 to 47 kDa (Figure 3.2 B, left panel) which are specifically recognised by either anti-VSV-G (Figure 3.2 B, central panel) or anti-HA antibodies (Figure 3.2 B, right panel). In contrast, the same procedure applied to BoNT/B was not successful and led to very limited expression of $H_c$ within inclusion bodies. We screened several E.coli strains with different genotypes for expression of a soluble BoNT/B $H_c$ fragment. When the TOPP3 strain was used, a soluble fusion protein was obtained which, after thrombin cleavage, gave a 48-kDa homogeneous band recognised by an anti-HA antibody (Figure 3.2 B, left and right panels).

3.1.2 Binding of recombinant TeNT and BoNT $H_c$s to neuronal cells

CNTs are known to bind to polysialogangliosides in vitro and this interaction is mediated by the $H_c$ domain (reviewed in Habermann and Dreyer, 1986; Halpern and Neale, 1995). We tested the competence of our recombinant $H_c$ fragments to bind polysialogangliosides
Figure 3.2 Expression of clostridial neurotoxin Hc fragments and binding to polysialogangliosides.

(A) Schematic representation of CNTs. The active holotoxin is composed of two chains (H and L) held together by a single disulfide bridge. The H chain can be further subdivided into two functional subdomains, Hc and Hn. The Hc fragments were tagged with the VSV-G (TeNT and BoNT/A) or with the HA epitope (BoNT/B and /E) at the amino terminus (star) and expressed as GST-fusion proteins. (B) SDS-PAGE profile of the recombinant Hc fragments stained with Coomassie Blue (left panel, 1 μg/lane) or after Western blotting with anti-VSV-G or anti-HA monoclonal antibodies followed by ECL. (C) The Hc fragments of BoNT/B and TeNT bind to polysialogangliosides in a dot-blot assay. Lipids (0.5 μg) were immobilised onto nitrocellulose, overlayed with TeNT and BoNT/B Hc (80 nM) and detected as described in Materials and Methods.
by using a dot-blot assay. As shown in Figure 3.2 C, TeNT \( H_c \) interacts with \( GT_{ib} \) and to a less extent to \( GD_{ib} \), whereas BoNT/B binds \( GT_{ib} \) and \( GQ_{ib} \). No interaction was observed with PC or the monosialilated ganglioside \( GM_1 \), thus confirming the preference of CNTs for a subset of gangliosides containing multiple sialic acid residues (Habermann and Dreyer, 1986; Halpern and Neale, 1995; Schengrund et al., 1991). BoNT/E \( H_c \) mirrors the binding behaviour of BoNT/B \( H_c \), whereas the BoNT/A \( H_c \) presents a much lower interaction with these glycolipids *in vitro* (not shown).

In order to test the \( H_c \) fragments in a more physiological context, we assessed their binding to mouse spinal cord cells, a mixed population of foetal spinal cord cells containing MNs, dorsal root ganglia, glial and stromal cells (Ransom et al., 1977).

The neuronal components of this culture display evoked neurotransmitter release which can be blocked by physiological concentrations of CNTs (Williamson et al., 1996), thus indicating the presence of functional CNT binding sites on their surface.

As shown in Figure 3.3, native TeNT and its recombinant \( H_c \) fragment bound to mouse spinal cord cells in culture with similar distribution. At 4°C, the immunoreactivities of both VSV-G tagged TeNT \( H_c \) fragment (Figure 3.3 A) and TeNT (Figure 3.3 B), present in the incubation medium at a final concentration of 80 nM, revealed a punctate surface distribution along neurites and cell bodies. The staining of TeNT \( H_c \) is specific as demonstrated by competition with an excess of native holotoxin (Figure 3.3 C). The absence of staining of the homogenous layer of non-neuronal supporting cells further demonstrated that TeNT and its \( H_c \) fragment bind selectively to neurons.

The binding of the recombinant \( H_c \) fragments of BoNT/A, B and E is very similar to that observed with TeNT, as shown in Figure 3.3 D-F. Staining is concentrated in distinct patches on the plasma membrane of neurites and the cell soma. Qualitatively, the extent of
Figure 3.3 Binding of clostridial neurotoxin Hc fragments to mouse spinal cord cells.
Spinal cord cells were incubated at 4°C with the Hc fragment of TeNT (A, C), native TeNT (B), the Hc fragments of BoNT/A (D), of BoNT/B (E) or of BoNT/E (F). In panel C, pre-incubation with a 200X excess of native TeNT was able to compete the binding of the TeNT Hc. Binding was detected using anti-VSV-G (A, C, D), anti-TeNT (B) or anti-HA (E, F) antibodies. Bar, 5 μm.
binding with the three BoNT \( H^s \) was lower than the one observed with TeNT. This may reflect a reduced number of membrane acceptors, which in turn could explain the higher concentration of native BoNTs required to observe a physiological intracellular effect (see below). Alternatively, the reduced staining seen with BoNT \( H^s \) could be due to an overall lower binding affinity of these fragments. Taken together, these results show that recombinant CNT \( H_C \) fragments retain their neurospecific binding ability.

### 3.1.3 CNT \( H_C \) fragments block the binding and intracellular activity of native CNTs

For our studies, it was essential to confirm that these recombinant fragments had the ability to recognise the receptor(s) of their parental neurotoxins. For this purpose, we tested whether the CNT \( H^s \) were able to inhibit the intracellular activity of the corresponding holotoxin in spinal cord cultures. As shown in Figure 3.4 A, spinal cord neurons express the SSV protein VAMP, which localises in bright clusters corresponding to synaptic contacts and to points of SSV accumulation. Treatment of the culture for 20 h at 37°C with 100 pM native TeNT caused the complete disappearance of VAMP immunostaining (Figure 3.4 B), due to its specific proteolysis by this neurotoxin (Schiavo et al., 1992a). As previously reported (Osen Sand et al., 1996; Williamson et al., 1996), TeNT-dependent VAMP ablation did not alter neuronal morphology nor affect cell survival.

Pretreatment of cells with TeNT \( H_C \) potently inhibited the proteolytic action of the neurotoxin in a dose dependent manner (Figure 3.4 C-F). Complete retaining of VAMP staining was achieved by pretreatment of the culture with 100 nM of TeNT \( H_C \). This result was confirmed by analysis of VAMP immunoreactivity after Western blotting of extracts from treated cells (Figure 3.4, top right inset).
Figure 3.4 Recombinant TeNT Hc blocks the binding and intracellular activity of the native neurotoxin. Mouse spinal cord cells were incubated for 20 hours at 37°C in the absence (A) or in the presence (B-F) of TeNT holotoxin. Before TeNT addition, samples C-F were treated with increasing amounts of the recombinant TeNT Hc (C, 1 nM; D, 10 nM; E, 100 nM; F, 1 μM). Intact VAMP was then immunodetected with an anti-VAMP-2 antibody. In parallel samples, cells were recovered and levels of VAMP-2 analysed by Western Blot (upper right panel). Bar, 5 μm.
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The effect of the $H_c$ fragment of BoNT/A, B and E on the intoxication mediated by the native neurotoxin was similarly tested. In this case, spinal cord cells were probed with an antibody against the substrate of the botulinum neurotoxin (VAMP-2 for BoNT/B and SNAP-25 for BoNT/A and E), whose staining disappears upon toxin cleavage (Blasi et al., 1993; Schiavo et al., 1993a; Schiavo et al., 1993b). As shown in Figure 3.5 A and G, SNAP-25 localises homogeneously along neurite membranes (Garcia et al., 1995). Cell treatment for 20 h at 37°C with 100 pM of BoNT/A (Figure 3.5 B) and 500 pM BoNT/E (Figure 3.5 H) abolished SNAP-25 immunostaining. SNAP-25 immunoreactivity was preserved when the cells were pre-incubated with 1 μM of the corresponding $H_c$ fragment (Figure 3.5 C and I). BoNT/B was less potent than the other CNTs on these cells and a concentration of 2.5 nM of BoNT/B was necessary to achieve complete loss of VAMP immunostaining (Figure 3.5 E). Under such conditions, only partial protection was elicited by preincubation of cells with BoNT/B $H_c$ (500 nM, Figure 3.5 F). This is consistent with a lower ratio between $H_c$ and native neurotoxin present in the medium. However, another explanation could be the presence of different conformational isoforms in the preparation of BoNT/B $H_c$, only a fraction of which are competent for binding. The immunofluorescence protection experiments were confirmed by Western blot using an anti-VAMP-2 antibody and, for SNAP-25, an antibody recognising both the intact protein and the cleaved fragment (data not shown, experiments performed in collaboration with Dr. J. Herreros).

3.1.4 CNT $H_c$ antagonise CNT-dependent blockade of neuromuscular transmission

BoNTs elicit an irreversible paralysis of the well-established system of the mouse phrenic nerve-hemidiaphragm preparation. The onset of paralysis (which is commonly expressed
Figure 3.5 The recombinant Hc fragments of BoNT/A, /B and /E block the intracellular activity of the corresponding native CNTs.

Mouse spinal cord cells were incubated for 20 hours at 37°C in the absence (A, D, G) or in the presence of native BoNT/A (B, C), BoNT/B (E, F) or BoNT/E (H, I). Prior to CNT addition, samples in C, F and I were treated with the corresponding Hc fragment (C, BoNT/A Hc, 1 μM; F, BoNT/B Hc, 0.5 μM; I, BoNT/E Hc, 1 μM). Target SNAREs were then detected with an anti-SNAP-25 antibody directed against the carboxy-terminus (panels A-C and G-I) or with an anti-VAMP-2 antibody (panels D-F). Bar, 5 μm.
as 50% of paralysis time) is dose-and temperature-dependent and ranges from 23 to 37 min (Table 3.1). Due to its site of action, TeNT is less potent on peripheral synapses with a longer (110 min) 50% of paralysis time (Schmitt et al., 1981; Simpson, 1984a; Simpson, 1984b). We tested the protective activity of the recombinant Hc fragments on the CNT-dependent inhibition of neuromuscular transmission by adding them to the electrophysiological bath (experiments performed in collaboration with Dr. O. Rossetto). All the recombinant Hc fragments antagonised the action of the parental CNT, resulting in a delay in the onset of paralysis (Table 3.1). The increase in the 50% of paralysis time ranged from 75% for BoNT/A and TeNT to more than 150% in the case of BoNT/B and E. This protective effect of Hc is strictly serotype-dependent. In fact, the Hc fragment of BoNT/B did not interfere with the paralysis induced by BoNT/E (not shown), as expected from serotypes not competing for the same cellular acceptors (Evans et al., 1986; Habermann and Dreyer, 1986).

3.2 p15, a putative protein receptor for TeNT

3.2.1 p15 in NGF-differentiated PC12 cells

Polysialogangliosides bind CNTs through the Hc domain with a Kd in the nanomolar range (Halpern and Neale, 1995; Helting et al., 1977; Schengrund et al., 1991; van Heyningen, 1974). The relatively low binding affinity to polysialogangliosides suggests however that these glycolipids are not the only CNT receptors. Additional protein receptor(s) with a higher affinity may participate in CNTs binding as suggested by the observation of protease-sensitive TeNT binding sites in different neuronal preparations (Bakry et al., 1991b; Parton et al., 1988; Pierce et al., 1986; Yavin and Nathan, 1986). Alternatively, a binary complex containing both protein and polysialogangliosides could represent the high
Table 3.1 Effect of recombinant CNT $H_C$ fragments on the 50% time of paralysis in mouse phrenic nerve-hemidiaphragm preparations intoxicated with TeNT and BoNTs.

<table>
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<th></th>
<th>Control CNT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CNT + $H_C$&lt;sup&gt;a&lt;/sup&gt; (+%)&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>BoNT/A</td>
<td>37 ± 6</td>
<td>65 ± 8 (+75)</td>
</tr>
<tr>
<td>BoNT/B</td>
<td>23 ± 5</td>
<td>60 ± 9 (+160)</td>
</tr>
<tr>
<td>BoNTE</td>
<td>24 ± 3</td>
<td>61 ± 7 (+154)</td>
</tr>
<tr>
<td>TeNT</td>
<td>111 ± 11</td>
<td>195 ± 20 (+75)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The values given are the 50% paralysis time values (in min) observed for preparations ($n = 3$) exposed to CNTs alone or in the presence of the correspondent recombinant $H_C$ fragment. Each value represents the mean ± S.D.

<sup>b</sup>Increase in the 50% of paralysis time following pretreatment with $H_C$ expressed as percentage of the control.
affinity receptor (Montecucco, 1986). Crosslinking experiments performed on NGF-differentiated PC12 cells suggested the presence of a putative 20-kDa receptor protein for TeNT (Schiavo et al., 1991). We sought to characterise this protein using the non-toxic recombinant TeNT H₃ fragment in NGF-differentiated PC12 cells, a widely used neuronal-like model system. After differentiation, these cells show extended neurites and an increased number of synaptic-like microvesicles (Tao-Cheng et al., 1995). In our investigation, we used a PC12 subclone that is sensitive to TeNT (Sandberg et al., 1989; Schiavo et al., 1991) and displays high-affinity receptors with pharmacological features similar to those found in mammalian brain (Sandberg et al., 1989). This subclone expresses complex polysialogangliosides before NGF-differentiation (Walton et al., 1988). However, TeNT-induced blockade of ACh release is strictly dependent upon NGF (Figliomeni and Grasso, 1985; Sandberg et al., 1989), suggesting the appearance of one or more TeNT-specific receptors upon NGF differentiation. In agreement with this hypothesis, our recombinant TeNT H₃ did not bind to undifferentiated PC12 cells (Figure 3.6 E), but gave a punctate, discontinuous staining of the plasma membrane in NGF-differentiated cells (Figure 3.6 A), similar to the surface distribution of TeNT or native H₃ (Figure 3.6 C and D). Binding of the recombinant TeNT H₃ is specific as it was completely abolished by pretreatment with an excess of native H₃ (Figure 3.6 B) and is functional, since is leads to internalisation of the toxin fragment into an endocytic compartment when the temperature is raised to 37°C (Figure 3.6 G).

To assess TeNT H₃ binding to putative protein receptors, binding and chemical crosslinking of [³²P]-labelled recombinant TeNT H₃ (200 pM) to intact NGF-differentiated PC12 cells were performed (experiments carried out by Dr. J. Herreros). In the presence of the homobifunctional crosslinker bis[2(succinimidylloxy)carbonyloxy]ethyl)sulphone
Figure 3.6 Binding and internalisation of TeNT and its fragments in NGF-differentiated PC12 cells.
Recombinant TeNT $H_c$ binding at 4ºC was detected with an antibody against the VSV-G tag (A, B and E-H), whereas binding of TeNT (C) and of its native $H_c$ (D) was detected with polyclonal antibodies recognising TeNT holotoxin. Note the similarity of the staining patterns using all forms of TeNT. In B, the interaction of the recombinant $H_c$ is competed by an excess (300X) of native $H_c$. Binding of $H_c$ to undifferentiated cells (E) is indistinguishable from the control without $H_c$ (F). (G) NGF-differentiated PC12 cells internalise recombinant TeNT $H_c$ incubated for 1 h at 37ºC in vesicular structures absent in control cells where the toxin fragment was omitted (H). The amount used for all proteins was 80 nM. G and H are 0.4 μm confocal z-sections. Bars: (A-F) and (G-H) 10 μm.
(BSOCOES), these experiments yielded a = 65-kDa radioactive band in addition to $^{32}$P-H$_C$ (Figure 3.7, lane 3, black arrow), indicating the interaction of H$_C$ with a protein of $\approx$ 15-20 kDa (p15). The 65-kDa crosslinking product was almost absent in undifferentiated PC12 cells (Figure 3.7, lane 1), indicating that the expression of p15 is strongly induced by NGF-differentiation. In similar experiments radiolabelled native H$_C$ generated a crosslinking product identical to that observed with the recombinant H$_C$ (Figure 3.7, lane 5). This result confirms that the recombinant H$_C$ behaves as the native fragment and that it is fully competent for binding to NGF-differentiated PC12 cells.

Additional crosslinking products running at $\approx$ 83 kDa (Figure 3.7, asterisk) and 90 kDa (not shown) were also obtained. Gel filtration experiments had shown that isolated TeNT H$_C$ has the ability to self-associate and form homodimers (Herreros et al., 2000b). The appearance after crosslinking of radioactive bands with molecular mass multiple of that of TeNT H$_C$ is therefore expected. Both the 83- and 90-kDa radioactive products were also observed in crosslinking experiments in the absence of cells (J. Herreros, unpublished observations). Based on these observations, the 65-kDa band appears to be the unique product formed after interaction of H$_C$ with a putative receptor protein.

Preincubation of cells with an excess of unlabelled recombinant, native H$_C$ or TeNT resulted in a strong inhibition of the $^{32}$P-H$_C$ binding and a total disappearance of the 65-kDa band (Figure 3.8, lanes 3-5). In contrast, equal amounts of recombinant BoNT/A or /B H$_C$-s did not affect H$_C$ binding nor the appearance of the crosslinking products (Figure 3.8, lanes 6-7). BoNT/E H$_C$ was the only BoNT fragment that weakly competed both the binding of TeNT H$_C$ and the appearance of the 65-kDa band (Figure 3.8, lane 8) (see Discussion). These experiments indicate that the 65-kDa crosslinking product results from the interaction of TeNT H$_C$ with a putative protein receptor highly specific for TeNT.
Figure 3.7 TeNT Hc interacts with a 15-kDa protein in NGF-differentiated PC12 cells.

Binding and crosslinking of $^{32}$P-recombinant Hc to undifferentiated (lane 1) and to NGF-differentiated PC12 cells (lanes 2-3) in the presence or absence of crosslinker (BSOCOES, bis[2(succinimidylcarboxyloxy)ethyl]sulphone). Binding and crosslinking of $^{125}$I-native Hc to NGF-differentiated PC12 cells (lanes 4-5). The formation of a $\approx 65$-kDa radioactive product (arrow) is observed after crosslinking using either Hc. The asterisk points to the band corresponding to Hc dimer (see text).
Figure 3.8 Characterisation of TeNT Hc binding to p15 in NGF-differentiated PC12 cells.
Binding and crosslinking of \[^{32}\text{P}]\cdot\text{Hc} to NGF-differentiated PC12 after preincubation with different cold competitors (2 \(\mu\text{M}\)) (\(\text{Hc}\), recombinant TeNT Hc; \(\text{nHc}\), native TeNT Hc; T, TeNT holotoxin; A, B and E, recombinant Hc fragments of BoNT/A, B or E, respectively). The appearance of the 65-kDa product (arrow) is competed by TeNT Hc fragments (lanes 3-4) and TeNT holotoxin (lane 5) and weakly by BoNT/E Hc (lane 8).
High-ionic strength washes and carbonate stripping experiments revealed that p15 behaves like an integral membrane protein (Herreros et al., 2000a). In addition, this putative TeNT receptor is N-glycosylated, since incubation of crosslinked samples with N-Glycosidase F (PNGase F), an enzyme able to efficiently remove N-linked glycans of glycoproteins (Tarentino and Plummer, 1994), caused a specific increase in the mobility of the 65-kDa crosslinking product (Herreros et al., 2000a).

### 3.2.2 p15 in rat MNs

TeNT enters the organism at the NMJ from where it is retrogradely transported to block neurotransmission at spinal cord inhibitory synapses. High affinity receptors at the NMJ account for an efficient uptake of very low amounts of TeNT from the extracellular fluid. Spinal cord MNs therefore represent the physiological targets for TeNT binding and intracellular sorting. To further characterise TeNT binding in this cell, we prepared a culture of purified MNs from E14 rat embryos, based on previously established protocols (Arce et al., 1999). The presence of this cell type in the culture was confirmed by immunostaining with antibodies against the MN markers Islet 1/2 (Ericson et al., 1992) (Figure 3.9). Typically, the purity of the culture was in the 60-90% range.

We first tested embryonic rat MNs for their ability to bind TeNT H<sub>c</sub> at different stages of differentiation in culture. Binding experiments performed at 4°C revealed an extensive punctate surface staining of the plasma membrane both on the MN cell body and along the neurites (Figure 3.10, top row), similar to that found in mouse spinal cord neurons and NGF-differentiated PC12 cells (Figures 3.3 A and 3.6 A). This staining was present from the first day in culture and did not vary in intensity or in pattern during in vitro differentiation. In a similar fashion, when cells were incubated with TeNT H<sub>c</sub> at 37°C the
Figure 3.9 Staining with Islet-1/2 confirms the presence of MNs in the culture.
2-day-old MNs were fixed, detergent-permeabilised and immunostained overnight with a mix of anti-Islet-1/2 antibodies. The positive nuclear staining confirms the presence of MNs in the culture (a). b shows the phase-contrast images of a, whereas c is a negative control where the primary antibodies were omitted. Bar, 10 μm.
Figure 3.10 MNs bind and internalise TeNT H₆ during differentiation in culture.

1-day-, 4-day-, or 8-day-old MNs were incubated with 40 nM VSV-G-tagged TeNT H₆ for 30 min at 4°C (a, c, e), or for 1 h at 37°C (b, d, f), fixed and immunostained with an anti-VSV-G antibody. Cells incubated at 37°C were permeabilised for TeNT H₆ detection. Binding of TeNT H₆ gives a punctate staining on the neuronal plasma membrane, whereas internalisation of the toxin fragment reveals a vesicular pattern diffused throughout the cells at all stages of development. Bar, 10 μm.
toxin fragment was internalised in vesicular structures independent of the number of days in culture (Figure 3.10, bottom row).

When MNs were used in TeNT $^{32}$P-Hc crosslinking experiments, a product of ~62-kDa was found in addition to the 83- and 90-kDa bands previously mentioned (Figure 3.11, lane 2) (experiments performed by Dr. J. Herreros). This crosslinking product was clearly observed from the 2nd-3rd day in culture (data not shown). The 62-kDa band displayed many features of the corresponding product in NGF-differentiated PC12 cells, providing indirect evidence that TeNT binding could be similar in the two cellular systems. In fact, the 62-kDa band also appeared to be highly specific for TeNT in rat MNs (Figure 3.11), since it could be competed by preincubation with cold Hc or with TeNT (Figure 3.11, lanes 3 and 4), but not by BoNT/A Hc (Figure 3.11, lane 5) or by BoNT/B Hc (not shown). Consistent with the results obtained in the NGF-differentiated PC12 cells, only BoNT/E Hc partially competed the binding of TeNT $^{32}$P-Hc to rat MNs, while inhibiting the formation of the 65-kDa band (Figure 3.11, lane 6), suggesting that this neurotoxin may interact with the same protein receptor of TeNT. In agreement with this observation, a crosslinking product equivalent to the one formed by TeNT $^{32}$P-Hc was obtained after binding of BoNT/E $^{32}$P-Hc to MNs after at least 10 days in culture (J. Herreros, data not shown).

Similar ~60-kDa and 62-kDa crosslinking products were also obtained after TeNT $^{32}$P-Hc binding and crosslinking to human neuroblastoma cells neuronally-differentiated with retinoic acid and to a mixed population of mouse spinal cord cells in culture (Herreros et al., 2000a). p15s from different cell types therefore display slight differences in their apparent mobility in SDS-PAGE. This result could be explained by cell- and species-

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Figure 3.11 TeNT H_c interacts with a 15-kDa protein in rat MNs.
Binding and crosslinking of [{superscript}32P]-H_c to one week old rat MNs with or without preincubation with cold competitors (abbreviations as in figure 3.8). [{superscript}32P]-H_c binding and the formation of the ≈ 62-kDa band (arrow) are significantly decreased by preincubation with H_c and TeNT holotoxin (lanes 3-4) and partially competed by BoNT/E H_c (lane 6).
specific glycosylation patterns and is supported by the finding that the mobility of the TeNT Hc-p15 complex is altered by glycosidases.

3.2.3 Identification of the minimal TeNT Hc binding domain

3.2.3.1 Expression and characterisation of TeNT Hc sub-domains

As shown by the structures of TeNT Hc and BoNT/A (Knapp et al., 1998; Lacy et al., 1998; Umland et al., 1997), CNT Hc fragments share a common structure consisting of two sub-domains of almost identical size predicted to fold independently. The amino-terminal sub-domain (HcN) is structurally related to plant lectins, whereas the carboxy-terminal sub-domain (HcC) exhibits a β-trefoil motif present in proteins involved in recognition and binding. To further clarify the contribution of HcN and HcC in TeNT neuronal binding and internalisation, we expressed them in a recombinant form, using the same type of approach previously adopted for the CNT Hc fragments.

We expressed TeNT HcC and HcN in E. coli and purified them as soluble GST fusion proteins. The sub-domains were amplified by standard PCR from TeNT Hc (see Material and Methods) and cloned into the pGEX-4T3-VSV-G vector containing the VSV-G epitope and a protein kinase A site (Figure 3.12 A). After cleaving the GST moiety, the two TeNT sub-domains run as 25 kDa (HcC) and 29 kDa (HcN) bands (Figure 3.12 B, left panel). The proteins can be radioactively phosphorylated in vitro (Figure 3.12 B, central panel) and can be detected by a monoclonal antibody against the VSV-G tag (Figure 3.12, right panel).
Figure 3.12 Expression of recombinant TeNT Hc and its sub-domains, Hc.C and Hc.N.
(A) Schematic representation of the recombinant TeNT Hc sub-domains expressed as GST-fusion proteins. Thrombin cleavage releases the protein containing a kinase site (P) for radiolabelling and the VSV-G tag at the amino-terminus. (B) Coomassie staining (3 μg/lane), [32P]-autoradiography (100,000 cpm/lane, 1.5 ng) and Western blotting (0.3 μg/lane) with an anti-VSV-G monoclonal antibody.
3.2.3.2 Binding of TeNT H_eN and H_eC to neuronal cells

We first tested if the recombinant H_eN and H_eC sub-domains were able to bind to the neuronal surface. Binding of H_e and H_eC to NGF-differentiated PC12 cells at 4°C revealed a very similar punctate pattern on the plasma membrane, both in cell bodies and neurites (Figure 3.13 A and B). In contrast, binding of H_eN to NGF-differentiated PC12 cells was negligible and comparable to control samples where the binding fragment was omitted (Figure 3.13 C and D). Similar results were also obtained with purified MNs (not shown), suggesting that the isolated H_eC domain contains the main determinants for TeNT neurospecific binding.

3.2.3.3 TeNT H_eC is sufficient for the interaction with p15

The ability of the recombinant TeNT sub-domains to interact with the putative protein receptor p15 was tested in crosslinking experiments. Preincubation of cells with cold H_eC potently inhibited the binding of radioactive H_e and abolished the appearance of the 65-kDa crosslinking product (Figure 3.14 A, lane 3). In contrast, H_eN did not alter the total binding of H_e nor its interaction with the putative 15-kDa protein receptor (Figure 3.14 A, lane 4). Consistently, the interaction of [^{32}P]-H_eN with the cellular membrane was extremely poor and did not generate any detectable crosslinking product (Figure 3.14 B, lanes 5-8). In contrast, [^{32}P]-H_eC binding to NGF-differentiated PC12 cells was similar to that of [^{25}P]-H_e and yielded a = 45-47- kDa crosslinking product (Figure 3.14 B, lane 2), likely to represent the interaction of [^{32}P]-H_eC with p15. Binding of radiolabelled H_eC and appearance of the crosslinking product were abolished by preincubation with cold H_eC (Figure 3.14 B, lane 3), but not by H_eN (Figure 3.14 B, lane 4). Preincubation with H_eN promoted the additional formation of a = 50-kDa product (Figure 3.14 B, lane 4, empty
Figure 3.13 Binding of TeNT Hc and of its sub-domains in NGF-differentiated PC12 cells.
Binding of 80 nM TeNT Hc (A), Hc-C (B), Hc-N (C) to NGF-differentiated PC12 cells detected with antibodies against the VSV-G tag.
Binding of Hc (A) and Hc-C (B) is very similar and gives a punctate staining on the plasma membrane of neurites and cell bodies. Binding of Hc-N (C) is negligible and identical to the control without toxin fragment (D). Bars: (A-B) and (C-D), 10 μm.
Figure 3.14 The carboxy-terminal sub-domain of Hc interacts with the putative 15-kDa TeNT protein receptor.

(A) Competition of the [32P]-Hc binding to NGF-differentiated PC12 cells by preincubation with cold HcN and HcC sub-domains. Addition of HcC completely abolishes [32P]-Hc binding (lane 3), whereas HcN is ineffective (lane 4). (B) [32P]-HcC (lanes 1-4) and [32P]-HcN (lanes 5-8) binding and competition by cold sub-domains. [32P]-HcC binds to p15 in NGF-differentiated PC12 cells and forms a ~45-kDa crosslinking product (dark arrow, lanes 2 and 4). After pre-treatment with cold HcN (lane 4), an additional crosslinking product of slightly higher molecular weight is formed (empty arrow) which is likely to correspond to the HcC/HcN heterodimer. Cold HcC completely abolishes [32P]-HcC binding (lane 3), whereas HcN has no effect (lane 4). [32P]-HcN binds very poorly (lanes 5-8) and the binding is not altered by the presence of cold HcC (lane 7).
Results

arrow) likely to represent HcC-HcN heterodimers. Taken together, our data demonstrate
that the HcC sub-domain is necessary and sufficient for the interaction with p15 and with
the neuronal surface. Photolabelling experiments and deletion analysis had previously
shown that the last 23-24 residues at the carboxy-terminus of TeNT interact directly with
polysialogangliosides (Halpern and Loftus, 1993; Shapiro et al., 1997). These
observations, together with the crosslinking results, strongly indicate that the HcC sub-
domain could be considered the core domain for TeNT neurospecificity, able to interact
with both polysialogangliosides and protein components on the neuronal membrane.

3.2.4 Characterisation of p15

3.2.4.1 p15 is a GPI-anchored protein

We focussed our efforts on the identification of the putative TeNT receptor p15. As
mentioned above, p15 is N-glycosylated and appears to be an integral membrane protein
in detergent-partitioning experiments (Herreros et al., 2000a). This behaviour could be due
to the presence of transmembrane domain(s) or to various forms of covalent lipid
modification. GPI-anchored proteins are abundant components of the plasma membrane,
which behave as integral membrane proteins. Their biochemical analysis is simplified by
the use of phosphoinositol specific-phospholipase C (PI-PLC), an enzyme that causes their
selective release from the membrane. We therefore tested the possibility that p15 is GPI-
anchored. Pretreatment with PI-PLC before TeNT Hc binding and crosslinking inhibited
the formation of the previously characterised ≈ 65-kDa crosslinking product in NGF-
differentiated PC12 cells in a dose-dependent manner (Figure 3.15, filled arrowhead)
(Herreros et al., 2001), whereas total binding was not significantly affected (Figure 3.15,
empty arrowhead). Formation of the corresponding crosslinking products in MNs and
Figure 3.15 p15, the putative TeNT protein receptor, is GPI-anchored. Binding and crosslinking of [$^{32}$P]-labelled TeNT Hc after pretreatment of NGF-differentiated PC12 cells, rat MNs and mouse spinal cord cells with PI-PLC. The empty arrowhead indicates cell-bound TeNT Hc and filled arrowheads point to the corresponding crosslinking products, which do not form after PI-PLC treatment.
spinal cord cells (Figure 3.15, filled arrowheads) was also inhibited by PI-PLC pretreatment. These results indicate that p15s, proteins interacting with TeNT Hc in our crosslinking assays, are GPI-anchored proteins in all three neuronal cell-types.

3.2.4.2 p15 is Thy-1 in NGF-differentiated PC12 cells

Among other strategies utilised to identify p15s, we searched for known proteins with the properties described above for the crosslinking product. Thy-1 (Williams and Gagnon, 1982), a major cell-surface GPI-anchored glycoprotein of 25-29-kDa present in thymocytes and brain and expressed in NGF-differentiated PC12 cells (Jeng et al., 1998; Richter-Landsberg et al., 1985), represented a possible candidate. Immunoprecipitation experiments performed using anti-Thy-1 antibodies pulled down the 65-kDa product in crosslinked NGF-differentiated PC12 cells, whereas mock antibodies or antibodies against prion protein, a GPI-anchored protein similar in size to Thy-1, failed to do so (Herreros et al., 2001). In addition, anti-Thy-1 but not mock antibodies immunoprecipitated the crosslinking product obtained after binding of 32P-labelled HcC to NGF-differentiated PC12 cells, consistent with the hypothesis that TeNT Hc and HcC are able to interact with the same protein component on the plasma membrane (Herreros et al., 2001). The direct interaction of TeNT Hc with Thy-1 in its membrane environment was further confirmed by fluorescence lifetime imaging microscopy (FLIM) (Herreros et al., 2001). Taken together, these results demonstrate that in NGF-differentiated PC12 cells the putative protein receptor p15 is Thy-1, a GPI-anchored protein able to specifically interact with TeNT Hc on the plasma membrane.
3.2.4.3 *p15 is not Thy-1 in spinal cord cells and MNs*

Based on the results obtained with NGF-differentiated PC12 cells, Thy-1 represented a good candidate as a protein receptor for TeNT in neuronal cells. However, several lines of evidence demonstrate that this protein is not essential for TeNT binding and internalisation in neurons. In fact, anti-Thy-1 antibodies were not able to immunoprecipitate the crosslinking product obtained after binding of TeNT Hc to the surface of either mouse spinal cord cells or rat MNs (J. Herreros, unpublished data). Moreover, crosslinking experiments performed in spinal cord cells isolated from Thy-1 knockout mice (Nosten-Bertrand et al., 1996) still showed the 65-kDa product observed in wild type cells (J. Herreros, unpublished data). Similarly, TeNT Hc binding and internalisation detected by immunofluorescence in the same Thy-1 knockout spinal cord cells did not show any apparent difference from wild type cells (Figure 3.16). Finally, Thy-1 knockout mice show only a very limited resistance to TeNT intoxication compared to wild type animals (J. Herreros and G. Schiavo, unpublished data). TeNT Hc therefore interacts with a GPI-anchored protein on the neuronal surface different from Thy-1.

3.2.4.4 *TeNT is found in lipid rafts in neuronal cells*

Many GPI-anchored proteins are preferentially localised in sphingolipid- and cholesterol-enriched membrane microdomains or lipid rafts (Simons and Toomre, 2000). TeNT interaction with Thy-1 or other GPI-anchored proteins could therefore be interpreted as an indication of the entry of the toxin in a raft environment and used as a probe to follow the recruitment of TeNT into lipid microdomains.

Isolation of detergent-insoluble microdomains (DIGs) is one of the most widely used methods for studying lipid rafts (Brown and London, 1998). To directly investigate
Figure 3.16 Thy-1 is not essential for binding and internalisation in neuronal cells.

15 day old spinal cord cells form wild type (wt) and Thy-1.2 knockout mice were incubated with 20 nM VSV-G-tagged TeNT Hc at 4°C, washed, fixed and immunostained to detect surface binding of the toxin fragment (left and centre panels, upper row). Alternatively, cells were washed after the incubation with TeNT Hc and warmed up to 37°C for 30 min before fixation. Internalised TeNT Hc was detected after detergent permeabilisation (left and centre panels, bottom row). All images are 0.4 μm confocal z-sections. The staining of wild-type and Thy-1-deficient neurons appears similar in both experiments and is specific, since control samples where the toxin fragment was omitted showed very limited background levels (right panels, both rows). Bar, 10 μm.
whether TeNT Hc binds to lipid microdomains, DIGs were prepared after binding TeNT Hc at 4°C to mouse spinal cord cells. Isolated DIGs contained ~ 60% of the total bound toxin fragment, suggesting that lipid rafts could indeed act as specialised domains for TeNT binding (Herreros et al., 2001). Consistent with this result, bound TeNT Hc displays a punctate pattern on the neuronal membrane (Figures 3.17 a), which is reminiscent of lipid raft markers (Harder et al., 1998; Mayor et al., 1994) and is similar to the surface staining for Thy-1 observed in NGF-differentiated PC12 cells (Jeng et al., 1998). Interestingly, Thy-1 also displays a punctate pattern on the surface of unpermeabilised MNs (Figure 3.17 c). Many proteins enriched in lipid rafts have been shown to rearrange in clusters upon antibody-mediated crosslinking (Harder and Simons, 1997). Fixation of MNs after incubation with primary and secondary antibodies caused the appearance of more intense and apparently larger TeNT Hc patches compared to cells fixed before antibody addition (Figure 3.17, compare a with b). Such change in surface staining cannot be ascribed to an artefactual variation in the immunogenic properties of the VSV-G tag by chemical modification with paraformaldehyde, since the VSV-G epitope lacks lysine residues. This finding suggests that TeNT Hc could interact with specialised microdomains able to rearrange upon antibody-mediated crosslinking. Thy-1, which is generally considered to be a neuronal raft marker (Madore et al., 1999) displays a similar behaviour (Figure 3.17, compare c with d). When cells were fixed at the end of the immunostaining procedure, the lack of colocalisation between TeNT Hc and Thy-1 was evident (Figure 3.17, e and f with Thy-1 in green and TeNT Hc in red). However, FESEM analysis of MNs where surface Thy-1 and bound TeNT Hc were immunodetected with gold particles of different size (Figure 1.18) showed that both proteins are concentrated in clusters on the neuronal membrane, suggesting a possible antibody-mediated recruitment
Figure 3.17 Antibody-mediated clustering of TeNT H<sub>c</sub> and Thy-1 on the neuronal surface.
MN<sub>s</sub> were incubated with 40 nM VSV-G-tagged TeNT H<sub>c</sub> at 4°C and either fixed immediately before immunostaining (<i>a</i>), or fixed at the end after incubation with primary and secondary antibodies (<i>b</i>). Alternatively, cells were fixed and stained for Thy-1 (<i>c</i>) or stained first for Thy-1 and then fixed (<i>d</i>). Both TeNT H<sub>c</sub> and Thy-1 show a brighter staining pattern concentrated in larger clusters on the neuronal surface when fixation is performed at the end, after incubation with primary and secondary antibodies (<i>b, d</i>). Antibody-mediated clustering in adjacent domains is visible when cells were stained for both TeNT H<sub>c</sub> and Thy-1 and subsequently fixed (<i>e, f</i>). All pictures are projections of a stack of confocal z-sections. Bars: (<i>a-b</i>) and (<i>c-e</i>) 10 μm; (<i>f</i>) 5 μm.
Figure 3.18 TeNT Hc and Thy-1 are found in clusters on MN neurites upon antibody-mediated crosslinking. MNs were incubated with 40 nM VSV-G-tagged TeNT Hc for 40 min at 4°C. Immunogold staining was performed with anti-Thy-1 and anti-VSV-G antibodies before fixation and processing for FESEM examination. (a) In the backscattering mode, gold particles appear as bright spots (here pseudocoloured in green) on the surface of MN neurites (pseudocoloured in red). (b) High-magnification detail of a neurite bundle. Gold particles (TeNT Hc, 5 nm gold, white arrows, Thy-1, 10 nm gold, yellow arrowheads) are distributed in clusters on the neuronal surface.
in a similar raft microenvironment. In addition, the fact that the two identified TeNT ligands, polysialogangliosides and GPI-anchored proteins, are enriched in lipid rafts (Jacobson and Dietrich, 1999; Prinetti et al., 2000; Simons and Toomre, 2000) suggests that these membrane microdomains could act as concentration platforms for TeNT binding as demonstrated for other toxins, like the GPI-protein-interacting aerolysin (Abrami and van der Goot, 1999).

A large body of work has established the use of cholesterol-sequestering drugs to disrupt membrane rafts (Simons and Toomre, 2000). Treatment of cells with these drugs has led to the conclusion that cholesterol plays a crucial architectural role in the organisation of lipid microdomains, although some raft components may behave differently according to the cell type and the concentration of the cholesterol-sequestering drug used (Abrami and van der Goot, 1999; Lipardi et al., 2000). One of the most commonly used compounds able to disrupt rafts is methyl-β-cyclodextrin (MCDX) (Neufeld et al., 1996).

In spinal cord cells, TeNT Hc binds to distinct lipid rafts sub-pools which display different sensitivities to MDCX (Herreros et al., 2001). The effect of MCDX on the recruitment of TeNT Hc to DIGs suggests that cholesterol might play an important role in the internalisation or intracellular sorting of TeNT. In spinal cord cells MCDX-mediated cholesterol depletion blocked TeNT Hc internalisation and prevented TeNT-induced VAMP cleavage in a dose-dependent fashion (Herreros et al., 2001). These observations suggest that cholesterol could play an important role not only in the surface interaction of TeNT with lipid microdomains, but also in ensuring its correct intracellular trafficking.
3.3 A retrograde transport assay in living MNs

3.3.1 Production and characterisation of a fluorescent TeNT Hc

In MNs, TeNT binding to the neuronal surface is followed by internalisation and retrograde transport to the cell body. Several reports have shown that TeNT Hc is responsible not only for the specific binding but also for retrograde transport of the full-length toxin in neurons (reviewed in Halpern and Neale, 1995; Schiavo et al., 2000). Indeed, TeNT Hc undergoes axonal retrograde transport in vivo, as shown by its accumulation in the CNS following intramuscular injection (Bizzini et al., 1977; Coen et al., 1997; Fishman and Carrigan, 1987; Morris et al., 1980; Sinha et al., 2000). We therefore decided to use TeNT Hc to create a novel and specific tool to investigate retrograde trafficking routes in living MNs. TeNT Hc sequence was excised from the pGEX-4T3-VSV-G vector and inserted into the pGEX-4T3-Cys expression vector (see Materials and Methods). This approach allowed the production of a recombinant TeNT Hc protein containing a small α-helical domain at the N-terminus (Figure 3.19, hatched box). This domain contains 4 cysteines with a spatial organization suitable for the binding of the biarsenical fluorescein derivative FLASH (Griffin et al., 1998). The cysteine-rich tag is also the preferred site of modification with other thiol-specific fluorescent reagents, such as the Alexa488, 546 or 594 maleimides. Multiple fluorophores can therefore be covalently coupled to this TeNT Hc fragment upstream of its coding sequence, thus avoiding the danger of perturbing the carboxy-terminal portion which is crucial for neurospecific binding (Halpern and Loftus, 1993; Sinha et al., 2000).

We tested whether fluorescent TeNT Hc still retains its binding and internalisation properties in MNs. At 4°C fluorescent TeNT Hc bound to MNs independent of the dye used for labeling (Figure 3.20 a and data not shown). This binding was specific as
Figure 3.19 Fluorescent labelling of Cys-TeNT Hc.
A recombinant TeNT Hc with a cysteine-rich domain at the N-terminus (hatched box) is expressed as a GST-fusion protein. The boxed segments are predicted to form α-helices with the thiol groups of cysteines (black boxes) favourably oriented to bind the reactive group of the fluorescein derivative FLASH (X = arsenic) or different Alexa fluorophores (X = maleimide).
Figure 3.20 Fluorescent TeNT H₂c specifically binds and is internalised into living MNs.

a and b show the projections of a stack of confocal z-sections of fixed cells, while c-h display living MNs imaged by low-light microscopy.

(a) 30 nM TeNT H₂c-Alexa488 binding at 4°C to rat MNs. (b) Plasma membrane staining is abolished by preincubation with a 100X excess of unlabelled TeNT H₂c. TeNT H₂c-FLASH (c), TeNT H₂c-Alexa488 (d), TeNT H₂c-Alexa 546 (e), TeNT H₂c-Alexa594 (f) (all 40 nM) are internalised in vesicular structures in MNs (arrowheads). (g) Binding and internalisation of TeNT H₂c-Alexa488 are competed by preincubation with a 100X excess of native TeNT. (h) Phase contrast picture of the correspondent image in g. Bars, 10 μm.
demonstrated by competition with an excess of unlabeled VSV-G-tagged TeNT He (Figure 3.20 b). When cells were warmed to 37°C, the differently-labeled He fragments were internalised in vesicular structures (Figure 3.20 c-f; arrowheads). Binding and internalisation were prevented by preincubation with an excess of TeNT (Figure 3.20 g and h), indicating that fluorescent TeNT He retains the ability to bind to the same surface acceptors of the native holotoxin and to be internalised in MNs.

3.3.2 Fluorescent TeNT He is retrogradely transported in living MNs

We chose to use TeNT He labelled with Alexa 488 to follow retrograde transport in living MNs since this dye was the brightest of the Alexa maleimides and because FLASH had a high photobleaching rate. After several trials, we chose a concentration of TeNT He-Alexa488 in the range 25-40 nM, based on the sensitivity of our imaging system and optimal exposure time to minimize phototoxicity. MNs were incubated with fluorescent TeNT He for 15 min at 37°C, washed and imaged by confocal or low-light microscopy. Typically, the plasma membrane appeared highly stained, particularly at neurite contacts and synaptic sites (see Figure 3.26 a). Cell bodies and dendrites were also brightly stained (Figure 3.21 a), suggesting that TeNT He binding sites are not restricted to the axonal surface in cultured MNs. Tubular compartments were visible in the soma especially at early time points (up to 45 min after the end of incubation with TeNT He). A closer analysis of dendrites revealed the presence of both round, mostly stationary and bi-directional tubular endosomes (video 1).

Internalisation of TeNT He in the somatodendritic domain is a fast process, since vesicular structures appeared immediately after removing the fluorescent protein, whereas fluorescent carriers appeared in axons approximately 20 min after washing the cells.
Figure 3.21 Visualisation of TeNT Hₐ retrograde carriers in living MNs.
Cells were incubated with 40 nM TeNT Hₐ-Alexa488 for 15 min at 37°C, washed and imaged by low-light time-lapse microscopy. (a) Low magnification image of a MN displaying vesicular staining. (b-g) Time series imaging of the axon in the boxed area in a. Intervals between frames are 5 s. Note the presence of both round vesicles (arrow) and tubular structures (arrowhead) travelling toward the cell body. See also videos 1 and 2. (h-j) Example of a tubular endosome bending during retrograde transport along a single axon (arrowhead). A slower round vesicle is also indicated (asterisk). The cell body is located out of view at the bottom of the picture. (k-m) Three different examples of "vesicle clusters" observed along the same axon (arrowheads). Bars: (a-g) 5 μm; (h-m) 2 μm.
Remarkably, the vast majority of these carriers moved in a retrograde fashion, while anterograde vesicles were extremely rare. This lag between binding and appearance of TeNT Hc may be due to a maturation phase of the endocytic compartment preceding the loading on transport tracks, as previously shown for ligands undergoing receptor-mediated endocytosis (Schmid et al., 1988; Ure and Campenot, 1997).

Low-light time-lapse microscopy revealed the presence of pleiomorphic TeNT Hc carriers (Figure 3.21 b-g; see also videos 1 and 2). We observed slow, usually saltatory, round vesicles and fast tubules with an apparently continuous movement. Only a small fraction of the vesicles were stationary or oscillating, and these appeared as bright round spots scattered along axons. Many vesicles seemed to stop briefly at the same points along axons before continuing their retrograde movement. We also observed long tubules bending while moving, suggesting a switch of transport tracks en route (Figure 3.21 h-j, arrowhead). In some cases the tubules appeared to collapse into round-oval structures during brief stationary periods, indicating that vesicular carriers could stretch into tubules during fast movement and then return to their original shape during pauses. However, many tubules (with an apparent length of up to 5 μm) retained their shape while pausing. Another typical feature was the presence of apparent “vesicle clusters” moving simultaneously (Figure 3.21 k-m). TeNT could therefore reach the MN cell body through pleiomorphic retrograde carriers.

3.3.3 Fluorescent TeNT Hc is internalised through a specific mechanism

We confirmed that the observed TeNT Hc carriers are endosomes by incubating MNs with TeNT Hc-Alexa488 and Texas Red-dextran (molecular weight 3000), a general endocytic marker (Nakata et al., 1998) (Figure 3.22). Simultaneous confocal observation in both
Figure 3.22 Fluorescent TeNT \( H_c \) is internalised in endocytic carriers.
MN were incubated with 40 nM TeNT \( H_c \)-Alexa488 together with 0.2 mg/ml Texas Red-dextran (mol wt 3000) for 30 min at 37°C. Cells were then washed and imaged with a confocal microscope. TeNT \( H_c \) (a) is present in endosomes that also contain Texas Red-dextran (b) (arrowheads). Note the presence of an endocytic vesicle that contains only dextran (b, arrow). (c) Merged image of a and b. (d) Corresponding DIC image. Bar, 5 \( \mu \)m.
Figure 3.23 Fluorescent TeNT Hc is internalised in living MNs. MNs were incubated with TeNT Hc-Alexa488, washed and imaged with low-light microscopy. (a) Vesicular compartments appear approximately 30 min after the end of incubation (arrowheads). (b) TeNT Hc endocytic organelles are still visible after effective quenching of plasma membrane staining with the addition of an anti-Alexa488 antibody. Bar, 10 μm.
green (Figure 3.22 a) and red channels (Figure 3.22 b) revealed colocalisation of TeNT Hc carriers with Texas Red-labeled vesicles (Figure 3.22 a-c, arrowheads). 44 % of the endosomes (on a total of 206) were positive only for Texas Red-dextran, suggesting that TeNT Hc-labelled organelles represent a major endosomal population in MNs at this stage of differentiation. Moreover, fluorescent TeNT Hc carriers could still be observed after incubation of MNs with an excess of anti-Alexa488 antibody that quenched the fluorescence due to toxin fragment still bound to the neuronal surface (Figure 3.23).

Several experimental findings indicate that TeNT Hc internalization is not due to nonspecific fluid phase endocytosis. First, incubation of MNs with fluorescent TeNT Hc at 4°C followed by washing and subsequent warming to 37°C still led to plasma membrane staining and appearance of retrograde vesicular carriers (see also below, Figure 3.33 a). Second, a complete lack of neuronal labelling was observed after incubation of cells with a protein carrier (trypsin inhibitor) labeled with Alexa488 (Figure 3.24 a) or with a similar amount of free fluorescent dye (Figure 3.24 c). Finally, we coincubated MNs for 20 min at 4°C with 40 nM TeNT Hc-Alexa488 and a 50X molar excess of Texas Red-BSA (1.8 μM). After washing, cells were warmed to 37°C and imaged by confocal microscopy. Only endosomes containing TeNT Hc were observed in axons (Figure 3.25, arrows), while no BSA-labeled vesicular structures could be detected (Figure 3.25 b). Taken together, these results indicate that fluorescent TeNT Hc is internalised in a subset of endocytic compartments through a specific uptake mechanism.

We examined the binding and internalisation of TeNT Hc in MNs by EM. Cells were incubated with TeNT Hc at 4°C, and subsequently immunogold-stained. We detected TeNT Hc on the axonal plasma membrane (Figure 3.26 b), coated membrane invaginations (Figure 3.26 c), synaptic sites and neurite contacts (Figure 3.26 d and e), as
Figure 3.24 Control proteins labelled with Alexa488 and free Alexa488 do not bind to living MNs.

MNs were incubated with trypsin inhibitor-Alexa488 (a, b) or free Alexa488 dye (c, d) in order to have in the medium a final concentration of fluorophore similar to the one used for TeNT H₄-Alexa488. In both cases MNs did not show any fluorescent staining. b and d are the corresponding phase images of a and c, respectively. Bar, 10 μm.
Figure 3.25 TeNT H₄ is internalised by a specific uptake mechanism. MNs were incubated with 40 nM TeNT H₄-Alexa488 and 2 μM Texas Red-BSA for 20 min at 4°C. Cells were then washed, warmed to 37°C and imaged. (a) Endosomes containing TeNT H₄-Alexa488 are visible (arrows), but they are not labelled by Texas Red-BSA (b). (c) Merged image of a and b. (d) Corresponding DIC image. The asterisk marks debris. Bar, 5 μm.
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observed with fluorescent TeNT Hc in living cells (Figure 3.26 a). When neurons were subsequently shifted to 37°C for 5 min, gold particles were associated with deep pits that pinched off forming coated vesicles (Figure 3.26 f-g). Upon warming to 37°C for 30 min, TeNT Hc was found in uncoated tubulo-vesicular and round endocytic structures (Figure 3.26 h-i), consistent with the pleomorphic retrograde carriers observed in living MNs (Figure 3.21), whereas only a few gold-decorated MVBs were observed (not shown). We could not detect structures similar to the “vesicle clusters” observed in living cells (Figure 3.21 k-m), possibly because the appearance of these compartments is due to the accumulation of fluorescent protein in different domains of the same tubular endosome. The diameter of the vesicular carriers varied between 50 and 100 nm, whereas the length of tubulo-vesicular structures was up to 2-3 μm. Similar results were obtained by using gold-conjugated TeNT Hc and are in agreement with a previous study performed using TeNT in mixed spinal cord cells (Parton et al., 1987).

3.3.4 Tracking of TeNT Hc carriers reveals different components of retrograde transport

The kinetics of retrograde transport in living MNs can be analysed in our experimental system by following the displacement of the TeNT Hc-labelled retrograde carriers. A representative analysis is shown in Figure 3.27 a. Two main groups of structures undergoing retrograde transport can be distinguished: tubules (Figure 3.27 a-b, filled circles), which showed a fast movement, and bright round vesicles (Figure 3.27 a and c, open squares) with a slower speed and frequent stationary periods. Other types were the “vesicle clusters” previously described (Figure 3.27 a and c, asterisks), rare bright oval bodies and, more frequently, faint round vesicles (Figure 3.27 a, half-filled squares), which displayed all types of movement previously described for the other groups of
Figure 3.26 Structures associated with TeNT $H_c$ binding and internalisation.

(a-e) Surface distribution of TeNT $H_c$ observed in living MNs and EM. (a) Still image of neurites and synaptic contacts stained with TeNT $H_c$-Alexa488 in living cells. The synaptic cleft and neurite contact points appear brightly stained.

(b-e) Cell surface labeling after incubation with TeNT $H_c$ for 20 min at 4°C revealed with 10 nm gold-conjugated secondary antibody. Gold is found along the neurite surface (b) and in forming coated pits (c). TeNT $H_c$ is also observed at synaptic sites (d) and neurite contacts (e). (f-i) Cells were treated as in b-e, washed and then warmed to 37°C for 5 min (f and g) or 20 min (h and i). TeNT $H_c$ is found in coated pits (f) and coated vesicles (g), while at later internalisation times gold particles are observed in tubular (h) and round organelles (i). Bars: (a) 5 μm; (b-i) 0.2 μm.
carriers. These latter structures (classified as “others” in Figure 3.27 a and e) tend to change their shape while moving, switching between vesicles to short tubules and vice versa. This behaviour posed problems of classification and could account for the apparent heterogeneity in average speed of this group of carriers. Tubules and vesicles together represent the majority of all TeNT Hc-labeled organelles (66%), with “vesicle clusters” and other types of carriers reaching 10% and 24% of the total, respectively (Figure 3.27 e).

The average speed distribution of all the retrograde carriers shows the presence of multiple components (Figure 3.27 d, open triangles). Deconvolution analysis confirmed 3 populations of carriers identified by Gaussian distributions with average speeds of 1.0, 1.6 and 2.1 μm/s, respectively. This tri-modal representation allowed the best fitting of the observed average speed curve with lower deviation (coefficient of determination $R^2=0.996$) than the corresponding bi- or uni-modal representations. Strikingly, this tri-modal distribution reflected the speed of the round vesicles and vesicle clusters, which show a single predominant peak at the slowest value of 1.0 ± 0.29 μm/s (Figure 3.27 d, open squares and asterisks, respectively), and of the faster tubules peaking at 1.5 ± 0.36 and 2.1 ± 0.13 μm/s (Figure 3.27 d, filled circles).

Other types of carriers displayed the same tri-modal distribution as the total frequency curve (not shown). In contrast, the rare carriers transiently moving in the anterograde direction presented an average speed of 0.23 μm/s (see Figure 3.49). The observed speed values are in the range previously reported for TeNT retrograde transport in vivo (0.8-3.6 μm/s) (Lazarovici et al., 1988; Stöckel et al., 1975). These results strengthen the concept of multiple retrograde carriers involved in TeNT Hc transport and reveal a correlation between their morphology and average speed.
Figure 3.27 Kinetic analysis of retrograde TeNT H$_2$ carriers.

The movement of visible organelles was tracked and plotted versus time. 

(a) Displacement of 15 carriers tracked during a representative experiment in an untreated neuron. Retrograde movement is conventionally shown as positive. Intervals between time points are 5 s. Note the presence of fast tubules (filled circles) and slower vesicles (empty squares). Asterisks indicate the displacement of clusters, while half-filled squares refer to the movement of other types of carriers not included in the previous classes (see text). (b) Displacement graphs of the tubules from a, setting the start of tracking as time = 0, show a constant movement with similar speed. (c) Displacement graphs of the remaining carriers from a show a slower and more discontinuous movement. (d) Distribution of the average speeds of all TeNT H$_2$ retrograde carriers (triangles; $n = 256$ carriers from 3 independent experiments). Round vesicles (empty squares, $n = 76$) have an average speed of 1.0 $\mu$m/s, whereas tubules (filled circles, $n = 93$) display a bimodal distribution peaking at 1.5 and 2.1 $\mu$m /s. The average speed of clusters is also shown (asterisks). (e) Percentage of the different types of carriers from the data collected in d.
3.3.5 TeNT and TeNT Hc-retrograde carriers display similar kinetic behaviour

We used our experimental system to compare the transport of TeNT Hc with that of full-length TeNT. We conjugated native TeNT with Texas Red in order to perform double-colour time-lapse observation with TeNT Hc-Alexa488. MNs internalised TeNT-Texas Red in pleiomorphic endocytic structures very similar to those observed with TeNT Hc-Alexa488 (Figure 3.28; also compare video 2 with video 3). Moreover, kinetic analysis of TeNT carriers revealed a distribution of speed values strikingly similar to the one observed for TeNT Hc (Figure 3.29). When MNs were simultaneously incubated with 40 nM of TeNT-Texas Red and TeNT Hc-Alexa488, a good colocalisation was observed between the two compartments, both in axons and in cell bodies (Figure 3.30 and video 4). Interestingly, cells displayed much lower plasma membrane staining of TeNT-Texas Red compared to fluorescent TeNT Hc. Indeed, TeNT-Texas Red axonal retrograde carriers were visible immediately after removing the toxin, whereas for TeNT Hc-Alexa488 they appeared generally after a lag phase of 20-30 minutes. Taken together, these results suggest that TeNT and its Hc fragment use the same retrograde compartments for axonal transport and that portions of the TeNT molecule different from the Hc domain could be necessary to ensure optimal recruitment to this trafficking route (see Discussion).

3.3.6 TeNT Hc-C and Hc-N trafficking in living MNs

The binding and crosslinking experiments previously described above had revealed that HcC represents the minimal TeNT binding domain. When MNs were incubated at 37°C to allow internalisation of the VSV-G-tagged sub-domains, only the HcC was internalised, giving a vesicular staining pattern similar to that of Hc, although quantitatively less intense (Figure 3.31 a and b). In contrast, the staining of cells incubated with HcN was
Figure 3.28 Fluorescent TeNT is retrogradely transported along axons of living MNs. Cells were incubated with 40 nM TeNT-Texas Red for 15 min at 37°C, washed and imaged by low-light time-lapse microscopy. Time series imaging of a MN axon (from left to right). The cell body is located out of view at the bottom of the picture. Intervals between frames are 5 s. Examples of retrograde carriers are visible (arrowhead, arrow, asterisk). See also video 3. Bar, 5 μm.
Figure 3.29 TeNT Hc and TeNT carriers show overlapping kinetic behaviour. Relative frequency of speed values observed between two consecutive frames (interval = 5 s) for TeNT Hc-Alexa488 (n = 364 vesicles, grey bars) and TeNT-Texas Red carriers (n = 88 vesicles, black bars). Retrograde movement is conventionally shown as positive.
Figure 3.30 Fluorescent TeNT Hc and TeNT colocalise in living MNs.
Cells were incubated with 40 nM TeNT Hc-Alexa488 and TeNT-Texas Red for 15 min, washed and imaged with double-colour low-light microscopy. Still image from a video taken 1 h after the end of the incubation. The two proteins colocalise in vesicular compartments in the somatodendritic domain (merge, left panel). Retrograde axonal carriers (right inset, arrowheads) can also be observed. See also video 4. Bar, 10 μm.
indistinguishable from control samples where toxin fragments were omitted (Figure 3.31 c and d).

We then sought to monitor the trafficking of TeNT H\textsubscript{c} sub-domains in living MNs. For this purpose we expressed recombinant TeNT H\textsubscript{c}C and H\textsubscript{c}N tagged at the N-terminus with the same cysteine-rich-domain that had previously allowed the fluorescent labelling of TeNT H\textsubscript{c} (Figure 3.19). MNs were incubated with 40 nM of fluorescent TeNT H\textsubscript{c}C- or H\textsubscript{c}N-Alexa488 for 15 minutes at 37°C, washed and imaged. The two sub-domains displayed a strikingly different behaviour. TeNT H\textsubscript{c}C showed a very discontinuous binding on the neurite plasma membrane, extending also to random areas outside synaptic contacts (Figure 3.31 f, arrowheads) and quite different form the initial homogeneous surface staining detected with TeNT H\textsubscript{c}. We could detect retrograde carriers similar in morphology and speed to TeNT H\textsubscript{c} organelles, although in lower amount (video 5). Cell bodies and dendrites were also brightly stained with a pattern similar to the one observed with TeNT H\textsubscript{c}. In contrast with the results obtained with the VSV-G-tagged variant, H\textsubscript{c}N-Alexa488 gave a faint vesicular staining apparently restricted to the somatodendritic domain (Figure 3.31 g and video 6). Further experiments are needed to address the reason of the contrasting results obtained with the two recombinant H\textsubscript{c}N domains. The somatodendritic staining obtained with the fluorescent H\textsubscript{c}N is reminiscent of that observed for transferrin receptor, which undergoes recycling in a compartment only found in neuronal cell bodies and dendrites (Mundigl et al., 1993). When cells were incubated with Texas Red-transferrin and TeNT H\textsubscript{c}N-Alexa488, no colocalisation was detected between the two proteins (Figure 3.32), suggesting that H\textsubscript{c}N could enter a somatodendritic endosomal system distinct from the known recycling compartment. Taken together, these results suggest that TeNT H\textsubscript{c}C still retains the determinants not only for binding, but also
Figure 3.31 TeNT $H_c$ and $H_cN$ show different behavior in internalisation experiments with MNs.

(a-d) MNs were incubated with 40 nM VSV-G-tagged TeNT $H_c$, $H_cC$, $H_cN$ or medium for 45 min at 37°C, fixed and permeabilised for immunodetection of internalised toxin fragments. Shown here are confocal z-sections. (e-h) MNs were incubated with similar concentration of the same toxin fragments labelled with Alexa488, washed and imaged with low-light microscopy about 30 min after the end of a 20 min incubation period. MNs internalise VSV-G-tagged TeNT $H_c$ (a) and TeNT $H_cC$ (b), but not the $H_cN$ (c) that gives a background staining similar to control cells where toxin fragment was omitted (d). TeNT $H_cC$-Alexa488 is internalised and retrogradely transported in living MNs. See also video 5. Note the spotted distribution along neurite membranes (f, arrowheads). TeNT $H_cN$-Alexa488 (g) is localised in faint vesicular structures, apparently restricted to the somatodendritic domain and absent in axonal processes as highlighted by the corresponding phase contrast image (h, arrow). See also video 6.

Bars: (a, d), (b, c, f), (e, g, h) 10 μm.
Figure 3.32 Fluorescent TeNT $H_c N$ enters a compartment distinct from recycling endosomes in living MNs. MNs were incubated with TeNT $H_c N$–Alexa488 (a) and transferrin-Texas Red (b) for 30 min, washed and imaged with double-colour low-light microscopy. TeNT $H_c N$–labelled organelles do not overlap with endosomes containing transferrin, as shown in the merged picture (c) and in the inset (far right). Bar, 10 μm.
for uptake and retrograde transport in neurons. On the other hand, the selective interaction of HcN with the plasma membrane of cell bodies and dendrites supports the presence of distinct axonal and somatodendritic endocytic systems in MNs.

### 3.3.7 Disruption of actin cytoskeleton blocks TeNT Hc endocytosis

Several studies have defined a role for actin in endocytosis, including clathrin-dependent and -independent endocytosis and macropinocytosis (Cremona and De Camilli, 2001; Qualmann et al., 2000; Skretting et al., 1999). We used our experimental system to determine whether the actin cytoskeleton is also involved in TeNT Hc internalisation. As shown above, untreated MNs displayed extensive TeNT Hc retrograde transport (Figure 3.33 a). However, no internalisation of TeNT Hc could be detected in cells pretreated with the actin monomer-sequestering drug latrunculin B (Lat B) before TeNT Hc addition. We observed only homogeneous plasma membrane staining on neurites, but no transport carriers (Figure 3.33 b; 52 neurites scored). We did, however, detect rare retrograde vesicles at the end of the observation period, beginning approximately 90 min after Hc incubation. This is possibly due to photodegradation of Lat B, which led to partial recovery from the drug effect. The inhibitory effect on TeNT Hc internalisation was also confirmed by treatment with two other actin-disrupting drugs, cytochalasin D and swinholide A (not shown), indicating a necessary role for F-actin in TeNT neuronal endocytosis.

### 3.3.8 TeNT Hc retrograde transport depends on both F-actin and MTs

Axonal retrograde transport is dependent on MTs and MT-dependent motors (Goldstein and Yang, 2000). However, other studies have shown actin-dependent organelle motility...
Figure 3.33 Disruption of actin cytoskeleton prevents TeNT H₄ endocytosis.
MNs were preincubated only with medium (Ctrl) (a) or with 0.5 μM Lat B (b) for 20 min at 37°C, then incubated for 20 min at 4°C with 40 nM TeNT H₄-Alexa488, washed, warmed to 37°C and imaged with low-light microscopy. In drug-treated samples, Lat B was kept throughout the entire observation time. (a) Time series imaging of an untreated axon. Retrograde vesicles (arrowhead), as well as tubules (arrow, asterisk) can be observed. (b) Time series imaging of an axon pretreated with Lat B. TeNT H₄ labels homogeneously the plasma membrane and no retrograde carriers are observed. The cell body is located out of view at the bottom. Intervals between frames are 10 s. Bar, 2 μm.
(Kuznetsov et al., 1992; Morris and Hollenbeck, 1995), leading to the hypothesis that the two cytoskeletal systems may cooperate in vesicle transport (Goode et al., 2000).

We therefore investigated the effect of F-actin or MT disruption on TeNT Hc retrograde transport. Whereas incubation of MNs with vehicle did not affect the retrograde movement of round and tubular organelles (Figure 3.34 a and video 2), treatment with Lat B after TeNT Hc endocytosis caused the great majority of the vesicles to stop or oscillate (Figure 3.34 b and video 7). We quantified this effect by counting the retrogradely-moving and the stationary/oscillating vesicles in untreated and Lat B-treated cells (Figure 3.35 a).

In untreated cells about 75% of the vesicles moved retrogradely, while 25% were stationary. Lat B treatment led to a two-fold increase in the number of resting organelles, while the moving carriers represented only 25% of the total vesicles observed. Both tubules and round vesicles were present in this residual population, which is characterised by a reduced average speed ranging between 0.1 and 0.7 μm/s. Upon Lat B treatment, the movement of TeNT Hc-positive endosomes in cell bodies also appeared to be limited to short-range oscillations (video 8). These results were confirmed using swinholide A (not shown). Phalloidin staining of control MNs shows F-actin accumulations along neurites (Figure 3.35 c) which disappeared after Lat B treatment (Figure 3.35 d). This effect was specific, since it did not alter MTs, as shown by immunostaining of Lat B-treated neurons with an anti-β-tubulin antibody (Figure 3.35 b) and EM morphometric analysis of neurite cross-sections (Figure 3.37 c, hatched bar).

Treatment with the MT-depolymerising drug nocodazole slowed but did not abolish retrograde movement. Nocodazole caused an increase in the frequency and duration of the stationary periods, without altering the number and morphology of the carriers. Retrograde organelles frequently stopped and then slowly resumed the movement while travelling.
MNs were incubated with 40 nM TeNT H$_c$–Alexa488 for 15 min at 37°C and then washed. After about 20 min, when retrograde carriers were visible, cells were treated with DMSO (Ctrl) (a) or with 0.5 μM Lat B (b) for 30 min before time-lapse imaging. Intervals between frames are 5 s. Cell bodies are located at the top for (a) and at the bottom for (b). (a) Control neurons display retrograde transport (arrowhead, arrow, asterisk). See also video 2. (b) Treatment with Lat B causes the majority of carriers to stop or oscillate (arrowhead). See also video 7. Bar, 5 μm.
Figure 3.35 Effect of F-actin disruption on TeNT Hc transport.
(a) Quantitative analysis of actin disruption on TeNT Hc transport. Carriers were classified as still/oscillatory when the extent of the movement did not lead to any significant progression. Results are expressed as percentage of the total carriers observed (control = 437, Lat B = 315). Bars represent the SD of 4 independent experiments. The differences observed between moving and still carriers in control and Lat B-treated samples have confidence intervals with p < 0.01. (b) Treatment with Lat B does not affect MTs visualized by anti-β-tubulin antibodies. (c) F-actin staining in untreated MNs shows a punctate distribution along neurites, which is abolished by Lat B treatment (d). Bars: (b) 10 μm; (c-d) 5 μm.
along axons (Figure 3.36 a). This effect was also clear after performing a quantitative analysis of speed values of TeNT Hc retrograde carriers (control = 256 carriers, nocodazole = 139). (Figure 3.36 b). Nocodazole treatment caused a general reduction in the frequency of the intermediate-high speed values, with a simultaneous three-fold increase in the frequency of stationary periods. When the extent of MT depolymerisation was assessed by EM morphometric analysis of neurite cross sections, nocodazole-treated samples showed a 75% decrease in MT density compared to control cells (Figure 3.37 c, compare grey and black bars).

Since the observed slowed movement of TeNT Hc carriers could be due to the nocodazole-resistant MTs, we analysed the effect of a more effective MT-disrupting treatment. The Vinca alkaloid vincristine (Vin) has been used to disrupt MTs in neurons without associated toxicity (Allison et al., 2000). Treatment with this drug greatly reduced retrograde movement (Figure 3.37 a and video 9). Quantitative analysis of TeNT Hc carriers from two independent experiments revealed a 3 fold increase in the number of stationary/oscillating vesicles compared to the control (Figure 3.37 b). The effect was clearly visible also in cell bodies, where TeNT Hc-labelled organelles almost completely stopped (video 10). Vin was clearly more efficient than nocodazole, since MTs were virtually absent in the EM morphometric analysis (1% residual MT density, compared to control) (Figure 3.37 c). The specific effect of Vin was confirmed by detecting MTs or F-actin in MNs incubated with this drug. Only tubulin paracrystals could be seen (Figure 3.37 d), while F-actin distribution was similar to untreated controls (Figure 3.37 e). Taken together, these data show that retrograde transport of TeNT Hc is dependent on both MT and F-actin.
Figure 3.36 Treatment with nocodazole slows TeNT Hc retrograde carriers.

(a) MNs were treated with 10 μg/ml nocodazole for 20 min after the end of incubation with TeNT Hc–Alexa488. Time-lapse imaging started 30 min after drug addition. Retrograde carriers display increased stationary pauses during transport (arrowhead). Intervals between frames are 5 s. The cell body is located at the top of the picture.
(b) Quantitative analysis of nocodazole effect on TeNT Hc transport. Shown here is the relative frequency of speed values observed in untreated (black bars) and nocodazole-treated cells (grey bars) from 3 independent experiments. Retrograde movement is conventionally shown as positive. A similar number of measurements for the two conditions (control = 2524, nocodazole = 2982) was analysed.
Figure 3.37 Treatment with vincristine leads to an efficient block of TeNT H₄ transport.

(a) MNs were incubated with TeNT H₄-Alexa488 and treated with 5 μM vincristine (Vin). The majority of carriers stops or oscillates (arrowhead, arrow). Intervals between frames are 20 s. See also video 9.

(b) Quantitative analysis of MT disruption on TeNT H₄ transport. Results are expressed as percentage of the total carriers observed (control = 364, Vin = 361). Bars represent the average of 2 independent experiments.

(c) Morphometric EM analysis of neurite cross sections shows that MTs are virtually absent in Vin-treated cells, whereas they are identical to control in Lat B-treated cells. Nocodazole treatment only leads to partial microtubule disruption. Data are expressed as percentage of the MT density observed in untreated MNs (control = 58; Vin = 36; Noc = 53; Lat B = 24 neurites). The same cells used in a were fixed and immunostained for β-tubulin. Treatment with Vin causes the accumulation of tubulin in paracrystals (d), but does not affect F-actin (e). Bars: (a) 5 μm; (d-e) 10 μm.
3.4 Characterisation of TeNT Hc endocytic carriers

3.4.1 TeNT Hc carriers are not characterised by known endocytic markers

In an effort to characterise the endocytic route followed by TeNT Hc, we asked if TeNT Hc carriers contain markers of the endosomal-lysosomal or recycling pathway. We performed experiments using both VSV-G-tagged TeNT Hc or TeNT Hc-Alexa488 and markers of EE, LE or RE. Preliminary EM analysis shows that in MNs TeNT Hc endocytosis appears to be mediated by coated pits in more than 80% of the cases observed. In some instances the electron-dense coat is reminiscent of clathrin (Marsh and McMahon, 1999). Surprisingly, immunofluorescence experiments performed using short TeNT Hc internalisation times failed to reveal colocalisation with clathrin (Figure 3.38). Future cryoimmunoEM studies will be necessary to clarify the nature of this coat.

The EE marker EEA1 was specifically found only in the somatodendritic domain of MNs, consistent with what reported for hippocampal neurons (Wilson et al., 2000). Surprisingly, confocal observation revealed a clear lack of colocalisation between the Rab 5 effector and TeNT Hc at early internalisation time points (Figure 3.39, top row). TeNT Hc did not colocalise with the transferrin receptor, also present only in cell bodies and dendrites (Figure 3.39, bottom row) (Mundigl et al., 1993). In line with this result, TeNT Hc-Alexa488 displayed only a limited overlap with tubular compartments labelled by Texas Red transferrin in dendrites of living MNs (Figure 3.40, bottom row) and only a minor fraction of TeNT Hc round endosomes colocalised with fluorescent transferrin in cell bodies (Figure 3.40, top row).

BFA has been shown to severely affect the structure and function of intracellular organelles, including the Golgi apparatus and REs (Peyroche et al., 1999). Treatment of MNs with 20 μg/ml BFA for at least 30 min completely disrupted the Golgi apparatus.
Figure 3.38 TeNT H does not colocalise with clathrin.
MNs were incubated with 40 nM TeNT H<sub>c</sub>–VSV-G at 4°C, washed, warmed up for 10 min at 37°C, fixed, permeabilised and immunostained with anti-VSV-G (red) and anti-clathrin (green) antibodies. No detectable colocalisation is observed. All images are 0.4 μm confocal z-sections except for a, which shows a projection of a stack of z-sections. a and c are low-magnification images of a MN and its neurites. The lack of colocalisation is highlighted also in the high-magnification image in d. Note that occasional fibroblast-like cells found in the MN culture are positive for clathrin, but not for TeNT H<sub>c</sub>, that is detected only in the overlapping axon (b, arrow). Bars: (a-b) 10 μm; (c-d) 5 μm.
Figure 3.39 TeNT $H_c$-labelled compartment does not colocalise with the early endosomal marker EEA1 nor with the recycling endosome marker transferrin receptor.

MN s were incubated with VSV-G-tagged TeNT $H_c$ at 4°C, washed, shifted to 37°C for 10 min (a-c) or 30 min (d-f). Cells were then fixed, permeabilised and immunostained to detect the toxin fragment (a, d) and EEA1 (b) or transferrin receptor (TfR, e). Very little colocalisation is observed in both experiments, as shown in the merged pictures (c, f). Both EEA1 and transferrin receptor are localised only in the somatodendritic domain (arrowheads), whereas TeNT $H_c$ is found also in axonal processes (arrows). Bars: (a-c) and (d-f) 10 μm.
Figure 3.40 TeNT \( H_c \) and transferrin enter distinct somatodendritic compartments.

MNs were incubated with TeNT \( H_c \)-Alexa488 (a, d) and Texas Red-transferrin (b, e) for 30 min at 37°C, washed and imaged with confocal microscopy after 15 min. The two proteins appear to be localised in compartments that show limited overlap in the somatodendritic domain (c, f and insets, right). (a-c) Confocal z-section of a MN cell body, (d-f) confocal z-section of a dendrite detail. The labelled structures appear mostly tubular in dendrites, and both tubular and vesicular in the cell body. Bars: (a-c) 10 μm; (d-f) 5 μm.
(Figure 3.41, compare d with e). Similar BFA treatment induced tubulation of both transferrin- and TeNT Hc-containing compartments that overall remained distinct (Figure 3.41, top row). Consistent with these results, only very limited colocalisation was observed in cell bodies with syntaxin 13, a marker of recycling compartments shared by transferrin receptor in the somatodendritic domain of hippocampal neurons (Prekeris et al., 1999) (Figure 3.42). We also considered the possibility that TeNT Hc compartment might be analogous to the APRE found in polarised epithelial cells and characterised by the presence of Rab11 (Casanova et al., 1999). In MNs Rab 11 displayed an abundant ubiquitous punctate staining but very low colocalisation with TeNT Hc was observed at different internalisation time points (from 30 min up to 2 hours) (Figure 3.43). Similarly, TeNT Hc and the ER-Golgi intermediate compartment marker Rab1 (Zerial and McBride, 2001) did not overlap (Figure 3.44). Taken together, these results indicate that TeNT Hc might use one or more alternative endocytic pathways able to bypass the classical endosomal system.

A previous study had suggested that TeNT might enter hippocampal neurons through SSV recycling (Matteoli et al., 1996). In contrast, MNs in culture internalise TeNT Hc before the development of synaptic contacts and in absence of depolarising conditions. Block of SSV exo-endocytosis by incubation of MNs with 10 nM BoNT/A for 20 h at 37°C did not prevent TeNT Hc internalisation (Figure 3.45). Moreover, both VSV-G-tagged TeNT Hc and TeNT Hc–Alexa488 showed very limited colocalisation with the SSV marker VAMP (Figure 3.46). These data are in agreement with studies demonstrating that TeNT uptake and retrograde transport are unaffected at NMJ in which neurotransmitter release has been inhibited (Habermann and Erdmann, 1978) and indicate that the uptake mechanisms of TeNT at the NMJ and in central neurons could be different.
Figure 3.41 TeNT Hc and transferrin-labelled compartments remain distinct after tubulation induced by BFA.

MNs were incubated with TeNT Hc—Alexa488 (a) and Texas Red-transferrin (b) for 30 min at 37°C, and for other 20 min in the presence of 20 µg/ml BFA. Cells were then washed and imaged keeping the drug throughout the observation time. Both the TeNT Hc- and the transferrin-positive compartments tubulate after BFA treatment, but they appear to remain distinct (merged picture in c and inset, far right). (d, e) BFA effectiveness was assessed by the ability to fragment the Golgi apparatus. (d) Untreated MNs immunostained for giantin. The Golgi structure is disrupted after treatment with 20 µg/ml BFA for 40 min (e). a-c are confocal z-sections, whereas d-e are projections of confocal z-stacks. Bars: (a-c) 5 µm; (d-e) 10 µm.
Figure 3.42 TeNT H₃-labelled structures are not stained for the recycling endosome marker syntaxin13.
MNs were incubated with VSV-G-tagged TeNT H₃ for 40 min at 37°C, washed and fixed. After permeabilisation, cells were immunostained with an anti-VSV-G antibody (a) and for the recycling endosome marker syntaxin 13 (b). Shown here is a 0.4 μm confocal z-section. Both proteins display a punctate staining, but very little colocalisation is visible in the merged picture (c and inset, far right). Bar, 10 μm.
Figure 3.43 TeNT Hc-positive organelles do not overlap with the Rab11-labelled compartment.

MN s were incubated with VSV-G tagged TeNT Hc for 25 min at 37°C, washed, left in medium at 37°C for 1 h and 45 min and then fixed. After permeabilisation, cells were immunostained for Rab11, a marker of the apical recycling compartment in polarised epithelial cells. Images are 0.4 μm confocal z-sections. TeNT Hc (a, d) and Rab11 (b, e) display distinct staining patterns in both cell body (a-c) and axon (d-f). The insets on the right correspond to the boxed areas in c and d. Bars: (a-c) 10 μm; (d-f) 5 μm.
Figure 3.44 TeNT H_c-containing vesicles do not colocalise with the ER-Golgi intermediate compartment marker Rab1 in MN cell bodies.

MNs were fixed, permeabilised and stained for Rab1 2 h after the end of the 20 min incubation with TeNT H_c-Alexa488. Shown here is a 0.4 μm confocal z-section. TeNT H_c (a) and Rab1 (b) appear to stain distinct compartments, as shown in the merged picture (c) and in the inset (far right). Bar, 10 μm.
Figure 3.45 Impairment of SSV exo-endocytosis does not prevent TeNT Hc internalisation in MNs.

MNs were incubated with 10 nM BoNT/A for 20 h at 37°C and with 20 nM VSV-G-tagged TeNT Hc in the last 50 min. Cells were then fixed, permeabilised and immunostained with an anti-VSV-G antibody. Vesicular staining is visible in cell bodies (a) and axonal processes (b). Bar, 10 μm.
Figure 3.46 TeNT Hc–labelled structures show little colocalisation with the SSV marker VAMP. 
(a–c) MNs were incubated with VSV-G-tagged TeNT Hc for 10 min at 37°C, fixed, permeabilised and immunostained for VSV-G to detect the toxin fragment (a) and for the SSV marker VAMP (b). Images are 0.3 μm confocal z-sections. The two proteins display very limited colocalisation (c and inset, top right). (d–f) MNs were incubated with TeNT Hc–Alexa488 (d) for 30 min at 37°C, fixed, permeabilised and immunostained for VAMP (e). Little colocalisation is observed at synaptic junctions (f and inset, bottom right). Bar, 10 μm.
**3.4.2 TeNT H<sub>C</sub> carriers do not colocalise with acidic organelles or lysosomes**

Conflicting results on the fate of TeNT after endocytosis have been reported. While some reports suggested that TeNT might escape lysosomal degradation *in vivo* (Critchley et al., 1985; Schwab and Thoenen, 1978), others found TeNT in MVBs and lysosomes in spinal cord neurons (Parton et al., 1987). To further characterise the fate of TeNT retrograde transport carriers, we performed double-color time-lapse microscopy in living MNs using the acidotropic membrane-permeable dye Lysotracker that stains acidic organelles and lysosomes. We observed a striking lack of colocalisation between TeNT H<sub>C</sub>-labelled endosomes and Lysotracker-stained round organelles, which oscillated or moved along axons mainly in a retrograde fashion (Figure 3.47 a-c and video 11).

Confocal time-lapse experiments and simultaneous DIC imaging revealed that Lysotracker was particularly concentrated in phase contrast-bright round structures (Figure 3.47 d and e, asterisk), which likely correspond to pre-lysosomal organelles (Kuznetsov et al., 1992), and were always distinct from the round or tubulovesicular TeNT H<sub>C</sub> carriers undetectable by DIC (Fig 3.47 d and e). Very low colocalisation between the two markers was also observed in cell bodies, even at late internalisation time points (Figure 3.48).

As a positive control, we checked that the Lysotracker-positive compartment could be accessible to the neuronal endocytic system by performing double-labelling experiment with Texas Red dextran. We observed colocalisation of the two dyes in vesicular compartments (Figure 3.47 f-h, arrowheads) and importantly, we also detected organelles stained by fluorescent dextran which were not acidic (Figure 3.47 f-h, asterisks). Some of these endocytic structures could participate to the retrograde route ensuring the transport of TeNT H<sub>C</sub> and, possibly, of other physiological ligands (see below).

A comparison of the motile behaviour displayed by TeNT H<sub>C</sub>- and Lysotracker-positive
Figure 3.47 Axonal TeNT \( H_c \) carriers do not colocalise with acidic organelles.

MN were incubated with TeNT \( H_c \)-Alexa488 and Lysotracker Red DND-99 for 20 min at 37°C. Cells were then washed and imaged with low-light microscopy. The cell body is located out of view to the right. Intervals between frames are 5 s. (a) Time series showing retrograde TeNT \( H_c \)-labeled endosomes (arrow and filled circle). (b) Corresponding frames showing Lysotracker-stained organelles (arrowheads). (c) Merged images of a and b. Note the lack of colocalisation between TeNT \( H_c \) and Lysotracker-stained organelles. See also video 11. (d-e) Detail from confocal observation of an axonal branch point. (d) DIC image. (e) Overlap of the green and red channels with the simultaneous DIC image. TeNT \( H_c \) (green) stains tubular and round carriers (arrows), while Lysotracker (red) labels distinct round vesicles (asterisk and arrowheads). An asterisk marks a phase-contrast-bright round organelle positive for Lysotracker, but negative for TeNT \( H_c \) (f-h) Lysotracker-positive organelles are accessible to endocytic tracers. MNs were incubated with Texas Red dextran overnight and with Lysotracker Green DND-26 for 30 min at 37°C. Cells were then washed and imaged by confocal microscopy. Lysotracker-positive organelles (f) are also stained by Texas Red dextran (g, arrowheads). (h) Merged image of f and g. Non-acidic organelles containing only dextran are also visible (asterisks). Bars, 5 \( \mu m \).
Figure 3.48 TeNT Hc-positive endocytic organelles show little colocalisation with acidic compartments in MN cell bodies. MNs were incubated with TeNT Hc-Alexa488 and Lysotracker Red DND-99 for 20 min at 37°C. Cells were then washed and imaged. Shown here is a 0.4 μm confocal z-section taken 2 h after the end of the incubation period. The lack of colocalisation between the TeNT Hc-labelled endosomes (a) and Lysotracker-positive organelles (b) is evident in the merged image (c) and at higher magnification (inset). Bar, 5 μm.
organelles further demonstrates that they are distinct compartments (Figure 3.49). The speed distribution for TeNT Hc carriers extends to values of more than 3 μm/s and is retrogradely biased, while the organelles stained by Lysotracker are slower and display a higher frequency of anterograde movement (Figure 3.49 a). Moreover, the Lysotracker vesicles changed direction more frequently compared to TeNT Hc-labelled organelles, as shown by the different incidence of reversal of the two compartments (Figure 3.49 b). These observations suggest that TeNT Hc carriers are not very acidic and that the toxin fragment could follow an axonal retrograde route able to bypass lysosomal targeting.

3.5 TeNT Hc and NGF share retrograde transport carriers
To gain insights into the possible physiological cargoes of this retrograde transport pathway, we analysed whether ligands known to undergo this trafficking route in vivo are recruited into TeNT Hc-containing carriers. Neurotrophins, which are crucial for neuronal growth and survival might share the same TeNT retrograde route. A large amount of work has indeed demonstrated that internalisation and retrograde transport of neurotrophin-receptor complexes is required to initiate cell body responses to neurotrophins (reviewed in Neet and Campenot, 2001). NGF represents the best-studied example of this phenomenon. This neurotrophin promotes neuronal survival and differentiation via activation of the receptor tyrosine kinase TrkA, assisted by the participation of the p75 neurotrophin receptor (p75^NTR), which can increase TrkA affinity of binding to NGF (Chao and Hempstead, 1995). In sympathetic neurons, NGF internalisation and retrograde transport from axon terminals is necessary for propagation of the neurotrophin signal leading to activation of cAMP response element-binding protein (CREB), a transcription factor implicated in NGF function (Riccio et al., 1997).
Figure 3.49 TeNT Hc carriers and Lysotracker-positive organelles show distinct motile properties.
(a) Relative frequency of speed values observed between two consecutive frames (interval = 5 s) for TeNT Hc carriers (n = 364 vesicles, black bars) and Lysotracker-containing organelles (n = 235 vesicles, grey bars). Retrograde movement is conventionally shown as positive. (b) The incidence of reversal (number of changes of direction per organelle) for Lysotracker-positive vesicles is 11 times higher than TeNT Hc carriers.
NGF is retrogradely transported along newborn MN axons (Yan et al., 1988) and has transport rates similar to TeNT in sensory and adrenergic neurons (Stöckel et al., 1975). MNs internalised both TeNT Hc-Alexa488 and Texas Red-labelled NGF when the two proteins were added directly at 37°C. The same result was obtained when TeNT Hc and NGF were first allowed to bind at 4°C, followed by washing and warming to 37°C. The NGF-containing retrograde compartments could be first detected approximately 45 min after the end of the incubation at 37°C, consistent with previous studies that used radioactive NGF in cultures of sympathetic neurons (Ure and Campenot, 1997). Using confocal time-lapse microscopy we detected partial colocalisation of TeNT Hc and NGF retrograde carriers (72 %, n = 106 carriers in 2 independent experiments). These organelles always corresponded to round vesicles (Figure 3.50, a-c and video 12). In contrast, we could not detect fluorescent NGF in TeNT Hc-labelled tubules (Figure 3.50 e-g). Interestingly, during development MNs express the neurotrophin receptor p75NTR, but not TrkA (Yan et al., 1993; Yan and Johnson, 1988). Experimental evidence showed that antibodies against p75NTR are retrogradely transported in MNs, and several reports have proposed that p75NTR might be a crucial component mediating NGF retrograde transport in these cells (Yan et al., 1993; Yan et al., 1988). Strikingly, more than 80% of TeNT Hc carriers colocalised with p75NTR-labelled compartments in axons (n = 572 organelles, 2 independent experiments) (Figure 3.51). Notably, this colocalisation was observed in absence of exogenous NGF. p75NTR therefore represents the first membrane marker of the retrograde endocytic pathway used by TeNT Hc. These results show that TeNT Hc and NGF share retrograde transport carriers and suggest that TeNT may enter the nervous system via an essential retrograde route used by physiological ligands, such as neurotrophins.
Figure 3.50 TeNT H$_c$ retrograde carriers partially colocalise with NGF-labeled compartments.
MNs were incubated with TeNT H$_c$-Alexa488 and Texas Red-NGF for 30 min at 37°C. Cells were then washed and imaged by time-lapse confocal microscopy. The cell body is located out of view to the right. (a) TeNT H$_c$-Alexa488 and (b) Texas Red NGF colocalise in retrograde carriers (arrowhead, asterisk, arrow). See also video 12. (c) Merged image of a and b. (d) Corresponding DIC image. TeNT H$_c$ (e) and NGF (f) are found only in round vesicles (arrowhead), while tubules appear to be labeled only by TeNT H$_c$ (asterisk). (g) Merged image of e and f. Bars, 5 μm.
Figure 3.51 Many TeNT Hc carriers colocalise with p75NTR.
MNs were incubated with TeNT Hc-Alexa488 for 30 min at 37°C (a), washed, fixed and immunostained for p75NTR (b). (c) Merged image. The two proteins colocalise in vesicular compartments (arrowheads). Bar, 5 μm.
3.6 Applications of the retrograde transport assay

3.6.1 Identification of TeNT Hc residues important for neuronal binding and retrograde transport

Our retrograde transport assay provides a useful experimental system to test the binding, internalisation and retrograde transport of TeNT Hc mutants. This could help to identify residues essential for interaction of TeNT with living target cells.

Structural and biochemical studies have shown that the carboxy-terminal β-trefoil domain of TeNT contains ganglioside-binding sites (Emsley et al., 2000; Fotinou et al., 2001; Halpern and Loftus, 1993; Shapiro et al., 1997). In collaboration with Dr. N. Fairweather (Imperial College, London), we performed some binding and competition experiments with TeNT Hc mutants in MNs and compared the results with the ability of the same proteins to bind to polysialogangliosides in vitro (Sinha et al., 2000).

The loops joining the β-sheets within TeNT Hc are exposed to the surface and therefore represent good candidate sites for the interaction with components of the neuronal plasma membrane (Umland et al., 1997) (Figure 3.52). Mutant M28 (Δ Gln 1274 – Pro 1279) lacks six residues in a loop joining two β-sheets within the β-trefoil domain (Figure 3.52, green). It displayed low binding both to GT1b and to MNs (table 3.2). Moreover, it was not retrogradely transported in vivo to the hypoglossal nucleus after injection in the tongue. To confirm this result, we preincubated MNs with a 100X excess of M28 before adding TeNT Hc-Alexa488. M28 did not compete the binding and retrograde transport of fluorescent TeNT Hc, suggesting that the loop region comprising residues 1274-1279 is important for binding of TeNT Hc to neurons (Figure 3.53, compare a with c). Interestingly, TeNT Hc-Alexa 488 displayed a more “patched” pattern on the plasma membrane after preincubation with M28 compared to control cells (Figure 3.53, inset). One possible
Fig. 3.52 Crystal structure of TeNT $H_c$ showing the location of the residues altered in the mutant fragments used.

Mutations in M28 ($\Delta$ Gln 1274 - Pro 1279) and M58 ($\Delta$ Asp 1214 - Asn 1219) are located in two surface-exposed loops joining $\beta$-sheets within the $H_c$ $\beta$-trefoil domain (green and magenta, respectively). Mutations in M57 (His 1293 Ala, $blue$) and M72 (Trp 1289 Ala, $light blue$) are instead located in a pocket shown to be important for toxin-ganglioside interaction.
explanation could be that this mutant is still able to bind with low affinity to some components on the neuronal surface, causing a change in the distribution of TeNT binding acceptors. As a positive control, preincubation of MNs with a 100X excess of wild type H_c completely abolished binding and transport of the fluorescent toxin fragment (Figure 3.53, compare a with b).

Another mutant, M58 (Δ Asp 1214 – Asn 1219), was used to analyse the role of a second loop exposed to the surface in the β-trefoil domain (Figure 3.52, magenta). This mutant bound very poorly to GT_1b and to MNs (Table 3.2), and was not retrogradely transported to the hypoglossal nucleus, indicating an important role also of this second loop in the interaction of TeNT H_c with the neuronal surface.

Photoaffinity labelling of TeNT H_c with derivatized gangliosides has shown that residues in the immediate vicinity of His 1293 are involved in binding to GT_1b (Shapiro et al., 1997) (Figure 3.52, blue). Moreover, the crystal structure of TeNT H_c complexed with a synthetic GT_1b analogue revealed that the region around residues His 1271, His 1293, Trp 1289 and Tyr 1290 forms a groove interacting with the Gal4-GalNAc3 group of GT_1b (Fotinou et al., 2001). The same region also interacts with lactose in the lactose-soaked TeNT H_c crystal (Emsley et al., 2000), suggesting a more general role of this TeNT H_c portion in polysialoganglioside recognition. We tested the importance of His 1293 by using mutant M57 (His 1293 Ala). M57 retained only 12.5% of wild-type binding to GT_1b, but quite surprisingly did not show a decreased binding to MNs (table 3.2). When these cells were preincubated with a 100X excess of M57, binding and transport of fluorescent TeNT H_c were greatly impaired (Figure 3.53 d), consistent with the ability of this mutant to reach the hypoglossal nucleus after intramuscular injection (Sinha et al., 2000). Therefore His 1293, although important for GT_1b recognition, is not crucial for TeNT
Figure 3.53 Competition of TeNT Hc–Alexa488 with different TeNT Hc mutants.
MNs were preincubated with medium (a, no comp) or 100X excess (4 μM) of wild-type TeNT Hc (b), M28 (c and inset), M57 (d), M72 (e) for 20 min at 37°C before adding TeNT Hc–Alexa488. M57 is the only mutant able to compete the binding and transport of fluorescent TeNT Hc, although not as efficiently as the wild-type toxin fragment. Cells preincubated with M28 display a patched distribution of TeNT Hc–Alexa488, especially along areas of neurite contact (inset, top right). Bars: (a, e and b-d) 10 μm; inset, 2 μm.
Table 3.2 Binding and transport properties of TeNT H$_C$ mutants.

<table>
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<tr>
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<th>In vitro GT$_{lb}$ binding</th>
<th>MN binding</th>
<th>Ret. Transport to hypoglossal nucleus</th>
<th>Competition with TeNT H$_C$–Alexa488</th>
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<tbody>
<tr>
<td>Wild-type TeNT H$_C$</td>
<td>100%</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M58 (ΔAsp1214 – Asn1219)</td>
<td>0.5%</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>M28 (ΔGln 1274 – Pro 1279)</td>
<td>5.2%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M57 (His 1293 Ala)</td>
<td>12.5%</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M72 (Trp 1289 Ala)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
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The properties of TeNT H$_C$ mutants are summarised and compared with wild-type TeNT H$_C$. ND, not determined.
binding and retrograde transport.

The crystal structure of TeNT \( H_c \) complexed with a synthetic \( G_{Tb} \) analogue shows that the hydrophobic faces of both galactose rings in the Gal4-GalNAc3 units are stacked against the indole ring of Trp 1289, suggesting an important role of this residue in ganglioside binding (Fotinou et al., 2001) (Figure 3.52, light blue). Mutant M72 (Trp 1289 Ala) did not compete fluorescent TeNT binding and retrograde transport in living MNs (Figure 3.53 e), suggesting that perturbing a residue directly involved in the interaction with sugars such as Trp 1289 can affect the overall binding ability of TeNT \( H_c \) to neuronal membranes. Taken together, these preliminary results indicate that the exposed Gln 1274 – Pro 1279 and Asp 1214 – Asn 1219 loops of TeNT \( H_c \) together with Trp 1289 are crucial for the initial interaction of TeNT with polysialogangliosides, a step important for toxin internalisation (Williamson et al., 1999). It will be interesting to compare the trafficking of fluorescent mutants still able to bind to the neuronal surface, such as M57 (His 1293 Ala), with that of wild-type TeNT \( H_c \) to gain a better understanding of the residues important for TeNT targeting to its neuronal retrograde route.
Chapter 4: Discussion
Chapter 4

4.1 CNT H\textsubscript{C}s as tools for the study of CNT neuronal binding

The identification of SNAREs as specific CNT targets has led to their extensive use in the dissection of regulated secretion and intracellular trafficking in a variety of cells (Schiavo and van der Goot, 2001). However, the molecular components involved in CNT neurospecific binding and trafficking are still largely unknown. One of the reasons for this can be found in the unavailability of specific biochemical tools. The CNT H\textsubscript{C} domain has been shown to be involved in the first step of CNT intoxication, consisting of the specific binding to neuronal membranes (Halpem and Neale, 1995; Herreros et al., 1999). Historically, CNT H\textsubscript{C} fragments had been obtained through papain cleavage of the native holotoxins (Bizzini et al., 1977; Helting and Zwisler, 1977). Only Halpem and Loftus had used a recombinant approach consisting of in vitro-translated wild-type and truncated TeNT H\textsubscript{C} fragments (Halpem and Loftus, 1993). Despite the importance of these studies for the preliminary characterisation of the binding ability of this domain, we felt that recombinant CNT H\textsubscript{C} fragments, available in biochemical amounts (mg) and epitope-tagged for specific radiolabelling and immunodetection would represent more flexible tools for the study of CNT neurospecific binding. We have described the expression and characterisation of such recombinant TeNT, BoNT/A, /B and /E H\textsubscript{C}s. In the past, the purification of CNT H\textsubscript{C} domains from bacteria had generally proven unsuccessful, due to low expression levels and relatively high insolubility of these fragments. The reason for these findings could be attributed to the presence of several rare codons in the CNT sequences (Makoff et al., 1989), leading to premature termination of translation and overall poor expression. Moreover, the low rate of translation may result in the association of H\textsubscript{C} with bacterial chaperones, leading to its targeting to inclusion bodies (Misawa and Kumagai, 1999). Using variants of the pGEX expression vector, we successfully
expressed TeNT, BoNT/A and /E Hc as soluble GST-fusion proteins in standard strains of E. coli. On the other hand, very limited expression of soluble protein was observed for BoNT/B Hc in strains based on the K-12 genotype. The reason for this lack of expression is not clear. Careful examination showed that all the BoNT Hc fragments are similar in number and type of rare codons. However, a possible explanation for the poor BoNT/B Hc expression could be due to the presence of multiple triplets of consecutive rare codons concentrated in the first third of the sequence, suggesting that the use of non K-12 bacterial strains could overcome the low expression problem. Indeed this was the case, since BoNT/B Hc of the expected molecular mass was successfully obtained in the TOPP3 strain.

The purified recombinant CNT Hc's bind to polysialogangliosides in vitro and, more importantly, antagonise the activity of the corresponding parental holotoxins in different experimental systems, indicating a functional interaction with the same acceptors of the native toxins (Lalli et al., 1999). All our Hc's displayed a binding preference for gangliosides of the G1b series, consistent with previous work showing that TeNT binds with the highest affinity to GT1b and GD1b, whereas both BoNT/B and E preferentially interact with GT1b (Halpern and Neale, 1995). The different binding affinity observed in vitro for BoNT Hc's is in line with the fact that distinct BoNT serotypes bind similarly but not identically to gangliosides (Black and Dolly, 1986; Kitamura et al., 1980; Ochanda et al., 1986). One possible reason for this observation may lie in the significant amount of divergence in the sequence of CNT binding domains. This is reflected in the slightly different orientation of the Hc portion observed in the CNT structures solved so far and, in the Hc-C domain, in a different organisation of the loop regions containing ganglioside binding sites (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000).
Our recombinant $H_c$s inhibit the intracellular proteolytic activity of the native neurotoxins, as shown by the protection of the CNT-mediated SNARE cleavage detected through immunofluorescence and immunoblotting. This is an established and sensitive method to test the intoxication by native CNTs at concentrations as low as picoMolar in a variety of cellular systems (Schiavo and M., 1997; Williamson et al., 1996). All fragments effectively prevented the proteolysis of the target mediated by the parental holotoxin in a concentration-dependent manner, indicating that our $H_c$s bind to functional acceptors responsible for effective toxin binding and internalisation. The ability of these recombinant domains to antagonise the intracellular targeting of the native holotoxins was also clearly demonstrated by the significant delay in the onset of paralysis (75-150%) of murine phrenic nerve-hemidiaphragm preparations. This system preserves the integrity of a fully developed NMJ and is widely used to study the effects of many different neurotoxins, including CNTs (Wohlfarth et al., 1997). Our results are in contrast with a previous study reporting no competition between the binding of native BoNT/A and its heavy chain prepared by chemical dissociation using similar murine NMJ preparations (Daniels-Holgate and Dolly, 1996). The reason for this discrepancy could be due to a loss of heavy chain biological activity during the isolation process or, alternatively, to a different conformation or aggregation state of the isolated fragments leading to varying biological function. In our experiments we had to use a higher concentration of TeNT compared to BoNTs to induce paralysis (10 nM versus 0.2 nM, respectively). This reflects the well-established fact that higher doses of TeNT compared to BoNTs are needed to cause paralysis at the neuromuscular junction (Simpson, 1984a; Simpson, 1985). Due to the large volume of the electrophysiological bath, a ratio between binding domain and holotoxin higher than 100:1 was possible only for BoNT $H_c$s that had to be applied in a
1,300X excess to observe a substantial delay in the onset of paralysis. Indeed, experiments performed with lower amount of BoNT H$_C$s resulted in a reduced level of protection, possibly due to the sequestration of non-specific low-affinity binding sites present on the tissue. These low-affinity sites would then release bound H$_C$ slowly, resulting in an apparently lower protecting effect from BoNT-induced paralysis.

Recent studies have pointed to the SSV proteins synaptotagmins as possible receptors of BoNT/A, B and E (Li and Singh, 1998; Nishiki et al., 1994; Nishiki et al., 1996a; Nishiki et al., 1996b). It was proposed that these BoNTs would enter neurons by binding to the lumenal domain of synaptotagmins during the SSV recycling process. However, the validity of this model and the fact that different BoNT serotypes interact with the same protein receptor are still debated. For example, antibodies against the lumenal domain of synaptotagmin do not prevent binding of BoNT/B to neuronal membranes nor do they protect the murine hemidiaphragm from toxin-induced neuromuscular blockade (Bakry et al., 1997). In addition, several studies have reported lack of competition among BoNT/A, /B and E serotypes in the binding process (Evans et al., 1986; Habermann and Dreyer, 1986). Consistent with these observations we observed a complete lack of competition with our recombinant BoNT/B H$_C$ on the neuromuscular paralysis induced by BoNT/E (O. Rossetto, unpublished results).

Our recombinant CNT H$_C$ fragments will help to address important questions, such as a full characterisation of the nature and distribution of CNT receptors and the possible presence of common components in the interaction of different CNTs with the neuronal membrane. The rest of this discussion will focus on the conclusions obtained from the studies performed with TeNT H$_C$ and their implications in neuronal endocytosis and trafficking.
4.2 A putative TeNT protein receptor in neuronal cells

Since the original demonstration of the interaction of TeNT with gangliosides present on the neuronal surface, there have been a large number of reports on the characterisation of TeNT binding to gangliosides in vitro and in vivo (Halpern and Neale, 1995; Schiavo et al., 2000). Although this is a well-documented fact, there are some concerns about the role of these lipids as unique TeNT receptors. The relative low affinity of toxin-ganglioside interaction and the presence of polygangliosides also in non-neuronal cells suggest that other membrane components may be involved in TeNT neurospecific binding process (Herreros et al., 1999; Pierce et al., 1986). Moreover, it is difficult to explain the different intracellular trafficking of TeNT and BoNTs only on the basis of the interaction with gangliosides, to which these neurotoxins bind with similar affinity (Critchley et al., 1986; Habermann and Dreyer, 1986; Ochanda et al., 1986; Schengrund et al., 1991). Additional specific co-receptors such as protein components on the plasma membrane could be responsible for binding of different CNTs and possibly lead to the distinct sorting of these neurotoxins at the synapse. Protease-sensitive TeNT-binding sites have been observed in different preparations ranging from rat brain membranes and synaptosomes to cerebral neurons in monolayer culture (Critchley et al., 1986; Pierce et al., 1986; Yavin and Nathan, 1986). In addition, crosslinking experiments using native [125I]-TeNT in NGF-differentiated PC12 cells had led to the discovery of a putative 15-kDa protein receptor for this neurotoxin (Schiavo et al., 1991). We confirmed this finding by using radiolabelled TeNT Hc. Importantly, our results indicate the existence of a 15-kDa protein receptor for TeNT also in mouse spinal cord cells and in MNs, the physiological TeNT target cells. p15 displays similar characteristics in all tested cellular systems: it behaves as an integral membrane protein and it is N-glycosylated (Herreros et al., 2000a). This finding is
consistent with the observation that sialic-acid specific lectins are able to antagonise TeNT intoxication at the murine phrenic nerve hemidiaphragm (Bakry et al., 1991a). Sialic acid-containing oligosaccharides are therefore essential for the functional interaction of TeNT with its receptor(s), which could then consist of both polysialogangliosides and glycoproteins, such as p15. In this regard, a previous report had shown the absolute requirements of gangliosides to mediate TeNT intoxication. Ganglioside-depleted mouse spinal cord cells are protected from the toxin action, which is restored upon re-addition of exogenous gangliosides (Williamson et al., 1999). In addition, mice lacking complex gangliosides, are less sensitive to TeNT intoxication (Kitamura et al., 1999). These findings do not exclude the presence of a protein component involved in toxin binding, but underline an essential role of gangliosides in delivering TeNT to its substrate. A possible model could envisage a role of polysialogangliosides in recruiting TeNT on the membrane in close vicinity to a specific high-affinity protein receptor. This could be facilitated by the ability of gangliosides to induce multivalent crosslinking of TeNT, based on the presence of multiple sugar-binding sites on TeNT Hc (Fotinou et al., 2001). Previous evidence supports the cooperation of protein-ganglioside components also for BoNT/B, since its binding to its putative protein receptor (synaptotagmin) is dependent on the presence of gangliosides GT1b and GD1a (Nishiki et al., 1994). One could thus speculate that CNT binding specificity would be the result of preferential interaction with some gangliosides of the 1b series coupled with a specific protein receptor(s). Interestingly, the binding of TeNT Hc to neuronal cells and to p15 is competed by TeNT or the native Hc, but is not affected by the BoNT/A and /B Hc-s, consistent with the fact that TeNT and several BoNT serotypes do not compete with each other for binding to rat brain preparations (Habermann and Dreyer, 1986). This would once more indicate that binding to
polysialogangliosides, a feature shared by all CNTs, is not sufficient to compete with the interaction of TeNT with neurons. On the other hand, BoNT/E Hc was able to decrease TeNT Hc binding to p15, an effect particularly visible in MNs (Herreros et al., 2000a). This indicates that TeNT and BoNT/E may share the same protein receptor on the neuronal plasma membrane, a result consistent with the reported TeNT Hc antagonism of BoNT/E action at the neuromuscular junction (Simpson, 1984a). Indeed, preliminary crosslinking experiments in MNs have revealed that BoNT/E binds to a protein with a mobility similar to p15, although less efficiently. A better characterisation of the different affinities of TeNT and BoNT/E for p15 should help clarify the determinants of the specificity of the two neurotoxins in the interaction with the neuronal surface.

4.3 TeNT binding to the neuronal membrane

4.3.1 Involvement of GPI-anchored proteins and lipid rafts

p15, the putative TeNT protein receptor, is GPI-anchored in different cells, including MNs (Herreros et al., 2001). In NGF-differentiated PC12 cells p15 is Thy-1, a small GPI-anchored molecule found in the nervous system and T-lymphocytes, where it is expressed in several distinct glycoforms (Morris, 1992; Parekh et al., 1987). Thy-1 has been involved in multiple processes, including neurite outgrowth (Tiveron et al., 1992), long-term potentiation (Nosten-Bertrand et al., 1996) and T-cell receptor signalling (Hueber et al., 1997). However, Thy-1 does not appear to be essential for development, survival nor fitness, since Thy-1 knockout mice display only a regional inhibition of long-term potentiation that does not affect normal spatial learning (Nosten-Bertrand et al., 1996).

Thy-1 expression is enhanced upon NGF-differentiation in PC12 cells (Richter-Landsberg et al., 1985), consistent with the pronounced appearance of the 65-kDa crosslinking
product and the bright TeNT surface immunostaining observed after NGF treatment. However, in neurons p15 is not Thy-1, as demonstrated primarily by the recovery of the crosslinking product in Thy-1 knockout mice. In addition, Thy-1 does not appear to be necessary for binding nor for intracellular trafficking of TeNT, since Thy-1 knockout mice are not resistant to TeNT intoxication. Interestingly, in MNs Thy-1 is ubiquitously present on the plasma membrane, differently from what reported in hippocampal neurons, where this GPI-anchored protein is almost exclusively located in the axonal domain (Dotti et al., 1991). This may suggest that distinct sorting pathways could be responsible for the delivery of Thy-1 to its final destination in the two neuronal cell types. On the MN surface, both Thy-1 and TeNT Hc appeared to concentrate in patches after antibody-mediated crosslinking, a possible indication of clustering in contiguous membrane microdomains. Some preliminary observations suggest that internalised TeNT Hc carriers may contain also Thy-1, consistent with the observation that anti-Thy-1 antibodies are retrogradely transported in the rat nervous system (LaRocca and Wiley, 1988). Further work will address the possibility that Thy-1 may represent a potential marker of at least a fraction of the retrograde organelles responsible for TeNT Hc transport.

The discovery that p15 is GPI-anchored points to a possible interaction of TeNT Hc with lipid rafts on the neuronal membrane. Although the segregation of cholesterol- and sphingolipid-enriched regions in artificial membranes is well established (Brown and London, 1998; Dietrich et al., 2001; Rietveld and Simons, 1998; Vyas et al., 2001), the detection of rafts in cell membranes has been the subject of intense controversy (Edidin, 1997; Galbiati et al., 2001; Jacobson and Dietrich, 1999; Mayor et al., 1994). Single particle tracking studies on GPI-anchored proteins and potentially raft-associated lipids have revealed differently sized regions of confined motion (Jacobson and Dietrich, 1999;
Pralle et al., 2000; Schutz et al., 2000), and functionally different GPI-anchored proteins have been shown to reside in different domains on neuronal membranes (Madore et al., 1999). Fluorescence resonance energy transfer (FRET) methodology (Varma and Mayor, 1998) and classical chemical cross-linking techniques (Friedrichson and Kurzchalia, 1998) have demonstrated that at least a fraction of GPI-anchored proteins are present in small clusters at the surface of living cells. However, other experiments using different FRET methodology failed to detect such clusters (Kenworthy and Edidin, 1998; Kenworthy et al., 2000). In addition, the role of sphingolipids in the organisation of GPI-anchored proteins in rafts remains to be fully investigated. We have obtained several results suggesting that TeNT Hc is recruited to lipid microdomains. First, a significant amount of the protein is recovered in DIG fractions upon binding in both NGF-differentiated PC12 and in spinal cord cells (Herreros et al., 2001). Second, the punctate plasma membrane staining observed after binding to a variety of cells, including MNs is reminiscent of the patched distribution of raft markers such as placental alkaline phosphatase (PLAP) or folate receptor detected in non-neuronal cells (Harder et al., 1998; Mayor et al., 1994). Notably, antibody-mediated crosslinking of the VSV-G-tagged TeNT Hc molecules bound to the neuronal surface caused a substantial clustering effect, leading to the appearance of more intense punctate staining. The antibody could coalesce and stabilise the lipid microenvironments (possibly enriched in G_{1b} gangliosides and the putative GPI-anchored receptor) with which TeNT Hc preferentially interacts. Finally, the association of TeNT Hc with Thy-1 in PC12 cells occurs in the DIG fraction (Herreros et al., 2001), suggesting that recruitment to rafts may facilitate the delivery of the toxin to a putative high-affinity receptor. It will be important to verify this interaction of TeNT Hc with the GPI-anchored p15 also in DIGs derived from neuronal cells. Interestingly, release of GPI-anchored
proteins from plasma membranes with PI-PLC did not affect TeNT $H_c$ total binding (Figure 3.15), indicating an important role of gangliosides as primary TeNT $H_c$ acceptors on the neuronal surface. However, this finding does not rule out the existence of a pool of GPI-anchored receptor resistant to PI-PLC treatment under our experimental conditions.

A crucial issue that requires further analysis is the role of p15 in binding and entry of TeNT into neurons. The determination of PI-PLC effect on toxin recruitment to rafts and subsequent internalisation could provide functional evidence for the involvement of p15 in TeNT intoxication. Treatment of mouse spinal cord cells with PI-PLC and cycloheximide (to prevent neosynthesis of GPI-anchored proteins) prevents TeNT-mediated VAMP cleavage, thus suggesting a crucial role of GPI-anchored proteins in TeNT trafficking (Munro et al., 2001). Until now the identification of p15 has been hampered by the low amount of crosslinking product obtained with primary neuronal cultures. The possible enrichment of this GPI-anchored protein in lipid rafts suggests alternative strategies, such as immunoprecipitation from DIG fractions after binding of VSV-G-tagged TeNT $H_c$.

A large body of work has demonstrated the involvement of lipid microdomains in the interaction of many pathogens such as bacteria and viruses with their target cells (van der Goot and Harder, 2001). Rafts provide a way to concentrate receptors for pathogens or toxins by increasing binding affinity or promoting toxin oligomerisation, as in the case of aerolysin from *Aeromonas hydrophila*, which specifically binds to GPI-anchored proteins (Abrami and van der Goot, 1999). Another example is cholera toxin that interacts with the ganglioside GM1, found on the plasma membrane but especially concentrated in rafts. This facilitates a multivalent, high-affinity interaction of the toxin pentameric binding subunit with its ganglioside receptor (Parton, 1994) (Wolf et al., 1998). Notably, disruption of rafts did not interfere with cholera toxin binding, but inhibited its
internalisation and subsequent cAMP accumulation in CaCo-2 human intestinal epithelial cells (Orlandi and Fishman, 1998). These results indicate that lipid microdomains could provide a preferential site for high-affinity toxin interaction with the target cell leading to toxin internalisation. A similar model could form the basis of TeNT binding to lipid rafts, which could act as concentrating platforms for the toxin on the plasma membrane. A multiple interaction with lipid and protein components enriched in lipid microdomains could confer TeNT the extremely high affinity and neurospecificity observed in vitro and in vivo (Montecucco, 1986) and would explain the apparent irreversibility of TeNT binding to the neuronal surface (Habermann and Dreyer, 1986; Schmitt et al., 1981). In addition, the presence of multiple ganglioside-binding sites in the H₃ domain (Emsley et al., 2000; Fotinou et al., 2001), together with the ability of TeNT to form homodimers (Ledoux et al., 1994) further support the concept of the multivalent nature of the toxin-raft interaction that would be characterised by low dissociation rates. TeNT could then enter passively through a default mechanism mediating the intracellular trafficking of lipid microdomains, or actively trigger endocytosis by interacting with signal molecules present in membrane rafts.

Interestingly, cholesterol depletion with MCDX caused the displacement of a fraction, but not all the TeNT H₃ from DIGs, suggesting the association of the toxin domain with different raft sub-pools. Importantly, the same treatment also prevented TeNT H₃ internalisation in spinal cord cells and protected from TeNT-induced VAMP cleavage (Herreros et al., 2001). These findings point to the existence of a specialised sub-pool of lipid rafts responsible for the successful binding and internalisation of TeNT, as shown for other pathogens, such as Simian Virus 40 (Pelkmans et al., 2001) or pathogenic E. coli strains (Baorto et al., 1997; Shin et al., 2000).
Cholesterol-sequestering drugs inhibit endocytosis from lipid rafts-caveolae in non-neuronal cells (Deckert et al., 1996; Orlandi and Fishman, 1998; Schnitzer et al., 1994). However, their selectivity for the disruption of lipid microdomains has been challenged by recent studies reporting inhibition of clathrin-mediated endocytosis caused by cholesterol depletion (Rodal et al., 1999; Subtil et al., 1999). Transferrin uptake in spinal cord cells treated with the same amount of MCDX that blocked TeNT intoxication was not significantly different from untreated cells (Herreros et al., 2001), indicating that the classical clathrin-mediated endocytosis was not impaired by MCDX addition.

These results and the almost complete exclusion between TeNT $H_C$ and clathrin in immunofluorescence experiments argue against a clathrin-mediated internalisation process. However, our results cannot conclusively rule out the involvement of clathrin in TeNT uptake. In this regard, TeNT is found in coated pits and vesicles in MNs, but the nature of the electron-dense coating still has to be determined. Further investigation including analysis of the effect of inhibition of clathrin-dependent endocytosis on TeNT $H_C$ internalisation and double cryoimmunogold labelling with anti-toxin and anti-clathrin antibodies should help address this unresolved issue.

### 4.3.2 TeNT internalisation and the actin cytoskeleton

Treatment of MNs with low amounts of Lat B prevented TeNT $H_C$ internalisation. Studies in budding yeast have established a clear relationship between actin and endocytosis that is still questioned in mammalian cells (Jeng and Welch, 2001). In fact, actin-disrupting agents have had variable effects, depending on the assay and the cell type (Fujimoto et al., 2000; Jeng and Welch, 2001). Actin has been implicated in different steps along the coated and uncoated endocytic pathways. These include membrane invagination,
membrane fission during vesicle formation and propulsion of the newly formed endocytic vesicle into the cytoplasm (reviewed in Qualmann et al., 2000). Recent evidence is strengthening a functional link between actin and endocytosis also in neuronal cells. The brain-enriched profilin II isoform, which promotes actin dynamics in vivo was shown to be able to interact with dynamin (Witke et al., 1998), as well as the F-actin-binding protein Abp1 (Kessels et al., 2001)

A large amount of biochemical and morphological studies have also pointed to an important role of the actin cytoskeleton in the SSV cycle. Synapsin, a major SSV-associated protein, binds to actin (Cremona and De Camilli, 2001), whereas disruption of synaptojanin 1 function at the synapse impairs SSV cycle and causes accumulation of actin around active zones (Cremona et al., 1999). This effect might be correlated with alteration in levels of PtdIns(4, 5)P$_2$, a potent regulator of both actin nucleation and endocytosis which is hydrolysed by synaptojanin (Rohatgi et al., 1999; Slepnev and De Camilli, 2000). Preliminary EM analysis of Lat B-treated cells revealed a dispersed membrane staining of TeNT H$_C$. This suggests that an intact actin cortex might be required to initiate membrane invagination and/or to localise the endocytic machinery to special areas of the neuronal plasma membrane. Indeed, the restriction of coated pit formation to specific sites has been demonstrated in mammalian non-neuronal cells (Gaidarov et al., 1999) and at Drosophila NMJ (Roos and Kelly, 1999). Moreover, the actin cytoskeleton has been recently involved in regulating dynamic association of raft components (Oliferenko et al., 1999). Occasionally, we have also detected TeNT H$_C$-labelled invaginations in Lat B-treated cells, suggesting the requirement of F-actin in vesicle fission from the plasma membrane. This possibility is supported by the link between actin function and the GTPase dynamin, which is shared by coated and non-coated endocytic
pathways in mammalian cells (Henley et al., 1998) (Oh et al., 1998; Slepnev and De Camilli, 2000).

4.4 Insights from a retrograde transport assay in living MNs

4.4.1 Two classes of retrograde carriers

Previous works demonstrated the ability of TeNT Hc to undergo axonal retrograde transport in vivo (Bizzini et al., 1977; Coen et al., 1997; Fishman and Carrigan, 1987; Morris et al., 1980). These studies have mainly relied on the accumulation of Hc at the site of nerve ligation after its intramuscular injection, followed by immunohistochemistry or autoradiography. Here we visualise for the first time the retrograde transport of TeNT Hc in living MNs. Our results highlight the presence of two major morphological classes of retrograde carriers: round vesicles and tubulo-vesicular organelles which have also been observed in EM studies. Other time-lapse microscopy studies had shown retrograde round carriers labelled by fluorescent dextran, a general endocytic marker in dorsal root ganglia neurons (Nakata et al., 1998). In hippocampal neurons, long tubules are used for the anterograde delivery of newly synthesised proteins to the neuronal periphery (Kaether et al., 2000; Nakata et al., 1998), while bi-directional tubulo-vesicular structures labelled by GFP-syntaxin13 appear to function as sorting/recycling compartment (Prekeris et al., 1999). Our study identifies a novel type of retrograde axonal carriers, thin tubules up to 5 \( \mu \text{m} \) long, characterised by a fast average speed (about 2 \( \mu \text{m/s} \)) with peak values of 3.6 \( \mu \text{m/s} \). A typical feature of these organelles is their apparently continuous movement persisting over long distances. Although a rigorous definition of continuous movement cannot rely on time-lapse imaging, even with the fastest acquisition rates allowed by our experimental conditions (3.3 frames/s for low-light fluorescence microscopy and 4.2
frames/s for confocal analysis) we still observed an apparently uninterrupted motion of TeNT Hc-labelled tubules. This type of progression could be clearly distinguished from the movement of the round TeNT Hc-positive carriers, characterised by slower speed values and stationary pauses. The fast mode of transport observed for the tubules might be due to a coordinated effort of the molecular motors linked to these organelles, resulting in a higher speed compared to the round saltatory carriers likely to be connected to fewer motors. At this stage it is still unclear if the round organelles and tubules are part of the same retrograde route or if they belong to two different transport pathways. Preliminary observations suggest that they may be linked, since the number of tubules tends to increase with time whereas the round organelles tend to disappear. In the current experimental conditions, the asynchronous internalisation of TeNT Hc in different regions of the MNs limits a chronological characterisation of the multiple transport carriers involved. It should be possible to resolve this issue with the use of compartmented cultures (Campenot et al., 1996) that would allow selective administration of TeNT Hc at specific sites on the MN surface (such as the axon tip). By avoiding the problem of multiple overlapping endocytic events, this system could facilitate the observation of the transport process to the cell body and help in the characterisation of the other types of retrograde organelles observed, like the faint carriers with variable shape and speed.

4.4.2 TeNT and TeNT Hc share retrograde carriers in living MNs

The speed values observed for TeNT Hc carriers are in the same range reported for fast axonal transport (1-5 μm/s) (Goldstein and Yang, 2000). Importantly, they are also consistent with the rates calculated for TeNT retrograde transport in vivo, ranging between 0.8 and 3.6 μm/s (Halpern and Neale, 1995; Lazarovici et al., 1988; Stöckel et al., 1975).
Conflicting views on the efficiency of retrograde transport of TeNT Hc compared to full-length TeNT have emerged from previous studies (Coen et al., 1997; Fishman and Carrigan, 1987; Morris et al., 1980; Weller et al., 1986). Importantly, in MNs TeNT retrograde carriers displayed a speed distribution almost identical to TeNT Hc-labelled organelles. This strongly suggests that the Hc fragment contains the determinants for the sorting to endosomes associated with specific motor proteins responsible for the type and direction of movement observed for the holotoxin. Our double-labelling experiments carried out by simultaneously applying fluorescent TeNT and the Hc domain to MNs provide some further insights into this matter. TeNT appeared to be internalised faster than Hc, since it already accumulated in vesicular endosomes at the end of a short incubation period, whereas the Hc at the same time still gave only an intense plasma membrane staining. This indicates that portions of the TeNT molecule other than Hc could participate in ensuring efficient toxin internalisation. Notably, at later time points, we could observe colocalisation of TeNT and Hc in both moving and stationary organelles, suggesting an overlap of endocytic routes and putative traffic stations during transport. We therefore conclude that, although the Hc fragment might not be as efficiently internalised as the holotoxin, it may share the same retrograde transport pathway of TeNT. Interestingly, preliminary observations have revealed an immediate uptake of both proteins in overlapping compartments in cell bodies, suggesting the involvement of distinct endocytic systems for the entry of the toxin in the axonal and somatodendritic domains.
4.4.3 TeNT H<sub>c</sub>C: the minimal binding (and trafficking) subdomain?

The β-trefoil carboxy-terminal domain of TeNT H<sub>c</sub> (H<sub>c</sub>C) has been shown to directly interact with polysialogangliosides (Emsley et al., 2000; Fotinou et al., 2001; Shapiro et al., 1997). We demonstrated that recombinant H<sub>c</sub>C is able to bind to neuronal cells and to the putative protein receptor p15 in crosslinking experiments (Herreros et al., 2000b). This sub-domain is internalised in both NGF-differentiated PC12 and MNs, as detected by immunostaining. However, the vesicular staining observed in fixed cells did not appear as intense as the one obtained after internalisation of similar TeNT H<sub>c</sub> molar amounts. This could be explained by a less efficient internalisation of TeNT H<sub>c</sub>C or by its mistargeting and partial degradation. Time-lapse experiments in living MNs showed that TeNT H<sub>c</sub>C is still internalised and transported along axons, but the number of retrograde carriers seems lower compared with TeNT H<sub>c</sub>. Quantitative kinetic analysis and double-labelling experiments with TeNT H<sub>c</sub> could help to clarify whether H<sub>c</sub>C shares the same retrograde carriers of H<sub>c</sub>. This could have important implications in the design of small vectors for neurospecific delivery of therapeutic agents. Interestingly, the H<sub>c</sub>C displayed a discontinuous membrane staining in living cells, possibly explained by the tendency of this sub-domain to oligomerise ((Herreros et al., 2000b) and J. Herreros and G. Schiavo, unpublished results). Future experiments addressing the recruitment of H<sub>c</sub>C to rafts will provide some insight on the interaction of this TeNT sub-domain with the neuronal surface.

TeNT H<sub>c</sub>N displayed a very different behaviour from H<sub>c</sub>C. Although the VSV-G-tagged version of H<sub>c</sub>N did not bind to NGF-differentiated PC12 cells and MNs, H<sub>c</sub>N-Alexa488 was internalised, although in low amounts, in somatodendritic regions. The latter result is consistent with the poor, but detectable binding of radiolabelled H<sub>c</sub>N in crosslinking
experiments (Figure 3.14 B, right panel) (Herreros et al., 2000b). One reason for this discrepancy could be that, although detectable in Western blot, the VSV-G tag of HcN might not be easily accessible to anti VSV-G antibodies in fixed cells. Notably, HcN did not appear to interact with p15, further strengthening the concept that HcC is uniquely responsible for the binding to the putative protein receptor. The striking exclusion of HcN-labelled organelles from axons is a further proof of distinct endocytic systems functioning in different neuronal domains. One could speculate that the lectin-like domain present in HcN might be able to mediate internalisation in a still uncharacterised somatodendritic compartment, possibly through interaction with carbohydrate units linked to lipids (such as gangliosides) or to proteins excluded from axons. In this regard, it is surprising that ganglioside-binding sites have been detected only in the HcC sub-domain but not in the HcN, which is structurally similar to sugar-binding proteins (Umland et al., 1997). One of the possible explanations could reside in the specificity of the HcN lectin-like domain for certain types of oligosaccharides present only in cell body and dendrites. HcN could therefore gain access exclusively to a somatodendritic compartment, similar to the recycling basolateral compartment found in polarised epithelial cells (Hauri et al., 2000).

4.4.4 Cytoskeletal requirements for TeNT Hc retrograde transport

Selective disruption of MTs or F-actin caused a major block of TeNT Hc retrograde carriers and the slowing of residual mobile organelles to speed values usually lower than 0.8 μm/s. These carriers could be simultaneously associated with MT- and actin-based motors acting in a coordinated fashion to ensure efficient retrograde transport. A dramatic example of this cooperation comes from the study of pigment granules (melanosomes) in melanophores. Melanosomes are transported bidirectionally along MTs by cytoplasmic
dynein and kinesin II, and are dispersed throughout the cytoplasm by moving along filamentous actin with the participation of myosin V (Rogers and Gelfand, 2000). Organelles in squid axoplasm, as well as mitochondria in living neurons, move along both actin microfilaments and MTs (Kuznetsov et al., 1992; Morris and Hollenbeck, 1995). A recent study showing a direct interaction of myosin V with conventional kinesin raised the possibility that MT- and actin-based motors might exist in multifunctional motor complexes transporting organelles along both cytoskeletal systems (Huang et al., 1999b). Furthermore, the fact that myosin Va and the intermediate chain of cytoplasmic dynein share an 8-kDa light chain (Mermall et al., 1998), and that this light chain self associates (Benashski et al., 1997), could indicate that these two motors may also interact. Myosin Va is present on bidirectional axonal vesicles and appears to be involved in local movement, especially in regions poor in MTs (Bridgman, 1999). The latter do not extend along the entire axonal length (Bearer and Reese, 1999), and actin filaments might act as “bridges”, ensuring a fast switch between MT tracks, as suggested by time-lapse observations. The striking retrograde bias of TeNT Hc carriers clearly indicates the association with minus-end directed motors, such as the KIFC2 kinesin (Hanlon et al., 1997; Saito et al., 1997) or the processive dynactin-dynein complex (King and Schroer, 2000). The putative association with the latter motor might be facilitated by an enrichment of acidic phospholipids in TeNT Hc-positive membranes allowing initial interaction with the PH domain of spectrin, recently shown to play an essential role in retrograde axonal transport (Muresan et al., 2001). Other unknown protein components of the vesicular carriers might also ensure more stable polyvalent interactions with spectrin. Our experimental system provides a useful assay for the identification of the motor molecules involved in TeNT Hc retrograde transport, a major goal for future research.
The substantial block of TeNT Hc movement in cell bodies observed after vin or Lat B treatment indicates the requirement of both MTs and actin microfilaments for endosomal trafficking also in the somatodendritic domain. MT disruption impairs cargo transport at different steps of recycling and transcytosis (Apodaca, 2001; Kamal and Goldstein, 2000). Endosomal Rabs could regulate interaction with MT-dependent motors (Echard et al., 1998), as recently shown for Rab5, able to increase minus-end directed motility by controlling a still unidentified kinesin (Nielsen et al., 1999) and for Rab7, that recruits dynein through its effector RILP (Jordens et al., 2001). The bidirectional movement of TeNT Hc in dendrites could possibly involve the participation of minus-end directed dynein and KIFC2 (Goldstein and Yang, 2000), whereas KIF21B, a novel kinesin member highly enriched in dendrites could represent a candidate plus-end directed motor (Marszalek et al., 1999). However, these possibilities can be tested only by the biochemical isolation of TeNT Hc-positive compartments or by observing the effect of the selective disruption of these motors. In addition, myosins have been implicated in secretory and endocytic traffic in yeast, plants and mammalian cells (Brown, 1999; Kamal and Goldstein, 2000). A close coordination between actin- and MT-dependent motors could therefore ensure correct organelle localisation also in MN cell bodies and dendrites.

4.5 A novel neuronal endocytic pathway

4.5.1 TeNT internalisation through SSV recycling

In vivo, TeNT enters MNs only at the NMJ, since axons are protected by the myelin sheath. Interestingly, in cultured MNs TeNT binds and is internalised not only in nerve terminals, but also along neurites and cell bodies, demonstrating the presence of functional receptors in different cellular membrane domains. In particular, the accumulation of the
TeNT H$_c$ in points of contact between neurites and at synaptic junctions could suggest the existence of preferred endocytic areas, possibly enriched in high-affinity receptor(s). TeNT internalisation through SSV recycling, as reported for hippocampal neurons (Matteoli et al., 1996), does not seem to be the main mechanism for entering MNs, as indicated by the very limited colocalisation with the SSV marker VAMP (Figure 3.46). Matteoli and collaborators showed that hippocampal neurons could internalise TeNT-Texas Red only under depolarising conditions (Matteoli et al., 1996). However, we could detect clear internalisation of both TeNT-Texas Red and TeNT H$_c$-Alexa488 in MNs in the absence of depolarisation. Moreover, the levels of internalised TeNT H$_c$-Alexa488 did not change upon BoNT/A treatment of MNs, in conditions of complete silencing of evoked neurotransmitter release and strong reduction of spontaneous release activity (Humeau et al., 2000). These findings are consistent with a previous work reporting unaltered TeNT uptake and retrograde transport at NMJ poisoned with BoNT/A (Habermann and Erdmann, 1978). Entry through SSV recycling would explain the old findings that animals injected with TeNT and exercised develop paralytic symptoms faster than animals at rest (Herreros et al., 1999). It is still possible that TeNT might exploit this mode of internalisation to get access to the inhibitory interneurons located in the spinal cord, after being retrogradely transported and released from MNs somas. This transcytosis process, which is one of the most interesting aspects of TeNT intoxication, is still an unresolved issue awaiting the establishment of proper experimental systems.

4.5.2 TeNT enters distinct somatodendritic and axonal endocytic pathways in MNs

The polarised localisation of the transferrin receptor and of the Rab 5 effector EEA1 indicates that MN somatodendritic and axonal regions represent two cellular domains with
different endocytic systems, as previously shown in hippocampal neurons (Mundigl et al., 1993; Wilson et al., 2000). BFA caused a clear tubulation of the TeNT $H_C$ somatodendritic compartment, whereas it did not affect the axonal carriers. The different sensitivity to this drug suggests the ability of the toxin fragment to enter at least two different endocytic pathways, one in cell bodies and one in axons. The somatodendritic TeNT endosomes have a tubulovesicular morphology similar to the compartments labelled by transferrin receptor-GFP (Burack et al., 2000) and positive for syntaxin 13 in hippocampal neurons (Prekeris et al., 1999). An extensive tubulovesicular endosomal network in dendrites had also been previously described (Mundigl et al., 1993; Parton et al., 1992). However, the very limited colocalisation observed with syntaxin 13, transferrin receptor and with fluorescent transferrin argue against the hypothesis of TeNT $H_C$ trafficking through classical recycling endosomes. Interestingly, TeNT $H_C$ internalisation appeared to be faster in the somatodendritic regions than along axons, again pointing to the existence of different endocytic mechanisms in the two neuronal domains. In cell bodies, it is likely that TeNT does not enter EEA1-positive early endosomes (Figure 3.39), suggesting the existence of still uncharacterised additional endocytic pathways.

The observed delay in the appearance of fluorescent TeNT $H_C$ axonal carriers could be due to the sensitivity limits of our system. Accumulation of enough fluorescent probe may be required for effective visualization of endosomal structures. This would suggest the possibility of endosome fusion and sorting events occurring along axonal processes. Indeed, we occasionally observed putative fusion and budding events of TeNT $H_C$-positive vesicles, similarly to what was previously shown for syntaxin 13 compartments in hippocampal neurons (Prekeris et al., 1999).
The peculiar itinerary of TeNT in MNs ending with a transcytosis process must involve trafficking through a compartment able to promote release of the toxin in the intersynaptic space. In our search for possible markers of TeNT Hc endocytic pathway, we considered the possible targeting of TeNT Hc to a compartment analogous to the apical recycling endosome, an organelle coordinating transcytosis in polarised epithelial cells and characterised by the presence of Rab11 (Casanova et al., 1999). Surprisingly, this GTPase displayed an abundant vesicular pattern throughout the entire MN. Rab11 might therefore participate in multiple trafficking pathways, instead of being restricted to specialised roles, as shown in polarised epithelial cells. Although conclusive evidence will need cryoimmunogold EM analysis, TeNT Hc did not appear to share compartments with Rab11, even two hours after internalisation. The conclusion emerging from these results points to the existence of one or more neuronal endocytic routes lacking classical endocytic markers that could be exploited by TeNT to enter MNs and to undergo transcytosis.

4.5.3 TeNT Hc retrograde carriers escape acidification during axonal transport

TeNT Hc-labelled organelles did not colocalise with acidic organelles and lysosomes stained by Lysotracker. Progression through the known degradative route includes acidification of early sorting endosomes (with a pH~6.2) in the trafficking to late endosomes (pH~5.0), up to lysosomes with lowest pH value (<5.0) (Gruenberg and Maxfield, 1995; Mellman et al., 1986). A previous report had analysed the distribution of endocytic organelle pH in axons of hippocampal neurons and found two main acidification steps occurring in the region located 50-150 μm from the growth cone, and in branching points, representing possible sorting stations for endosomes (Overly and
Hollenbeck, 1996). The striking exclusion of TeNT $H_c$ from acidic organelles and lysosomes strongly suggests that TeNT might use a retrograde route escaping acidification to reach the MN cell body. This conclusion is further supported by the distinct kinetic behaviour of the two compartments emerging from the tracking analysis. Overall, Lysotracker-labelled organelles are still retrogradely biased, but they are much slower than TeNT $H_c$ carriers (Figure 3.49 a), suggesting the association of the two compartments with a different repertoire or number of retrograde motors. Moreover, the increased incidence of reversal of the acidic organelles could indicate their association with functional anterograde motors and their involvement in trafficking pathways distinct from the one(s) of TeNT $H_c$. Our results are consistent with a previous study using colloidal gold-labelled TeNT *in vivo* showing most of the internalised toxin within smooth membranous structures different from electron-dense lysosomes (Schwab and Thoenen, 1978). Other reports had presented evidence of little degradation of TeNT in spinal cord cultures (Critchley et al., 1985; Habig et al., 1986). Also, the majority of TeNT $H_c$-positive organelles did not acidify in cell bodies, at least after 2 hours following incubation with the toxin fragment. The lack of targeting to lysosomes could have important functional consequences since TeNT has to be protected from acidification and degradation during transport to reach the adjacent interneuron in a fully active form. In this regard, Simian virus 40, *E. coli* and the malaria parasite *Plasmodium falciparum* were recently shown to use endocytic routes able to bypass the endosomal-lysosomal system in non-neuronal cells, thereby allowing their survival or productive infection (Lauer et al., 2000; Pelkmans et al., 2001; Shin et al., 2000). Interestingly, rafts have been involved in the internalisation of all these pathogens, similarly to TeNT $H_c$ (Herreros et al., 2001). In addition to providing a concentration platform on the membrane, cholesterol-enriched
micrdomains might also lead to an "alternative" endocytic pathway escaping degradation. It is tempting to speculate that the putative GPI-anchored protein receptor might contribute to this sorting, as recently shown by studies on the endocytic itinerary followed by GPI-GFP (Nichols et al., 2001), and even regulate retention for long times in endocytic compartments (Chatterjee et al., 2001).

4.6 TeNT Hc enters a retrograde endocytic pathway shared by NGF and p75

Endogenous ligands, like neurotrophins, might enter a retrograde transport pathway similar to the one used by TeNT to escape degradation and to reach intact the neuronal cell body. Indeed, NGF injected intramuscularly accumulates in murine spinal cord MNs without being degraded (Yan et al., 1988). Similarly, most of \(^{125}\)I-NGF applied to distal axons of sympathetic neurons in compartmented cultures is found intact in cell bodies, as shown by SDS-PAGE analysis (Ure and Campenot, 1994).

Importantly, we found that NGF shares retrograde vesicular compartments with TeNT Hc in MNs. Similar retrograde transport rates in the range of 0.8 - 1.5 \(\mu\)m/s for TeNT and NGF had been reported in adrenergic neurons in vivo (Stöckel et al., 1975), consistent with the speed calculated from our time-lapse experiments. We observed NGF in the TeNT Hc-labelled round organelles, but not in the tubules. There could be several explanations of this result. The sensitivity of our system may not allow the detection of NGF-Texas Red less concentrated in the larger volume of the tubules. Alternatively, the growth factor could be preferentially sorted in the round vesicles. Finally, NGF might be able to enter a tubular compartment at later stages of the retrograde endocytic route that we failed to monitor. Double-colour time-lapse analysis performed at longer chase periods should help address this issue. A significant lag between receptor binding and loading
onto the retrograde transport system has been previously reported for $^{125}$I-NGF in cultured rat sympathetic neurons (Ure and Campenot, 1997). Consistent with this observation, steady-state accumulation of $^{125}$I-NGF in cell bodies/proximal axons is reached in 8 hours in these cells (Claude et al., 1982). Before delivery to the retrograde transport compartment, NGF-containing organelles could possibly undergo a maturation step, as described for other receptor-internalised ligands, like EGF (Schmid et al., 1988; Stoorvogel et al., 1991).

Several reports have described the interaction of NGF with its receptor tyrosine kinase TrkA to form retrograde complexes finally resulting in nuclear responses (Ricco et al., 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). However, MNs do not express trkA (Yan et al., 1993), but they express $p75^{NTR}$. It has therefore been proposed that this protein might mediate the NGF retrograde transport observed during development (Yan et al., 1993; Yan et al., 1988).

The extensive colocalisation between TeNT $H_c$ carriers and $p75^{NTR}$ in MN axons strongly indicates that $p75^{NTR}$ could represent a marker of the TeNT $H_c$ retrograde route. Interestingly, this colocalisation was observed in the absence of NGF, since MNs had been extensively washed and incubated in serum- and growth factor-free medium. This would raise the possibility that $p75^{NTR}$ is internalised and retrogradely transported even in the absence of bound NGF. Such a constitutive transport of $p75^{NTR}$ is also supported by the fact that the IgG-192 antibody raised against the rat extracellular domain of $p75^{NTR}$ is retrogradely transported in sympathetic neurons and MNs (Taniuchi and Johnson, 1985; Yan et al., 1988). TeNT $H_c$ might therefore enter the $p75^{NTR}$ endocytic route to reach the MN cell body. However, it is still unclear when the TeNT $H_c$ and $p75^{NTR}$ trafficking
pathways overlap. Localised administration of TeNT H_c and simultaneous monitoring of p75^{NTR} traffic in living cells should give some clues to answer this important question.

The biological roles of p75^{NTR} appear to be very diverse. This membrane glycoprotein is structurally homologous to the members of a large gene family including tumor necrosis factor (TNF), CD40 and Fas (Smith et al., 1994). p75^{NTR} has an intriguing pattern of expression in the developing nervous system, including major populations of neurons projecting axons over long distances (Yan and Johnson, 1988). At maturity, expression is downregulated in MNs but persists in some motor pools (Koliatsos et al., 1993) and is dramatically upregulated after axotomy (Ernfors et al., 1989). The involvement of this receptor in modulating axonal outgrowth is also supported by the discovery of its possible interaction with the GTPase RhoA, a modulator of actin cytoskeletal assembly (Yamashita et al., 1999). In addition, the recent discovery of another p75^{NTR} splice variant lacking the extracellular neurotrophin binding domain, but with an intact intracellular signalling domain raises more questions about other possible biological functions, including blood vessel formation (von Schack et al., 2001). Besides increasing the affinity of neurotrophin binding to Trks and modulating the subsequent signalling responses, (Bibel et al., 1999; Hantzopoulos et al., 1994; Neet and Campenot, 2001), p75^{NTR} is also involved in apoptosis of different neuronal types (Fraade and Barde, 1998; Neet and Campenot, 2001). However, the expression of p75^{NTR} by many surviving neurons suggests that this receptor is likely to participate in multiple biological functions, including the internalisation/retrograde transport of BDNF and NT-4 (Curtis et al., 1995). TeNT H_c could therefore exploit a retrograde route used by physiological ligands such as neurotrophins to enter the nervous system.
Although the endocytic compartment responsible for p75<sup>NTR</sup> trafficking has not been characterised yet, recent studies with PC12 cells and brain plasma membrane preparations highlighted p75<sup>NTR</sup> ability to associate with caveolae-like domains (Huang et al., 1999a; Wu et al., 1997). It is tempting to imagine that specialised lipid domains could be involved also in p75<sup>NTR</sup> endocytosis and targeting to a retrograde route, which avoids degradation and is possibly shared by pathogens such as TeNT.

### 4.7 A model of TeNT H<sub>c</sub> binding, internalisation and transport in MNs

The work presented in this Ph.D. thesis was focussed on the use of a non-toxic TeNT binding fragment to investigate neuronal endocytic and retrograde transport pathways. Although we are far from a complete understanding of these processes, our results can be summarised in a hypothetical model presented in figure 4.1.

TeNT initially binds with low affinity to polysialogangliosides on the neuronal membrane (primarily GT<sub>1b</sub> and GD<sub>1b</sub>). The possible enrichment of these lipids in specialised membrane microdomains, together with their ability to induce multivalent crosslinking of distinct TeNT molecules (Fotinou et al., 2001) could help in recruiting the toxin in close proximity to a high-affinity putative GPI-anchored protein, also enriched in rafts (Herreros et al., 2001). These multiple lipid- and protein- interactions would facilitate the internalisation process requiring cholesterol and the recruitment of a still unidentified coat.

In this regard, it has recently been reported that in mouse cerebral cortex clathrin has also been found in DIGs together with other conventional raft markers (Parkin et al., 1999). Actin polymerisation could be required to initiate membrane invagination and ensure the fission of the endocytic pits. The possible presence of lipid microdomains enriched in GPI-anchored proteins in TeNT endosomes could favour their sorting in compartments.
Figure 4.1 A model of neuronal binding and internalisation of TeNT.
TeNT (red circles) binds to low-affinity polysialogangliosides on the neuronal membrane (1). The enrichment of gangliosides in lipid microdomains (blue), together with their ability to induce multivalent crosslinking of distinct TeNT molecules may help in recruiting TeNT to a putative high-affinity GPI-anchored protein receptor (green) (2). Lipid rafts can act as concentration platforms facilitating TeNT endocytosis, which involves F-actin (yellow) (3). The GPI-anchored receptor and the lipid composition of TeNT endosomes could help in sorting the toxin to retrograde organelles, which contain also p75NTR (purple) and escape degradation (4). Efficient retrograde transport requires both intact microtubules (red lines) and F-actin. Microtubule-dependent retrograde motors (brown ellipse) could ensure fast transport, whereas F-actin-dependent motors (yellow dot) may provide a fast switch between microtubule tracks on bridging actin filaments (yellow).
bypassing the classical recycling/degradative pathways. The lipid composition of the TeNT membrane compartments may also play a role in recruiting retrograde motors. Efficient transport of TeNT carriers could be ensured by the presence of both MT- and actin-based motors. TeNT-endosome fusion/sorting events could occur during axonal transport, but these compartments appear to reach the neuronal cell body without undergoing acidification. The presence of p75NTR in the TeNT Hc-positive compartments suggests that physiological ligands like neurotrophins could share the same retrograde route to escape degradation and reach the soma.

4.8 Future perspectives

This study sheds some light on a peculiar neuronal endocytic route tightly linked with axonal retrograde transport. Identification of the TeNT putative GPI-anchored protein receptor remains a crucial point to address, since it represents a fundamental step for the understanding of TeNT neurospecific binding and trafficking. Cell pretreatment with PI-PLC in DIGs preparations and in the retrograde transport assay might help clarify the functional role of this putative receptor and to establish its absolute requirement not only for recruitment to rafts, but also for efficient toxin internalisation and retrograde transport. Another important issue to investigate is the nature of the TeNT endocytic compartment. TeNT Hc carriers do not appear to contain the classical markers of recycling or degradative pathways. A biochemical approach consisting of the isolation of the organelles containing TeNT Hc linked to magnetic beads is currently underway and could provide valuable insights into the molecular composition of these endosomes.

The identification of the motors involved in TeNT retrograde transport also represents an essential aspect to investigate further. The retrograde transport assay described in this
thesis could be applied to cells where the function of motor proteins is altered, for example by microinjection of specific antibodies or dynamitin for dynein disruption (Ahmad et al., 1998). In addition, the possibility to expand our assay to mouse MNs could allow the analysis of retrograde transport in different transgenic lines such as the *dilute* (myosin V null) mice (Bridgman, 1999) or in murine models of spinal pathologies (Gurney et al., 1994; Mitumoto et al., 1994).

Finally, the use of fluorescently-tagged TeNT H\(_C\) sub-domains and mutants in the retrograde transport assay could provide a molecular analysis of the residues crucial for toxin trafficking. This approach could be helpful for a better understanding of the functions of the H\(_C\)N sub-domain and for the identification of additional regions in the H\(_C\)C able to promote endocytosis. Information derived from these studies could be of potential interest for the design of new drug delivery strategies in neuronal diseases affecting the spinal cord, such as MN pathologies and ALS.
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The binding fragment of Tetanus Neurotoxin: a probe to study neuronal endocytosis and retrograde transport

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