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Internalization of a GFP-tetanus toxin C-terminal fragment fusion protein at mature mouse neuromuscular junctions

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The distribution, dynamics, internalization, and retrograde axonal traffic of a fusion protein composed of green fluorescent protein (GFP) and the atoxic C-terminal fragment of tetanus toxin (TTC) were studied after its in vivo injection. Confocal microscopy and immunogold electron microscopy revealed that the fusion protein (GFP-TTC) rapidly clustered in motor nerve terminals of the neuromuscular junction. Clathrin-coated pits, and axolemma infoldings located between active zones appeared to be involved in the internalization of the fusion protein. Biochemical analysis of detergent-extracted neuromuscular preparations showed that the GFP-TTC fusion protein was associated with lipid microdomains. We suggest that GFP-TTC clustering in these lipid microdomains favors the recruitment of other proteins involved in its endocytosis and internalization in motor nerve terminals. During its retrograde trafficking, GFP-TTC accumulated in different axonal compartments than those used by cholera toxin B-subunit suggesting that these two proteins are transported by different pathways and cargos.

Introduction

Tetanus neurotoxin (TeNT), produced by the anaerobic bacterium *Clostridium tetani*, is one of the most potent, naturally occurring bioactive compounds known, and it is responsible for the severe neurological illness known as tetanus. TeNT enters the nervous system at the neuromuscular junction (NMJ), where it is internalized and retrogradely transported along the motoneuron axons by an unknown mechanism (Schiavo et al., 2000). After reaching the cell body, TeNT is transcytosed into adjacent inhibitory interneurons where it blocks the release of inhibitory neurotransmitters (i.e., γ-aminobutyric acid and glycine), leading to spastic paralysis. In its active form, TeNT is a dichain protein, consisting of an ~100-kDa heavy chain and an ~50-kDa light chain associated with a single disulfide bond and non-covalent interactions, that targets the vesicle-associated membrane protein (VAMP/synaptobrevin), an essential element of the neuroexocytosis apparatus (reviewed by Schiavo et al., 2000). The three-dimensional crystal structure of TeNT's carboxy-terminal domain has been determined (Umland et al., 1997), which has provided a basis for understanding the relationship between TeNT's structure and mechanism of action. The N-terminal domain of TeNT's heavy chain is responsible for the membrane translocation of the toxin's light chain into the neuronal cytosol, and the C-terminal domain is mainly responsible for the toxin's neurospecific binding (Montecucco and Schiavo, 1995; Schiavo et al., 2000). It has been well documented that TeNT binding to neurons occurs within particular regions of the plasma membranes that are enriched in cholesterol, gangliosides, and glycophosphoinositol (GPI)-anchored proteins (Herreros et al., 2000a,b; Munro et al., 2001). Therefore, it is likely that TeNT uses constitutive mechanisms for its internalization and traffic. TeNT has been suggested to use the synaptic vesicle recycling pathway for its internalization in cultured hippocampus neurons (Matteoli et al., 1996). In differentiated PC-12 cells and cultured spinal cord neurons, the C-terminal fragment of TeNT or TTC fragment displays a punctuate binding pattern, which is similar to the one displayed by lipid raft markers (Harder et al., 1998), and associates with

Abbreviations: α-BTX, α-bungarotoxin; β-gal, β-galactosidase; β-gal-TTC, fusion protein of β-gal and C-terminal fragment of tetanus toxin; CT-B, AlexaFluor 594-conjugated B-subunit of cholera toxin; GFP, green fluorescent protein; GFP-TTC, fusion protein of GFP and C-terminal fragment of tetanus toxin; NMJ, neuromuscular junction; TeNT, tetanus neurotoxin; TTC, C-terminal fragment of tetanus toxin.

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lipid microdomains (Herreros and Schiavo, 2002; Herreros et al., 2001; Vyas et al., 2001). Fused to a reporter gene such as LacZ or the green fluorescent protein (GFP), the atoxic TTC fragment can traffic retrogradely and trans-synaptically inside a restricted neural network either after direct injection of the hybrid protein (Coen et al., 1997; Miana-Mena et al., 2002; Sapir et al., 2004) or when expressed as a transgene in mice (Maskos et al., 2002; Sakurai et al., 2005). Therefore, since the in vivo mechanisms involved in the neuronal internalization and trafficking of GFP-TTC are still poorly understood, we decided to examine its retrograde traffic after its intramuscular injection and to compare it with that of the cholera toxin B-subunit (CT-B). In the present study, we show that GFP-TTC is associated with lipid microdomains and rapidly clusters in motor nerve terminals of the NMJ through a process involving clathrin-coated pits and axolemma infoldings located between the active zones. Although GFP-TTC and CT-B colocalized at motor nerve ending, they appear to use different cargos for their retrograde trafficking to the spinal cord.

Results

GFP-TTC rapidly localizes in motor nerve terminals of the NMJ

In order to characterize GFP-TTC's distribution in the NMJ, a single injection of the fusion protein was administered in the immediate vicinity of the *levator auris longus* (LAL) muscle and, at various times postinjection, the muscle was removed and examined as a whole mount. The LAL muscle is thin and flat and consists of only a few layers of fibers; therefore, the whole neuromuscular preparation with associated nerves could be examined by confocal microscopy. GFP-TTC rapidly concentrated in the NMJs which were identified by the staining of muscle nicotinic acetylcholine receptors (AChRs) with TRITC-conjugated α-bungarotoxin (TRITC-α-BTX) (Fig. 1A, the results are representative of 5 different experiments for each condition). A patchy clustering of GFP-TTC in NMJ was observed 5 min after subcutaneous injection of the protein at the immediate vicinity of the LAL. After 30 min, a more diffuse staining that persisted for

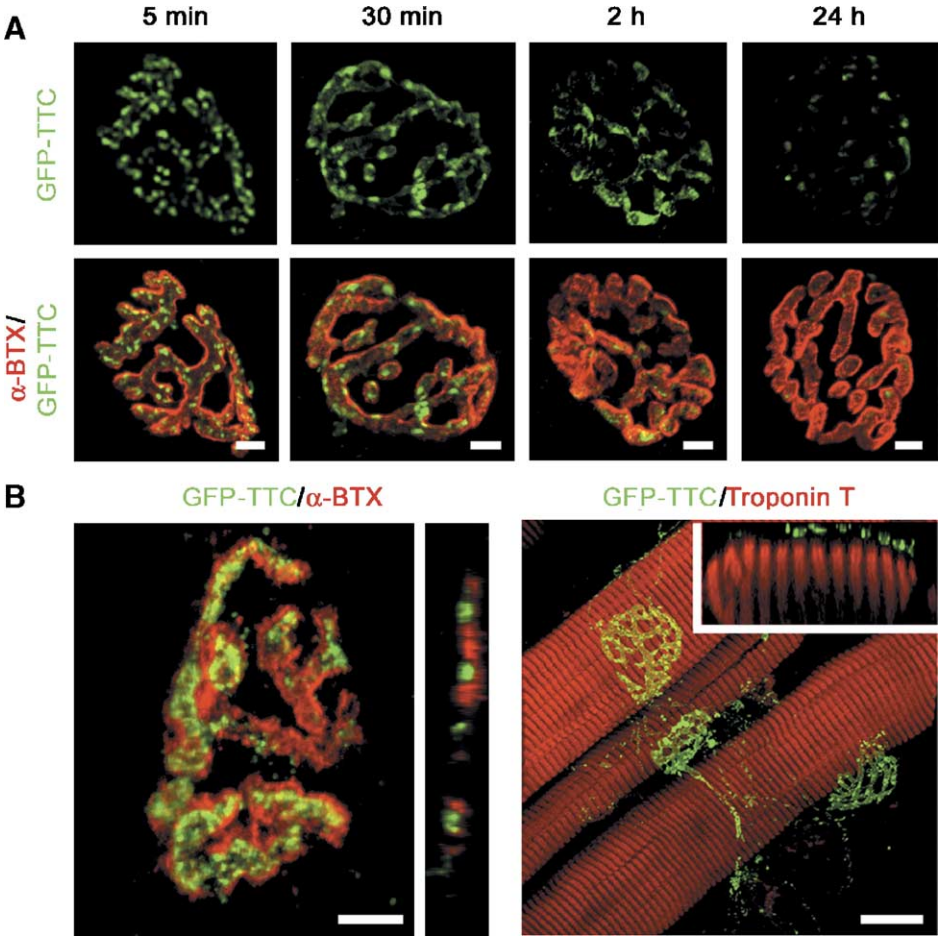

Fig. 1. Confocal fluorescence demonstrating GFP-TTC localization in mature mouse LAL NMJs. (A) GFP-TTC staining (green) of motor nerve endings of NMJs, identified by TRITC-α-BTX labeling (red), at various times after its subcutaneous injection into the vicinity of the LAL muscle. GFP-TTC staining alone (top panel) and associated with TRITC-α-BTX labeling (lower panel) are shown on the same NMJ. (B) Left panel: GFP-TTC and TRITC-α-BTX labeling of the NMJ 2 h postinjection of GFP-TTC (same conditions as for panel A). Note on the side view image that GFP-TTC staining is exclusively presynaptic, since no colocalization is observed with TRITC-α-BTX labeling of AChRs. Right panel: immunostaining of the muscle cytoskeletal troponin T protein, visualized using a Cy3-conjugated secondary antibody (2 h postinjection of GFP-TTC). Note the presynaptic GFP-TTC staining, in the side view image. Scale bars: panel A = 5 μm; panel B, GFP-TTC/TRITC-α-BTX = 5 μm, GFP-TTC/Troponin T = 20 μm.