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Ciliary neurotrophic factor is not required for terminal sprouting and compensatory reinnervation of neuromuscular synapses: Re-evaluation of CNTF null mice

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Abstract

Loss of synaptic activity or innervation induces sprouting of intact motor nerve terminals that adds or restores nerve–muscle connectivity. Ciliary neurotrophic factor (CNTF) and terminal Schwann cells (tSCs) have been implicated as molecular and cellular mediators of the compensatory process. We wondered if the previously reported lack of terminal sprouting in CNTF null mice was due to abnormal reactivity of tSCs. To this end, we examined nerve terminal and tSC responses in CNTF null mice using experimental systems that elicited extensive sprouting in wildtype mice. Contrary to the previous report, we found that motor nerve terminals in the null mice sprout extensively in response to major sprouting-stimuli such as exogenously applied CNTF *per se*, botulinum toxin-elicited paralysis, and partial denervation by L4 spinal root transection. In addition, the number, length and growth patterns of terminal sprouts, and the extent of reinnervation by terminal or nodal sprouts, were similar in wildtype and null mice. tSCs in the null mice were also reactive to the sprouting-stimuli, elaborating cellular processes that accompanied terminal sprouts or guided reinnervation of denervated muscle fibers. Lastly, CNTF was absent in quiescent tSCs in intact, wildtype muscles and little if any was detected in reactive tSCs in denervated muscles. Thus, CNTF is not required for induction of nerve terminal sprouting, for reactivation of tSCs, and for compensatory reinnervation after nerve injury. We interpret these results to support the notion that compensatory sprouting in adult muscles is induced primarily by contact-mediated mechanisms, rather than by diffusible factors.

Keywords: Endplate; Inactivity; Neuromuscular junction; Nodal sprouting; Paralysis; Partial denervation; Terminal Schwann cell; Terminal sprouting; Reinnervation

Introduction

Paralysis or partial denervation elicits reactive sprouting of intact axons and nerve terminals in adult muscles known as nodal and terminal sprouting (Hoffman, 1950; Duchen and Strich, 1968). These sprouts add or restore synaptic contacts on inactive (Snider and Harris, 1979; Angaut-Petit et al., 1990; Meunier et al., 2002) or denervated muscle fibers, which improve the motor deficits associated with peripheral nerve injury (Brown et al., 1981; Wernig and Herrera, 1986; Tam and Gordon, 2003a) or spinal cord injury (Yang et al., 1990; Marino et al., 1994; Thomas et al., 1997). Despite its significance for repair of the adult nervous system, the molecular and cellular events leading to the compensatory process remain elusive.

Several neurotrophic factors, including Ciliary Neurotrophic Factor (CNTF), elicit terminal sprouting when they are administered exogenously to intact muscles (Caroni and Grandes, 1990; Gurney et al., 1992; Caroni et al., 1994; Kwon and Gurney, 1994; Funakoshi et al., 1995; Tarabal et al., 1996; Trachtenberg and Thompson, 1997; Siegel et al., 2000). Although the physiological roles of most of these diffusible factors remain unclear, an earlier genetic analysis of CNTF null (−/−) mice provided evidence for the particular importance of CNTF as a key mediator of the sprouting responses; Siegel et al. (2000) reported that paralysis, partial denervation, and exogenous CNTF all failed to elicit significant sprouting in the null mice and concluded that CNTF is required for motor nerve sprouting. The same group subsequently proposed that CNTF, released from Schwann cells after paralysis or denervation, could induce terminal sprouting indirectly through an action on muscle fibers (English, 2003).

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Two issues prompted us to reexamine the sprouting responses of CNTF−/− mice. First, Siegel et al. might have underestimated the sprouting competence of the mice. Because their experimental system induced only moderate sprouting even in wildtype mice, their conclusion relied to a large extent on a statistical assessment. Moreover, we have recently discovered that exogenously administered CNTF induces primarily intrasynaptic sprouting of terminals that remain dramatically trapped within the parental endplates (Wright et al., submitted for publication). This finding raises the possibility that the conventional evaluation of terminal sprouting, which is based solely on extrasynaptic growth, might underestimate the extent of sprouting.

Second, although terminal Schwann cells (tSCs) have been strongly implicated in terminal sprouting (Son et al., 1996; Kang et al., 2003; Koirala et al., 2003), whether sprouting defects in CNTF null mice are due to abnormal reactivity of tSCs has not been addressed. Indeed, tSCs become reactive in response to paralysis or denervation (Reynolds and Woolf, 1992), and extend processes that appear to induce and guide terminal sprouts from innervated- to denervated muscle fibers (Son and Thompson, 1995a; Ko and Chen, 1996; Lubischer and Thompson, 1999; O'Malley et al., 1999; Love et al., 2003; Tam and Gordon, 2003b). Schwann cells are also the only cells in the peripheral nervous system that are known to express CNTF (Stockli et al., 1989; Friedman et al., 1992; Rende et al., 1992; Sendtner et al., 1992). We therefore were attracted by the possibility that nerve terminals in CNTF−/− mice fail to sprout because tSCs fail to extend processes. Here, we show that, in contrast to the earlier report, CNTF is dispensable for the reactive plasticity of both motor neurons and tSCs, and that compensatory reinnervation by axonal sprouts proceeds normally in CNTF−/− mice after nerve injury.

Materials and methods

Animals

Two-month-old female mice with homogeneous disruption of the *Cntf* gene (Masu et al., 1993) and age-matched wild type (+/+) controls (C57BL/6; Jackson Laboratories, Bar Harbor, Maine) were used. Three animals were typically used in each experimental group. CNTF null (−/−) mice reared in a colony maintained at the Drexel University College of Medicine (DUCOM) were routinely genotyped by PCR analysis (Masu et al., 1993) and the genotype of all mice used in the present experiments was additionally confirmed. All experimental procedures were performed in accordance with DUCOM's Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

CNTF and BoTX administration

Mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and xylazine (8 mg/kg; Lloyd Laboratories, Shenandoah, Iowa). To evaluate the reactivity of nerve terminals and tSCs to exogenously applied CNTF, recombinant rat CNTF (200 ng/25 g mouse; R&D Systems, Minneapolis, MN), dissolved in 50 μl vehicle containing 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), was injected subcutaneously over the right Levator auris longus (LAL) muscle under aseptic conditions. CNTF was administered every 12 h, for up to 14 days. To assess reactivity of nerve terminals and tSCs to muscle paralysis, botulinum toxin A (BoTX; 2.0 pg/25 g mouse; Sigma-Aldrich, St. Louis MO), dissolved in 50 μl sterile saline, was injected subcutaneously over the right LAL muscle. Similar dosages of BoTX have been shown to induce complete paralysis of mouse LAL muscles for up to 4 weeks (Juzans et al., 1996; Angaut-Petit et al., 1998). In order to assure prolonged paralysis of LAL muscles, BoTX was administered every fourth day for up to 14 days.

Partial or complete denervation

To examine sprouting and reinnervation after partial denervation, the L4 and L5 spinal roots were exposed under aseptic conditions and the right L4 spinal root was transected to denervate extensor digitorum longus (EDL) muscles by ca. 80% (Albani et al., 1988; Tam et al., 2001). The proximal segment of the transected L4 root was tied with 10.0 suture and the distal segment reflected laterally to prevent reinnervation by L4 axons. Fourteen days after partial denervation, mice were euthanized with an overdose of pentobarbital sodium (300 mg/kg; Abbott Laboratories, North Chicago, IL) and EDL muscles were harvested for immunocytochemistry. To study CNTF expression in denervated tSCs, sciatic nerve was exposed proximal to the branching of the sural nerve, transected and the proximal nerve stump was ligated with 7-0 suture to prevent regeneration. Mice were euthanized 10 days later and EDL muscles ipsilateral to the lesion were harvested for immunohistochemistry. Tails were collected from all euthanized CNTF−/− mice and processed for PCR verification of genotypes.

Immunohistochemistry

The procedures used for immunostaining have been described in detail previously (Burns et al., 2007). Briefly, muscles were postfixed in 4% paraformaldehyde for 30 min and rinsed in phosphate-buffered saline (PBS; pH 7.4) containing 0.1 M glycine. Muscles were then incubated for 15 min with rhodamine-conjugated α-bungarotoxin (Molecular Probes, St. Louis, MO), diluted 1:200 in PBS, to label acetylcholine receptors (AChRs). The muscles were then permeabilized in 20 °C methanol for 5 min and blocked for 1 h in PBS containing 0.2% Triton and 2% BSA. The muscles were subsequently incubated overnight at 4 °C in a cocktail of primary antibodies diluted in the blocking solution. Axons and nerve terminals were labeled with mouse monoclonal antibodies to neurofilaments (SMI 312; Sternberger Monoclonals, Baltimore, MD), diluted 1:1000 and to a synaptic vesicle protein, SV2 (Developmental Studies Hybridoma Bank, Iowa), diluted 1:10. Schwann cells in intact muscles were labeled with rabbit anti-cow S-100 polyclonal antibody (Dako, Carpentaria, CA),