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Short Communication

Muscle ciliary neurotrophic factor receptor α helps maintain choline acetyltransferase levels in denervated motor neurons following peripheral nerve lesion

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ABSTRACT

Systemic ciliary neurotrophic factor (CNTF) administration protects motor neurons from denervating diseases and lesions but produces non-neuromuscular side effects. Therefore, CNTF related therapeutics will need to specifically target motor neuron protective receptor mechanisms. Expression of the essential ligand binding subunit of the CNTF receptor, CNTF receptor α (CNTFRα), is induced in skeletal muscle by denervating lesion and in human denervating diseases. We show here, with muscle-specific *in vivo* genetic disruption, that muscle CNTFRα makes an essential/non-redundant contribution to maintaining choline acetyltransferase levels in denervated motor neurons following nerve crush, suggesting the muscle CNTFRα induction is an endogenous denervation-induced neuroprotective response that could be enhanced to treat nerve lesion and denervating diseases. Notably, unlike motor neuron gene expression, skeletal muscle gene expression can be specifically targeted with human gene therapy vectors already approved for market.

1. Introduction

A wide range of diseases and lesions disrupt motor neuron (MN) innervation of skeletal muscle with profound functional consequences. The pressing clinical challenge is to maintain and regenerate functional MNs confronted by such denervating insults. Systemic CNTF administration protects MNs from denervating diseases and lesions but produces non-neuromuscular side effects (Sendtner, 2014) indicating therapeutic intervention will need to specifically target the endogenous MN protective receptor mechanisms.

In the neuromuscular system, the essential ligand binding subunit of the CNTF receptor, CNTFRα, is found in MNs and muscle (MacLennan et al., 1996). Clinically approved gene therapy vectors specifically targeting MNs are unavailable, so enhancing MN CNTFRα expression is not a current option. However, skeletal muscle CNTFRα expression is greatly upregulated with denervating lesions (Davis et al., 1993; Lee et al., 2013) and data suggest similar upregulation in all human denervating diseases, including ALS (Weis et al., 1998). When we previously inhibited this increase following lesion with muscle-specific CNTFRα gene disruption, it had no effect on the muscles themselves but surprisingly impaired axon regeneration (Lee et al., 2013), indicating the increased CNTFRα expression is an endogenous response supporting MN axons challenged by this insult, and further suggesting a similar MN supportive role for the muscle CNTFRα induction in denervating diseases. If so, enhancing this neuroprotective/regenerative response may be therapeutic. Muscle is a particularly promising target since expression in muscle, unlike MNs, can be *specifically* modified in humans with *current*, *approved for market* gene therapy techniques (Ylä-Herttuala, 2012).

MN choline acetyltransferase (ChAT) is essential for acetylcholine synthesis and therefore absolutely required for neuromuscular transmission. Consequently, maintaining MN axons without maintaining MN Chat levels is of little functional/clinical benefit. Here we use muscle-specific CNTFRα disruption and find endogenous muscle CNTFRα makes an essential/non-redundant contribution to ChAT maintenance in MNs following denervation. This surprising denervation-induced interplay between muscle and MNs supports the idea of clinically targeting muscle CNTFRα to maintain denervated MNs.

2. Materials and methods

Floxed CNTFRα (Lee et al., 2013) and mlc1f-Cre (Bothe et al., 2000; from Dr. Steven Burden [NYU]) mice were PCR genotyped from tail biopsy and maintained on a 129SvEvBrd background. CNTFRα knockdown mice (mlc1f-Cre$^{+/-}$/flxCNTFRα$^{+/+}$) and non-floxed littermate controls (mlc1f-Cre$^{+/-}$/flxCNTFRα$^{-/-}$) of both sexes were generated

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through mlc1f-Cre+/−/flxCNTFRα+/− X mlc1f-Cre−/−/flxCNTFRα+/− breeding and, at 2.5–4 months, processed in parallel (blind to genotype) through all procedures including, nerve crush, perfusion, immunohistochemistry and image analysis. This controlled for variation in *in utero* and postnatal rearing environments, age, and all reagents.

t-tests were used for direct *a priori* comparisons of 2 groups. These were then confirmed by a 2-way ANOVA analysis followed by appropriately protected Bonferroni post-hoc comparisons, all conducted with GraphPad Prism 5 (RRID:SCR_002798). University of Cincinnati IACUC approved all animal procedures.

2.1. Unilateral sciatic nerve crush

Following ketamine/xylazine (100 mg/kg) anesthesia, mid-thigh skin was opened and a 5 mm longitudinal cut in the biceps femoris exposed the underlying nerve passing superficial to the obturator internus tendon. The nerve was freed from connective tissue and crushed for 10 s with Dumont #5 Biologic Tip forceps (Fine Science Tools). This procedure lesions all nerve axons, as we have demonstrated (Lee et al., 2013).

2.2. Motor neuron ChAT analysis

Following overdose with avertin (20 mg/ml; IP), mice were perfused with 4 °C saline and then 4 °C, 4% paraformaldehyde. Spinal cords were post-fixed in 4% paraformaldehyde overnight at 4 °C and then incubated for at least 48 h in 30% sucrose/2.5 mM sodium azide before sectioning. Every fourth 30um, coronal section was collected with a cryostat throughout the L5 cord, which contains the sciatic nerve projecting MNs. Sections were processed free-floating according to described immunohistochemistry procedures (MacLennan et al., 1996), and with a 4-day, 4 °C incubation in rabbit anti-ChAT polyclonal primary antiserum (Chemicon [Millipore]; Cat. # AB143; RRID:AB2079760) at 1:6000. Label was visualized with ABC signal amplification (Vector Laboratories; cat. # PK-6100) and cyanine-3 tyramide (Perkin Elmer; cat. # SAT704B).

The ChAT antiserum has been widely used and extensively characterized through immunohistochemistry and western blot (see Stillman et al., 2009). Chat-labeled MNs were stereologically counted in accordance with Hyman et al. (1998). In addition to their characteristic ChAT expression, MNs were identified by their characteristic location in the ventrolateral horn, irregular shape, and large nuclei. Counts were multiplied by four to estimate total labeled MNs since every fourth section was assayed. In addition, MetaMorph; RRID:SCR_002368) was used to outline the cytoplasm of each labeled MN (total of over 7500 MNs analyzed) and determine its average cytoplasmic ChAT labeling intensity. As with all procedures, this analysis was conducted by individuals *unaware of genotype.*

Images (Fig. 1A–F) were captured with a DXM1200 camera and Nikon E800 microscope, and were identically adjusted with CorelDRAW.

3. Results

In contrast to early postnatal MNs, adult MNs survive and regenerate axons following peripheral nerve crush (*e.g.*, Lee et al., 2013). Therefore, this model is widely used in the search for endogenous mechanisms that support adult MNs challenged by denervating insult.

CNTF receptors contain the essential ligand binding subunit, CNTFRα, as well as leukemia inhibitory factor receptor β and gp130, with only CNTFRα being unique to CNTF receptors, required for all CNTF receptor signaling, and not involved in other signaling (Elson et al., 2000). Therefore, CNTFRα disruption best reveals endogenous CNTF receptor function. To specifically disrupt the CNTFRα gene in muscle, we crossed floxed CNTFRα mice (Lee et al., 2013) with mice carrying a Cre recombinase (Cre) gene inserted into the myosin light chain *1f* locus (mlc1f-Cre) (Bothe et al., 2000), a locus expressed very selectively in skeletal muscle cells. Our previous work, and that of others, indicate that mlc1f-Cre functions as designed in that it excises floxed sequence in all adult skeletal muscles tested, with no excision in other tissues including brain, spinal cord, sciatic nerve, liver, heart or

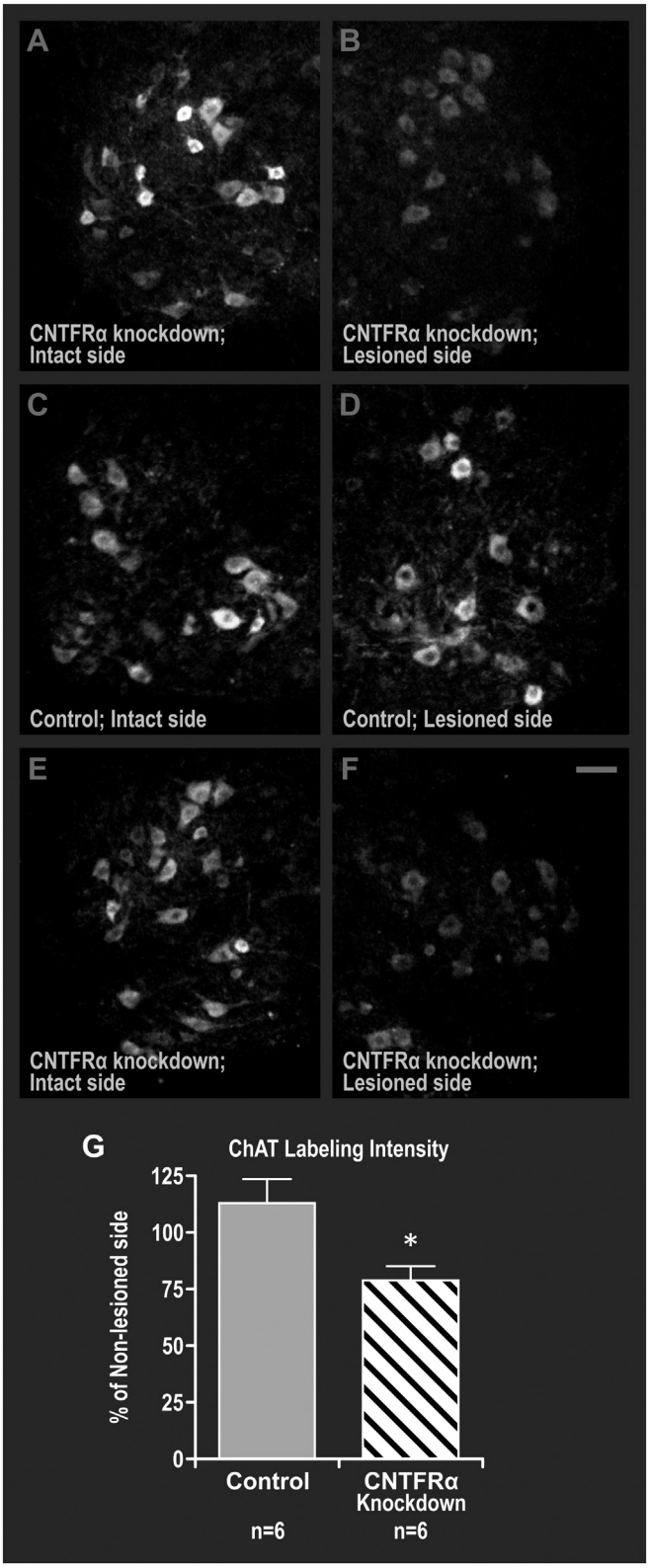

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Fig. 1. Muscle-specific CNTFRα knockdown leads to decreased motor neuron choline acetyltransferase (ChAT) levels following sciatic nerve crush. Muscle-specific CNTFRα knockdown mice and littermate controls received a unilateral sciatic nerve crush. Motor neuron ChAT levels were assessed one week post-lesion with ChAT immunohistochemistry and image analysis. (A–F) ChAT immunohistochemistry examples. ChAT-labeled spinal cord ventral horn motor neurons in CNTFRα knockdown (A, B, E, F) and control (C, D) mice. Lesioned side on right (B, D, F); dorsal to top. The CNTFRα knockdown mice display a lesion-induced decrease in ChAT label not seen in control mice. Scale bar = 50 μm. (G) Quantification of motor neuron ChAT labeling intensity indicated labeling intensity was not affected by the nerve crush in control mice but was significantly reduced in CNTFRα knockdown mice. For each side of each mouse, the labeling intensities of all labeled motor neurons in the many sections collected (see Methods) were determined and used to calculate an average intensity. * $p < .01$ Bonferroni *post hoc* comparison to control mice.

stomach (Bothe et al., 2000; Lee et al., 2013).

Here we compared "muscle CNTFRα knockdown" mice (mlc1f-Cre+/−/flxCNTFRα+/+) with non-floxed, littermate controls (mlc1f-Cre+/−/flxCNTFRα−/−). We have previously shown by CNTFRα PCR that this knockdown decreases CNTFRα RNA levels by approximately 80% in all muscles tested, including denervated muscles following the same lesion used here (Lee et al., 2013). This large but incomplete decrease is consistent with previous mlc1f-Cre work (Bothe et al., 2000) and may result from Cre expression in most but not all the many nuclei in each myofiber.

Muscle CNTFRα knockdown and control mice received a unilateral sciatic nerve crush. We have shown this lesion axotomizes all MN axons in the nerve, which then regenerate and re-innervate the denervated neuromuscular junctions 2–3 weeks post-lesion (Lee et al., 2013). In order to determine what role, if any, muscle CNTFRα plays in helping the lesioned MNs maintain their ChAT levels during denervation, muscle CNTFRα knockdown and control mice were perfused for ChAT immunohistochemistry one week post-lesion at complete denervation.

Neither the lesion nor the CNTFRα knockdown affected the *number* of ChAT-labeled MNs per mouse (mean ± SEM: control intact side, 1484 ± 42.9; knockdown intact side 1636 ± 126.2; control lesion side, 1684 ± 74.34; knockdown lesion side, 1585 ± 143.1; all comparisons $p > .05$), consistent with our previous quantification of cresyl violet stained MNs in this same model that indicated muscle CNTFRα does not regulate MN survival following this lesion (Lee et al., 2013).

In contrast, analysis of ChAT labeling *intensity* revealed a lesion-induced decrease in ChAT that was present only in CNTFRα knockdown mice. We first directly compared intact MNs in the control mice with those in the knockdown mice. While this between mouse comparison is not as sensitive as the within mouse lesion *vs* intact comparisons, there was clearly no knockdown-induced decrease in Chat (mean ± SEM [MetaMorph arbitrary units]: control, 40.2 ± 5.9; CNTFRα knockdown, 48.8 ± 5.1; $p = .29$; $n = 6$). We then directly compared the lesioned and intact MNs within the control mice and found no effect (Fig. 1G left bar, $p = .27$), which indicated MNs normally maintain their ChAT levels following the lesion. However, the same comparison in the knockdown mice revealed a significant lesion-induced reduction in ChAT (Fig. 1G right bar, $p = .021$), indicating that, in contrast to the control mice, knockdown mice were unable to maintain ChAT levels. As expected, ANOVA analysis of all the data revealed a significant interaction ($p = .012$) and appropriately protected Bonferroni *post hoc* tests found a significant effect of the CNTFRα knockdown in lesioned MNs ($p < .01$) and no effect in intact MNs ($p > .05$).

4. Discussion

We report here that muscle-specific CNTFRα knockdown does not affect ChAT levels in intact MNs, but with denervating lesion this same knockdown leads to a decrease in MN ChAT. Therefore, muscle CNTFRα makes an essential/non-redundant contribution to ChAT levels in the injured MNs in that no other mechanisms were able to fully compensate for the loss of muscle CNTFRα. The greater role for muscle CNTFRα after the denervating insult is consistent with the large increase in muscle CNTFRα gene expression following this insult (Davis et al., 1993; Lee et al., 2013). Together the data suggest that the muscle CNTFRα gene induction is an endogenous neuroprotective response to denervation that helps MNs maintain their functionally critical neurotransmitter synthesizing enzyme, ChAT.

Several considerations suggest that the present data, while revealing a significant effect, likely also underrepresent the magnitude of muscle CNTFRα's contribution to MN ChAT. First, the muscle-specific knockdown used, although the best available, only reduces muscle CNTFRα by ~80% (Lee et al., 2013), such that the remaining 20% can still contribute to MN ChAT. Second, other compensatory mechanisms have most likely evolved to at least partially maintain this critical enzyme. Third, we quantified ChAT labeling in L5 segment MNs because this spinal cord region contains MNs projecting through the sciatic nerve and therefore axotomized by the lesion. However, not all L5 MNs are sciatic MNs and the tissue fixation conditions required for their identification through retrograde labeling were not compatible with the ChAT labeling conditions, such that multi-labeling was not workable. Therefore, we quantified ChAT in all L5 MNs, recognizing this included some MNs not axotomized and likely not affected by muscle CNTFRα. In summary, a clear knockdown effect was observed despite the above three factors that would be expected to each reduce its magnitude. Therefore, muscle CNTFRα likely makes a bigger contribution to ChAT than detected here.

The idea that *muscle* CNTFRα regulates *MN* ChAT is somewhat counterintuitive. This muscle-MN interaction does not result from muscle CNTFRα simply maintaining muscle health since our previous work with the same muscle-specific CNTFRα knockdown used here found no effect on muscle denervation atrophy, regeneration, fiber type or contractility following the same lesion (Lee et al., 2013). However, muscle CNTFRα may promote muscle CNTF receptor signaling leading to release of unidentified muscle factor(s) acting directly or indirectly on MNs to maintain ChAT. Moreover, following nerve lesion, muscle CNTFRα is released in a soluble, functional form (Davis et al., 1993) that could potentially enhance ChAT-supportive CNTF receptor signaling in MNs by diffusing to regenerating axons for retrograde transport to MN soma, or diffusing directly to MN soma.

The significant distance between the lesion at mid-thigh and the denervated lower limb muscles may seem to suggest against factors from these muscles acting directly on axotomized MNs. However, the rapid, dramatic increase in muscle CNTFRα expression is sustained for at least several days post-lesion (Davis et al., 1993), so the CNTFRα and/or CNTFRα-dependent factors from denervated muscles should have time to diffuse to regenerating axons and MN soma.

CNTFRα disruption, as done here, is the most comprehensive way to identify *in vivo* functions of endogenous CNTF receptors because CNTFRα is required for all CNTF receptor signaling, regardless of the ligands involved (Elson et al., 2000). As a result, the present data do not reveal which CNTF receptor ligand(s) play a role in muscle CNTFRα's contribution to MN ChAT. CNTF may be involved since it is thought to be released from Schwann cells at peripheral nerve lesion sites (Sendtner, 2014) and therefore may combine with CNTFRα released from denervated muscle (Davis et al., 1993). The complex of cardiotrophin-like cytokine/cytokine-like factor-1 (CLC/CLF) also acts as a CNTF receptor ligand (Elson et al., 2000), but studies are needed to determine how CLC/CLF is expressed in the adult neuromuscular system, particularly following nerve lesion. The ligands may also act together. Regardless, conditional disruption of individual ligands, with and without muscle CNTFRα knockdown, will be required to address this issue.

The data here and our previous work (Lee et al., 2013) indicate muscle CNTFRα gene upregulation promotes MN ChAT maintenance and axon regeneration following denervating nerve lesion. Other data

suggest that muscle CNTFRα gene expression is similarly upregulated in all denervating human diseases, including ALS (Weis et al., 1998), thereby also suggesting, along with our data, that the upregulation is an endogenous neuroprotective response to denervation. If so, enhancing this endogenous response by further increasing muscle CNTFRα expression may promote MN maintenance and regeneration in cases of denervating trauma and disease. Notably, in contrast to MN gene expression, skeletal muscle gene expression can be *specifically* enhanced in humans with gene therapy techniques currently *approved for market* (Ylä-Herttuala, 2012). Moreover, while several identified muscle factors are known to work across the neuromuscular synapse to help maintain MNs, the present and previous (Lee et al., 2013) nerve lesion studies suggest muscle CNTFRα can promote MN maintenance and regeneration over the much greater distances from denervated muscle to lesion site. This suggests that muscle CNTFRα enhancement may help maintain and regenerate those MNs in denervating diseases, including ALS, that have lost contact with muscle. If so, this would be particularly useful for treating ALS in that it is generally first diagnosed in the late stages of the disease when many MNs have lost muscle contact.

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Conflict of interest

The authors declare no conflicts.

Author contributions

Study concept and design: AJM. Data acquisition: NL, HAW. Data analysis/interpretation: NL, AJM. Drafting manuscript: AJM. Reviewing and editing manuscript: NL, HAW.

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