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Adult Ciliary Neurotrophic Factor Receptors Help Maintain Facial Motor Neuron Choline Acetyltransferase Expression *In Vivo* Following Nerve Crush

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Abstract

Exogenous ciliary neurotrophic factor (CNTF) administration promotes the survival of motor neurons in a wide range of models. It also increases the expression of the critical neurotransmitter enzyme, choline acetyltransferase (ChAT), by *in vitro* motor neurons, likely independent of its effects on their survival. We have used the adult mouse facial nerve crush model and adult onset conditional disruption of the CNTF receptor α (CNTFRα) gene to directly examine the *in vivo* roles played by endogenous CNTF receptors in adult motor neuron survival and ChAT maintenance, independent of developmental functions. We have previously shown that adult activation of the CreER gene construct in floxed CNTFRα mice depletes this essential receptor subunit in a large subset of motor neurons (and all skeletal muscle, as shown here) but has no effect on the survival of intact or lesioned motor neurons, thereby indicating that these adult CNTF receptors play no essential survival role in this model, in contrast to their essential role during embryonic development. We show here that this same CNTFRα depletion does not affect ChAT labeling in non-lesioned motor neurons, but it significantly increases the loss of ChAT following nerve crush. The data suggest that while neither motor neuron nor muscle CNTF receptors play a significant, non-redundant role in the maintenance of ChAT in intact adult motor neurons, the receptors become essential for ChAT maintenance when the motor neurons are challenged by nerve crush. Therefore, the data suggest that the receptors act as a critical component of an endogenous neuroprotective mechanism.

Graphical Abstract

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Resources Cited

Rabbit anti-ChAT polyclonal antiserum (Chemicon [Millipore]; Cat. # AB143; RRID:AB_2079760)

GraphPad Prism 5 software (http://www.graphpad.com/scientific-software/prism/RRID:SCR_002798)

MetaMorph image analysis software (https://www.moleculardevices.com; RRID:SCR_002368)

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Role of authors: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AJM. Acquisition of data: NL, CER, MSR, DST. Analysis and interpretation of data NL, CER, MSR, DST, AJM. Drafting manuscript: AJM. Reviewing and editing manuscript: NL, CER, MSR, DST.

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Using adult onset CNTF receptor disruption and the mouse facial nerve crush model, this study shows that adult CNTF receptors help maintain expression of the critical neurotransmitter enzyme, choline acetyltransferase in lesioned motor neurons. Image shows choline acetyltransferase immunohistochemistry of motor neurons with the lesion side on the right.

Keywords

CNTF Receptor α; CNTFRα; inducible Cre recombinase; mouse; RRID:AB_2079760; RRID:SCR_002798; RRID:SCR_002368

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a potent neuroprotective factor for embryonic motor neurons *in vitro* (Lindsay et al., 1994). *In vivo,* CNTF administration protects motor neurons after early postnatal peripheral nerve lesion (Sendtner et al., 1990), in genetic models of motor neuron disease (Sendtner et al., 1992a; Ikeda et al., 1995; Sagot et al., 1995), and during developmental naturally-occurring motor neuron death (Oppenheim et al., 1991), suggesting that endogenous CNTF receptor signaling may maintain motor neurons during development and in adulthood.

To directly determine the role(s) of endogenous CNTF receptors in motor neuron maintenance, the receptors must be manipulated *in vivo*. CNTF receptor α (CNTFRα) is an essential ligand binding subunit of the CNTF receptor, which is composed of CNTFRα, leukemia inhibitory factor receptor β (LIFRβ) and gp130 (Davis et al., 1993a). While LIFRβ and gp130 are found in other related receptors, CNTFRα is unique to CNTF receptors and is required for all known forms of CNTF receptor signaling, regardless of the ligands or signaling pathways involved (Davis et al., 1993a; Elson et al., 2000; Derouet et al., 2004). Therefore, CNTFRα disruption is the best method to comprehensively determine endogenous CNTF receptor function.

Unconditional disruption of the CNTFRα gene in mice leads to uniform death within 24 hours of birth accompanied by motor neuron loss (DeChiara et al., 1995). Thus, endogenous CNTF receptor signaling is essential for embryonic motor neuron survival/development. However, it is particularly important to identify and characterize the endogenous mechanisms maintaining *adult* motor neurons because such mechanisms may serve as therapeutic targets for adult onset motor neuron diseases and trauma. The perinatal death of the unconditional CNTFRα knockout mice precludes their use for adult studies. Moreover, in order to identify adult functions, independent of potential developmental effects, adult onset disruption is required. Therefore, we have used *Cre/loxP* technology to deplete CNTFRα in adult facial motor neurons by combining an adult inducible Cre recombinase (Cre) gene construct and floxed CNTFRα mice (Lee et al., 2013a). We found that, in

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contrast to the effects of CNTFRα disruption during development, adult disruption did not affect motor neuron survival (Lee et al., 2013a), indicating the motor neuron CNTF receptors do not play an essential role in the survival of non-insulted adult motor neurons.

Adult facial motor neurons, unlike early postnatal motor neurons, do not die following nerve crush (Gilad et al., 1996; Kuzis et. al, 1999; Lee et al., 2013a) and this thereby serves as a model to identify endogenous mechanisms maintaining adult motor neurons following insult. Our adult CNTFRα disruption did not affect motor neuron survival following nerve crush (Lee et al., 2013a), indicating that the receptors are not essential for the survival of the lesioned motor neurons.

However, several studies indicate that CNTF promotes expression of choline acetyltransferase (ChAT) in cultured motor neurons (Magal et al., 1991; Glicksman et al., 1993; Wong et al. 1993; Kato and Lindsay, 1994; Zurn, 1994), likely independent of its effects on motor neuron survival (Wong et al. 1993; Zurn, 1994). These data raise the interesting possibility that endogenous CNTF receptors, while not essential for adult motor neuron survival, may play an important role by helping maintain this functionally critical neurotransmitter enzyme in adult motor neurons.

To directly address this question in adult motor neurons *in vivo*, the experiments reported here again used the same adult onset CNTFRα disruption model with facial nerve crush. The effect of CNTFRα disruption on ChAT was examined in intact and nerve crush lesioned motor neurons.

MATERIALS AND METHODS

General design

For all experiments, homozygous floxed CNTFRα mice and non-floxed littermate controls were processed in parallel (by individuals kept blind to genotype) through the complete procedure, including tamoxifen injection, nerve crush, perfusion, immunohistochemistry/histology and image analysis. This approach controlled for variability in *in utero* and postnatal rearing environments, as well as any variation in genetic background, age, and all reagents. For statistical analysis we used a student's t-test (for the qRT-PCR comparison of CNTFRα in floxed vs control mice) or a 2-way ANOVA analysis followed by Bonferroni post-hoc comparisons (for the ChAT analysis), all conducted with GraphPad Prism 5 software (http://www.graphpad.com/scientific-software/prism/RRID:SCR_002798).

The floxed CNTFRα gene was bred as heterozygote by heterozygote crosses, whereas all other constructs were bred in heterozygote by wild-type crosses. The University of Cincinnati Institutional Animal Care and Use Committee approved all the animal procedures.

Mouse lines

We have previously described the floxed CNTFRα mice and their characterization (Lee et al., 2008). Cre activity in these mice leads to functional inactivation of the floxed CNTFRα gene. Crossing them with a "deleter" line that produces universal, Cre-dependent floxed

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gene excision, results in the designed excision of the CNTFRα gene (confirmed by Southern blot) as well as the expected motor neuron loss and perinatal death of all homozygous floxed mice (Lee et al., 2008), as seen with unconditional, universal CNTFRα gene disruption (DeChiara et al., 1995). CreER mice were generated and initially characterized by Guo et al., (2002) and were purchased for this study from Dr. C. Lobe (Sunnybrook and Women's College, Toronto). ROSA26 reporter mice (Soriano, 1999) were purchased from Jackson Laboratories. All mice (male and female) were genotyped by PCR analysis of tail biopsy DNA and backcrossed at least 5 generations onto a 129SvEvBrd background.

Tamoxifen administration

Mice (3–4 months of age) were injected I.P. with 200 mg/kg tamoxifen (Sigma; cat. #:T5648) made up at 20mg/ml in corn oil (Sigma; cat. #: C8267).

Facial nerve crush

The previously published protocol of Kuzis et al., (1999) was used to produce unilateral facial nerve crush lesions. Mice were anesthetized with ketamine/xylazine (100mg/kg). Skin and muscle were opened by the ear to expose the facial nerve which was then freed from fascia and crushed (for 10 seconds) with Dumont #5 Biologic Tip forceps (Fine Science Tools; cat. # 11251-20).

Anatomical procedures

Following overdose with avertin (20 mg/ml; IP), mice were perfused with 4°C saline and then with 4°C 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C and then cryoprotected for at least 48 hours in 30% sucrose with 2.5 mM sodium azide before sectioning. Muscle was similarly postfixed and cryoprotected. Thirty micron cryostat sections of muscle were stained slide-mounted with standard Xgal histology for the ROSA26 reporter study.

For ChAT immunohistochemistry, every fourth 30 micron coronal cryostat section was collected throughout the complete rostral caudal extent of the facial motor nucleus and processed free-floating with previously described immunohistochemistry procedures (MacLennan et al., 1996) using a rabbit anti-ChAT polyclonal antiserum (Chemicon [Millipore]; Cat. # AB143; RRID:AB_2079760) at 1:6000 and visualized with ABC signal amplification (Vector Laboratories; cat. # PK-6100) and cyanine-3 tyramide (Perkin Elmer; cat. # SAT704B).

ChAT labeled facial motor neurons were stereologically counted. To correct for cells potentially split in the Z dimension, all motor neurons in focus at the top border of the sections were excluded (optical dissector) in accordance with the stereological methods described in Hyman et al., (1998) and Hatton and von Bartheld, (1999). Counts were multiplied by 4 to estimate total labeled motor neurons given that every fourth section was assayed and counted. MetaMorph image analysis software (https://www.moleculardevices.com; RRID:SCR_002368) was used to outline the cytoplasm of each labeled motor neuron and determine its *average* cytoplasmic ChAT labeling intensity. As with all procedures, this analysis was conducted by individuals unaware of genotype. Motor

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neurons were categorized into ten ChAT labeling intensity ranges, determined *a priori*, with "0" equal to background and "100" corresponding to the most intensely labeled motor neuron in the entire study. The 9th and 10th categories were then combined for graphical presentation because so few motor neurons were found in these highest labeling intensity categories.

Antibody characterization

The ChAT antiserum has been widely used and extensively characterized. Its pattern of immunolabeling is confined to areas known to express detectable levels of ChAT (German et al., 1985; Peterson et al., 1990; Smith ML et al., 1993, 1994). The immunolabeling also corresponds to that seen with other anti-ChAT antibodies (Stillman et al., 2009; McKenna et al., 2013). Moreover, preadsorption of the antiserum with recombinant rat ChAT protein abolished labeling (Stillman et al., 2009). Finally, western blot characterization of the antiserum with human brain and placental tissue produced a distinct band at 68 kDa that corresponds to the molecular weight of enzymatically active ChAT (Bruce et al., 1985).

Microscopy

A 12 megapixel DXM1200 camera and Nikon E800 microscope with a 10X (NA=0.45) lens were used to capture images. The brightness and contrast of the images for figures were adjusted with Coral Draw software.

Quantitative real-time PCR (qRT-PCR)

TaqMan CNTFRα qRT-PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR system using GAPDH as a normalizing control. Mouse CNTFRα and GAPDH specific primers were from ThermoFisher Scientific (Mouse CNTFRα; cat. # 4331182 [Mm00516693_m1], GAPDH; cat. # 4331182 [Mm99999915_g1]).

RESULTS

To disrupt facial motor neuron CNTF receptors starting in adulthood, we bred together the floxed CNTFRα gene and a gene construct ("CreER") enabling tamoxifen-inducible Cre activity (Guo et al., 2002). We have previously shown, with a reporter gene, that adult induction of CreER leads to Cre activity (i.e., floxed gene excision) in approximately half of facial motor neurons (Lee et al., 2013a), and that it also leads to the corresponding loss of all detectable CNTFRα in approximately half of the facial motor neurons in homozygous floxed CNTFRα mice (referred to hereafter as floxed CNTFRα mice; Lee et al., 2013a), as expected since the floxed CNTFRα gene is designed to be disrupted by Cre activity. This loss of motor neuron CNTFRα in the floxed CNTFRα mice is also consistent with all our previous work with these same floxed mice and several different Cre lines, in which Cre activity, as indicated by reporter signals, reliably disrupted CNTFRα in a variety of cell types (Stefater et al., 2012; Lee et al., 2013b, 2013c). Since the CreER construct does not lead to Cre activity prior to the tamoxifen induction (Guo et al., 2002; Lee et al., 2013a), the effects of this adult induction reflect adult CNTFRα loss. It is worth noting that there are no methods that we are aware of that lead to adult onset floxed gene disruption in *all* motor neurons.

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We have subsequently discovered that the adult CreER induction also leads to reporter visualized Cre activity in all skeletal muscle fibers, including those in all facial muscles (e.g., Figure 1). Skeletal muscle contains CNTF receptors (Helgren et al., 1994) which could play a role in motor neuron maintenance (see Discussion). Therefore, we used qRT-PCR to directly confirm that the same adult CreER induction protocol used in our previous motor neuron survival study (Lee et al., 2013a) reduces facial muscle CNTFRα RNA in floxed CNTFRα mice, as expected from the reporter data. Floxed CNTFRα mice and non-floxed littermate controls (all CreER+) were treated with tamoxifen at three to four months of age and temporal muscle was dissected four months later. The floxed CNTFRα muscle contained 32.7±.4.6% as much CNTFRα RNA as controls; ($t=3.43$; $P=0.011$; $n=3$ floxed CNTFRα; 6 controls). This degree of CNTFRα RNA depletion without complete removal is consistent with what we find with other gene constructs driving Cre activity in skeletal muscle of the floxed CNTFRα mice (Lee et al., 2013b) and what others find targeting different floxed genes with Cre in skeletal muscle (Bothe et al., 2000, Wredenberg et al., 2002). It may result from Cre induction in most but not all of the many nuclei in each myofiber, such that at least some reporter signal is generated in all myofibers (as we observe) but not all the many copies of the floxed CNTFRα gene in each myofiber are excised. Regardless, the depletion of muscle CNTFRα in the floxed CNTFRα mice with CreER, considered in the context of our previous finding that motor neuron survival is not affected in these mice, even after nerve lesion (Lee et al., 2013a), suggests that neither motor neuron *nor muscle* CNTFRα play a significant, essential role in the *survival* of adult intact or nerve crush lesioned motor neurons (see also Discussion).

We next used the same CreER/floxed CNTFRα model to determine if *in vivo* CNTF receptors play an essential role in maintaining adult motor neuron ChAT. Floxed CNTFRα mice and non-floxed littermate controls were administered tamoxifen at three to four months of age. They then received a unilateral facial nerve crush four months later. The mice were perfused for ChAT immunohistochemistry three weeks after the lesion (e.g., Figure 2). Image analysis consisted of counting ChAT labeled motor neurons in each facial motor nucleus and determining the average cytoplasmic ChAT labeling intensity of each motor neuron (see Experimental Procedures).

The adult onset depletion of CNTFRα clearly did not decrease ChAT labeling in *intact* facial motor neurons, in that the CNTFRα depleted mice displayed at least as many ChAT labeled intact motor neurons as controls. If anything, there was a trend toward an increase in ChAT labeled motor neurons in some labeling intensity ranges (Figure 3A). However, with appropriate multiple comparison post-hoc tests, this trend did not reach significance at any labeling intensity (Figure 3A).

Comparison of the intact and lesioned facial motor neurons within the controls indicated that the nerve crush produced a small (statistically insignificant) decrease in ChAT labeling (Figure 3B), consistent with previous cranial nerve crush data (Armstrong et al., 1991). In contrast, the identical nerve crush in the CNTFRα depleted mice produced a large, highly significant decrease in ChAT labeling (Figure 3C). The between animal comparison of lesioned motor neurons in control versus CNTFRα depleted mice likewise indicated significantly less ChAT labeling in the CNTFRα depleted mice (Figure 3D), although the

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difference was not as great as seen with the within animal comparisons given the trend to less ChAT labeled motor neurons in the controls. Finally, the overall ANOVA genotype by lesion interaction term also indicated that the lesion had a significantly larger effect on ChAT labeling in the CNTFRα depleted mice than in the controls, even when the data were collapsed across all labeling intensities ($F=5.14$; $P<0.05$).

DISCUSSION

The present report describes more of our continuing effort to determine the *in vivo* roles played by endogenous CNTF receptors in maintaining adult motor neurons. We used floxed CNTFRα mice and CreER to produce an adult onset CNTFRα gene disruption. We have previously shown that this eliminates CNTFRα labeling in approximately half of facial motor neurons, but has no effect on motor neuron survival, even following nerve crush, suggesting that adult motor neuron CNTF receptors do not play an essential role in adult motor neuron survival under these conditions (Lee et al., 2013a). The present finding that the identical CreER/floxed CNTFRα manipulation also depletes skeletal muscle CNTFRα suggests that adult muscle CNTF receptors likewise do not make a significant, essential contribution to the survival of intact or nerve crush lesioned adult motor neurons. The substantial depletion, but not complete removal of muscle CNTFRα, leaves open the possibility that muscle CNTFRα contributes to motor neuron survival, such that even a small amount of muscle CNTFRα is sufficient to provide maximal effect. However, it seems most likely that if muscle CNTFRα were to play a significant, essential role, its substantial depletion would produce at least a trend toward an effect on survival, which was not seen (Lee et al., 2013a). Moreover, as discussed below, this same manipulation produces a large effect on ChAT in lesioned motor neurons, indicating that any role adult CNTF receptors play in motor neuron survival is at least much less than their contribution to motor neuron ChAT maintenance.

We find that the depletion of adult motor neuron and muscle CNTFRα does not decrease motor neuron ChAT labeling in intact motor neurons, suggesting that CNTF receptors do not play a significant essential role in maintaining ChAT levels in intact adult motor neurons. However, the same CNTFRα depletion leads to a decrease in ChAT labeling in nerve lesioned motor neurons, which is significantly larger than that seen in controls, suggesting that, with this insult, motor neuron and/or muscle CNTF receptors become essential for maintaining motor neuron ChAT levels. In other words, the data suggest that while adult CNTF receptors are not essential to maintain motor neuron ChAT levels in the absence of insult, they adopt such a role when the motor neurons are challenged by nerve crush.

It is theoretically possible that the depletion of CNTFRα from roughly half of the motor neurons does not affect ChAT in the non-lesioned motor neuron population because the remaining receptor activity in the motor neurons with CNTFRα somehow maintains ChAT in the motor neurons with depleted CNTFRα through some sort of non-cell autonomous process. However, this unlikely scenario would be more of a concern if we were to have not observed any effect of the CreER/floxed CNTFRα manipulation and were concluding that adult CNTFRα does not play any role in motor neuron ChAT maintenance. Instead we observe a large effect of the CreER floxed CNTFRα manipulation in lesioned motor

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neurons, which indicates that even if an unlikely non-cell autonomous process is involved, it is not enough to entirely compensate for the loss of CNTFRα. It remains remotely possible that a non-cell autonomous process masks a role for CNTFRα in maintaining ChAT in non-lesioned motor neurons but is not enough to mask the larger role of CNTFRα in lesioned motor neurons.

While the mechanisms by which CNTFRα helps maintain ChAT are not currently known, adult motor neuron CNTFRα could obviously contribute to motor neuron CNTF receptor signaling that helps maintain motor neuron ChAT, as suggested by the *in vitro* data (see below). Muscle CNTFRα could contribute to muscle CNTF receptor signaling leading to increased release of one or more yet to be identified muscle factors that act on motor neurons to maintain ChAT. Another interesting possibility involves the release of muscle CNTFRα itself. CNTFRα is attached to the extracellular surface of the plasma membrane by a glycosyl-phosphatidylinositol linkage. Following nerve lesion, muscle CNTFRα is released in a soluble, functional form (Davis et al., 1993b) that could potentially, over time, diffuse to axons proximal to the lesion and enhance motor neuron CNTF receptor signaling. Unfortunately, the optimal conditions for CNTFRα and ChAT immunohistochemistry were not compatible for double labeling. Therefore, we were unable to determine whether the effect of the CreER/floxed CNTFRα manipulation on motor neuron ChAT levels is primarily restricted to the subpopulation of motor neurons lacking CNTFRα, as expected if motor neuron CNTFRα is primarily responsible for maintaining motor neuron ChAT, or occurs independent of which motor neurons are CNTFRα depleted, as expected if muscle CNTFRα plays a significant role.

Although motor neurons and muscle are the only cell types expressing CNTFRα in the neuromuscular system (Davis et al., 1991; MacLennan et al., 1996; Lee et al., 1997) we cannot exclude the unlikely possibility that motor neuron ChAT is somehow indirectly maintained by CNTFRα in some class of non-neuromuscular cells which may also be affected by CreER induction in floxed CNTFRα mice. In addition, it is worth noting that CNTFRα RNA has been detected in peripheral nerve by some (Kirsch et al., 1998) but not others (Mata et al., 1993; Kirsch and Hofmann, 1994) and never anatomically localized to nerve soma (Kirsch et al., 1998). Immunohistochemistry detects nerve CNTFRα protein exclusively in axons (MacLennan et al., 1999), suggesting that the CNTFRα RNA nerve signal reflects axonal CNTFRα RNA, consistent with several reports of motor neuron RNA in peripheral nerve axons (Jung et al., 2012). However, even if nerve soma express some CNTFRα RNA, the effect of the CreER/floxed CNTFRα manipulation here unlikely results from disrupting such expression since CreER induction does not lead to Cre activity in peripheral nerve soma (data not shown).

Regardless of the cell types involved, the present data demonstrate for the first time that adult CNTF receptors can help maintain motor neuron ChAT *in vivo*. Clearly, future cell type specific, adult onset CNTFRα gene targeting will be needed to definitively determine the cellular location(s) of the critical receptors.

We targeted CNTFRα because CNTFRα disruption is the most comprehensive approach to determining the *in vivo* functions of endogenous CNTF receptor signaling. Thus, CNTFRα

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is required for all forms of CNTF receptor signaling, regardless of which ligands and signaling pathways are involved (Davis et al., 1993a; Elson et al., 2000; Derouet et al., 2004). Consequently, the present data do not address which individual ligand(s) or pathways are responsible for the motor neuron ChAT maintenance following nerve crush. CNTF may play a role given that it is apparently released from peripheral nerve Schwann cells at the lesion site (Sendtner et al., 1992b) and could act on either motor neuron (axonal) and/or muscle CNTF receptors. Motor recovery following sciatic nerve crush is decreased in unconditional CNTF knockout mice (Yao et al., 1999) consistent with endogenous CNTF contributing to neuromuscular maintenance/recovery following the injury. However, this unconditional knockout study did not address the cellular source of the CNTF or whether the knockout affects motor neuron ChAT levels. The complex of cytokine-like factor-1/cardiotrophin-like cytokine (CLC/CLF) also serves as a CNTF receptor ligand (Elson et al., 2000) and is another viable candidate for participation in motor neuron ChAT maintenance. Unlike CNTF, it plays an essential role in maintaining motor neurons during development. Thus, complete unconditional knockout of cytokine-like factor-1 (Alexander et al., 1999; Elson et al., 2000; Forger et al., 2003), cardiotrophin-like cytokine (Zou et al., 2009), or CNTFRα (DeChiara et al., 1995), but not CNTF (Masu et al., 1993; DeChiara et al., 1995), leads to substantial perinatal motor neuron loss. However, adult neuromuscular CLC/CLF expression has yet to be examined. Both CNTF and CLC/CLF may contribute to the adult ChAT maintenance found here and may act together. Adult onset disruption of the individual ligands is needed to definitively address this issue, with cell type specific disruption identifying the cellular source of each ligand.

The present data showing no loss of motor neuron ChAT with adult onset CNTFRα disruption in intact (non-lesioned) motor neurons may at least initially appear to contrast with the multiple findings that exogenous CNTF can help maintain ChAT in embryonic cultured motor neurons (Magal et al., 1991; Glicksman et al., 1993; Wong et al. 1993; Kato and Lindsay, 1994; Zurn, 1994). While this difference may result from distinctive properties of embryonic versus adult motor neurons and/or the *in vivo* versus *in vitro* conditions, it is worth noting that the cultured motor neurons were, of course, axotomized and perhaps a better model for the lesioned adult motor neurons in the present mice, in which we found a decrease in ChAT when the CNTF receptor was disrupted, consistent with the *in vitro* data.

Motor neuron ChAT is a critical enzyme absolutely required for neuromuscular transmission. Therefore, motor neuron ChAT loss has significant functional consequences. The present data showing adult CNTF receptors maintain *in vivo* ChAT levels in motor neurons challenged by insult raise the possibility that specific targeting of these receptors may be therapeutic in adult neuromuscular diseases such as human amyotrophic lateral sclerosis in which motor neurons challenged by the disease display decreased ChAT prior to their eventual death (Kato, 1989; Oda et al., 1995). Enhancing adult motor neuron CNTF receptor signaling may delay or block this ChAT loss and the resulting functional impairment. It also remains possible that the ChAT loss is a precursor event leading to the subsequent motor neuron degeneration such that promoting motor neuron ChAT maintenance through CNTF receptor signaling could significantly slow the progression of the disease. Finally, an analogous argument could be made for CNTF receptor based therapeutics and the treatment of peripheral nerve injury.

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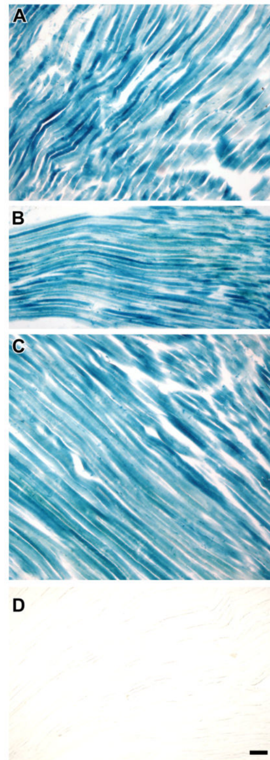

Figure 1. The CreER gene construct enables adult onset disruption of floxed genes in skeletal muscles, including those innervated by the facial nerve

Adult CreER induction leads to floxed gene excision in facial muscles, as revealed by ROSA26 reporter Xgal histology (blue signal)in: **(A)** temporal muscle, **(B)** nasolabial muscle, and **(C)** masseter muscle of a CreER+, ROSA26 reporter+ mouse injected with tamoxifen as an adult and perfused for histology two months later. **(D)** temporal muscle of a similarly treated CreER-negative control mouse. Scale bar = 100μm.

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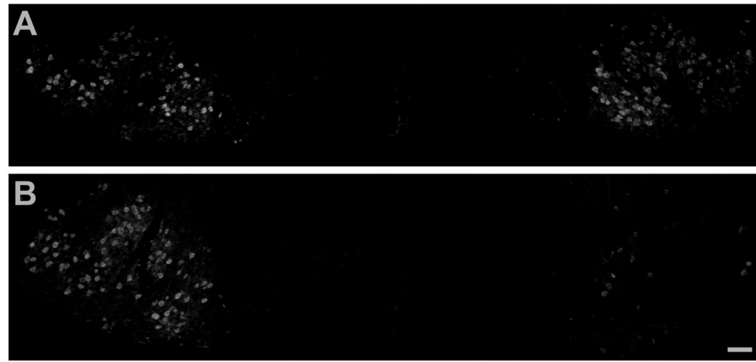

Figure 2. Adult onset CNTF receptor α (CNTFRα) gene disruption with CreER decreases facial motor neuron choline acetyltransferase (ChAT) labeling following nerve crush

Homozygous floxed CNTFRα mice and littermate non-floxed controls, all containing CreER, were injected with tamoxifen at three to four months of age to deplete motor neuron and muscle CNTFRα specifically in the floxed mice (see text). Four months later they all received a unilateral facial nerve crush. Three weeks after the lesion, their facial motor nuclei were examined by ChAT immunohistochemistry. Examples of sections from control, non-floxed **(A)** and CNTFRα-depleted, floxed **(B)** mice are presented with the lesioned side on the right. The clusters of labeled motor neurons correspond to the facial motor nuclei. This qualitative decrease in ChAT labeling was observed throughout the lesion side, CNTFRα-depleted facial motor nuclei (every 4th section assayed; see Materials and Methods). Quantification is presented in Figure 3. The images in A and B were identically captured and adjusted for brightness and contrast. Scale bar=100μm.

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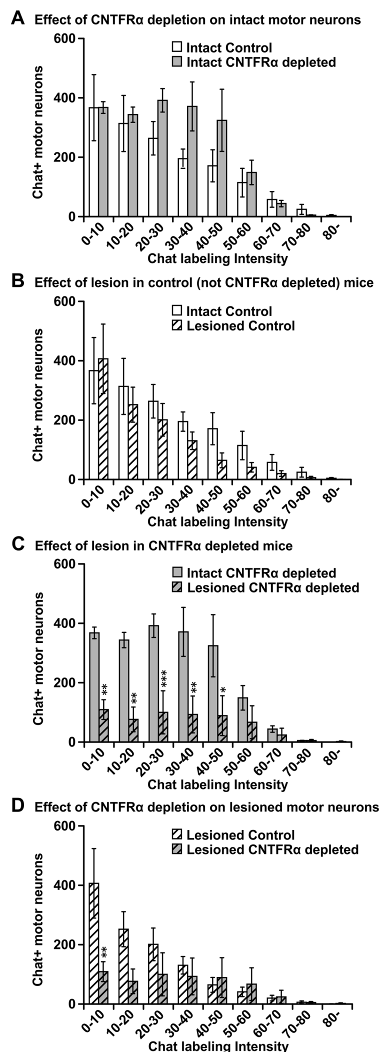

Figure 3. Quantitative analysis of facial motor neuron ChAT labeling following nerve crush in control and CNTFRα depleted mice

Following adult onset CNTFRα-depletion, nerve crush and ChAT immunohistochemistry (see Figure 2), the average cytoplasmic ChAT labeling intensity was determined with MetaMorph software for each labeled facial motor neuron. Motor neurons were then categorized into ten ChAT labeling intensity ranges, determined *a priori*, with "0" equal to background and "100" corresponding to the most intensely labeled motor neuron in the entire study. The 9th and 10th categories are combined here for graphical presentation because so few motor neurons were found in these categories. **(A)** CNTFRα depletion did not decrease ChAT labeling in intact (i.e., non-lesioned) motor neurons. The CNTFRα depleted mice displayed a trend toward increased labeled neurons in some labeling intensity ranges, but none reached significance with appropriate multiple comparison post-hoc statistics. **(B)** Nerve crush in control (i.e., not CNTFRα depleted) mice led to a trend toward decreased ChAT labeling which was not significant at any labeling intensity. **(C)** In contrast, nerve crush in CNTFRα depleted mice led to a large, highly significant decrease in ChAT labeling. **(D)** The between mice comparison of ChAT labeling in lesioned motor neurons of

controls vs CNTFRα depleted mice also revealed a significant decrease in ChAT labeling with CNTFRα depletion, even with the trend for less overall ChAT labeling in the control mice. Finally, an ANOVA analysis of all the data, collapsing across all labeling intensities, also indicated that the lesion had a significantly larger effect on ChAT labeling in the CNTFRα depleted mice than in the controls (see Results). n = 4 CNTFRα-floxed mice; 7 control mice. Means±SEMs presented. Bonferroni post hoc tests: *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$ compared to intact CNTFRα depleted motor neurons in C and lesioned control motor neurons in D.

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Table 1

Primary Antibody Used in This Study

Antibody	Immunogen	Source	Concentration
Choline Acetyltransferase	Purified human ChAT	Millipore (cat. # AB143) RRID: AB_2079760 Lot #: LV1583387 rabbit polyclonal	1:6000