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Absence of axonal sprouting following unilateral lesion in 125-day-old rat supraoptic nucleus may be due to age-dependent decrease in protein levels of ciliary neurotrophic factor receptor alpha

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Abstract

Within the supraoptic nucleus (SON) of a 35-day-old rat we previously demonstrated a collateral sprouting response that reinnervates the partially denervated neural lobe (NL) after unilateral lesion of the hypothalamo-neurohypophysial tract. Others have shown a decreased propensity for axonal sprouting in an aged brain; therefore, in order to see if the SON exhibits a decreased propensity for axonal sprouting as the animal ages, we performed a unilateral lesion in the 125-day-old rat SON. Ultrastructural analysis of axon profiles in the NL of the 125-day-old rat demonstrated an absence of axonal sprouting following injury. We previously demonstrated that ciliary neurotrophic factor (CNTF) promotes process outgrowth from injured magnocellular neuron axons *in vitro*. Thus, we hypothesized that the lack of axonal sprouting in the 125-day-old rat SON may be due to a reduction in CNTF or the CNTF receptor components. To this point, we found that as the rat ages there is significantly less CNTF receptor alpha (CNTFRα) protein in the uninjured, 125-day-old rat compared to the uninjured, 35-day-old rat. We also observed that protein levels of CNTF and the CNTF receptor components were increased in the SON and NL following injury in the 35-day-old rat, but there was no difference in the protein levels in the 125-day-old rat. Altogether, the results presented herein demonstrate that the plasticity within the SON is highly dependent on the age of the rat, and that a decrease in CNTFRα protein levels in the 125-day-old rat may contribute to the loss of axonal sprouting following axotomy.

Abstract

Following unilateral lesion in the 35-day-old rat, CNTF protein levels increased in the contralateral, sprouting SON and lesion NL concomitant with a collateral sprouting response. However, unilateral lesion in the 125-day-old rat did not result in a collateral sprouting response nor increases in CNTF protein levels.

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1. INTRODUCTION

Unlike the post-injury responses that occur in the peripheral nervous system, axonal regeneration following injury in the central nervous system (CNS) is rare. In order for the post-injury responses to occur in the CNS, the combination of several important factors need to be considered, including neuronal and glial intrinsic factors, extrinsic factors, and the age of the animal. One region that has shown the propensity for plasticity following injury in the CNS is the supraoptic nucleus (SON) of the hypothalamus. The magnocellular neurosecretory system (MNS) is composed of magnocellular neurons in the SON and their axons that project via the infundibulum to terminate in the neural lobe (NL; posterior pituitary), and surgical removal of the NL (Kawamoto & Kawashima, 1985; Moll, 1957; Moll & de Wied, 1962; Polenov, Ugrumov, Propp, & Belenky, 1974; Raisman, 1973), transection of the infundibulum (Adams, Daniel, & Prichard, 1964; Kiernan, 1970, 1971), and unilateral lesion of the axons in the hypothalamus (Watt et al., 1999; Watt & Paden, 1991) all result in post-injury regenerative growth of axons. However, it remains to be determined what promotes the axonal plasticity. We have hypothesized that the collateral sprouting response following unilateral lesion is due to a protein that is inherent to astrocytes in the SON, ciliary neurotrophic factor (CNTF). In hypothalamic organotypic cultures, CNTF promotes neuron survival (Askvig et al., 2013; Askvig & Watt, 2015; Rusnak, House, Arima, & Gainer, 2002; Vutskits, Bartanusz, Schulz, & Kiss, 1998) and axonal sprouting (Askvig & Watt, 2015) following axotomy. Additionally, CNTF protein levels increase in the SON during the collateral sprouting response (Askvig, Leiphon, & Watt, 2012; Watt, Bone, Pressler, Cranston, & Paden, 2006), further suggesting that it may play a role in promoting the axonal sprouting from uninjured neurons following unilateral lesion.

When axonal sprouting does occur in the CNS, there is a maturational decline in the propensity for the functional reorganization (Bates & Stelzner, 1993; Cotman & Scheff, 1979; Crutcher, 1990; Scheff, Benardo, & Cotman, 1980; Scheff, Bernardo, & Cotman, 1978). Remarkably, the adult SON is a highly plastic system capable of activity-dependent morphological changes in both neurons and glia (Armstrong & Hatton, 1978; Chapman, Theodosis, Montagnese, Poulain, & Morris, 1986; El Majdoubi, Poulain, & Theodosis, 1997; Hatton, 1986, 2004; C. Montagnese, Poulain, Vincent, & Theodosis, 1988; C. M. Montagnese, Poulain, Vincent, & Theodosis, 1987; Theodosis, Chapman, Montagnese, Poulain, & Morris, 1986; Theodosis & Poulain, 1984) suggesting the possibility that the adult SON may be able to functionally reorganize following injury. Thus, we sought to determine how the maturing SON responds to unilateral lesion of the hypothalamo-neurohypophysial tract and the effects that the injury has on CNTF and CNTF receptor component protein levels in the SON and NL of 125- day-old rats.

2. MATERIALS AND METHODS

2.1 Animals

Male Sprague-Dawley rats were purchased from Harlan Laboratories (Minneapolis, MN) and housed in the University of North Dakota Center for Biomedical Research Facility, an AAALAC accredited facility, under a 12L:12D light cycle with ad lib access to lab chow and tap water throughout the investigations unless otherwise noted. Experimental protocols utilized in these studies followed the guidelines in the NIH guide for the care and use of laboratory animals and were approved by the UND Institutional Animal Care and Use Committee. Animals used in this study were 35–40 days of age (75–100 grams), 60–65 days of age (200–250 grams), and 120–130 days of age (450–550 grams).

2.2. Antibody characterization

Quantification of CNTF protein in the rat SON was achieved using rabbit anti-CNTF antibody (1:5000; #AAR21; Bio-Rad/AbD Serotec, Raleigh, NC; RRID:AB_2276485). Antibody specificity was previously documented in our lab by performing pre-adsorption control with rat recombinant CNTF (rrCNTF) (Askvig et al., 2012). The mouse anti-CNTFRα (1:20,000; #558783; BD Biosciences, Franklin Lakes, NJ; RRID:AB_397120) antibody was utilized for the quantification of CNTFRα protein levels in the SON. While the anti-CNTFRα antibody utilized in the present study was raised against human CNTFRα, others have demonstrated that there is a 94.1% homology between human and rat CNTFRα amino acid sequence (Beltran et al., 2003), we previously documented antibody specificity when the pre-adsorption control resulted in the complete absence of protein bands using rat SON tissue (Askvig et al., 2012). Quantification of gp130 protein in the rat SON was performed using rabbit anti-gp130 (1:5000; #sc-655; Santa Cruz Biotechnology, Santa Cruz, CA; RRID:AB_631590) that recognizes the C-terminus of gp130 of human origin (manufacturer's technical information). However, manufacturer's technical information indicates cross-reactivity with rat gp130 and we have previously demonstrated gp130 protein bands in rat SON (Askvig et al., 2012). Protein quantification of LIFRß in the rat SON was achieved using rabbit anti-LIFRß (1:5000; #sc-659; Santa Cruz Biotechnology; RRID:AB_2136105), which recognizes the C-terminus of LIFRß of human origin (manufacturer's technical information). However, manufacturer's technical information indicates cross-reactivity with rat LIFRß and we have previously demonstrated LIFRß protein bands in rat SON (Askvig et al., 2012). The ß-actin antibody (1:50,000; #A2228; Sigma-Aldrich; RRID:AB_476697) recognizes an epitope located on the N-terminal end of the rat ß-isoform of actin (Gimona et al., 1994). ß-actin was utilized as a tissue loading control for our Western blot analyses and the anti-ß-actin antibody consistently recognized a band at approximately 42 kDa in all rat tissue samples analyzed.

For immunocytochemical analyses we utilized guinea pig anti-oxytocin (1:2000; #T-5021.0050; Peninsula Laboratories, San Carlos, CA; RRID:AB_518526) and -vasopressin (1:2000; #T-5048; Peninsula Laboratories; RRID:AB_518680) antibodies that recognize the neurohormones oxytocin and vasopressin, respectively, with no reported cross reactivity for other neurohormones (manufacturer's technical information). The reported distribution of oxytocin- and vasopressin-immunoreactivity, as well as the morphology of

the oxytocinergic and vasopressinergic magnocellular neurons of the rat SON (Silverman & Zimmerman, 1983) are comparable to those observed in the present study.

2.3 Unilateral Lesion of Hypothalamo-Neurohypophysial Tract

Animals were either 35–40 or 120–130 days of age at the time a unilateral hypothalamic knife cut of the hypothalamo-neurohypophysial tract was performed. Briefly, the animals were placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL) and using a tabletop anesthesia apparatus (Matrx Quantiflex Low Flow V.M.C.; Matrx, Orchard Park, NY) equipped with an isoflurane Vaporizer (Matrx VIP 3000; Matrx), the animals were kept under constant isoflurane anesthesia (2.5%; Abbot Laboratories; Abbott Park, IL). Transection of the hypothalamo-neurohypophysial tract was performed with a wire knife constructed of HTX-33-gauge tubing that passed through the entire length of the hypothalamus as previously described (Watt & Paden, 1991). The knife tract extended to just dorsal to the ventral surface of the brain, entering the optic chiasm lateral to the third ventricle and medial to the ipsilateral SON, but passing through the lateral aspect of the ipsilateral PVN. Stereotaxic lesion coordinates were 0.6 mm (for 35-day-old rats) or 0.8 mm (for 125-day-old rats) lateral to the midsagittal suture, and the lesion extended from −4.0 mm to +4.0 mm anterior-posterior from bregma. This results in complete transection of the ipsilateral hypothalamo-neurohypophysial tract. For TEM and immunocytochemical analyses, lesion tracts were confirmed histologically using cresyl violet, while for Western blot analyses, the lesion tract was visually confirmed under a dissecting microscope during tissue collection. Only rats with a complete transection of the hypothalamo-neurohypophysial tract were included in these studies. All efforts were made to minimize the numbers of rats used in this study and their suffering.

2.4 Tissue preparation for ultrastructural analysis

For transmission electron microscope (TEM) analysis, rats were initially perfused with a 0.1M PO_4 solution (pH 7.3) followed by a 2% glutaraldehyde/4% paraformaldehyde solution in 0.1M PO_4 with pH set at 7.3. Neural lobes were excised en toto and post-fixed overnight, followed by 4% OsO_4 for 60 minutes at 4°C (Ted Pella). NL's were then dehydrated in increasing concentrations of ethanol, cleared in propylene oxide (Ted Pella), then vacuum infiltrated in a 100% Epon/Araldite mixture (50:50, v/v; Ted Pella), overnight. Subsequent polymerization was performed at 58°C for 72 hrs. Coronal 1 mm serial sections of the intact NL were collected from a rostral to caudal direction and all NL samples were mounted on formvar-coated slot grids, then contrast stained with Reynold's lead citrate uranyl acetate in an LKB Ultrastainer and viewed on a Zeiss TEM 10C at 60 kV. Nine sampled fields from each of three intact NL cross sections were collected at 5000X.

2.5 Quantification of axon numbers

After photographic enlargement of original TEM micrographs to 13,500x, an unbiased counting frame was positioned over each micrograph, and all axon profile not in contact with the exclusion sides were identified by the presence of neurofilaments and neurosecretory vesicles to distinguish axon profiles from pituicyte processes. The areal densities were then calculated for the right, left, and central regions of each cross section of the NL. A single mean value was then calculated for each cross section. The total number of

axons in each cross section was then determined by multiplying the mean areal density by the total cross-sectional area that was determined from the adjacent 1 mm thick plastic section stained with toluidine blue. In this manner the number of axon profiles was estimated separately for the rostral, middle, and caudal regions of the NL of each animal. No statistically significant differences in the areal density of axons were observed between the three regions of the NL in any group, permitting a single mean value to be calculated for each animal.

2.6 Metabolic analysis

Animals were individually housed in Nalgene metabolic cages throughout the experimental period. Daily drinking and urine excretion volumes were determined directly from graduated drinking and urine collection tubes, respectively. Urine samples were taken at a consistent time each day and then centrifuged to remove any precipitate prior to analysis using a Wescor vapor pressure osmometer.

2.7 Gel electrophoresis and Western blot analysis

Following unilateral lesion, 35 and 125-day-old rats included in Western blot analysis were sacrificed 10 days post lesion (dpl), along with age-matched uninjured control rats, as previously described (Askvig et al., 2012). Briefly, the rats were placed under isoflurane anesthesia, were decapitated, and their brains were removed intact. The brain was coronally sectioned to take approximately a 500 μm section containing the SON and the SON was then dissected away from the brain parenchyma and meninges under a dissecting microscope. SON from 6 rats were pooled (30 total rats, n=5 groups of 6 pooled rat SON) in a solution of radioimmuno-precipitation assay (RIPA) buffer containing 20 mM Tris (pH 7.5 Sigma-Aldrich; St. Louis, MO), 150 mM NaCl (Sigma-Aldrich), 1% nonidet P-40 (Roche Diagnostics; Indianapolis, IN), 0.5% sodium deoxycholate (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 0.1% SDS (Pierce; Rockford, IL), 1% protease inhibitor (Protease Inhibitor Cocktail; Sigma-Aldrich) and 1% phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich). The SON samples were then homogenized in RIPA buffer and centrifuged at 10,000 rpm for 20 mins at 4ºC. Supernatant from each sample was stored at −80ºC until needed. SON protein content was determined using the bicinchoninic acid (BCA) colorimetric detection assay (Pierce BCA Protein Assay; Pierce).

For gel electrophoresis, each lane was loaded with 50 μg of protein and separated by a 12% SDS-PAGE gel (Precise Protein Gels; Pierce) at 90 V for approximately 1.5 hrs and then electrophoretically transferred to a PVDF membrane (0.2 μm; Bio-Rad, Hercules, CA) at 70 V for 2 hrs. After blocking non-specific binding sites (5% nonfat milk in phosphate-buffered saline (PBS) plus 0.1% Tween-20; blocking buffer; Bio-Rad), the membranes were incubated overnight at 4°C in rabbit anti-CNTF (1:5000). The membranes were then washed repeatedly for 1 hr in PBS-Tween and incubated for 2 hrs in the appropriate HRP-conjugated secondary antibody (1:100,000; Santa Cruz Biotechnology). Following PBS washes for 2 hrs, the bands were subsequently visualized using the West Femto chemiluminescent detection kit (Pierce) with high performance chemiluminescence film (Amersham Hyperfilm ECL; GE Healthcare; VWR; West Grove, PA) on an AGFA CP1000 film processor (DMS Health Group; Fargo, ND). Subsequently, bound antibodies were removed with stripping

buffer (pH 2.2; 15g glycine; Sigma-Aldrich, 1 g SDS; Bio-Rad, 10 ml Tween-20; Bio-Rad in 1 L ultrapure water) in 2×10 min incubations and the steps were repeated to sequentially reprobe the membrane for the following antibodies; mouse anti-CNTFRα (1:20,000), rabbit anti-gp130 (1:5000), rabbit anti-LIFRß (1:5000), and mouse anti-ß-actin (1:50,000).

Densitometric analysis of immunoblot signals was performed using MCID image analysis software (Version 7.0, Imaging Research Inc.). Briefly, digitized Western blot images were opened in MCID and the protein bands of interest were outlined. The band area and relative optical densities (ROD) were then determined and the area of the band was then multiplied by the density value. The ROD of all bands was normalized to the respective ROD of ß-actin bands to obtain ratios. Analysis was repeated on 3 separate samples per group resulting in mean ratio values for each group that were used for statistical analysis as described below.

2.8 Immunocytochemistry

At 10 dpl, 125-day-old rats were deeply anesthetized with isoflurane and perfused transcardially with 0.9% saline followed by periodate-lysine-paraformaldehyde fixative (PLP; 3.2% paraformaldehyde, 2.2% lysine, 0.33% sodium- (meta) periodate; Sigma-Aldrich) prepared immediately before use (McLean & Nakane, 1974). Brains were then removed and post-fixed overnight before cryoprotection in 20% sucrose in PBS. Serial cryosections (16 μm) were thaw-mounted on gelatin-coated slides and stored at −20ºC until use.

Sections were washed with PBS containing 0.3% Triton X-100 (PBS-T; Sigma-Aldrich) in 3×10 minute intervals before and after all incubations. Non-specific staining was alleviated by treatment with 4% of the appropriate normal serum (Vector, Burlingame, CA) in PBS-T (blocking buffer) for 1 hr at room temperature followed by an overnight incubation at 4°C in guinea pig anti-oxytocin (1:2000) or guinea pig anti-vasopressin (1:2000). Sections were then incubated in biotinylated donkey anti-guinea pig IgG (1:500; Vector) for 1 hr followed by incubation in avidin-biotin complex (ABC *Elite* kit, Vector). Binding of ABC reagent was visualized with a reaction in 0.05% diaminobenzidine (DAB; 200 mg glucose, 40 mg ammonium chloride, 50 mg DAB per 100 mL PBS; Sigma-Aldrich) using glucose oxidase (0.3%; Sigma-Aldrich) to generate hydrogen peroxide (Itoh et al., 1979). Following PBS washes, sections labeled with anti-oxytocin and anti-vasopressin were counterstained with hematoxylin (Vector) for 30 seconds prior to dehydration in a graded series of alcohol.

2.9 Vasopressin and oxytocin immunoreactive profile counts

To count vasopressinergic and oxytocinergic immunoreactive profiles in the SON, we performed immunocytochemical counts as previously described (Askvig et al., 2012). To ensure that the same immunoreactive profile was not counted twice, adjacent sections were stained for oxytocinergic and vasopressinergic magnocellular neurons and a minimum of five sections (80 μm) were skipped before the next sections were processed. A third-party blind to experimental conditions randomly coded the slides before analysis and immunopositive neurons containing a counterstained nucleolus were counted using a drawing tube attached to an Olympus BX51 microscope. In order to account for the rostral-caudal size difference of the SON, we determined the numbers of cells per unit area of SON

by obtaining the total area of each SON using Image J. Data were expressed as the percentage of the age-matched controls. A minimum of six sections containing a well-defined SON were taken at rostral to caudal levels matched across animals for analysis.

2.10 Statistical analysis

The Kolmogorov-Smirnov test was used to test distribution normality of each group of data (GraphPad InStat, version 3.06 for Windows, San Diego California). Student's *t* test or one-way ANOVA with post *hoc* Tukey's tests (GraphPad InStat) were used, where appropriate, to compare groups with $p<0.05$ considered statistically significant. Results are expressed as the group means ± SD.

3. Results

3.1 Absence of Sprouting in 125-day-old SON Following Unilateral Lesion

We previously demonstrated that unilateral hypothalamic lesion in 35-day-old rats resulted in a 42% decrease in neurosecretory axons in the NL at one-week post-surgery followed by a collateral sprouting response that returns the neurosecretory axons to control levels at four weeks post-surgery (Watt et al., 1999; Watt & Paden, 1991). Furthermore, the unilateral lesion in the 35-day-old rat resulted in a chronic hyperosmolality with a concomitant decrease in daily water intake and urine excretion volume (Watt et al., 1999; Watt & Paden, 1991). However, our results demonstrated that there was an absence of a sprouting response following unilateral hypothalamic lesion in 125-day-old rats (Figure 1) and no increase in the neurosecretory activity following the injury in the 125-day-old rat as measured by urine osmolarity. At one-week post-surgery, there is a statistically significant reduction of 41.8% in the number of axons in the lesion NL compared to the intact NL, and in spite of the previously documented increase in size of the NL with age (Galabov & Schiebler, 1978; Krisch, 1980; Watt et al., 1999), the number of axons in the injured NL at four weeks post-surgery was 47.5% below intact levels (Figure 1). Additionally, following unilateral lesion in the 125-day-old rat, urine osmolarity was significantly decreased throughout the four weeks post-surgery compared to the response that was observed in the 35-day-old rat following unilateral lesion (Figure 2).

At 10 dpl the unilateral lesion resulted in the decrease of 88% and 93% of the immunoreactive profiles indicative of oxytocinergic and vasopressinergic magnocellular neurons, respectively, in the axotomized SON of the 125-day-old rat ($p<0.0001$; Figure 3). Moreover, there was no significant difference in the number of magnocellular neurons as determined by oxytocin- ($p=0.6941$) or vasopressin-staining ($p=0.9851$) in the SON contralateral to the injury (Figure 3). These results are consistent to the effect that the unilateral lesion has on the neurons in the 35-day-old rat, where the injury resulted in the decrease of 85% and 90% of the immunoreactive profiles for oxytocin and vasopressin, respectively, in the axotomized SON (Askvig et al., 2012).

3.2 Protein Levels of CNTF and CNTF Receptor Components in the Maturing Magnocellular Neurosecretory System

Western blot analysis demonstrated that within the uninjured, control SON (*p=0.07*; Figure 5b) and uninjured, control NL (*p=0.16*; Figure 5c) there was no significant difference in the CNTF protein levels between 35, 60, or 125-day-old rats. However, CNTFRα protein levels in the uninjured 125-day-old rat were decreased by 31% compared to the uninjured 60-day-old rat (*p=0.0087*; Figure 5a) and by 38% compared to the uninjured 35-day-old rat SON (*p=0.0029*; Figure 5a). There was no significant difference in the CNTFRα protein levels in the uninjured NL as the rat matures (*p=0.2978*; Figure 5c). Additionally, there was no statistical difference in the protein levels of LIFRß in the maturing SON (*p=0.10*; Figure 5b) or NL (*p=0.27*; Figure 5c) or gp130 in the maturing SON (*p=0.28*; Figure 5b) or NL (*p=0.15*; Figure 5c). Together, these data indicate that as the rat matures there was less CNTFRα protein in the SON.

3.3 Protein Levels of CNTF and CNTF Receptor Components in the Injured Magnocellular Neurosecretory System

We next sought to determine how unilateral hypothalamic lesion in 125-day-old rats alters protein levels of CNTF and the CNTF receptor components (Figure 4). We previously demonstrated that unilateral lesion in the 35-day-old SON resulted in a 241% increase in CNTF, a 437% increase in CNTFRα, a 82% increase in LIFRß, and a 258% increase in gp130 protein levels in the axotomized SON (Askvig et al., 2012). Similarly, the axotomy in 125-day-old rats resulted in a 81% increase in CNTF (*p=0.014*), a 311% increase in CNTFRα (*p=0.0013*), a 33% increase in LIFRß (*p=0.04*), and a 251% increase in gp130 (*p<0.0001*) protein levels in the 125-day-old lesion SON compared to age-matched uninjured SON (Figure 6). However, in contrast to the response in the 35-day-old rat SON where we previously found significant increases of 131% in CNTF, of 288% in CNTFRα, of 34% in LIFRß, and of 176% in gp130 protein levels in the SON contralateral to injury (Askvig et al., 2012), there was no significant difference in CNTF (*p=0.23*), CNTFRα (*p=0.075*), LIFRß (*p=0.07*), or gp130 (*p=0.36*) protein levels in the 125-day-old rat SON contralateral to the injury at 10 dpl compared to age-matched, uninjured, control SON (Figure 6).

Next, we quantified the change in CNTF and the CNTF receptor components in the NL following unilateral hypothalamic lesion in the 35- and 125-day-old rat. Our results demonstrate that at 10 dpl in the 35-day-old rat CNTF protein levels increased by 72% (*p=0.002*), CNTFRα protein levels increased by 160% (*p<0.0001*), LIFRß protein levels increased by 69% (*p=0.0003*), and gp130 protein levels increased by 108% (*p=0.018*) compared to age-matched control NL (Figure 7). In contrast, there was no significant difference in CNTF (*p=0.68*), CNTFRα (*p=0.24*), LIFRß (*p=0.45*), or gp130 (*p=0.99*) protein levels in the axotomized 125-day rat NL (Figure 8) when compared to age-matched control NL.

4. DISCUSSION

Axonal sprouting has many beneficial effects on the nervous system; however, the developmental and maturational decline in the plasticity of the CNS precludes these positive effects in the adult. Multiple reports demonstrate that the brain is less plastic with age; thus, our data demonstrating a lack of axonal sprouting in the 125-day-old rat SON is not surprising. While our research compares the plasticity in the SON between 35- and 125-day-old rats (approximately 1- and 4-month old rats), the majority of published reports showing a loss of brain plasticity occurs in older animals, commonly between 'young' highly plastic animals that are 2–4 months old and 'aged' animals that are 12–24 months old (Buga, Dunoiu, Balseanu, & Popa-Wagner, 2008; Buga, Sascau, et al., 2008; Jaerve, Schiwy, Schmitz, & Mueller, 2011; Luo et al., 2010; Shetty & Turner, 1998). Thus, our results in the SON are unique in that the magnocellular neurons appear to have a very small period of time during which they can elicit a collateral sprouting response following injury.

The MNS has been a model system to study plastic changes in the adult rat specifically because of the reversible changes that occur in the SON during increased neurosecretory activity in the absence of injury; changes that facilitate neurosecretion including, neuronal changes that increase dendritic synapses (Hatton, Perlmutter, Salm, & Tweedle, 1984; Perlmutter, Tweedle, & Hatton, 1984; Salm, Modney, & Hatton, 1988) and somatic contact to facilitate intercellular communication (Hatton, Yang, & Cobbett, 1987; Theodosis, 2002; Tweedle & Hatton, 1977). The first reports of plasticity in the SON of the rat following injury was demonstrated following hypophysectomy (Billenstien & Leveque, 1955). Although it has been repeatedly demonstrated that the magnocellular axons are able to undergo axonal sprouting following hypophysectomy, these reports are with rats that are younger (smaller) than 125-day-old rats (450–550 grams) used in our present study, ranging from 115–200 gram rats (Billenstien & Leveque, 1955; Scott, Wu, Slusser, Depto, & Hansen, 1995; Villar, Meister, & Hokfelt, 1994; Wu & Scott, 1993) to 200–250 gram rats (Ishikawa et al., 1995; Yuan, Scott, So, & Wu, 2006, 2007). Therefore, our results are the first reporting a lack of axonal plasticity following axotomy in the SON of mature rats and we believe that understanding the mechanisms involved in the collateral sprouting response in the SON will provide valuable insight into understanding the decrease in brain plasticity as the rat ages.

It remains to be determined what changes facilitate the age-dependent decline in plasticity in the CNS. Several hypotheses exist for the decrease in the propensity for axon sprouting in the adult CNS, many of which are focused on the increase or decrease in anti-sprouting or pro-sprouting molecules, respectively. For example, reports demonstrate that the adult CNS environment possesses axon growth inhibitory molecules (Filbin, 2003; He & Koprivica, 2004; Luo et al., 2010), while others have shown decreases in adequate trophic supply, such as neurotrophic or growth factors, that occur with age (Buga, Dunoiu, et al., 2008; Popa-Wagner, Buga, & Kokaia, 2011). However, it appears that adult CNS neurons maintain their ability to exhibit plasticity. Multiple reports demonstrate that when adult CNS neurons are placed in an environment that mimics the neonatal environment, the adult CNS neurons can extend processes (Crutcher, 1989; David & Aguayo, 1981; Richardson, McGuinness, & Aguayo, 1980; Schnell & Schwab, 1990), suggesting that within the adult CNS there are

changes in environmental conditions that do not allow axonal remodeling to occur, contributing to the age-dependent reduction in brain plasticity.

Our lab is interested in understanding the mechanisms of the collateral sprouting response in the MNS. We previously demonstrated that CNTF promotes process outgrowth of injured magnocellular neurons *in vitro* (Askvig & Watt, 2015). Additionally, in the CNS, CNTF promotes axon regeneration of injured retinal ganglion cells (Jo, Wang, & Benowitz, 1999; Leibinger, Andreadaki, Diekmann, & Fischer, 2013; Leibinger et al., 2009; Park, Luo, Hisheh, Harvey, & Cui, 2004; Vigneswara et al., 2014), and others have demonstrated an increase in astrocytic CNTF and CNTFRα expression during the period of entorhinal cortex (Lee, Deller, Kirsch, Frotscher, & Hofmann, 1997) and hippocampal sprouting (Guthrie, Woods, Nguyen, & Gall, 1997), providing further evidence for a possible involvement of CNTF in promoting post-injury axonal sprouting. Since it has been hypothesized that axon growth is not the default function of a surviving neuron following injury and instead the neuron may need to be specifically signaled for axonal sprouting to occur (Goldberg et al., 2002), we hypothesized that the reduced sprouting efficacy observed in the 125-day-old rats was due to an age-dependent reduction in the protein levels of CNTF or the CNTF receptor complex components in the SON, which could reduce the signaling effectiveness of CNTF. To this point, we demonstrated that as the rat matures there is significantly less CNTFRα protein in the SON. Since CNTFRα is the specific receptor for CNTF and CNTF signal transduction is unable to occur with only LIFRß and gp130 (Davis et al., 1993), a reduction in CNTFRα could specifically reduce the efficacy of CNTF signaling in the SON of the 125-day old rat while not affecting the signaling capabilities of other cytokines (interleukin-6, interleukin-11, leukemia inhibitory factor, oncostatin M, and cardiotrophin-1) that utilize either LIFRß, gp130, or both (Hirano, Matsuda, & Nakajima, 1994). The reduction in CNTFRα in the SON as the rat ages may prevent the collateral sprouting response that occurs in the 35-day-old rat from occurring following unilateral lesion in the 125-day-old rat. Additionally, if the significant reduction in CNTFRα in the SON does prevent the collateral sprouting response from occurring, these data suggest that the collateral sprouting response is initiated in the SON since the mature NL exhibited consistent CNTFRα protein levels as the rat ages.

We previously found that protein levels of CNTF and all of the CNTF receptor components increased in the axotomized SON and the contralateral SON, which is the SON from which the collateral sprouting response arises, following unilateral lesion in the 35-day-old rat (Askvig et al., 2012). While the unilateral lesion in the 125-day-old rat resulted in increased protein levels of CNTF and all of the CNTF receptor components in the axotomized SON, the protein levels of CNTF and the CNTF receptor complex did not increase in the contralateral SON following unilateral hypothalamic lesion in the 125-day-old rat, suggesting that the CNTF signaling response is not elevated in the SON that contains the magnocellular neurons from which the sprouting response would arise, possibly due to the observed decrease in CNTFRα protein levels. In addition, while we found an increase in the protein levels of CNTF and all of the CNTF receptor components in the 35-day-old axotomized NL, there was not an increase in CNTF or the receptor components in the axotomized NL of the 125-day-old rat, which is the site of the degeneration of the axotomized axons. These data suggest that the increases in CNTF and the receptor

components in the axotomized 35-day-old NL were due to the sprouting event, and not the degeneration of the axotomized axons. However, the precise role of CNTF in the NL is still not known. To this point, there is evidence indicating that CNTF-immunoreactive cells of the NL are perivascular cells that may be neuronophagic during the axonal degeneration that follows partial denervation of the 35-day-old rat NL (Lo, SunRhodes, & Watt, 2008) suggesting that CNTF may play a role in axonal degeneration, as opposed to axonal sprouting in the NL.

In summary, these data indicate that the plasticity of the magnocellular neurons of the SON to reinnervate the NL following injury in the 35-day-old rat is not preserved in the 125-day-old rat SON and the lack of axonal sprouting in the mature rat may be due to a decrease in CNTFRα protein levels. Moreover, while injury in the 125-day-old rat results in increases in CNTF and CNTF receptor component protein levels that is comparable to the increases observed following axotomy in the 35-day-old rat SON, the 35-day-old rat elicits a dramatic increase in CNTF and the CNTF receptor components in the sprouting SON (Askvig et al., 2012) and NL that is not observed within the SON contralateral to injury in the 125-day-old rat SON or axotomized NL. Future research will aim to determine if the decrease in CNTFRα protein levels in the 125-day-old rat SON prevents axonal sprouting following unilateral lesion.

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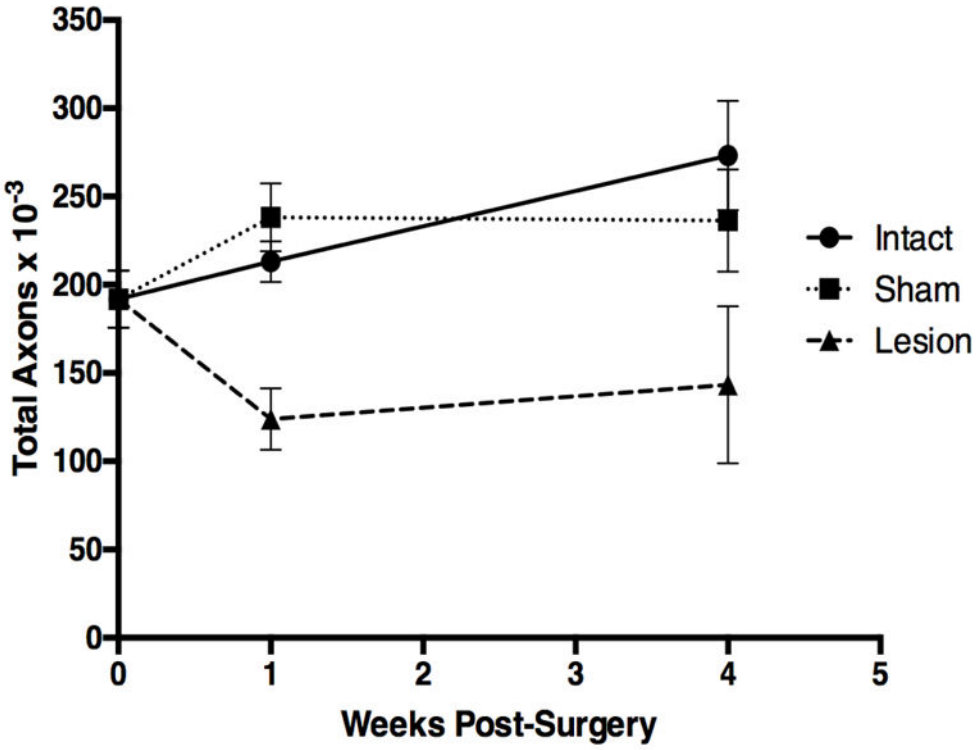

Figure 1.

Failure of lesion-induced axonal sprouting in 125-day-old rat. Following unilateral lesion of the hypothalamo-neurohypophysial tract, the number of axons is significantly decreased at one-week post-surgery compared to intact and sham-control ($p<0.0001$). Moreover, the number of axons remains significantly decreased in the lesion NL at four-weeks post-surgery compared to intact and sham-control ($p<0.0001$).

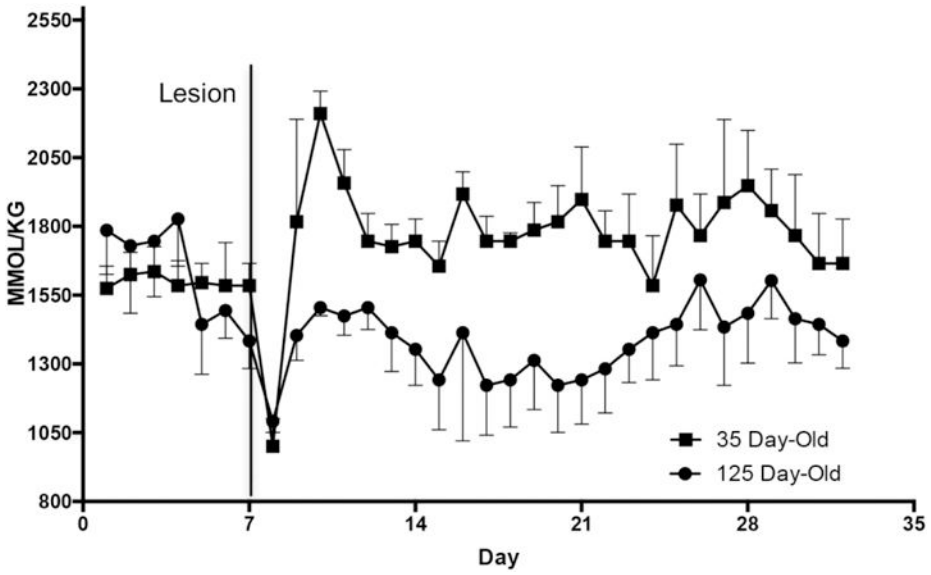

Figure 2.

Urine osmolarity remains reduced following unilateral lesion in the 125-day-old rat. Following unilateral lesion, urine osmolality in the 125-day-old rat was significantly decreased ($p<0.01$) compared to the response following unilateral lesion in the 35-day-old rat. The decrease is first observed two-days post-surgery and persisted throughout the entire three-week post-surgery period. The elevated urine osmolarity in the 35-day-old rat post-lesion is indicative of heightened neurosecretory activity of the magnocellular neurons.

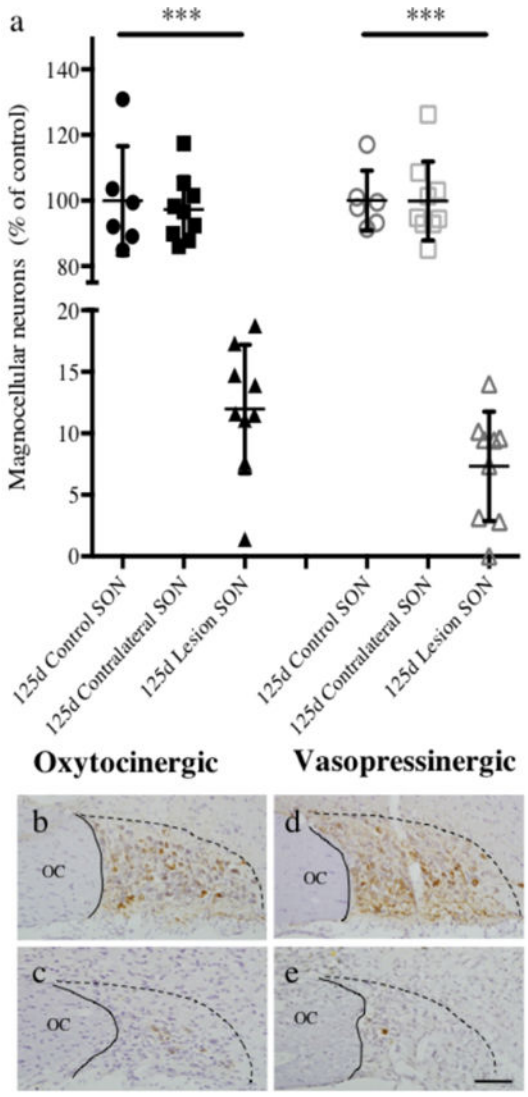

Figure 3.

Unilateral lesion results in less oxytocinergic and vasopressinergic immunoreactive profiles in the 125-day SON. Cell counts demonstrated no significant difference in the number of immunoreactive profiles indicative of oxytocinergic (*p=0.6941*) or vasopressinergic (*p=0.9851*) neurons in the SON contralateral to the unilateral hypothalamic lesion. However, at 10 dpl the numbers of immunoreactive profiles indicative of oxytocinergic and vasopressinergic neurons were reduced by 88% and 93%, respectively, in the lesion SON (a). Representative images demonstrate the decrease in oxytocinergic neurons between uninjured control (b) and lesioned SON (c) and the decrease in vasopressinergic neurons between uninjured control (d) and lesioned SON (e). The oxytocin- and vasopressin-immunoreactive profiles in b-e are in brown while the purple represents the cresyl violet-labeled nucleoli. Each shape on the graph indicates an individual data point with the lines representing the mean and SD. Each data point is comprised of a minimum of six sections sampled from each of [n] animals. The SON is outlined by the dashed line. OC = optic chiasm. Magnification bar = 100μm. ****p<0.0001.*

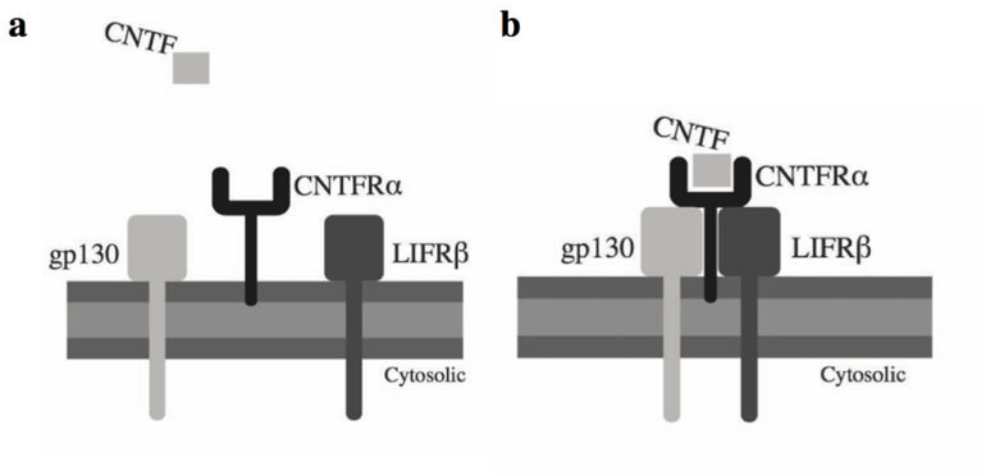

Figure 4.

Illustration of CNTF and CNTF receptor complex. CNTF requires three signaling molecules to transduce the signaling response intracellularly, CNTFRα, LIFRß, and gp130. CNTFRα is GPI-anchored to the extracellular membrane while LIFRß and gp130 are transmembrane receptors. The LIFRß and gp130 receptor molecules are not associated with CNTFRα until CNTF binds to CNTFRα (a). When CNTF binds CNTFRα, it causes a translocation of LIFRß and gp130 to interact with CNTFRα and LIFRß and gp130 are then able to transduce the signal intracellularly (b). Images created with BioRender.

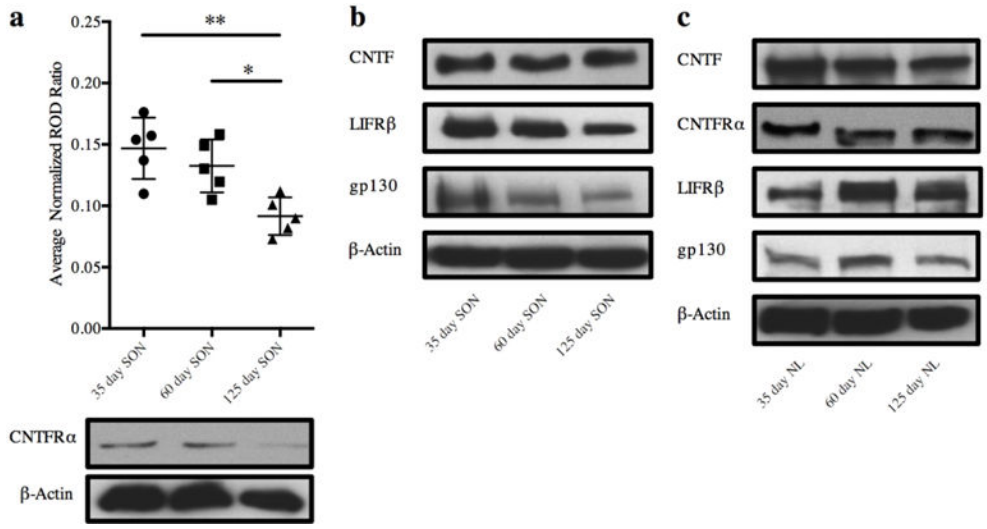

Figure 5.

CNTFRα protein levels decreased in the uninjured SON with age. Western blot analysis revealed a significant decrease in CNTFRα protein levels in the control SON from 35- to 125-days of age (a), but no change in CNTFRα protein levels in the maturing NL (c; *p=0.2978*). Analysis revealed no change in protein levels of CNTF in the maturing SON (b; *p=0.07*) or maturing NL (c; *p=0.16*), LIFRß in the maturing SON (b; *p=0.10*) or maturing NL (c; *p=0.27*), or gp130 in the maturing SON (b; *p=0.28*) or maturing NL (c; *p=0.15*). Each shape on the graph indicates an individual data point with the lines representing the mean and SD. Each data point represents isolated SON pooled from six rats with all experiments repeated in triplicate. *$p<0.05$, **$p<0.01$

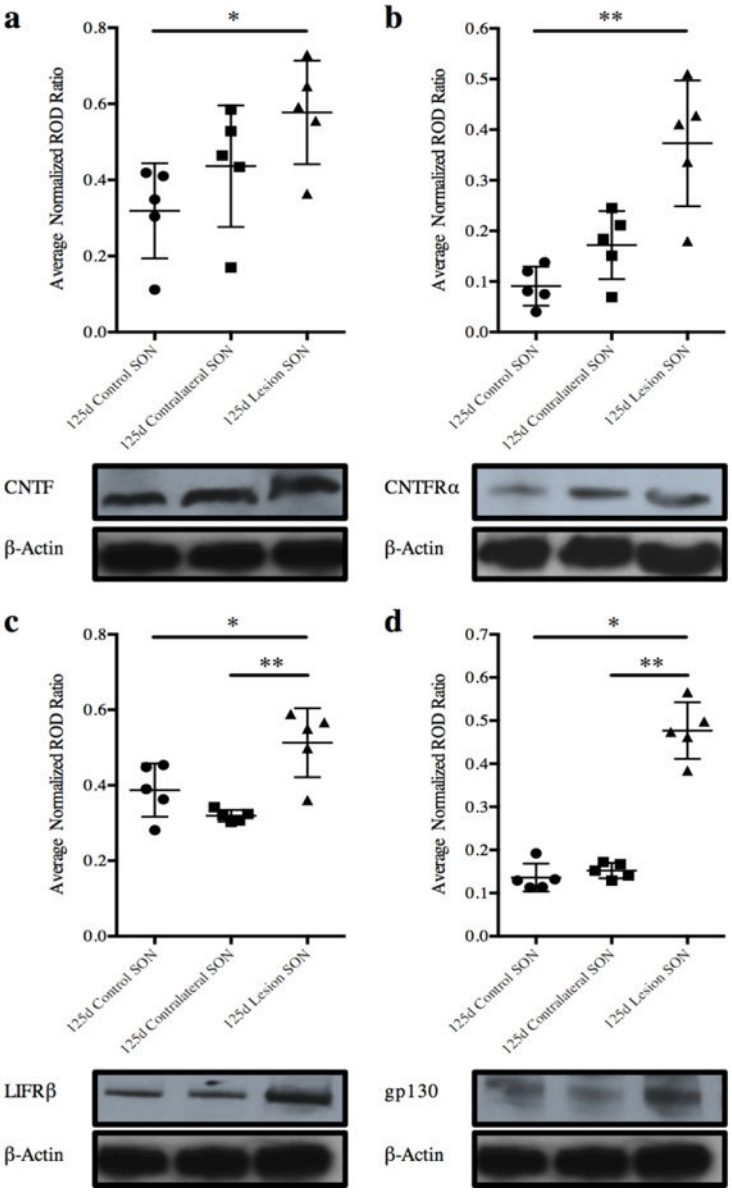

Figure 6.

Unilateral lesion results in increased levels of CNTF and CNTF receptor complex proteins in the 125-day-rat SON. Western blot analysis demonstrated a significant increase in CNTF (a; *p=0.014*), CNTFRα (b; *p=0.0013*), LIFRß (c; *p=0.04*), and gp130 (d; *p<0.0001*) protein levels in the injured (lesion) SON at 10 dpl. However, there was no significant difference in the protein levels in the SON contralateral to injury compared to age-matched, uninjured, control for CNTF (a; *p=0.23*), CNTFRα (b; *p=0.075*), LIFRß (c; *p=0.07*), and gp130 (d; *p=0.36*). Each shape on the graph indicates an individual data point with the lines representing the mean and SD. Each data point represents isolated SON pooled from six rats with all experiments repeated in triplicate. **p<0.05, **p<0.01, ***p<0.0001.*

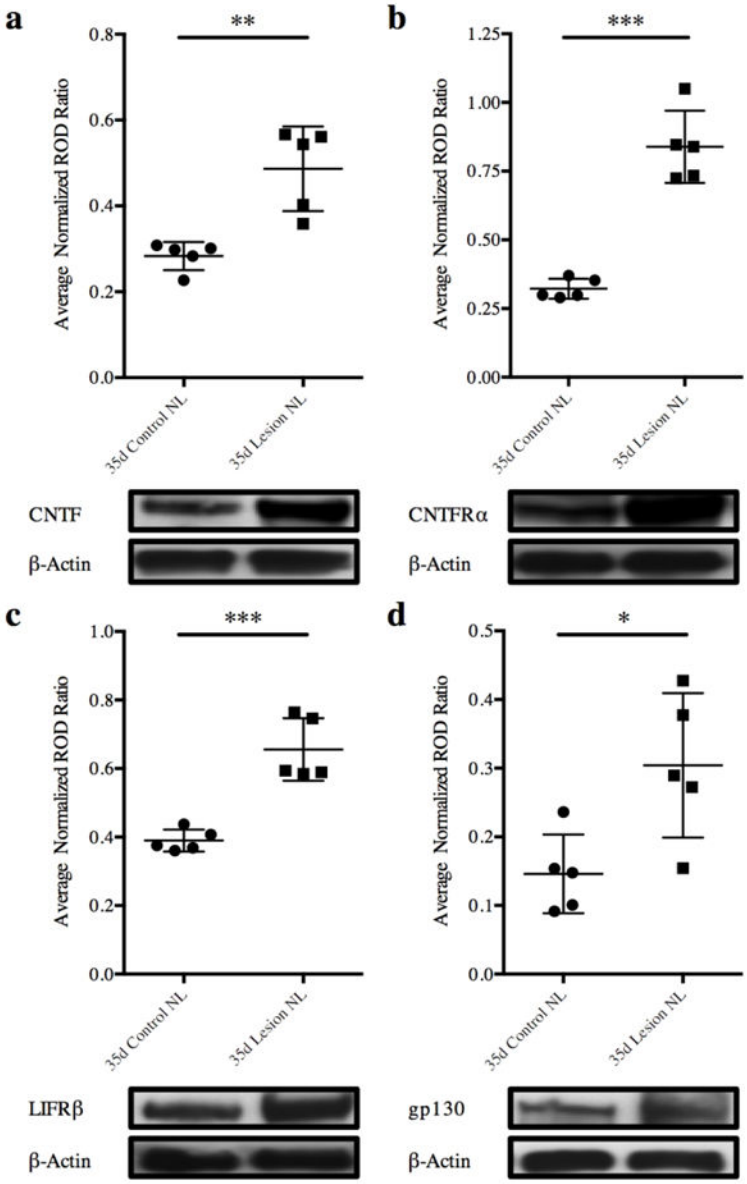

Figure 7.

Protein levels of CNTF and CNTF receptor complex increase in the 35-day rat NL following unilateral lesion. Western blot analysis demonstrated a significant increase in CNTF (a; *p=0.002*), CNTFRα (b; *p<0.0001*), LIFRß (c; *p=0.0003*), and gp130 (d; *p=0.018*) protein levels in the lesion NL compared to age-matched control. Each shape on the graph indicates an individual data point with the lines representing the mean and SD. Each data point represents isolated SON pooled from six rats with all experiments repeated in triplicate. *$p<0.05$, **$p<0.01$, ***$p<0.0001$.

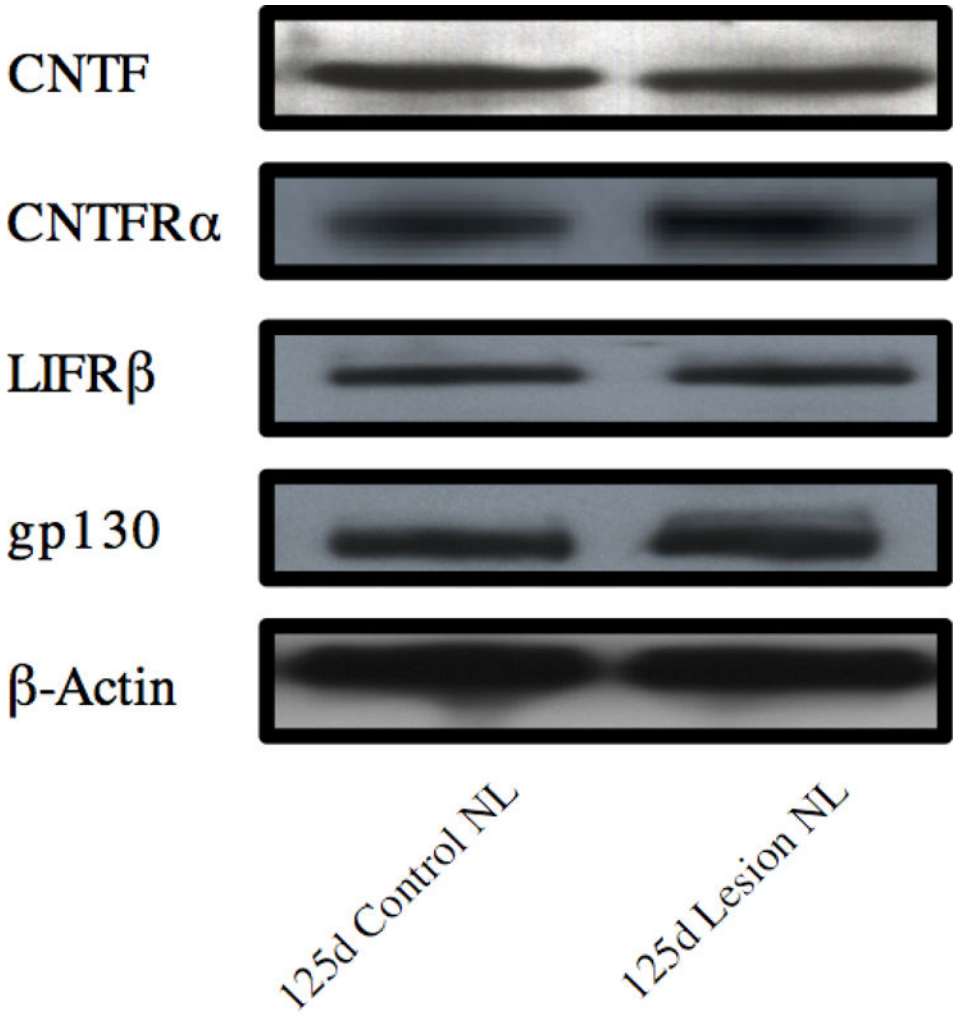

Figure 8.

Protein levels of CNTF and CNTF receptor complex did not change in the 125-day rat NL following unilateral lesion. Western blot analysis demonstrated no significant difference in CNTF *(p=0.68),* CNTFRα *(p=0.24),* LIFRß *(p=0.45),* and gp130 *(p=0.99)* protein levels in the 125 day lesion NL compared to age-matched control. Images are representative protein bands with all experiments repeated in triplicate.