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The effect of age and tongue exercise on BDNF and TrkB in the hypoglossal nucleus of rats

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1.1 Introduction

Aging is associated with muscle weakness and fatigue, a condition that has been termed sarcopenia [1–3]. The cause of sarcopenia is likely multifactorial and includes a variety of potential mechanisms including neuromuscular changes, decreased nutrition, hormonal changes, and inactivity [4–6]. Sarcopenia affects all elderly individuals to some extent because it is a consequence of normal aging [4]. In addition, sarcopenia has clinical relevance because the loss of muscle mass and strength in the aging musculature has functional consequences. Functional deficits have been well characterized in the limb musculature and include changes in balance and gait, increased risk of falls, and decreased independence [7, 8]. Recent data suggest that similar age-related changes occur in the cranial musculature involved in swallowing, and may be associated with the age-related changes seen in the swallow of elderly individuals [9–11]. Elderly individuals swallow more slowly and have increased residue in the pharyngeal cavity following the swallow, both of which may result from reduced strength in tongue and pharyngeal musculature [12, 13].

Due to the complex nature of sarcopenia, a variety of treatments have been suggested to decrease the functional consequences of this condition. Of the possible treatments proposed for limb musculature, resistance exercise appears to be the most promising [14–17]. In addition, resistance exercise training of the tongue musculature has been shown to have beneficial effects on tongue muscle strength and swallowing function in elderly individuals [18, 19]. Therefore, tongue exercise protocols are currently being used in clinical practice to strengthen the lingual musculature and to improve age-related declines in swallowing function [20, 21].

While evidence suggests that resistance exercise is a beneficial treatment for sarcopenia, the underlying mechanisms responsible for the increases in muscle strength and function associated with exercise are unknown. In addition to the changes in musculature with exercise, there is evidence to suggest that exercise has a neuroprotective component [22–24] mediated by neurotrophins in both the central and peripheral nervous systems [25]. Neurotrophins are a family of proteins that, when activated through binding with tropomysoin-related kinase (Trk) receptors, initiate signaling cascades that promote the development, survival, and function of neurons [26–28]. Given that the neurotrophin receptors TrkB and TrkC are decreased in the spinal motoneurons of aged rats [29], it appears that decreases in neurotrophin levels may also have a role in the limb deficits seen

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with aging. Results of previous studies in the brain and spinal cord support the hypothesis that neurotrophins act as a therapeutic agent in cases of neurodegenerative disease and nerve injury [30–32]. In addition, neurotrophins appear to be regulated in an exercise dependent manner. Vaynman and colleagues found that mRNA of brain-derived neurotrophic factor (BDNF) and its receptor TrkB was up regulated in the hippocampus of rats after 3 and 7 days of wheel running [33]. The same group also found that both mRNA and protein levels of BDNF were increased in the spinal cord following 5 days of wheel running exercise [34]. Other work has shown that BDNF is important for plasticity in respiratory regions of the spinal cord after intermittent hypoxia, which is used as a method of inducing long term facilitation in phrenic and hypoglossal motor outputs [35, 36]. Therefore, previous work has demonstrated a link between neurotrophins, aging, and activity-dependent neuroplasticity in the limb sensorimotor system.

No studies, however, have examined changes in neurotrophin levels in the cranial sensorimotor system with either age or exercise. Currently, our laboratory is using an animal model to study the underlying changes to the tongue musculature associated with progressive resistance exercise of the tongue [9, 10]. We have shown that tongue exercise induces changes in muscle fiber size and variability in the genioglossus muscle of aged rats that are associated with increased protrusive tongue forces. We found that there was a trend toward an increase in muscle fiber size with tongue exercise and a significant increase in muscle fiber size variability with tongue exercise [9]. In addition, we have shown that neuromuscular stimulation results in a reduction in age-related changes to the morphology of the neuromuscular junction in aged rats [10]. In this study we used our previously described animal model to examine changes in neurotrophin levels in the hypoglossal nucleus of rats with both age and exercise. We hypothesized that levels of BDNF and TrkB immunoreactivity would be decreased with age and increase with exercise. Therefore, the purpose of our study was to examine the levels of BDNF and TrkB in the hypoglossal nucleus of rats at different ages, in both control and exercise conditions, to determine the effect of age and exercise on BDNF and TrkB in the cranial nucleus that controls the tongue musculature involved in swallowing.

1.2 Methods

All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin. A total of 48 (16 young, 16 middle-aged, 16 old) male Fischer 344/Brown Norway (F344/BN) rats were obtained from the National Institute of Aging colony. At study completion, the animals were in one of three age groups: young (9–10 months), middle-aged (24–25 months), or old (32–33 months). The median life expectancy of F344/BN rats is 36 months [37]. Every effort was made to minimize the number of animals used and their suffering. Thus, tissues from these animals were assigned to more than one experiment [38]

1.2.1 Exercise

Animals were housed in pairs in standard polycarbonate cages on a 12:12 hour light-dark reversed light cycle. Rats were obtained 8 weeks prior to the start of the experiment to allow acclimation to the animal care facility, reversal of light cycle, water restriction, and familiarization to the tongue force operandum. Food was given ad libitum. Water was restricted to 3 hours per day to encourage the animals to press a disk for a water reward. Experimental methods for tongue press measurements in rats have been detailed previously [9, 39] but are discussed briefly below.

Throughout the experiment, animals were placed individually into a polycarbonate cage resembling the home cage, but equipped with a 1 × 1 centimeter (cm) aperture and force operandum that delivered aliquots of water based on tongue press behaviors.

After familiarization with the task, baseline tongue force measurements were obtained for the rats in the exercise group allowing for a measurement of baseline maximum force (g). Following baseline testing the animals in the exercise group underwent an 8-week training paradigm. Throughout the 8 weeks of training the force required for a water reward was increased to mimic a progressive resistance training program. For the first two weeks of training the animals were required to press at 50% of their estimated maximum press (EMP) force, which was established during baseline testing. During the second two weeks the force was increased to 60% of their EMP force, then 70%, and finally 80%. After the 8 weeks of training, post-exercise maximum force (g) values were obtained.

The control rats were placed on a water restriction protocol and light/dark cycle reversal identical to the exercise-treatment rats. However, they were not given access to the operandum enclosure and did not receive any exercise treatment. Instead, they were placed in an enclosure that resembled the operandum enclosure and were permitted to drink water ad libitum from a water dish for 3 hours.

1.2.2 Perfusion

Rats were anesthetized with isoflurane followed by sodium pentobarbital (120 mg/kg i.p.). Anesthetized rats were transcardially perfused with 200ml of heparinized saline (10,000 units/liter) followed by 400 mL of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (PB) (pH 7.4). Brains were removed and postfixed for 1 hour at 4°C, then cryoprotected for 24–36 h at 4°C with 20% sucrose and 5% glycerol in 0.1M phosphate buffer. Sections were cut coronally (40μm) and stored in 0.1 M phosphate buffer containing 0.02% sodium azide at 4° C.

1.2.3 Immunocytochemistry

Two sections through the hypoglossal nucleus from each animal were immunochemically reacted for the presence of BDNF. A separate pair of adjacent sections were reacted for the presence of TrkB. Specifically, sections were selected from the junction between middle-caudal (to be known as caudal sections) and middle-rostral (to be known as rostral sections) of the hypoglossal nucleus in the medulla. A dilution series was conducted to identify the optimal dilution for each antibody. Sections were washed in Tris-buffered saline (TBS), then in 0.1% Triton X-100 in TBS (TBST). After 2 h in blocking solution (5% normal donkey serum in TBST), primary antibodies were applied for 24 h at room temperature in blocking solution. Primary antibodies were used at 1:50: anti-BDNF (Santa Cruz, sc-546, Santa Cruz, CA), and 1:200 anti-TrkB (Santa Cruz, sc-8316, Santa Cruz, CA). An Alexa-Fluor conjugated secondary antibody (594 donkey anti-rabbit IgG. Invitrogen, Eugene, OR) was used for both BDNF and TrkB staining at 1:500 in 5% normal donkey serum in TBST. All sections were reacted at the same time. Negative controls were reacted simultaneously with the omission of either the primary or secondary antibody. Sections were mounted and coverslipped with Vectashield Hard Set mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). There were no labeled cells in negative control sections from all behavioral states.

1.2.4 Analysis

All images were acquired during the same session using SPOT (Advanced version) computer software and SPOT RT Slider camera (Diagnostic Instruments, Sterling Heights, MI, USA) attached to a Nikon Eclipse E600 microscope. In each section, one image was

taken from each side of the ventral middle hypoglossal nucleus (Fig. 1A) for both BDNF and TrkB immunoreactivity using the 40X objective, resulting in 8 images from each animal (4 of BDNF, 4 of TrkB). Images were analyzed using ImageJ [40]. To separate signal from background an Otsu thresholding algorithm was applied to each image. The average fluorescent intensity of each image, captured in relative fluorescent units (RFU), was then measured and used for statistical analysis.

We used an analysis of variance (ANOVA) to compare tongue forces between age groups. The pre-to-post exercise change in force was assessed within each age group using a paired t-test. The impact of age, exercise, and region (caudal vs. rostral) on average fluorescent intensity within the specified area of the hypoglossal nucleus was examined using a mixed model analysis for both BDNF and TrkB independently. Age, exercise, and region were included as fixed variables, and the rat itself was included as a random variable to account for the multiple measures taken from each animal. Post-hoc testing was completed on all significant interactions found during the mixed model analysis using a Fisher's LSD analysis to examine individual group differences. In addition, Spearman Correlation was used to examine the relationship between BDNF and TrkB immunoreactivity in the different age, exercise, and region groups. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC). P-values less than 0.05 were considered as significant.

1.3 Results

1.3.1 Force

Tongue exercise was associated with increased maximum tongue force (g) at all ages compared with baseline values (Fig. 2; $F_{2, 21}$=4.23, p=.03.) Posthoc testing revealed that middle aged and old rats had significantly greater gains in maximum tongue forces than young rats ($p < .05$). No difference in force gains was found between middle aged and old rats.

1.3.2 BDNF Immunoreactivity

BDNF immunoreactivity was found in both the rostral and caudal regions of the hypoglossal nucleus in all age groups. BDNF staining was diffuse throughout the ventral medial portion of the hypoglossal nucleus, with the areas of high intensity staining concentrated in cell bodies (arrows in Fig.1B).

Significant regional differences in staining intensity were found ($F_{(2, 42)} = 9.71$, $p = 0003$). Specifically, BDNF immunoreactivity was significantly higher in the caudal region than in the rostral region in both young exercised animals and in middle-aged control animals (Fig. 3A; $p = 0.003$, $p = 0.002$), respectively.

1.3.3 Age and Exercise-associated changes in BDNF Immunoreactivity

BDNF levels in the ventral medial portion of the hypoglossal nucleus were not affected by age. BDNF immunoreactivity was similar in all age groups in both the caudal and rostral regions of the hypoglossal nucleus (Fig.3B). BDNF levels were, however, affected by exercise ($F_{(2, 42)} = 9.71$, $p = .0003$), but this effect was only seen in the young rat group in the caudal portion of the hypoglossal nucleus (Fig.3C). Specifically, a significant increase was found in BDNF immunoreactivity with exercise in young animals in the caudal region ($p = 0.03$). However, no differences were found in any other age group or region.

1.3.4 TrkB Immunoreactivity

TrkB immunoreactivity was present in both the rostral and caudal regions of the hypoglossal nucleus in all age groups. In contrast to BDNF, TrkB staining was punctate and appeared to stain axons (double arrow heads in Fig.1C) and synaptic vesicles (arrows in Fig.1C).

Significant regional differences in staining intensity were detected and were more widespread for TrkB than BDNF. Regional differences in TrkB immunoreactivity were present across conditions ($F_{(1,38)} = 5.32$, $p = 0.03$) and age groups ($F_{(2,38)} = 5.11$, $p = 0.01$). Specifically, TrkB immunoreactivity was greater in the caudal region than the rostral region for both the exercise group and the control group (Fig.4A; $p < 0.0001$, $p = 0.0096$) and in each of the three age groups (Fig.4B; $p = <0.0001$, $p = 0.01$, $p = 0.05$).

1.3.5 Age and Exercise-associated changes in TrkB Immunoreactivity

TrkB levels in the hypoglossal nucleus were affected by age ($F_{(2,38)} = 5.11$, $p = 0.01$). Post-hoc testing revealed a significant decrease in TrkB immunoreactivity with age in both the caudal and rostral regions of the hypoglossal nucleus (Fig.4C). In the caudal region, there was a significant decrease in TrkB in old animals compared with young animals ($p = 0.0001$), and in old animals compared with middle-age animals ($p = 0.02$). No difference was found between middle-age animals and young animals. In the rostral region, there was a significant decrease in TrkB in the old animals compared with young animals ($p = 0.02$) and in old animals compared with middle-age animals ($p = 0.03$). TrkB levels in the hypoglossal nucleus were not affected by exercise at any age.

1.3.6 Relationship between BDNF and TrkB Immunoreactivity

Both BDNF and TrkB must be present to exert the downstream signaling effects that cause synaptic changes in the cell [28, 41, 42]. Thus, we examined the relationship between BDNF and TrkB immunoreactivity within age and exercise conditions. Spearman correlation analysis revealed that BDNF and TrkB immunoreactivity were positively correlated following exercise in both the caudal and rostral regions in young and middle-age animals (Fig.5; young caudal: rho = 0.59, $p = 0.03$; young rostral: rho = 0.62, $p = 0.02$; middle-age caudal rho = 0.58, $p = 0.02$; middle-age rostral rho = 0.52, $p = 0.04$). Negative or weak correlations were found in control animals at all ages. In addition, BDNF was negative or weakly correlated with TrkB immunoreactivity in old animals in both the exercise and control groups (Fig. 5).

1.4 Discussion

The hypothesis guiding this study was that the neurotrophin BDNF and its receptor TrkB would be decreased in the hypoglossal nucleus of rats in an age-dependent manner and would increase following a tongue exercise regime of eight weeks. We found that TrkB immunoreactivity was decreased with age in both the caudal and rostral regions of the hypoglossal nucleus and that BDNF was increased with exercise in caudal region of the hypoglossal nucleus in young rats. However, BDNF did not decrease with age, and exercise-induced changes in BDNF and TrkB were not found in the middle aged and old rats despite significant increases in tongue force.

Our results are similar to previous work that reported age-related reductions in TrkB in spinal cord motor and sensory neurons [29, 43, 44]. BDNF sparing in the presence of TrkB reductions with aging has also been found in the pituitary [45]. Because both the neurotrophin and its receptor must be present to allow downstream signaling cascades leading to synaptic changes within the neuron [28, 41, 42], age related changes to TrkB receptors alone may be sufficient to affect synaptic function. Therefore, reduced TrkB

immunoreactivity with age in the hypoglossal nucleus suggests that there are age-related changes to the neurotrophin system that may interfere with normal synaptic function. It may not be necessary for both the neurotrophin and its receptor to manifest reductions with age for the entire system to be affected.

TrkB functions as a high affinity receptor for neurotrophins other than BDNF [28, 42, 46]. Specifically, TrkB is also a receptor for muscle-derived NT4/5 neurotrophin [41]. Accordingly, the lack of age-related decline in BDNF in this study may suggest that another neurotrophin, for instance NT4/5, may be reduced with age. In this study, only BDNF immunoreactivity was measured. Given that NT4/5 has been shown to be important for neuromuscular plasticity in the uninjured animal throughout the lifespan [29, 47], NT4/5 may serve as an important neurotrophin to focus on in future studies.

Our results indicated that BDNF immunoreactivity was increased with tongue exercise in the caudal region of young animals, and represents the first indicator that targeted tongue training can result in increased neurotrophin levels in the cranial sensorimotor system. Previous work in the limb sensorimotor system has shown that direct administration of BDNF into the spinal cord can promote growth and survival of damaged neurons [36, 48] and can lead to improved recovery after spinal cord injury [49]. In addition, BDNF and NT4/5 have been shown to improve synaptic transmission at the neuromusclular junction by binding with the TrkB receptors to increase release of synaptic vesicles [50]. Our results show that targeted tongue exercise may be a less invasive therapeutic method of increasing BDNF levels in the cranial motor system and further support the neuroprotective effects of exercise.

Additional support for the neuroprotective effects of exercise is provided by the results of our correlative data, in which we found a moderate positive correlation between TrkB and BDNF immunoreactivity in both sections of the hypoglossal nucleus in young and middle-age animals following exercise: the immunoreactivity levels of both BDNF and TrkB in young and middle-age animals increased following exercise. However, these increases were not large enough to lead to statistically significant changes, except in the caudal section of young animals. As stated previously, both the neurotrophin and receptor need to be present to initiate the down-stream signaling cascades that result in improved synaptic transmission [41]. Therefore exercise may serve as a method to regulate the levels of both BDNF and its receptor to promote more effective ligand to receptor binding. However, this does not appear to be the case in the old animals, as TrkB and BDNF immunoreactivity were weakly correlated in both the control and exercise conditions in old animals.

In a recent systematic review of the literature examining the effect of exercise on peripheral (serum or plasma) levels of BDNF in human subjects, it was shown that increases in BDNF levels following exercise were dependent on the type of exercise performed and appeared to be somewhat transient in nature [51]. In our study only one type of exercise (strength training) was evaluated, and there was a time delay between the end of our exercise protocol and our data collection. Therefore the lack of significant increases in BDNF and TrkB immunoreactivity observed in our study in middle age and old animals may be due to these factors. Future studies should examine the effect of different forms of exercise on levels of BDNF and TrkB in the cranial sensorimotor system, such as acute exercise vs. prolonged training, and should also measure neurotrophin levels immediately following the end of the exercise protocol in an attempt to capture more transient increases in neurotrophins and receptors following exercise.

Tongue forces increased following exercise in all age groups. Despite an increase in BDNF expression in young rats following exercise, there was not a concomitant increase in BDNF

immunoreactivity in the middle aged and old rats. It is possible that different mechanisms and or neurotrophins are involved in the functional changes seen in older animals. For example, Ying and colleagues found increases in NT-3 mRNA and protein in the lumbar spinal cord after 7 days of treadmill running, and increases in TrkC mRNA in both lumbar spinal cord and soleus muscle after exercise. However, increases in NT-3 mRNA and not in protein were observed in the soleus muscle [52]. Similarly, Gomez-Pinilla and colleagues found that exercise has a differential effect on BDNF and NT-3 in both the spinal cord and muscle [34]. Based on the results of these studies it will be necessary to look at changes in mRNA and protein levels of several different neurotrophins and their receptors in both the hypoglossal nucleus and the tongue musculature before we can rule out the possibility that neurotrophins play a role in the increased tongue forces seen in old animals.

1.5 Conclusion

This study is the first to examine age and exercise-induced effects on neurotrophins in the cranial sensorimotor system. Our results show that the cranial system undergoes age related neurotrophic changes, specifically manifested as a decrease in TrkB receptors in the hypoglossal nucleus with age. In addition, our results show that exercise results in an increase in BDNF immunoreactivity in the hypoglossal nucleus of young animals. However the role of exercise induced neurotrophic up-regulation in older animals requires further study. Based on the results of this initial study it appears necessary to continue to explore age and exercise induced changes in neurotrophic factors in the tongue musculature and hypoglossal nucleus to further elucidate the neuroprotective role of exercise as it relates to disorders of the cranial sensorimotor system.

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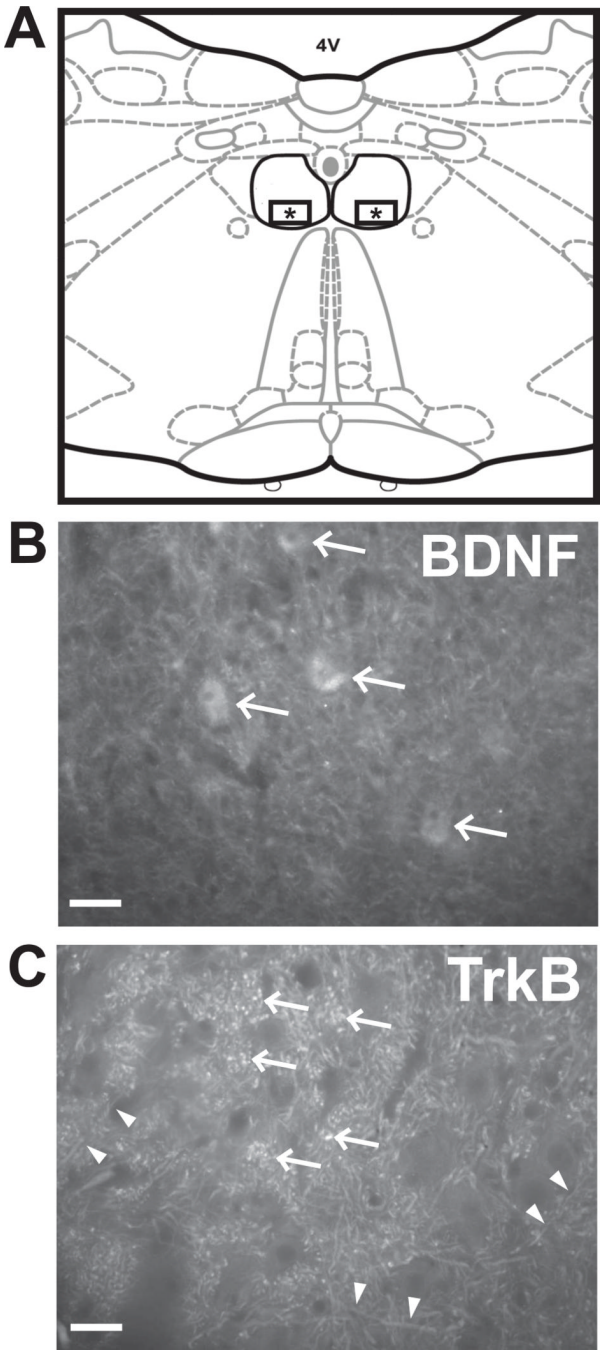

Fig 1.

(A) Diagram of a representative coronal section through the caudal medulla showing the location of images used for measurement of fluorescent signal for BDNF and TrkB. The hypoglossal nucleus is outlined in black. The squares containing asterisks within the ventral half of the nucleus depict the areas imaged. (B) Representative image of BDNF immunoreactivity showing diffuse staining throughout the nucleus. Staining was more intense in cell bodies (arrows). (C) Representative image of TrkB immunoreactivity showing punctuate staining of axons (double arrow heads) and synaptic boutons (arrows). Scale bar = 50 μm.

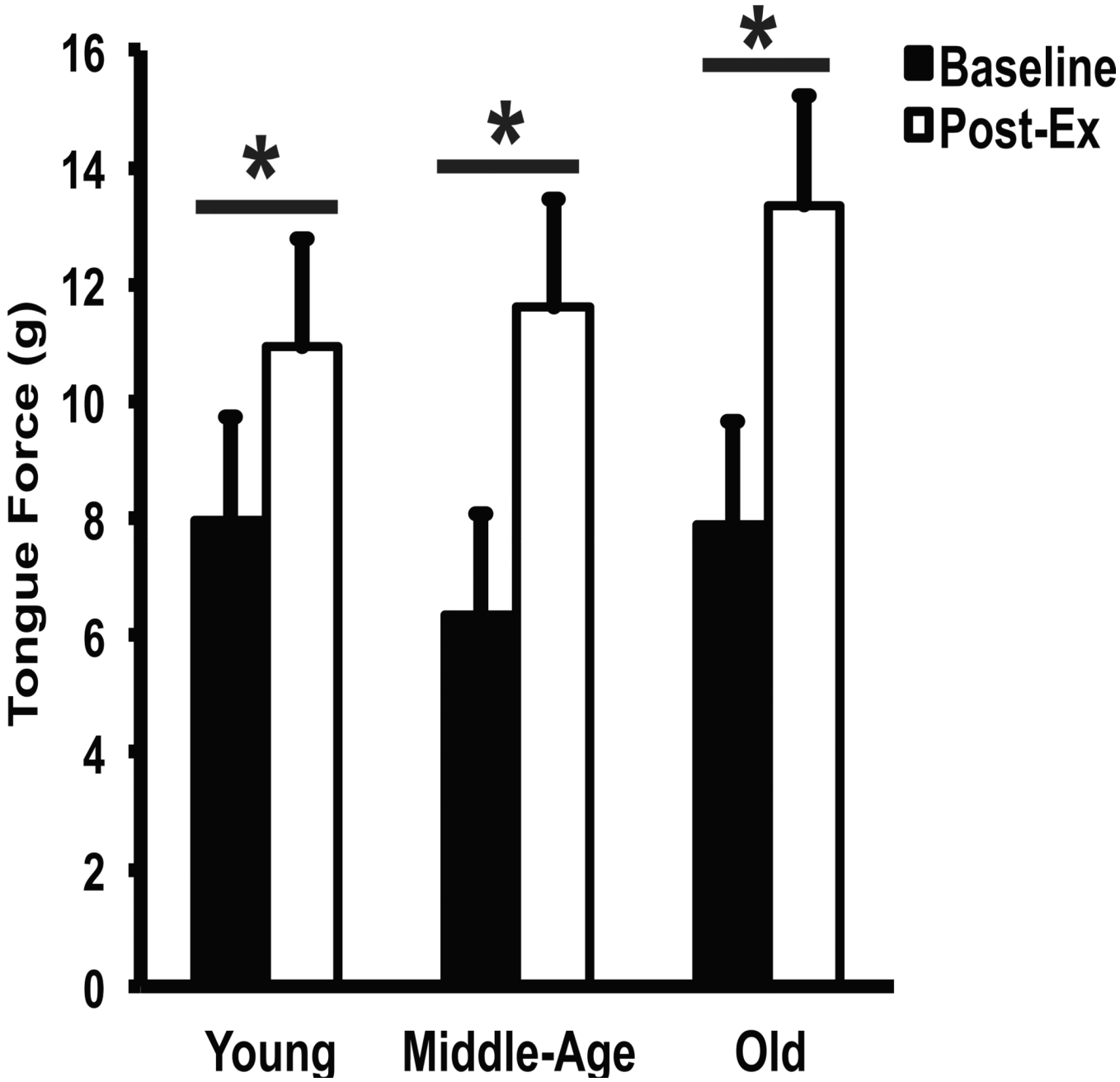

Fig 2.

Effect of tongue exercise on behavioral tongue forces at different ages. Age groups (young, middle-age, and old) are represented along the horizontal axis and tongue force (in grams) is represented along the vertical axis. Black bars represent baseline tongue forces and white bars represent tongue forces following 8-weeks of tongue exercise. A significant increase ($p < 0.05$) in tongue force was seen in all age groups. * denotes significant values; error bars represent standard error of the mean.

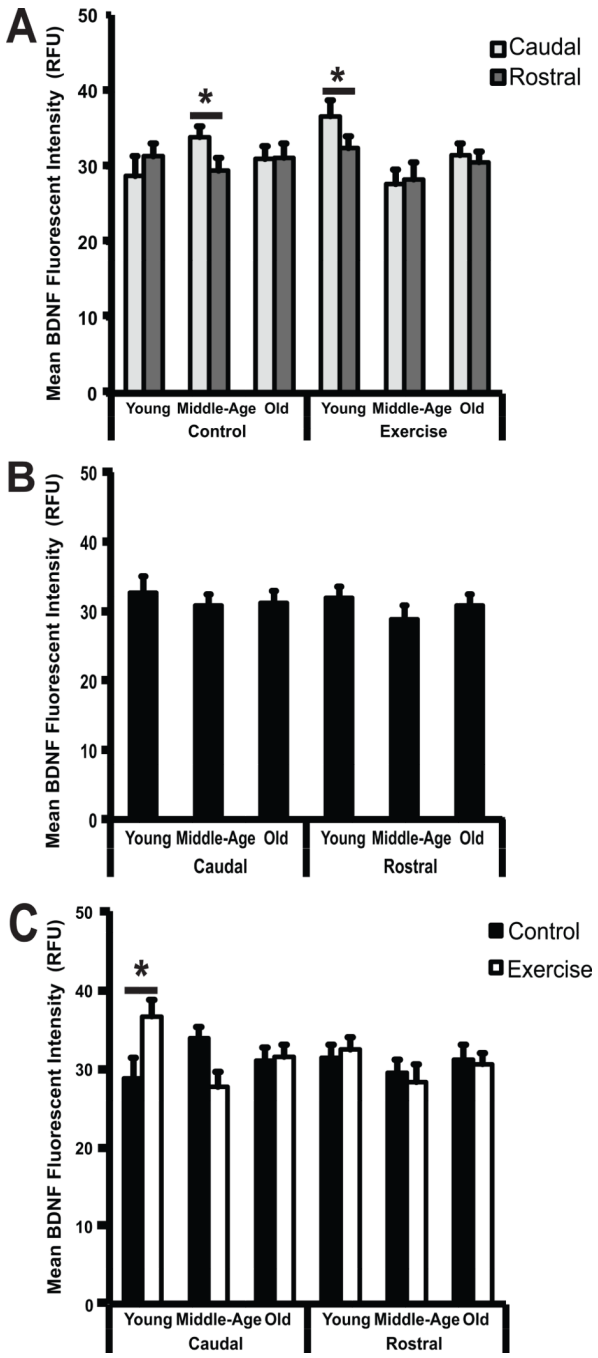

Fig 3.

(A–C) Changes in BDNF immunoreactivity by region (A), age (B), and exercise (C). (A) Mean BDNF fluorescent intensity, expressed in relative fluorescent units (RFU), is represented along the vertical axis. Age groups (young, middle-age, and old), divided into control and exercise conditions, are represented along the horizontal axis. BDNF immunoreactivity in the caudal portion of the hypoglossal nucleus is shown in light gray and BDNF immunoreactivity in the rostral portion of the hypoglossal nucleus is shown in dark gray. A significant increase ($p < 0.05$) in BDNF immunoreactivity was seen in the caudal portion of the hypoglossal nucleus compared to the rostral portion of the hypoglossal nucleus in middle-aged animals in the control group, and in young animals in the exercise

group. (B) Mean BDNF fluorescent intensity, expressed relative fluorescent units (RFU) is represented along the vertical axis. Age groups (young, middle-age, and old), divided into control and exercise conditions, are represented along the horizontal axis. No significant differences ($p < 0.05$) in BDNF immunoreactivity were seen across age groups in either the caudal or rostral regions of the hypoglossal nucleus (C) Mean BDNF fluorescent intensity, expressed in relative fluorescent units (RFU) is represented along the vertical axis. Age groups (young, middle-age, and old), divided into control and exercise conditions, are represented along the horizontal axis. A significant increase ($p < 0.05$) in BDNF immunoreactivity was seen in the young age group in the caudal portion of the hypoglossal nucleus with exercise. * denotes significant values; error bars represent standard error of the mean.

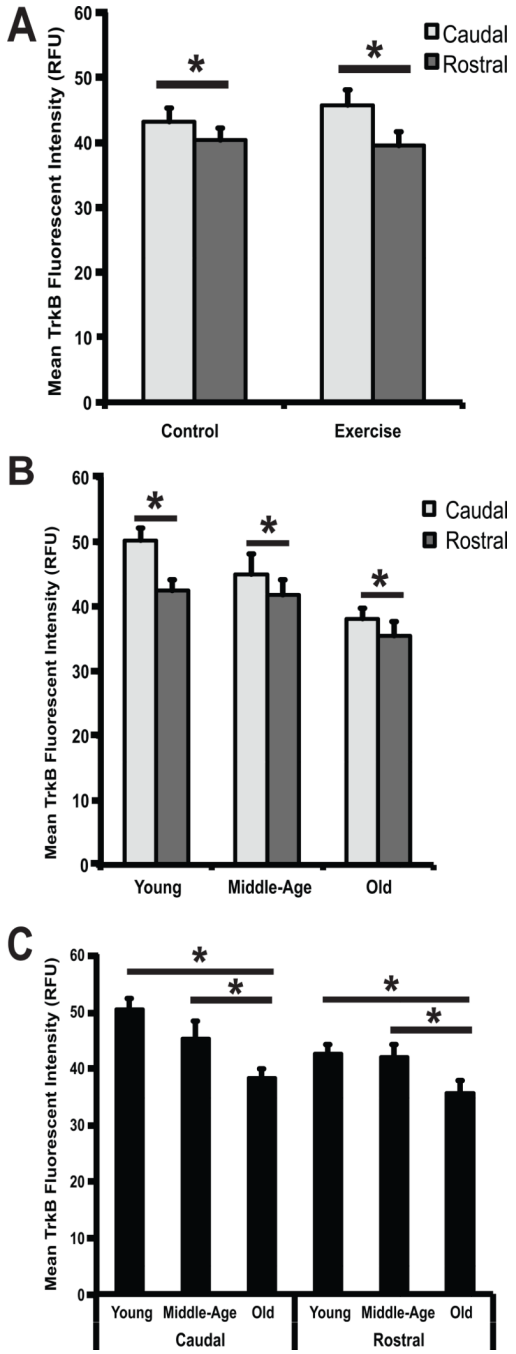

Fig 4.

(A–C) Changes in TrkB immunoreactivity by region (A), region and age (B) and age (C). (A) Mean TrkB fluorescent intensity, expressed in relative fluorescent units (RFU), is represented along the vertical axis. Control and exercise conditions are represented along the horizontal axis. TrkB immunoreactivity in the caudal portion of the hypoglossal nucleus is shown in light gray and TrkB immunoreactivity in the rostral portion of the hypoglossal nucleus is shown in dark gray. Significantly greater TrkB immunoreactivity ($p < 0.05$) was seen in the caudal portion of the hypoglossal nucleus compared to the rostral portion of the hypoglossal nucleus in both the control and exercise conditions. (B) Mean TrkB fluorescent intensity, expressed in relative fluorescent units (RFU) is represented along the vertical axis.

Age groups (young, middle-age, and old) are represented along the horizontal axis. TrkB immunoreactivity in the caudal portion of the hypoglossal nucleus is shown in light gray and TrkB immunoreactivity in the rostral portion of the hypoglossal nucleus is shown in dark gray. Significantly greater TrkB immunoreactivity ($p < 0.05$) was seen in the caudal portion of the hypoglossal nucleus compared to the rostral portion of the hypoglossal nucleus in all age groups. (C) Mean TrkB fluorescent intensity, expressed in relative florescent units (RFU), is represented along the vertical axis. Age groups (young, middle-age, and old), divided into caudal and rostral regions, are represented along the horizontal axis. A significant decrease ($p < 0.05$) in TrkB immunoreactivity was seen in the old age group compared to the young and middle-age age groups in both the caudal and rostral portions of the hypoglossal nucleus. * denotes significant values; error bars represent standard error of the mean.

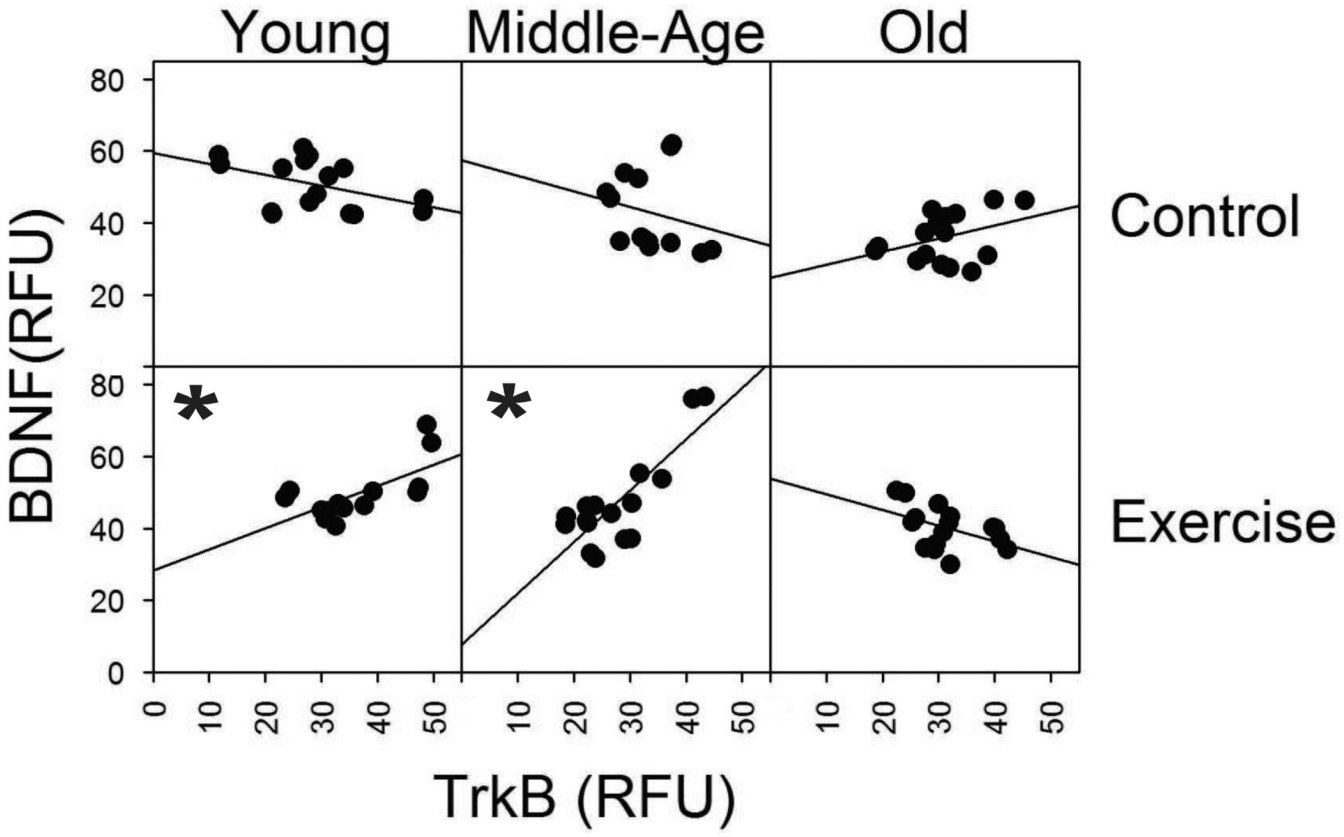

Fig 5.

Correlation between BDNF and TrkB immunoreactivity with exercise in young and in age groups. Each square represents a different age group and condition. The control condition is represented in the first row and the exercise condition represented in the second row. Age groups (young, middle-age, and old) are represented across the three columns, respectively. Within each square, mean TrkB intensity expressed in relative fluorescent units (RFU), is represented along the horizontal axis and mean BDNF intensity along the vertical axis. Each data point represents one animal. With both caudal and rostral data combined for each age group and condition. The slope of the line indicates the correlation between BDNF and TrkB immunoreactivity. A significant positive correlation ($p < 0.05$) between TrkB and BDNF was seen in the young and middle-age groups with exercise. * denotes significant values.