The Neuroplastic Potential of Exercise in the Cranial Sensorimotor System

By

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Abstract

Exercise-based therapies are currently used to treat voice and swallowing disorders without a clear understanding of the mechanisms that alter the cranial neuromuscular system. The recent application of principles of neuroplasticity to rehabilitation has revolutionized how we think about treatment, highlighting the need for change in both behavior and neural substrates to create lasting benefits. It is difficult, however, to study neural substrates in human patients while controlling for factors that may influence plasticity such as genetic and environmental differences. The use of a rat model allows these controls. This dissertation research aims to further our understanding of the neuroplastic potential of exercise in the cranial sensorimotor system with the ultimate long-term and future goal of guiding care of individuals with voice and swallowing problems.

Neurotrophins are mediators of neuroplasticity. Results from Study 1 show age-related decreases in neurotrophins in the cranial sensorimotor system and increases in tongue force in all age groups following 8 weeks of tongue exercise. Study 1 also found neurotrophin up-regulation in the hypoglossal nucleus in young adult groups, but not in old groups. Results from Study 2 again show age-related decreases in neurotrophins. However, with the inclusion of a no-exercise control group and detraining groups, Study 2 found that tongue exercise paired with a water swallow resulted in increased behavioral tongue force, regardless of age. Study 2 also found that BDNF concentration in the hypoglossal nucleus is increased in old exercise groups compared to old control and 2- and 4-week detraining groups.

This work is significant because it examines the neuroplastic potential of exercise in the cranial sensorimotor system in both muscle and the central nervous system, along with the enduring effects of exercise (detraining) with the long term and future goal of using our results to guide current therapy timelines and protocols used in clinical populations with voice and swallowing problems.

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I. Review of the Literature

1.1 Introduction

Voice and swallowing disorders are prevalent in elderly people and result in significant quality of life impairments and increased health care costs (Chen et al., 2009; Cohen et al., 2012a; Cohen et al., 2012b; Golub et al., 2006; Irles Rocamora et al., 2009; Kocdor et al., 2015; Lindgren and Janzon, 1991; Marik and Kaplan, 2003; Merrill et al., 2011; Ney et al., 2009; Niederman et al., 1998; Tian et al., 2013; Tibbling and Gustafsson, 1991; Turley and Cohen, 2009; Verdonck-de Leeuw and Mahieu, 2004). A number of neurodegenerative changes occur in the cranial sensorimotor system with aging and have direct effects on voice and swallowing function (Davids et al., 2012; Logemann et al., 2000; Robbins et al., 1995).

In this review of literature, neurodegenerative changes are discussed at multiple levels within the cranial sensorimotor system including muscles involved in voice and swallowing, motor neurons and nerves innervating specific muscles, and the connection between nerves and muscles, the neuromuscular junction. When degenerative changes across multiple levels are considered, a complex etiologic picture is formed to explain age-related functional changes in voice and swallow (For review, see Jaradeh, 1994; Kays and Robbins, 2006; Schindler and Kelly, 2002). That is, etiologies can include, motor impairments alone manifested as changes in strength resulting in reduced bolus propulsion and oral or pharyngeal stasis, or sensorimotor impairments related to deficits in timing, coordination, and reduced sensation, resulting in delayed triggering of the swallow, penetration, and in some cases aspiration.

1.2 Age-related changes in voice and swallowing

Older individuals, even those without dysphagia, swallow more slowly overall with increased oral and pharyngeal transit times and decreased hyolaryngeal movement (Cook et al., 1994; Robbins et al., 1992; Sonies et al., 1988; Tracy et al., 1989). As a result of the global slowing of the swallow, older individuals are at risk for more dangerous complications, like aspiration pneumonia, because of the

increased time in which the open airway is exposed to the bolus during the swallow (Nicosia et al., 2000). While aspiration pneumonia is not commonly seen in healthy older adults, secondary factors, such as a disease or injury that further tax the system are likely to result in dysphagia that causes serious health complications (Jaradeh, 1994).

There are also global changes to the voice in many elderly people. The term "presbyphonia" describes the age-related change in vocal function in elderly people, and is characterized by breathiness (Linville, 2002), reduced intensity, reduced phonation duration, and "rough" vocal quality, mostly likely due to asymmetric vocal fold vibration (For review, see Baken, 2005; Kendall, 2007; Pontes et al., 2005). The age-related changes described above result in physical and emotional difficulties and reduce the quality of life of elderly individuals (Costa and Matias, 2005; Golub et al., 2006; Roy et al., 2007; Verdonck-de Leeuw and Mahieu, 2004).

The current state of knowledge concerning changes in cranial muscles, motor neurons, nerves, and the neuromuscular junction related to voice and swallow function in elderly people is reviewed in the following sections. Many of the same cranial muscles mediate voice, swallowing, and respiratory actions (McCulloch et al., 1996; Palmer et al., 2008; Perlman et al., 1999) and receive input from similar neural control elements (McFarland and Tremblay, 2006; McFarland and Paydarfar, 2011; Nishio and Niimi, 2004). The focus of this dissertation is on the tongue and larynx because muscles within these structures mediate both voice and swallowing actions and are common therapeutic targets for age-related decrements in these critical cranial functions (Hewitt et al., 2008; Logemann et al., 1992; McCulloch et al., 1996; Perlman et al., 1999; Pressman, 1941; Robbins et al., 2005; Steele, 2012; Stuart, 1891; Van Daele et al., 2005; Yeates et al., 2008).

1.2.1 Altered cranial muscle structure and function with aging

It is well documented that there are age-related reductions in muscle mass in limb skeletal muscles in humans, specifically in type II muscle fibers, and that the reductions in mass are linked with a

reduction in strength, a condition known as sarcopenia (Doherty, 2003; Rosenberg, 1997). Specific changes to the human tongue musculature with age are also documented (For review, see Logemann, 1990). These findings coincide with a putative reduction in tongue muscle mass with age and an increase in fat and additional connective tissue (For review, see Miller et al., 2002). Interestingly, some studies deny age-related atrophy of the tongue, claiming that MRI studies provide no evidence that atrophy of the tongue is based on age alone when looking at measures of volume, perimeter, and area (Mahne et al., 2007; Ohnishi et al., 1992). However, these studies did not consider differences in muscle mass versus fat and connective tissue, and instead looked only at overall measures of tongue size. Considering the amount of adipose tissue in the tongue is particularly important, because the human tongue is found to increase its percentage of adipose tissue by 2.7% each decade (Rother et al., 2002). Therefore, studies that do not take the percentage of fat and connective tissue into account may skew their size measurements because the tongue may stay the same size or grow in size overall with age, but the actual percentage of muscle mass has been shown to decrease (Rother et al., 2002). These reductions in muscle mass coincide with reports of decreased tongue strength. For example, reductions in maximum isometric tongue pressures have been observed in elderly individuals (Nicosia et al., 2000; Robbins et al., 1995; Youmans et al., 2009). A recent study provided correlative evidence for the relationship of tongue strength, self-reported swallowing function, nutritional and anthropometric parameters, and sarcopenia in an elderly group of individuals (Maeda and Akagi, 2015). Results showed that maximum tongue pressure was significantly associated with age, albumin concentration, and presence of sarcopenia. In addition, the presence of dysphagia was significantly associated with a diagnosis of sarcopenia (Maeda and Akagi, 2015). Accordingly, reductions in tongue muscle mass and strength may contribute to the swallowing deficits observed in elderly people.

Animal studies examining the tongue musculature also report reductions in tongue strength with age. For example, research using a rat model has shown that there are reductions in protrusive tongue force with age (Nagai et al., 2008; Schaser et al., 2012). In addition, timing differences have been found

in the contractile properties of the aged rat tongue (Becker et al., 2015; Ota et al., 2005). Interestingly, older animals were found to have comparable maximum tongue forces to young animals following stimulation of the whole hypoglossal nerve (Ota et al., 2005), as well as during stimulation of the retrusive tongue muscles (Becker et al., 2015). However, older rats required increased contraction times to achieve maximum tongue forces (Ota et al., 2005). Accordingly, studies involving animal models of age-related tongue force and timing measures found deficits in tongue strength and timing consistent with data from human subjects. As a result, these studies indicate that a rat model is appropriate for future studies, such as this dissertation, attempting to determine the underlying cause of functional age-related swallowing difficulties.

Reductions in muscle mass are also seen in the human larynx with age. For example, 25% of adults with voice complaints over the age of 65 were found to have vocal fold atrophy in a retrospective chart review (Davids et al., 2012). In addition, qualitative changes in laryngeal postures were identified within the senescent larynx using laryngeal imaging, with an increased percentage of vocal fold bowing and vocal process prominence found in geriatric versus younger patients (Pontes et al., 2006). Animal studies have also found age-associated changes in the thyroarytenoid (TA) muscle. For example, a studying looking at histological markers and in vivo contractile properties of the rat TA, found the TA to be "weaker, slower, and more fatigable with age" (McMullen and Andrade, 2006). In addition, muscle fiber analysis determined a reduced number of muscle fibers in the TA with age in the rat. However, this reduction in number of muscle fibers was not accompanied by a significant reduction in muscle fiber diameter (Nishida et al., 2013). As stated previously, one of the proposed mechanisms for degradation in vocal function with age in humans is atrophy of the thyroarytenoid (TA) muscle (Pontes et al., 2006). That is, the age-related changes observed in voicing, such as breathiness, roughness, and reduced intensity described above, may be the result of reduced glottal closure and vocal fold vibratory asymmetry directly related to atrophy of the TA muscle.

Biochemical changes within contractile proteins in cranial muscles may underlie deficits observed with age in strength and mass. The distinct myosin heavy chain (MyHC) isoforms collectively represent most of the myosin protein structure, which is the main contractile protein within the muscle (Bottinelli and Reggiani, 2000; Pette and Staron, 1990). MyHC isoforms are closely linked to muscle fiber type and changes in MyHC isoforms have been shown to correspond to changes in muscle contraction speed (Bottinelli et al., 1994). As found in limb muscles (For review, see Pette and Staron, 2000), a transition from fast to slow MyHC isoforms has been reported in extrinsic tongue muscles (Schaser et al., 2011) and the intrinsic larynx (Suzuki et al., 2002) in rats. Therefore, the shift from fast to slow contracting isoforms may have a role in the functional changes seen with age in voice and swallowing, or could serve as a compensatory strategy to allow functional swallowing or voicing to occur in the setting of other age-related changes.

In summary, previous work has shown that aging results in reduced muscle mass, strength, and contractile speed at the functional and biochemical level in both the tongue and laryngeal musculature, which could contribute, in part, to observed age-related changes in voice and swallowing.

1.2.2 Altered motor neuron and nerve structure and function with aging

It has been hypothesized that age-related changes within motor neurons may result in denervation-like changes within muscle targets (Campbell et al., 1973). Specifically, a reduction in motor neuron number has been shown with aging, with a corresponding increase in motor unit size as adjacent motor neurons are recruited to innervate muscle fibers that have lost their innervation (Brooks and Faulkner, 1994; Campbell et al., 1973; Larsson, 1995). This change in innervation is manifested as a change in the structure of the muscle. That is, with denervation/reinnervation patterns, there is abnormal grouping of similar muscle fiber types that disrupts the normal mosaic pattern of the musculature (For review, see Lexell, 1995). Because motor neuron firing controls the contractile properties of different muscle fiber types (Liu et al., 2014b), a change in innervation will also change the MyHC isoform characteristics of the muscle. For example, studies involving cross reinnervation and artificial stimulation of the motor neuron have shown that it is the motor neuron firing pattern that drives muscle fiber type (For review, see Edstrom and Grimby, 1986). Denervation and reinnervation patterns have also been shown to result in increased proportions of hybrid muscle fiber types, suggesting a transition in MyHC isoform properties from one type of motor neuron firing pattern (slow) to another (fast) or vice versa (For review, see Ingalls, 2004; Pette and Staron, 2001). While continued work is needed to examine the motor neuron numbers, motor unit size changes, and innervation patterns within the cranial sensorimotor system, initial studies in aging rat models have shown reductions in motor neuron number in the nucleus ambiguous (Basken et al., 2012) and primary dendrite number in hypoglossal nucleus (Schwarz et al., 2009). Therefore, it is possible that similar changes exist in humans and could be responsible for the change in speed and coordination seen in senescent swallowing and vocalizations.

Age-related changes have also been found in cranial nerves. After the age of 60 there is a reduction in the speed of nerve conduction in the recurrent laryngeal nerve (RLN) in humans, as shown through EMG testing (Takeda et al., 2000). The RLN innervates the muscles of the larynx involved in

voice and swallowing function. A reduction in conduction speed could result in altered coordination of phonatory function, airway protection, and a safe swallow.

1.2.3 Altered structure and function at the neuromuscular junction with aging

Age-related changes to the neuromuscular junction (NMJ) have also been found in the cranial system. Specifically, there is a morphological change to the structure of the NMJ with age in both the tongue and larynx in animal studies. Aging NMJs are larger and less densely packed in animals models (Hodges et al., 2004; Johnson and Connor, 2011; Johnson et al., 2013), a change that is also seen in the aging human limb musculature (Li et al., 2011; Valdez et al., 2010). However, further work is needed to examine if the above changes in structure result in altered physiological properties of the NMJ as well the functional changes seen in older individuals' swallow and vocalizations.

1.2.4 Neurotrophic factors may combat age-related decline throughout the neuromuscular system

While further examination of age-related changes at each level of the cranial sensorimotor system is needed, a more efficient strategy may be to explore candidate proteins or growth factors that interact at each level of the cranial sensorimotor system. Neurotrophic factors, or neurotrophins, represent one such molecular candidate (Barbacid, 1995; Carrasco and English, 2003; Garcia et al., 2010). It was recently proposed that neurotrophins are responsible in part for changes in MyHC isoform composition, specifically in response to high-intensity exercise (Liu et al., 2014a). Neurotrophins are necessary for the proper development of muscle fiber phenotypes and muscle fiber type transitions during early development (Carrasco and English, 2003), and are important for synapse growth, maintenance, and survival, (For review, see Dechant and Neumann, 2002). Specifically, neurotrophin binding and receptor signaling is necessary for NMJ stability, morphological integrity, and function (Loeb et al., 2002; Mantilla et al., 2014). Neurotrophins also contribute to maintaining and restoring synaptic function in neurons of both the central and peripheral nervous systems, and have been proposed as a mechanism of activity-dependent change in the limb sensorimotor system (Barde, 1990; Gomez-Pinilla et al., 2002;

Hennigan et al., 2007; Mantilla et al., 2004; Zhan et al., 2003). Therefore, neurotrophins represent a potentially important candidate to examine as a possible mediator of change during age and exercise related studies of the cranial sensorimotor system, because they have been shown to act at each level within the system. Thus, studies focusing on neurotrophins may provide a better understanding of how system-wide decrements occur with aging and how they may be altered with exercise.

1.3 Neurotrophic Factors

Neurotrophic factors are a family of proteins that induce signaling cascades to improve the development, growth, maintenance, and survival of neurons (For review, see Bothwell, 1995). They act by binding to either a high affinity receptor, Tyrosine receptor kinase (Trk) or a low affinity receptor, p75 (Barbacid, 1995). During development, binding of the neurotrophin to p75 is especially important because p75 mediates both excitatory and inhibitory signaling cascades to assist in proper growth and pruning within the developing nervous system, and is an important player in cell apoptosis (cell death) that needs to occur to induce proper development (For review, see Hennigan et al., 2007). After the developmental stage is complete, the action of the high affinity Trk receptor becomes more important for the growth, maintenance, and survival of the neuron (For review, see Hennigan et al., 2007). The action of neurotrophic factors is carried out once the neurotrophin binds to the high affinity Trk receptor, initiating endocytosis of the neurotrophin and its receptor, and inducing signaling cascades to improve the maintenance and survival of individual neurons (Chao, 1992). Specifically, ERK (Loeb et al., 1992) and phospholipase C gamma I (Widmer et al., 1993) signaling pathways are initiated and lead to increased production of transcription factors such as CREB to promote protein signaling of different synaptic proteins like synapsin I and synaptotagmin that improve synaptic efficiency (For review, see Leslie and Nedivi, 2011).

1.4 Neuroplasticity

Creating both a behavioral change and an underlying change to the structure and/or function of a neural substrate should be the goal of our therapy protocols, because this is the way to create a lasting neuroplastic change (Kleim and Jones, 2008; Ludlow et al., 2008; Robbins et al., 2008). Neuroplasticity is defined as any change in neuronal structure or function that can be shown either at the individual level by studying a single neuron or that can be inferred by looking at a population of neurons (Warraich and Kleim, 2010). In addition, this change in function and structure should be accompanied by a behavioral change and should be brought about by external factors (For review, see Kleim and Jones, 2008). An example of neuroplasticity at the individual neuronal level occurs when a high frequency stimulation (external stimuli induced in a scientific experiment) causes a functional change, such as an excitatory post-synaptic potential (EPSP) and over time can result in long term changes to the structure of the neuron such as, increased axonal arborization, increased number of synapses, increased dendritic spine density, increased receptor density, or localization of specific receptors to increase synaptic efficiency (For review, see Warraich and Kleim, 2010). In addition, changes in populations of neurons can be examined after exercise or after a learning paradigm has been put into place (external factor). The structure and function of specific populations of neurons, such as those within the motor cortex, can be examined through motor mapping, functional magnetic resonance imaging (fMRI) or additional imaging techniques to determine if a structural (increase gray matter density or structural thickness) and/or functional (increased signaling) change has occurred (For review, see Warraich and Kleim, 2010). It is only when both an underlying structural or functional change and a behavioral change takes place that the change can be determined a neuroplastic change.

It is important to remember that not every behavioral change can be considered neuroplastic. For example, compensatory treatments can often result in behavioral changes, but don't necessarily cause changes at the neuronal level. Specific to the swallowing literature, compensatory treatments such as positional changes can result in a patient being able to eat safely or at a more favorable consistency level. However, there is no evidence available to show that this behavioral improvement stems from changes at

the neuronal level (Robbins et al., 2008). We do know that there are structural and functional changes at the peripheral level that occur within the swallowing musculature with exercise, such as increased muscle bulk and increased strength and pressure generation during the swallowing (Robbins et al., 2005; Steele, 2012; Yeates et al., 2008). However, we currently lack the evidence to show that a neuronal change also occurs with exercise. Studies using animal and exercise models, such as those reported in this dissertation are important to determine if neuroplastic changes are also occurring.

In general, neuroplasticity is thought to occur in two phases, a short term phase that does not require protein synthesis and a long term phase that requires protein synthesis (Leslie and Nedivi, 2011). Neuroplasticity results from simultaneous firing of neurons, creating a connection between the neurons on additional stimulation, termed Hebbian synapse based on the work of Hebb who stated that "neurons that fire together, wire together" (For review, see Berlucchi and Buchtel, 2009). The increase in activity within neurons that have been excited at the same time is known as Long term potentiation or LTP (Bliss and Lomo, 1973; Cooke and Bliss, 2006; Lomo, 2003). The mechanisms of LTP have been thoroughly studied by Kandel and his work in *aplysia* and in the mammalian hippocampus (Siegelbaum and Kandel, 1991). In addition, the opposite effect can occur and signaling between neurons can be depressed, a term known as Long Term Depression (LTD) (Linden, 1994). While Kandel has elegantly described the mechanisms of LTP and LTD in *aplysia* and within the hippocampus, his work has been focused on small populations of neurons and the initial increase in firing within the synapse has been induced by experiment stimulation or by reflexive learning and memory strategies within the *aplysia*. Since the work of Kandel, others have expanded this work and have looked at the mechanisms of LTP in learning and memory that occur within larger populations of neurons and with more advanced species (Bliss et al., 2004). However, it is still unclear if the mechanisms of LTP involved in learning and memory are the same as those involved in experience dependent plasticity, specifically with regard to exercise.

II. Statement of the Problem

2.1 Introduction

It has been proposed that neuroplasticity can be induced through activity dependent mechanisms, such as exercise or specific learning based paradigms, through the signaling of neurotrophic factors (Lu, 2003). However, the mechanisms of inducing these changes within the cranial sensorimotor system are currently unknown. While exercise may lead to neuroplastic change, optimal exercise types, exercise durations, and intensities, how specific the exercise needs to be, and at what point within the lifespan the exercise should optimally occur are currently unknown (Ludlow et al., 2008). Therefore, it is necessary to use animal models to examine the underlying structural and functional neuroplastic changes as they relate to the disorders seen in our clinical populations. In addition, a dearth of information exists about the role of neurotrophins within the cranial sensorimotor system. As a first step towards development of mechanistically based therapy approaches for age-related voice and swallowing, this dissertation examines one possible neuroplastic target, neurotrophins, in the cranial sensorimotor system and their response to age and exercise.

2.2 Exercise as a way to induce a neurotrophic response in the cranial sensorimotor system

Exercise-based interventions for dysphagia and dysphonia are currently within the armamentarium of the speech-language pathologist. However, little is known about mechanisms that underlie functional changes associated with exercise and the effects of exercise and age on the cranial sensorimotor system (Ludlow et al., 2008; Robbins et al., 2008). Exercise-based therapy programs focused on strength and coordination of the voice and swallowing musculature represent a minimally invasive approach to treatment and have been used by speech-language pathologists throughout the history of clinical practice (For review, see Stathopoulos and Felson Duchan, 2006). Activity-based treatment approaches hold theoretic promise because they are known to result in significant alterations in the brain and within limb muscles (Fluck, 2006; Gomez-Pinilla et al., 2002; Kempermann et al., 2000;

Knaepen et al., 2010; Marais et al., 2009; Mattson, 2000; Ploughman, 2008; Smith and Zigmond, 2003; Vaynman and Gomez-Pinilla, 2005; Zoladz and Pilc, 2010). However, the potential of exercise-based treatment to create underlying changes in the cranial sensorimotor system has been largely unexplored. Current review articles found in the swallowing and voice literature have suggested that exercise-based treatments result in improved outcomes for patients (Burkhead et al., 2007; Kays and Robbins, 2006; Robbins et al., 2005; Shaker et al., 2002; Steele, 2012; Stemple et al., 1994; Tay et al., 2012). Yet, there is little to no evidence that current exercise therapies result in changes to the underlying structure and function of neural substrates within the cranial sensorimotor system. (Kays and Robbins, 2006; Ludlow et al., 2008; Robbins et al., 2008)

Neurotrophins were examined as the neural substrate within the cranial sensorimotor system in this dissertation. As stated above, neurotrophins are the molecular mediators of synaptic plasticity. They are up-regulated with exercise and provide system-wide benefits in the central (CNS) and peripheral (PNS) nervous systems (Gomez-Pinilla et al., 2001; Pedersen et al., 2009; Ploughman, 2008; Vaynman and Gomez-Pinilla, 2005). In addition, previous research has shown that the amount and presence of neurotrophins in the CNS is dependent on age (Johnson et al., 1996; Johnson et al., 1999) and that exercise differentially up-regulates neurotrophins in aging models (Hopkins et al., 2011). However, the effects of age and exercise on neurotrophin levels in the cranial sensorimotor system are currently unknown.

2.3 Aims

2.3.1 Study 1:

Previous research in our laboratory has shown that 8 weeks of tongue exercise leads to increased tongue forces across the lifespan in a rat model (Behan et al., 2012; Connor et al., 2009), and that aging results in degenerative changes to the nucleus ambiguus, hypoglossal nucleus, and muscles involved in voice and swallowing (Basken et al., 2012; Hodges et al., 2004; Ota et al., 2005; Schaser et al., 2011; Schwarz et al., 2009; Suzuki et al., 2002).

However, prior to Study 1 it was unknown if: 1) neurotrophins were present in the cranial sensorimotor system involved in voice and swallowing and varied with age, and 2) the behavioral changes seen with tongue exercise in the rat were accompanied by underlying changes in neurotrophin levels.

Aim 1: To determine the effect of age on neurotrophins in the hypoglossal nucleus. ***Experiment 1a***: We quantified the amount of BDNF and its receptor TrkB in the hypoglossal nucleus using immunohistochemical methods from young adult and old rats.

Hypothesis: We hypothesized that levels of BDNF and TrkB immunoreactivity would be decreased with age.

Aim 2: To determine the effects of tongue exercise on the hypoglossal nucleus. ***Experiment 1b***: We quantified the amount of BDNF and its receptor TrkB in the hypoglossal nucleus using immunohistochemical methods from young adult and old rats that underwent 8 weeks of tongue exercise and compared values to control rats.

Hypothesis: We hypothesized that levels of BDNF and TrkB immunoreactivity would be increased with exercise.

2.3.2 Study 2:

The degree to which specificity and exercise follow-up are required to produce enduring changes in behavioral measures of voice and swallowing, and the associated neuroprotective effects have been largely unexplored prior to this dissertation. Therefore, the goal of Study 2 was to obtain a better understanding of the effects of age, exercise specificity, and detraining on neurotrophic factors and behavioral measures within the cranial sensorimotor system. Study 2 expands upon the work completed in Study 1, includes examination of specific muscles involved in voice and swallowing, and included the neurotrophins NT-4, BDNF, and receptor TrkB.

Aim 1: To determine the neuroplastic and cross-activation effects of tongue exercise on behavioral measures, brainstem nuclei, and muscles involved in voice and swallowing. ***Experiment 1***: We quantified behavioral measures of larynx and tongue muscle function (vocalization acoustics and tongue forces), and the amount of BDNF and NT4 and their receptor TrkB in the hypoglossal nucleus, nucleus ambiguus, genioglossus muscle (GG; tongue), and thyroarytenoid muscle (TA; larynx) from young adult and old rats that underwent 8 weeks of tongue exercise and a no-exercise control condition.

Hypothesis: Based on the principles of specificity and saliency (Kleim and Jones, 2008) it was hypothesized that changes in physiological and underlying neural substrates would be greater in measures involving the tongue (genioglossus (GG) muscle, tongue forces, and hypoglossal nucleus) following tongue exercise than for those involving the larynx (thyroarytenoid (TA) muscle, vocalization acoustics and nucleus ambiguous).

Aim 2: To determine the durability of exercise-based therapies by examining the effect of de-training on tongue force measures, vocalization acoustics, and BDNF, NT4/5 and TrkB in brainstem nuclei and GG and TA muscles. Experiment 2: We compared neurotrophin measures, tongue force measures, and vocalization acoustics from young adult and old rats that received 8 weeks of tongue exercise followed by either 2 or 4 weeks of detraining.

Hypothesis: It was hypothesized that all outcomes would decrease following detraining, with greater reductions at 4 weeks versus 2 weeks, based on the "use it or lose it" and timing principles of neuroplasticity (Kleim and Jones, 2008).

2.4 Summary

Without a clear understanding of the changes to underlying neural substrates with exercise and age, clinicians lack the physiologic and mechanistic evidence needed to support their decision to formulate a specific treatment protocol. Thus, the goal of this dissertation was to use an animal model to systematically examine therapy parameters and the resulting changes to neurotrophins after exercise-based therapy to provide clinicians with the evidence needed to better guide clinical decision-making and gain maximum therapeutic benefit. The ultimate translational goal of this work is to contribute to the future development of new exercise-based therapies for dysphagia and dysphonia.

III. Study 1

The Effect of Age and Tongue Exercise on BDNF and Trkb in the Hypoglossal Nucleus of Rats

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Abstract

Age-associated changes in tongue musculature may contribute to dysphagia. One possible treatment is tongue exercise. Exercise induces synaptic plasticity by increasing neurotrophic factors in spinal cord and limb musculature. However, effects of exercise on neurotrophic factors in the cranial sensorimotor system are unknown. Our purpose was to examine the effects of age and exercise on brain-derived neurotrophic factor (BDNF) and its receptor TrkB in the rat hypoglossal nucleus.

Young, middle-aged, and old rats were assigned to exercise or no-exercise control conditions. Exercise animals were trained to perform a licking task for 8 weeks. Samples from the hypoglossal nucleus were analyzed for BDNF and TrkB immunoreactivity.

Baseline maximum tongue forces were similar in all age groups and increased significantly following exercise. BDNF immunoreactivity did not show a significant decrease with age in control group. However, in the exercise group, BDNF was significantly increased in young animals. TrkB immunoreactivity decreased significantly with age in control group, but did not change with exercise. BDNF and TrkB immunoreactivity levels were positively correlated with exercise in young and middle-aged animals, but were negatively or weakly correlated with exercise in old animals and with a lack of exercise in no-exercise controls.

Tongue exercise was associated with increased tongue forces in rats at all ages. While increases in BDNF and TrkB levels associated with exercise may play a role in mechanisms contributing to increased tongue forces in young and middle-aged rats, other mechanisms may be involved in increased tongue forces observed in old rats.

Key Words: Exercise, Aging, Neurotrophic Factors, Hypoglossal Nucleus, Tongue Muscle

3.1 Introduction

Aging is associated with muscle weakness and fatigue, a condition that has been termed sarcopenia (Doherty, 2003; Janssen, 2010; Rosenberg, 1997). The cause of sarcopenia is likely multifactorial and includes a variety of potential mechanisms including neuromuscular changes, decreased nutrition, hormonal changes, and inactivity (Marcell, 2003; Roubenoff, 2001; Thomas, 2010). Sarcopenia affects all elderly individuals to some extent because it is a consequence of normal aging (Roubenoff, 2001). 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 (Dutta, 1997; Lang et al., 2010). 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 (Connor et al., 2009; Johnson and Connor, 2011; Schaser et al., 2011). 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 (Cook et al., 1994; Nicosia et al., 2000).

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 (Kirkendall and Garrett, 1998; Liu and Latham, 2009; Peterson et al., 2011; Waters et al., 2010). 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 (Kays and Robbins, 2006; Robbins et al., 2005). 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 (Logemann, 2005; Yeates et al., 2008).

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 (Grondard et al., 2005; Mattson, 2000; Smith and Zigmond, 2003) mediated by neurotrophins in both the central and peripheral nervous systems (Ebadi et al., 1997). Neurotrophins are a family of proteins that, when activated through binding with Tyrosine receptor kinase (Trk) receptors, initiate signaling cascades that promote the development, survival, and function of neurons (Poo, 2001; Schinder and Poo, 2000; Skaper, 2008). Given that the neurotrophin receptors TrkB and TrkC are decreased in the spinal motoneurons of aged rats (Johnson et al., 1996), it appears that decreases in neurotrophin levels may also have a role in the limb deficits seen with aging. Results of previous studies in brain and spinal cord support the hypothesis that neurotrophins act as a therapeutic agent in cases of neurodegenerative disease and nerve injury (Lindsay, 1996; Yuen and Mobley, 1995; Yuen, 2001). 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 (Vaynman et al., 2003). 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 (Gomez-Pinilla et al., 2001). 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 (Baker-Herman et al., 2004; Wilkerson and Mitchell, 2009). 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 (Connor et al., 2009; Johnson and Connor, 2011). 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 (Connor et al., 2009). 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 (Johnson and Connor, 2011). 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.

3.2 Methods

All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of Wisconsin Animal Care and Use Committee. 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 (Turturro et al., 1999). 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 (Behan et al., 2012).

3.2.1 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 licking measurements in rats have been detailed previously (Ciucci and Connor, 2009; Connor et al., 2009) 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 x 1 centimeter (cm) aperture and force operandum that delivered aliquots of water based on licking 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.

3.2.2 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 4º C.

3.2.3 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) 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.

3.2.4 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 (Abramoff et al., 2004). To separate signal from background an Otsu thresholding algorithm was applied to each image. The average fluorescent intensity of each image 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 pixel 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.

3.3 Results

3.3.1 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.

3.3.2 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 (arrow heads 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.

3.3.3 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.

3.3.4 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 (arrow head in Fig.1C) and synaptic vesicles (arrow 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; $p = <0.0001$, $p = 0.01$, $p = 0.05$).

3.3.5 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.

3.3.6 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 (Barbacid, 1995; Bothwell, 1995; Skaper, 2008). 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).

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 arrowheads) and synaptic boutons (arrows). Scale bar = 50 μm.

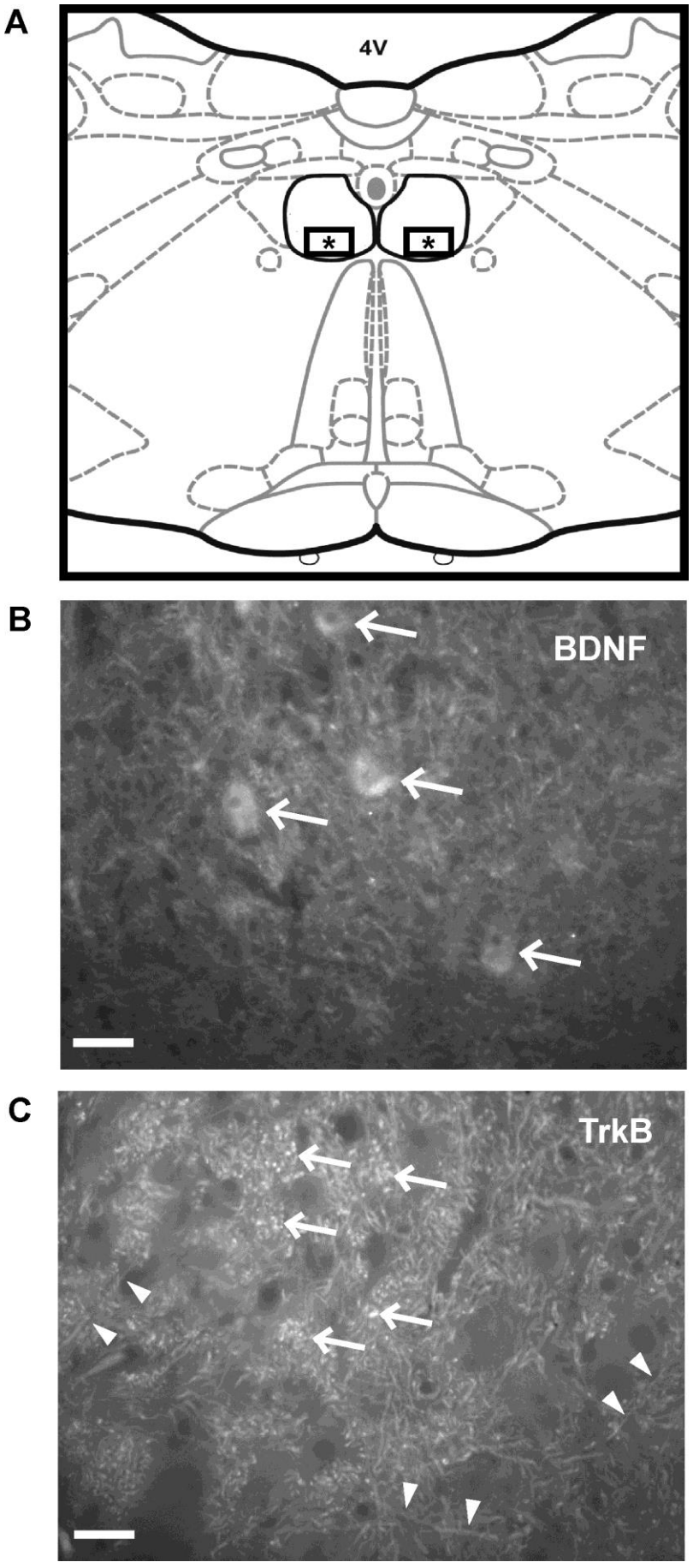

A
4V
*
*
B
BDNF
C
TrkB

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.

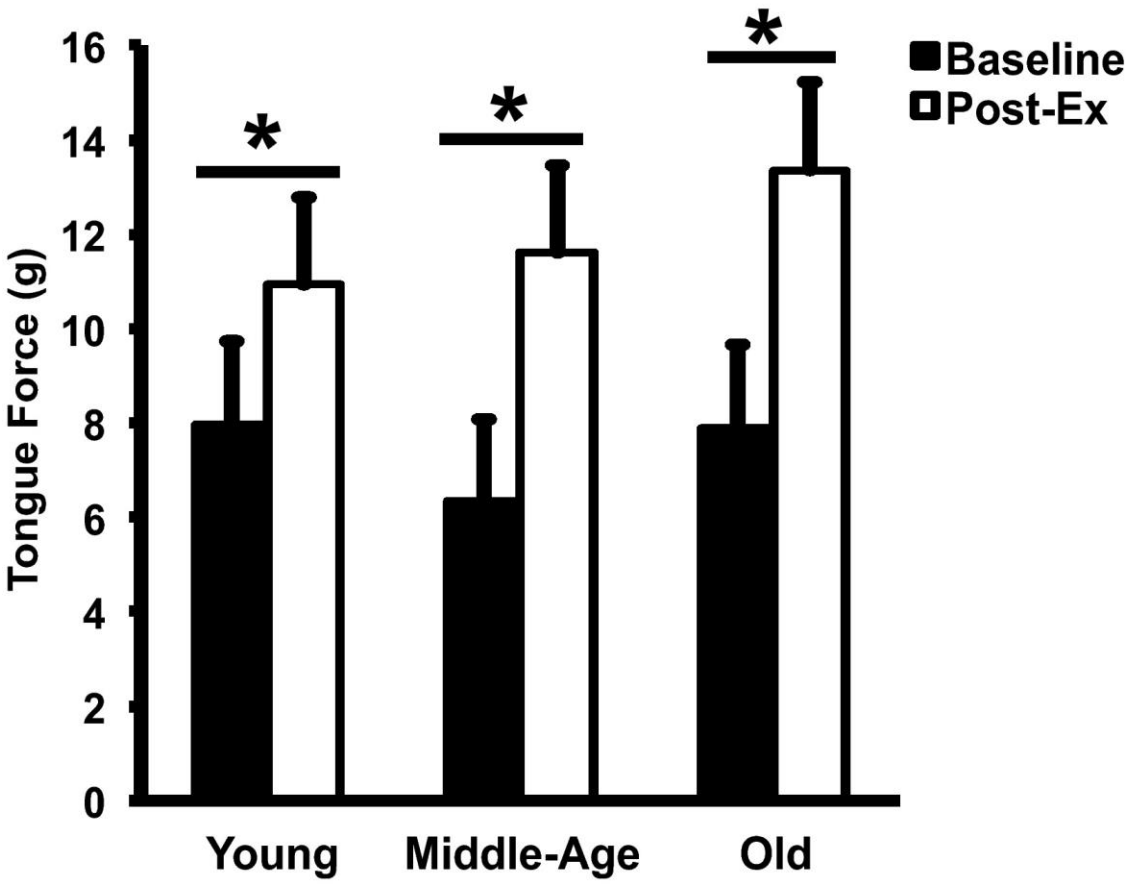


Fig. 3. (A-C) Changes in BDNF immunoreactivity by region (A), age (B), and exercise (C). (A) Mean BDNF fluorescent intensity, expressed in relative fluorescent units (RFU), is represented along the vertical axis. Age groups (young, middle-age, and old), divided into control and exercise conditions, are 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.

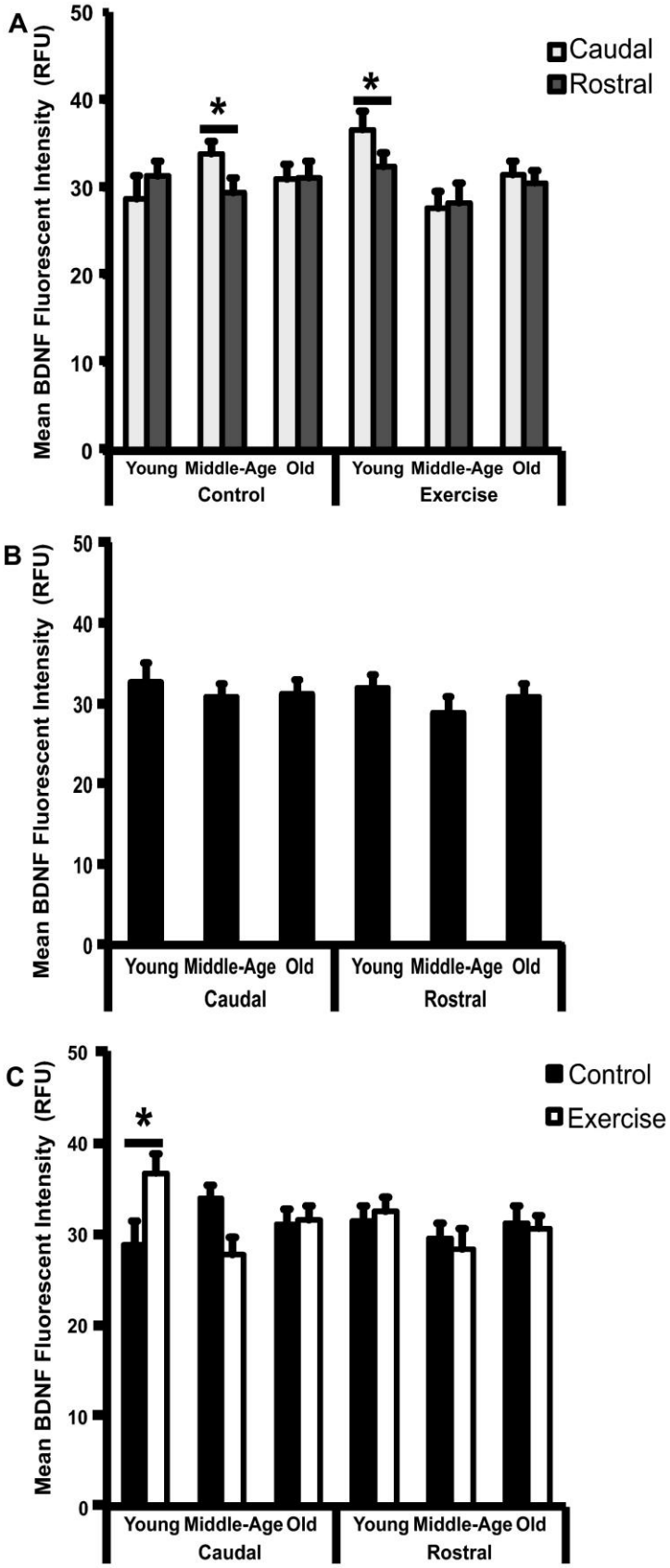
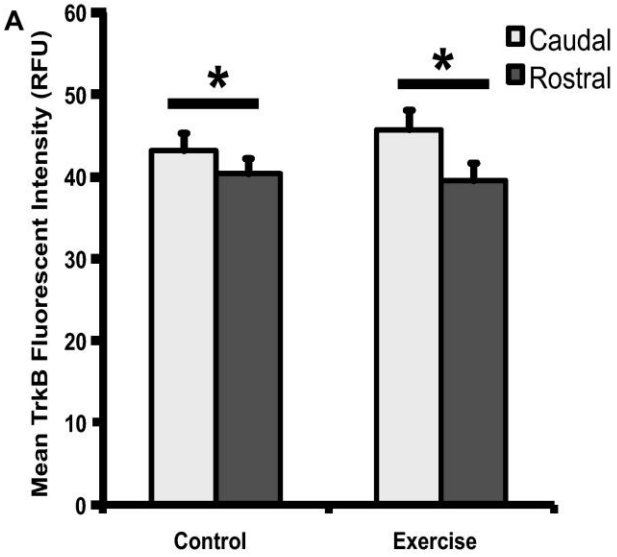
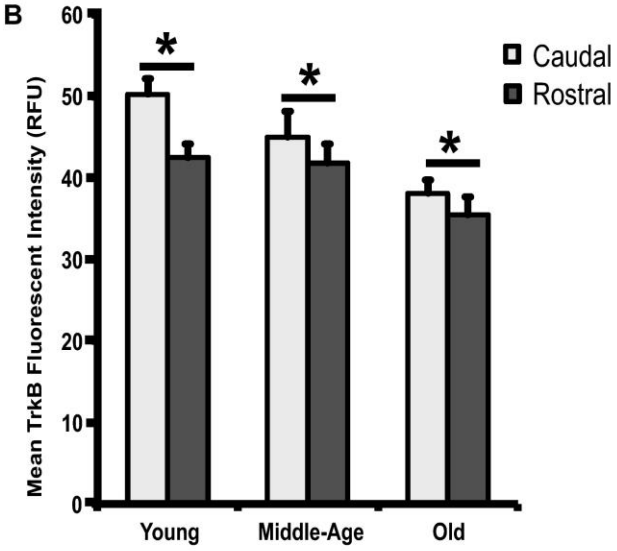

A
Mean BDNF Fluorescent Intensity (RFU)
50
40
30
20
10
0
Caudal
Rostral
*
*
Young Middle-Age Old
Control
Young Middle-Age Old
Exercise
B
Mean BDNF Fluorescent Intensity (RFU)
50
40
30
20
10
0
Young Middle-Age Old
Caudal
Young Middle-Age Old
Rostral
C
Mean BDNF Fluorescent Intensity (RFU)
50
40
30
20
10
0
Control
Exercise
*
Young Middle-Age Old
Caudal
Young Middle-Age Old
Rostral

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.


A
Mean TrkB Fluorescent Intensity (RFU)
60
50
40
30
20
10
0
Caudal
Rostral
*
*
Control
Exercise


B
Mean TrkB Fluorescent Intensity (RFU)
60
50
40
30
20
10
0
Caudal
Rostral
*
*
*
Young
Middle-Age
Old

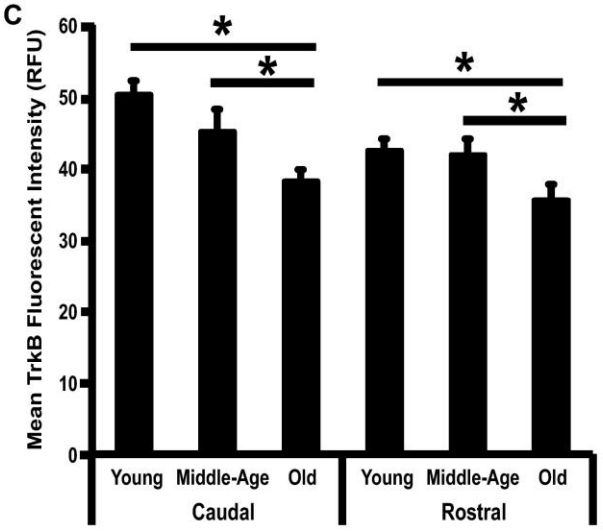
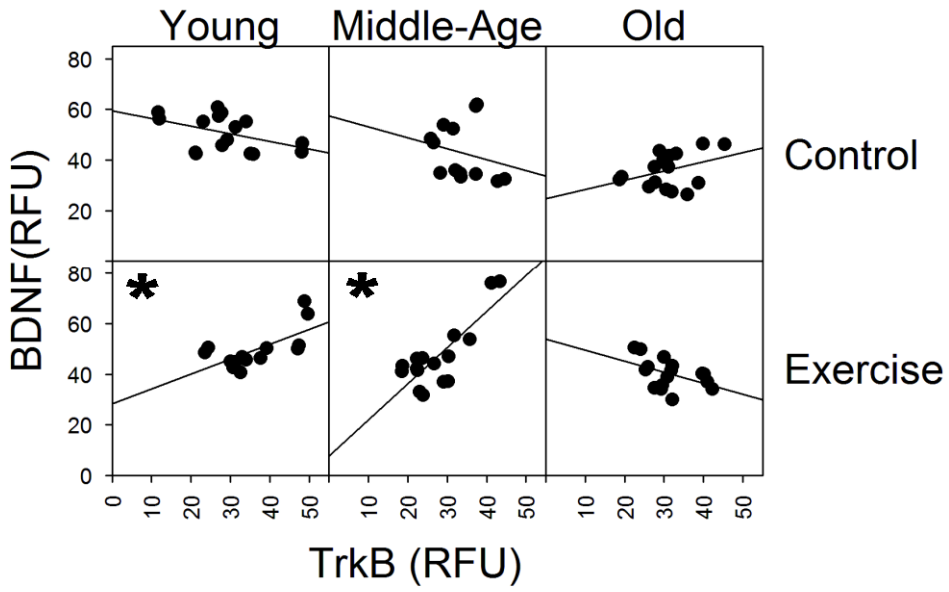

C
Mean TrkB Fluorescent Intensity (RFU)
60
50
40
30
20
10
0
*
*
*
*
Young
Middle-Age
Old
Caudal
Young
Middle-Age
Old
Rostral

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.



3.4 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 (Bergman et al., 1996; Johnson et al., 1996; Johnson et al., 1999). BDNF sparing in the presence of TrkB reductions with aging has also been found in the pituitary (Rage et al., 2007). Because both the neurotrophin and its receptor must be present to allow downstream signaling cascades leading to synaptic changes within the neuron (Barbacid, 1995; Bothwell, 1995; Skaper, 2008), 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 (Barbacid, 1995; Patapoutian and Reichardt, 2001; Skaper, 2008). Specifically, TrkB is also a receptor for muscle-derived NT4/5 neurotrophin (Bothwell, 1995). 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 (Funakoshi et al., 1995; Johnson et al., 1996), 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 (Friedman et al., 1995; Wilkerson and Mitchell, 2009) and can lead to improved recovery after spinal cord injury (Jakeman et al., 1998). In addition, BDNF and NT4/5 have been shown to improve synaptic transmission at the neuromuscular junction by binding with the TrkB receptors to increase release of synaptic vesicles (Zhan et al., 2003). 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 (Bothwell, 1995). 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 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 (Knaepen et al., 2010). 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 lumbar spinal cord and soleus muscle. However, increases in NT-3 mRNA and not in protein were observed in rat soleus muscle (Ying et al., 2003). Similarly, Gomez-Pinilla and colleagues found that exercise has a differential effect on BDNF and NT-3 in both the spinal cord and muscle (Gomez-Pinilla et al., 2001). 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.

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.

Acknowledgements

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IV. Study 2

The Cross-activation and Detraining Effects of Tongue Exercise Paired with a Water Swallow in Aged Rats.

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Abstract

Purpose: Lingual and laryngeal sensorimotor systems are impaired with aging and can manifest as voice and swallowing deficits. Tongue exercise is used clinically as a treatment for swallowing disorders. In addition to activating lingual muscles, tongue exercise paired with a water swallow also activates laryngeal muscles. It was hypothesized that: 1) tongue exercise paired with a water swallow would result in improved behavioral outcomes and increased neurotrophic factor concentration with greater effects in lingual versus laryngeal structures, and 2) exercise effects would diminish with detraining.

Methods: Forty young adult (9m) and 40 old (32m) male rats were randomly assigned to a tongue exercise group, 2- or 4-week detraining groups, or control group. Exercise and detraining animals performed a licking task paired with a water swallow for 8 weeks. Detraining animals underwent 2 or 4 weeks of detraining, respectively. Tongue forces and ultrasonic vocalizations were recorded at all time-points. Following training, laryngeal and lingual muscles and brainstem nuclei of interest were removed for analysis of neurotrophic factors. Two-way analyses of variance were performed to examine effects of age and training condition on tongue force, ultrasonic vocalizations, and neurotrophic factors. T-tests were performed to examine the effects of detraining. In addition, Spearman correlations were performed to examine the relationship between exercise participation, tongue force, and neurotrophic factor concentration.

Results: Significant main effects of age ($p < 0.001$) and condition ($p < 0.001$) were seen for tongue force. Post-hoc analyses revealed that with exercise both age groups increased tongue force, with tongue force increasing to a greater extent with tongue exercise in the old group. A detraining effect was found only in old animals after 4 weeks. Results of the ultrasonic vocalization data showed a significant main effect of age ($p < 0.05$), and a significant increase in intensity with exercise in one training group ($p=0.04$). NT4 was reduced with aging in all brainstem nuclei studied ($p < 0.05$) and BDNF was reduced with aging in the laryngeal muscle in one training group ($p=0.03$). In addition, an age and condition interaction effect

($p=0.04$) was found for BDNF in the hypoglossal nucleus. Post-hoc testing revealed that BDNF concentration in the hypoglossal nucleus in the old group alone was associated with tongue exercise.

Conclusions: Tongue exercise paired with a water swallow was associated with increased tongue forces at all ages and gains were maintained after detraining only in the young group. Age-related changes in vocalizations, NT4, and BDNF were shown. However, cross-system activation effects were only observed on one vocalization measure (intensity), which is logical based on the activation of the laryngeal and respiratory structures during the swallow. In addition, concentration of BDNF in the hypoglossal nucleus appears to be related to behavioral improvement in tongue forces in old animals. Thus, this research showed that tongue exercise paired with a swallow led to specific, neuroplastic benefits in the old animals in that both behavioral improvements and an underlying change at the level of the hypoglossal nucleus was observed through up regulation of BDNF. Therefore, tongue exercise paired with a water swallow represents a therapeutic modality that could be developed, studied, and optimized in human clinical studies for the treatment of swallowing and voice disorders in elderly people.

4.1 Introduction

Exercise-based interventions are currently used to treat a variety of cognitive and sensorimotor impairments that occur as a result of aging and/or neurodegenerative disorders (Ding et al., 2011; Dishman et al., 2006; Intlekofer and Cotman, 2013; Vaynman and Gomez-Pinilla, 2005). In addition, exercise-based interventions are an often-used tool for the treatment of dysphagia and dysphonia by the speech-language pathologist (Behrman et al., 2008; Burkhead et al., 2007; Carnaby and Harenberg, 2013; Fox et al., 2006; Green and Bavelier, 2008; Robbins et al., 2007; Roy et al., 2003; Stemple et al., 1994). However, treatment efficacy has not been optimized because the underlying functional changes associated with exercise and the effects of exercise and age on neuroplastic mechanisms within the cranial sensorimotor system are currently unknown. As a result, clinicians have limited evidence upon which to choose an exercise method based on the specific signs and symptoms of their patients. Clinicians are currently required to try a plethora of "possible" exercises to see if one of them will result in improvement, resulting in increased therapy time and a lack of evidenced-based therapy guidelines (Carnaby and Harenberg, 2013; Ludlow et al., 2008; Robbins et al., 2008). Neuroplasticity is defined as "any change in the structure or function of a neuron that can be shown either at the individual neuronal level or that can be inferred by looking at a population of neurons" (Warraich and Kleim, 2010) *and* is accompanied by a behavioral change (Berlucchi and Buchtel, 2009; Warraich and Kleim, 2010). Without a clear understanding of changes to underlying neural substrates with exercise and age, clinicians are at a disadvantage because they lack physiological and mechanistic evidence to support the decision to use a specific exercise, to decide if training of behaviors that share neuromuscular substrates will have cross-activation effects, or to determine when and if exercise follow-up should occur (Carnaby and Harenberg, 2013; Cramer et al., 2011). Thus, it is imperative that research systematically examines therapy parameters and the resulting changes to neural substrates after exercise-based therapy to provide clinicians with the evidence needed to better guide clinical decision-making and maximize beneficial neuroplasticity. While work in this study is performed in an animal model and is not directly related to

clinical care, the level of evidence provided in this dissertation will serve as the foundation for future clinical studies and will provide the evidence needed to direct more focus hypotheses for future human clinical studies.

Neurotrophic factors, or neurotrophins, represent molecular candidates for neural substrates responsible for neuroplastic change. Neurotrophins are the molecular mediators of synaptic plasticity within the motor system (Zhan et al., 2003), contribute to maintaining and restoring synaptic function in neurons of both the central and peripheral nervous systems (Barde, 1990; Hennigan et al., 2007), and have been proposed as a mechanism of activity-dependent change in the limb sensorimotor system (Gomez-Pinilla et al., 2002; Gomez-Pinilla et al., 2011). However, a dearth of information exists about the potential for neuroplastic change within the cranial sensorimotor system and the role of neurotrophins in this context, making development of mechanistically based neuroplastic therapy approaches for voice and swallowing challenging.

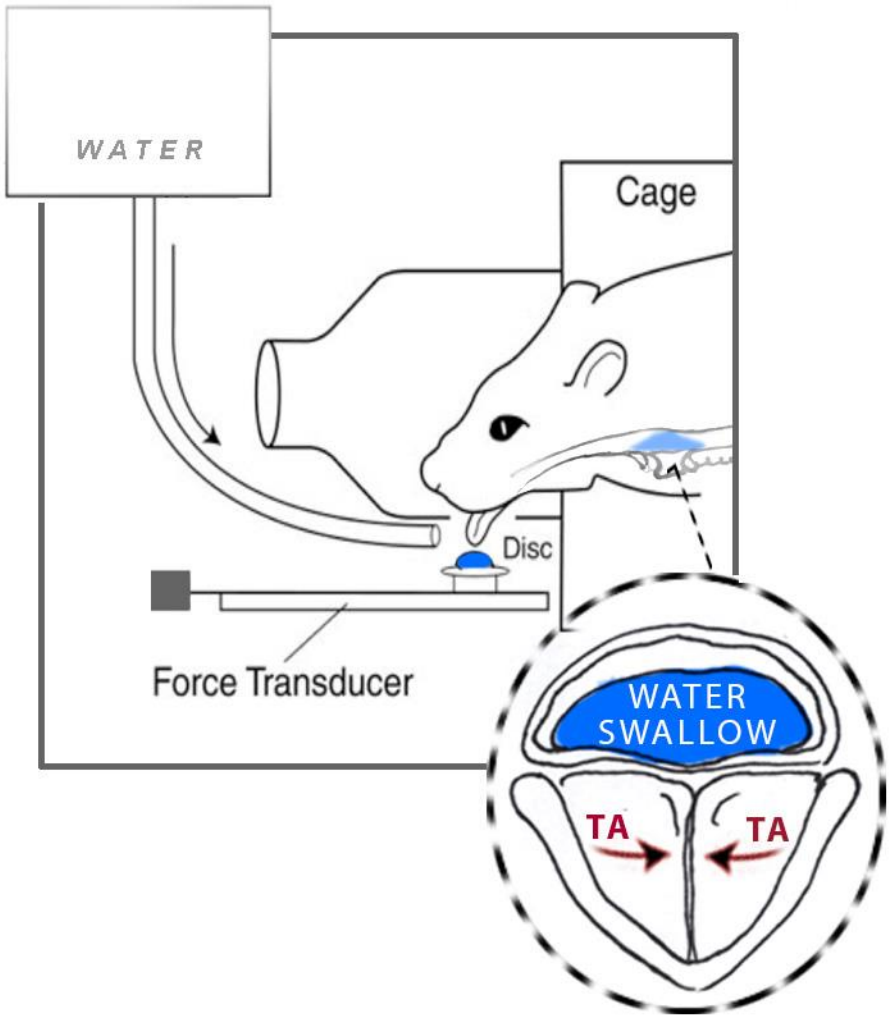
Previous research in our laboratory has shown that 8 weeks of tongue exercise leads to increased tongue forces across the lifespan in a rat model (Behan et al., 2012; Connor et al., 2009; Schaser et al., 2012), and that aging results in degenerative changes to the nucleus ambiguus (NA), hypoglossal nucleus (HN), and muscles involved in voice and swallowing (Basken et al., 2012; Hodges et al., 2004; Ota et al., 2005; Schaser et al., 2011; Schwarz et al., 2009; Suzuki et al., 2002). In addition, our previous research suggests that brain derived neurotrophic factor (BDNF) and its receptor TrkB may be a putative mechanism underlying age and exercise related change in tongue function, because the HN showed decreased TrkB with age and increased BDNF with exercise in adult rats (Schaser et al., 2012). However, questions still exist regarding the enduring benefits (effects of detraining), generalizability (cross-activation potential) of tongue exercise paired with a water swallow, how neurotrophic factor protein may mediate adaptations in motor sensorimotor function with age and exercise, and how other neurotrophic factors, such as NT4 (Barbacid, 1995) may exert effects through the TrkB receptor.

The degree to which specificity and exercise follow-up are required for producing enduring changes in behavioral measures of voice and swallowing, and the associated neuroprotective effects have been largely unexplored. Therefore, the goal of this study was to obtain a better understanding of the effects of age, exercise specificity, and detraining on neurotrophic factors and behavioral measures within the cranial sensorimotor system. Although this work is performed in an animal model, the ultimate translational goal of this work is to focus development of new exercise-based therapies for future human clinical studies related to dysphagia and dysphonia.

To examine exercise specificity and cross-activation in our study, tongue exercise paired with a water swallow was used as the exercise-based therapy intervention. The concept of cross-activation during training as a method to induce treatment efficiency is already being explored in the clinical world (Easterling, 2008; El Sharkawi et al., 2002). For the exercise task trained in the current study, muscles of both the tongue and larynx were activated during the exercise to allow for a safe and accurate swallow (Fig. 6). Thus, this task had the potential to affect both lingual and laryngeal structures and their sensorimotor outputs because the larynx is known to elevate and the vocal folds are known to close to protect the airway during the swallow (Fink, 1974; Logemann et al., 1992; McCulloch et al., 1996; Perlman et al., 1999; Pressman, 1941; Stuart, 1891; Van Daele et al., 2005). In addition, muscles involved in both vocalization and swallowing receive input from similar neural control elements and are both involved in respiration (McFarland and Tremblay, 2006; McFarland and Paydarfar, 2011; Nishio and Niimi, 2004). Therefore, it is theoretically possible and reasonable that an exercise targeting a specific component, such as the tongue, within a larger sensorimotor framework (swallowing) may provide cross-system benefits to other components not specifically targeted but activated, such as the larynx (McFarland and Tremblay, 2006). However, based on the principles of specificity and saliency proposed in a seminal paper outlining the 10 principles of neuroplasticity (Kleim and Jones, 2008), it was hypothesized that changes in physiological and underlying neural substrates would be greater in measures involving the

tongue (genioglossus (GG) muscle, tongue forces, and HN) following tongue exercise than for those involving the larynx (thyroarytenoid (TA) muscle, vocalization acoustics and NA).

Fig. 6. Schematic of tongue force operandum. Image demonstrates tongue exercise followed by a water swallow in the rat. Image highlights activation of the thyroarytenoid muscle (TA) to protect the airway, following licking, during the water swallow. Image adapted by TG.



To examine the lasting benefits of exercise in the cranial sensorimotor system and to provide clinical evidence to support the need for maintenance following exercise-based therapy, a detraining protocol was also used in this study. Periods of 2 and 4 weeks of detraining were chosen because they represent both short term (2 weeks) and long term (4 weeks) detraining (Mujika and Padilla, 2000a; Mujika and Padilla, 2000b). It was hypothesized that all outcomes would decrease following detraining, with greater reductions at 4 weeks versus 2 weeks, based on the "use it or lose it" and timing principles of neuroplasticity (Kleim and Jones, 2008). This hypothesis is also supported by previous clinical studies that have shown that tongue forces were decreased following 2-4 weeks of detraining following a 9-week lingual strengthening protocol (Clark et al., 2009).

4.2 Methods

All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals, Eighth Edition (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, 2011) and approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee. A total of 80 Fischer 344/Brown Norway male rats were obtained from a National Institute on Aging (NIA) aging animal colony. This inbred strain of rat is used most frequently in aging research because they are genetically identical and are raised in identical conditions. Two age groups were studied: 40 young adult (9-month old) and 40 old (32-month old). The 32-month old group represents advanced senescence because the median lifespan for this strain of rats is 33 months (Turturro et al., 1999). Only male rats were used because the female estrus cycle may affect vocalization acoustics (Matochik et al., 1992). The rats were equally and randomly assigned to either a tongue exercise group (n=20; 10 young adult and 10 old), 2-week detraining group (n=20), 4-week detraining group (n=20) or a no-exercise control group (n=20). Measurement variables are found in Table 1.

Table 1. Summary of training groups, tissues analyzed and measurement variables.

Training Group	Tissue Analyzed	Measurement Variables
8 weeks of tongue exercise	Hypoglossal Nucleus (HN) Nucleus Ambiguus (NA) Genioglossus muscle (GG) Thyroarytenoid muscle (TA) Control Tissue: 2 tissue slices at level of spinal cord + 2 tissue slices in medulla rostral to NA (Brainstem Control (BSC)) & Extensor Digitorum Longus Muscle (EDL)	Tongue Force Maximum and Δ tongue force (mN) Vocalization Acoustics Total and Δ number of calls Average and Δ peak frequency (kHz) Average and Δ bandwidth of calls (kHz) Average and Δ call intensity (dB) Average and Δ call duration (seconds) Neurotrophic Factors (BDNF, NT4, TrkB) Concentration (pg/ml) Neurotrophin Protein: Enzyme Linked Immunosorbent Assay (ELISA)
8 weeks of no-exercise (skill maintenance)		
8 weeks of tongue exercise followed by Detraining: Short-term (2 wks) & long-term (4 wks)		

4.2.1 Behavioral Tongue Exercise and Tongue Force Acquisition

Rats had access to water gradually restricted to only 3 hours a day over the course of 14 days (Toth and Gardiner, 2000). The rats also had their light and dark cycles reversed to ensure that exercise was provided at the time of most activity. All rats were trained during a 2-week acclimation period to lick with 2.0 mN of force against an 18 mm aluminum disk fitted with a force transducer on the shaft of the disk (Sensotec load cell, 0-250 g range) to receive an approximately 0.10 mL water reward, using a variable ratio 5 reinforcement schedule (VR5; one reinforcement for approximately every 5 licks). Following a 10 minute training session per day, rats were removed from the operandum enclosure (Fig. 6) and given water ad libitum in a water dish for 3 hours. This program of water delivery is in current use in our laboratory and was consistent in this study throughout the acclimation period, exercise program, detraining program, and control condition (Connor et al., 2009; Schaser et al., 2012).

Following the 2 weeks of preliminary behavioral training, each rat was tested to determine an individual maximum tongue force value by averaging the 10 highest force values for each rat over 3 days. Each rat was placed in the operandum enclosure and force targets were progressively increased within 3 consecutive daily sessions to determine these maximum values. Maximum tongue force testing was performed for all exercise, detraining, and control rats at baseline and following the 8 week experimental period.

The progressive resistance exercise program began immediately following the 2 weeks of preliminary behavioral training. The exercise period lasted 8 weeks and was performed 5 days per week. Work in our laboratory has shown that 8 weeks of tongue exercise is necessary to produce maximum changes in tongue forces (unpublished data). At the end of each 2-week exercise period, the tongue force threshold required to for receipt of a water reward was progressively increased.

A. For the first 2 weeks, the criterion for receiving a water reward was set at 50% of the maximum tongue force, defined individually for each rat at baseline.

B. For weeks 3 and 4, the threshold for receipt of a water reward was increased to 60% of the baseline maximum tongue force. At the end of Week 4, maximum tongue force values were re-assessed to account for possible increases in tongue strength with exercise.

C. Based on the individualized data for each rat, tongue force thresholds for weeks 5 and 6 were increased to 70% of the new maximum tongue force determined at the end of Week 4.

D. For the final 2 weeks (weeks 7 and 8) the tongue force threshold was increased to 80% of the maximum tongue force determined at the end of Week 4.

Within an exercise session, rats were required to produce at least 20 licks greater than or equal to the target threshold for that day to be counted as a successful training session. If an initial training session was not successful, rats were placed in the training cage for subsequent attempts (maximum of 2 repeat trials per day). If the rat did not produce 20 licks greater than or equal to the target threshold for 3 consecutive days of training, the threshold value was reduced and the new threshold was documented. Values for percent of maximum force and number of resistance trials within a session are consistent with recommendations for strength training for humans by the American College of Sports Medicine (Kraemer et al., 2002). Post-testing identical to that performed at baseline revealed maximum tongue forces associated with training. Testing was also performed in an identical manner for animals in the detraining groups.

The no-exercise control rats were placed on a water restriction protocol and light/dark cycle reversal identical to the exercise treatment rats. To allow collection of baseline tongue force data, the no-

exercise control rats also underwent 2 weeks of behavioral training using the operandum without any applied resistance. In this manner, the control rats had a threshold of 2.0 mN for receiving a water reward. These rats did not receive progressive resistance exercise during the 8-week experimental duration, but instead entered the cage and licked from the operandum without any resistance for less than 5 reinforcements per session to ensure skill maintenance. In addition, no-exercise control rats received water for 3 hours per day throughout the experimental duration. Post-testing identical to that performed at baseline revealed maximum tongue forces associated with skill maintenance alone in the no-exercise control group.

4.2.2 Vocalization Acoustics

Rats produce several types of ultrasonic vocalizations (USVs) in the 50 kHz range. These calls have been studied extensively in previous research and are semiotic in nature in that they have symbolic reference and are capable of changing the behavior of the communication partner (Bialy et al., 2000; Blanchard et al., 1992; Brudzynski, 2005; McGinnis and Vakulenko, 2003; Riede, 2014; Wohr et al., 2008). USVs in rats are produced by a constriction in the larynx that occurs through fine motor control of intrinsic laryngeal muscles (Johnson et al., 2010; Riede, 2011). Although this constriction produces a high frequency, whistle-like acoustic output unlike human phonation, rat USVs provide a good model for addressing our research questions because they are produced during egressive airflow with intrinsic laryngeal muscle activity (Johnson et al., 2010; Riede, 2011). Thus, rat USVs serve as a good model for studying laryngeal muscles, associated neuromuscular pathways, and vocalization behaviors in our specific context (Ciucci et al., 2007; Ciucci et al., 2008; Ciucci et al., 2009; Ciucci et al., 2010; Russell et al., 2010).

Baseline and post-intervention acoustic measurements of USVs from all rats were obtained using an existing mating paradigm (Ciucci et al., 2008). The procedure involved: (1) pairing a male rat with a receptive female in estrus, evidenced by lordosis, darting, and ear wiggling in the female rat; (2) allowing

the male to mount the female; and (3) putting both rats into the test cage and removing the female after the male exhibits further interest, such as chasing and vocalization (mounting was not allowed during this stage to control for reduced mounting behavior in aged rats), which induced further vocalization by the male and allowed USVs to be recorded from the male rat in isolation (Ciucci et al., 2008). Once the female was out of the range of the microphone, two minutes of calling was recorded onto a Windows-based personal computer using an ultrasonic recording system (Avisoft, Germany) with an appropriate frequency response range (10 to 180 kHz) and the capability of producing spectrograms in real time. All calls within the 50 kHz range, collected during the 2 minute recording period, were analyzed for total number of calls. For subsequent USV characteristic analyses (see Table 1) only frequency modulated (operationally defined as calls with a bandwidth greater than 10 kHZ) calls were included. Offline acoustic analyses were performed with a customized automated program using SASLab Pro (Avisoft, Germany). Spectrograms were built from each waveform with the frequency resolution set to an FFT of 512 points, frame size of 100%, flat top window, and the temporal resolution set to display 75 % overlap. Measures are found in Table 1. (For video example and review of previous studies, see Ciucci et al., 2009; Johnson et al., 2011; Johnson et al., 2013).

4.2.3 Detraining

The two detraining rat groups were placed on a water restriction protocol and a light/dark cycle reversal identical to the exercise rats and no-exercise control rats. They underwent the same exercise treatment protocol as the exercise rats. Baseline and post exercise maximum tongue force measures and vocalization acoustic measures were obtained as described previously (Table 1). Following collection of the post exercise measures, the 2- and 4-week detraining animals remained on the water restriction protocol. During the 2-week no-exercise period, the 2-week detraining group entered the cage and licked from the operandum without any resistance for a total of less than 5 reinforcements per session, to ensure skill maintenance. After the 2-week no-exercise period, post detraining tongue force and vocalization data

were obtained. Identical procedures were applied to the 4-week detraining rats following a 4-week period of no-exercise.

4.2.4 Tissue Collection

All rats were deeply anesthetized with 5% isoflurane until unresponsive to toe pinch or corneal reflex, were humanely euthanized, and their brains and muscle were dissected for analyses. Brain tissue was snap-frozen and embedded in optimum cutting temperature compound (OCT) and stored at -80 degrees. Brains from the level of the spinal cord through the medulla were sliced into 300μm thick slices with a freezing microtome (Leica 2000R, Bannockburn, IL, USA). Using known anatomical markers and based on studies previously conducted in our laboratory (Behan et al., 2012; Schaser et al., 2012; Schwarz et al., 2009) the hypoglossal nucleus and nucleus ambiguous were grossly microdissected and stored for downstream protein analysis. Neural control tissue from the spinal cord and medulla at levels above and below the nuclei of interest was also collected (Brainstem Control (BSC)). The GG and TA muscles were dissected out, as well as the extensor digitorum longus (EDL) muscle in the hind limb to serve as muscular control tissue. The specific nuclei and muscles were chosen because of their role in voice and swallowing behaviors. The EDL was chosen because it contains largely rapidly-contracting muscle fiber types analogous to the tongue and larynx (Connor et al., 2008). The HN is the brainstem nucleus that controls the tongue musculature, including the GG muscle (Nowinski et al., 2012). The GG is the main protrusive muscle of the tongue, is active during bolus manipulation and transport (Fregosi and Fuller, 1997; Gilliam and Goldberg, 1995; Miller, 2002; Sauerland and Mitchell, 1975), and is active throughout the swallow (Perlman et al., 1999). The NA is the brainstem nucleus that controls the intrinsic laryngeal musculature, including the TA (Nowinski et al., 2012). The TA is active during the swallow to protect the airway (Fink, 1974; Gay et al., 1994; Logemann et al., 1992; Pressman, 1941; Stuart, 1891), and creates tension in the vocal folds during vocalization (Story and Titze, 1995). Muscle tissue was placed in an Eppendorf tube and frozen in liquid nitrogen. Samples were stored at -80^{o}C until use.

4.2.5 Measurement of Neurotrophins

Neurotrophins levels were measured in the HN, NA, GG, TA, and control samples. Enzyme Linked Immunosorbent Assays (ELISA) were used to detect protein levels. Whole muscles were homogenized in ice cold PBS buffer. Homogenates were centrifuged and supernatants collected. Overall protein concentration levels were normalized using wet tissue weight (w.t.w) mg/volume (µl). BDNF, NT4, and TrkB protein levels were quantified using selected ELISA kits, (BDNF Emax ImmunoAssay System Kit (Promega, Madison, WI); Rat Neurotrophin 4 (NT4) ELISA Kit (Cusabio); Rat TrkB (Tyrosine receptor kinase B) ELISA Kit (MyBioSource), as per the manufacturer's protocol. Unknown protein concentrations were compared with known protein concentrations using a standardized calibration curve provided by the respective manufacturers.

4.2.6 Statistical Analysis

A two-way analysis of variance (ANOVA) was used to examine main and interaction effects of age (2 levels: young adult and old) and training group (2 levels: tongue exercise and no-exercise controls) on the variables listed in Table 1. For the behavioral data, t-tests were performed on the Δ variables from the post-exercise time point to each respective detraining time point to examine the effect of detraining on tongue force and USV characteristics. In addition, Spearman correlations (ρ) were used to examine the relationship between average participation during tongue training, Δ tongue force, and neurotrophin concentration for the different neurotrophins, age groups, training groups, and regions of interest. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC) or Sigma Plot. An α-level of less than 0.05 was established as the criterion for statistical significance. An α-level correction was not used in this study, due to the limited number of multiple comparisons (6) and the increased chance of type two errors with more conservative α-levels . Dr. Glen Leverson, a biostatistician in the UW-Department of Surgery, served as a consultant for all statistical analyses.

For some measures, missing data resulted in a smaller sample sizes for some comparisons, which is reflected in the degrees of freedom for particular tests. Specifically, unexpected animal expiration accounted for some missing tongue force, USV, and neurotrophin data (n=3). The USV analyses also contained missing data when rats did not vocalize in a testing session (n=10-19, depending on the time point). For the neurotrophin analyses, missing data also occurred when ELISA assays could not be used because the standard curve needed for a plate was not at an acceptable level (n=4-17, depending on the measure). Laryngeal neurotrophin analyses were not performed within the 2- and 4-week detraining groups, with the exception of the intensity measure, because significant differences were not found as a function of exercise, and this smaller sample size is reflected in the degrees of freedom for those tests (overall n=32-38). All other available data were used in statistical analyses.

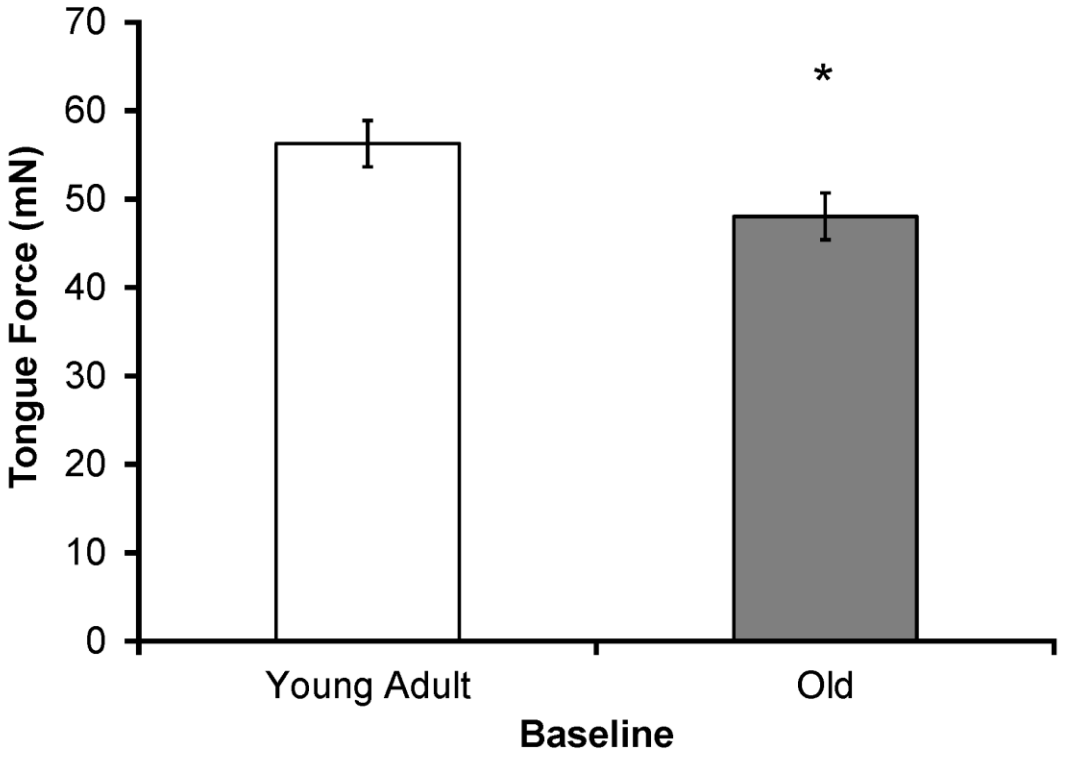
4.3 Results

4.3.1 Tongue Force

4.3.1.1 Age-related Deficits in Tongue Force

Tongue force (mN) was significantly reduced in the old group compared with the young adult group at baseline (Fig. 7; $F_{1,73}$ =4.91, p=0.03), suggesting an age-related tongue weakness in this cohort of aged rats.

Fig. 7. Reduced tongue force in the old group at baseline. Significantly lower (p=0.03) tongue force was found in the old group than the young adult group. * Denotes significant values; error bars represent standard error of the mean.



4.3.1.2 Increased Tongue Force with Exercise

The change in tongue force (Δ mN) from baseline to the post exercise time point was significantly greater in old group compared with young adult group (Fig. 8A, $F_{1, 73} = 12.73$, $p < 0.001$) and in the exercise groups than in the no-exercise control group (Fig. 8B, $F_{1, 73} = 192.02$, $p < 0.001$). This finding indicated that progressive-resistance tongue exercise was associated with increased tongue force versus skill maintenance of the task alone, regardless of age.

4.3.1.3 Decreased Tongue Force with 4 weeks of Detraining in the Old

In the old group alone, tongue force (Δ mN) was significantly decreased at the 4-week detraining time point relative to immediate post exercise levels (Fig. 9A, $p=0.02$). However, as shown in Figure 9B, tongue force at the 2-week and 4-week time points was significantly greater than baseline tongue forces in both age groups ($p < .001$). In combination, these results suggest that detraining did not eliminate the improved tongue forces found following tongue exercise paired with a water swallow, but that the old group experienced a relative decrement in maintenance of post-exercise tongue force levels.

Fig. 8A. Tongue exercise resulted in a greater increase in tongue force in old group. Significantly larger increases ($p < 0.001$) in tongue force were found in the old group. * Denotes significant values; error bars represent standard error of the mean.

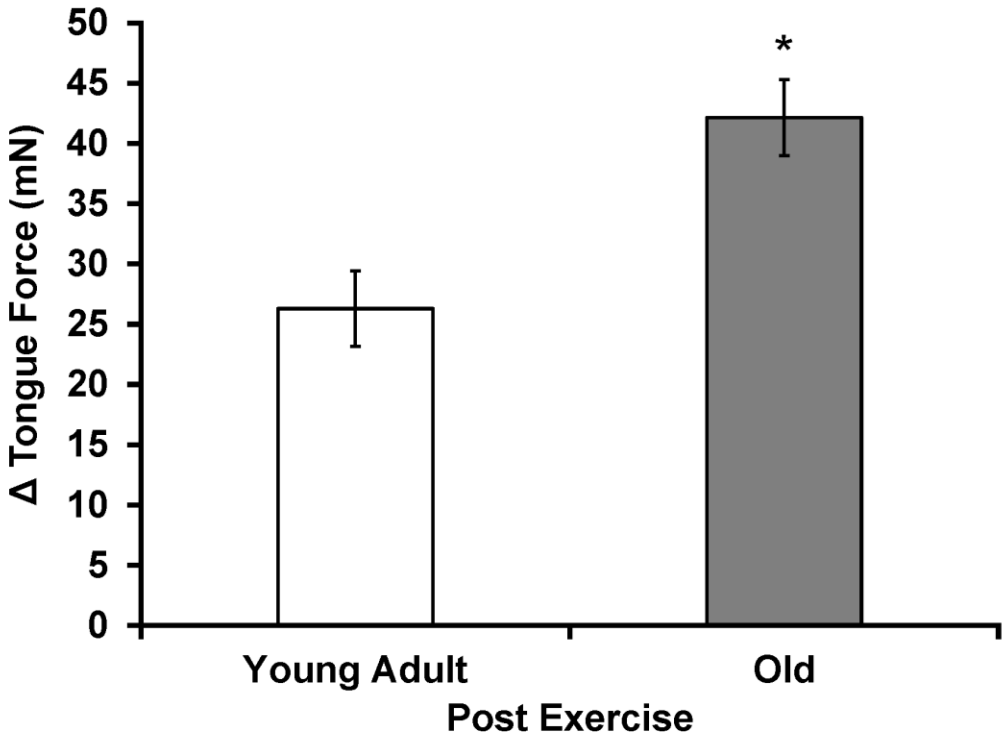


Fig. 8B. Tongue exercise resulted in greater increases in tongue force than skill maintenance (control). A significantly larger increase ($p < 0.001$) in tongue force was observed for both ages in the exercise group than the control group. * Denotes significant values; error bars represent standard error of the mean.

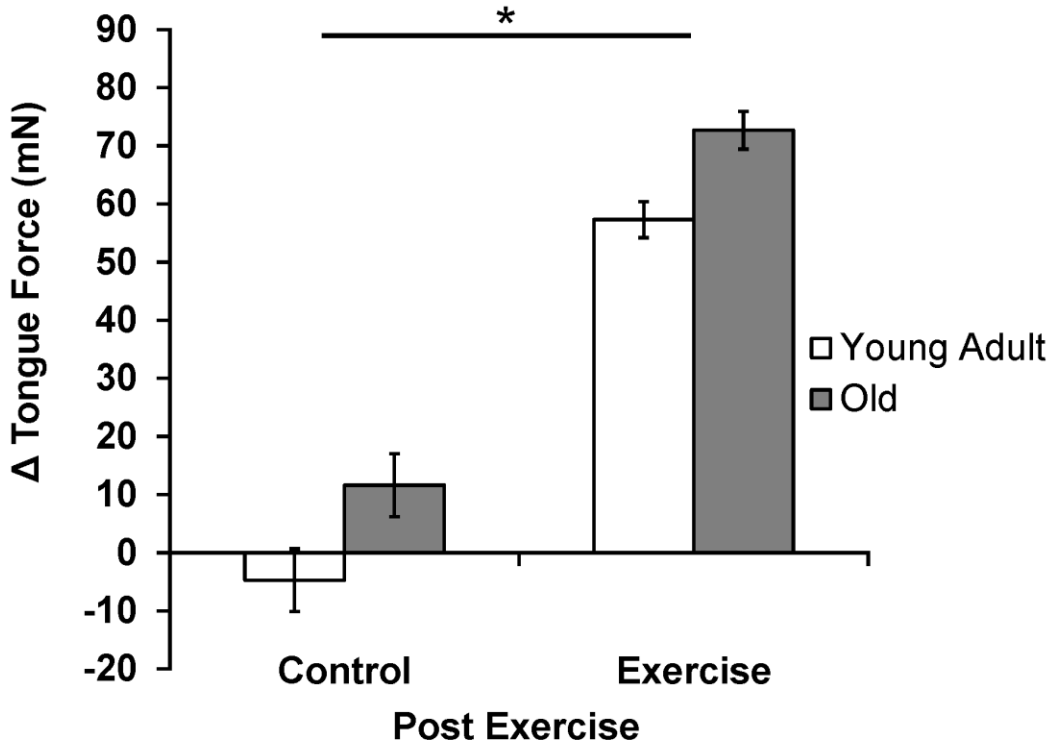


Fig. 9A. Decreased tongue force following 4 weeks of detraining in old group. A significant decrease ($p=0.02$) in tongue force was observed in the old 4-week (4W) detraining group alone. * Denotes significant values; error bars represent standard error of the mean.

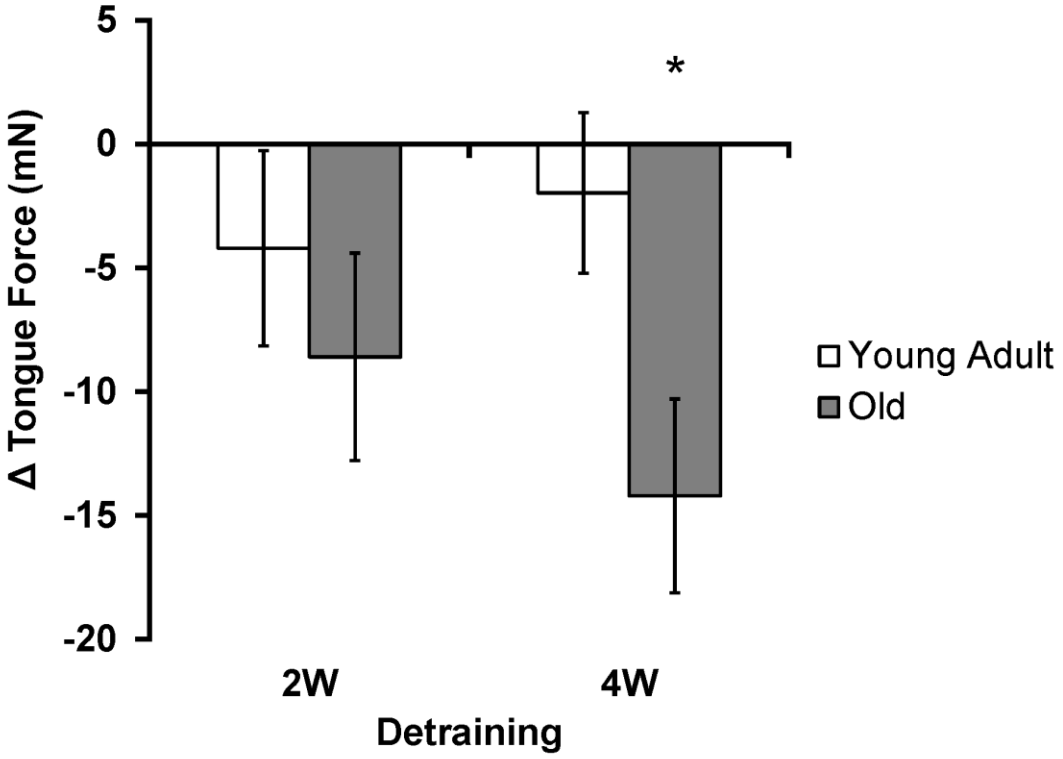
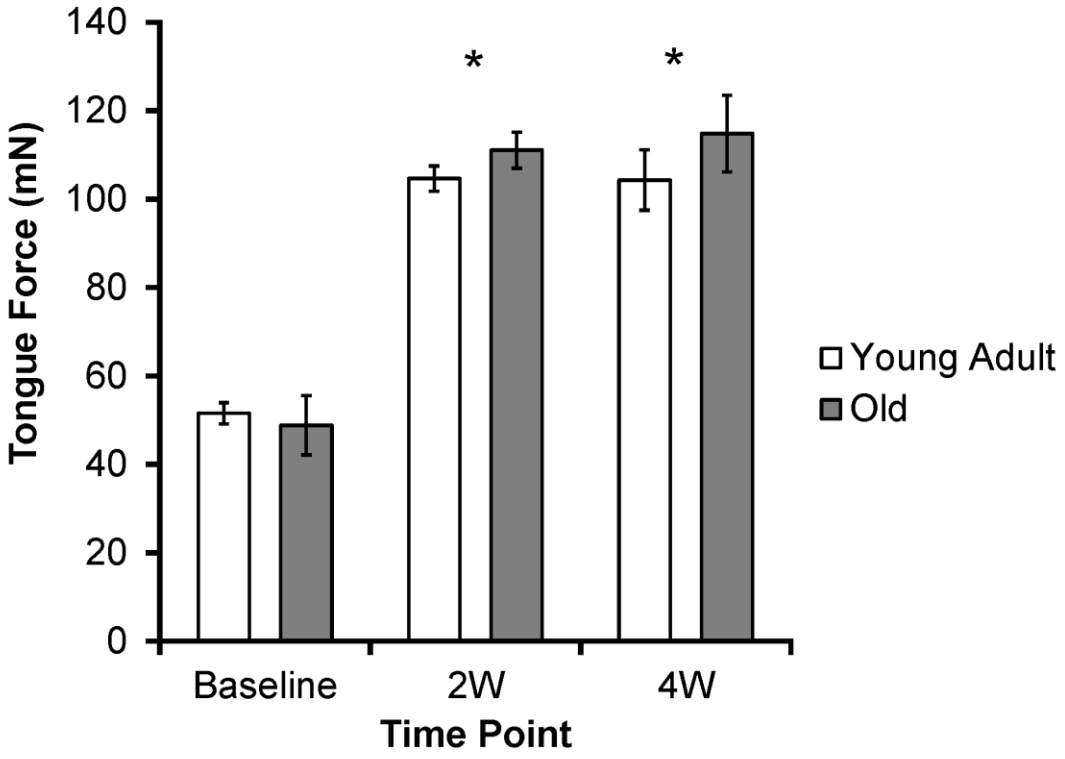


Fig. 9B. Increased tongue force was found after 2 and 4 weeks of detraining compared to baseline. Tongue force at the 2-week (2W) and 4-week (4W) time points was significantly greater than tongue force at baseline ($p < 0.001$). * Denotes significant values; error bars represent standard error of the mean.



4.3.2 Ultrasonic Vocalizations (USVs)

4.3.2.1 Age-related Changes in USVs

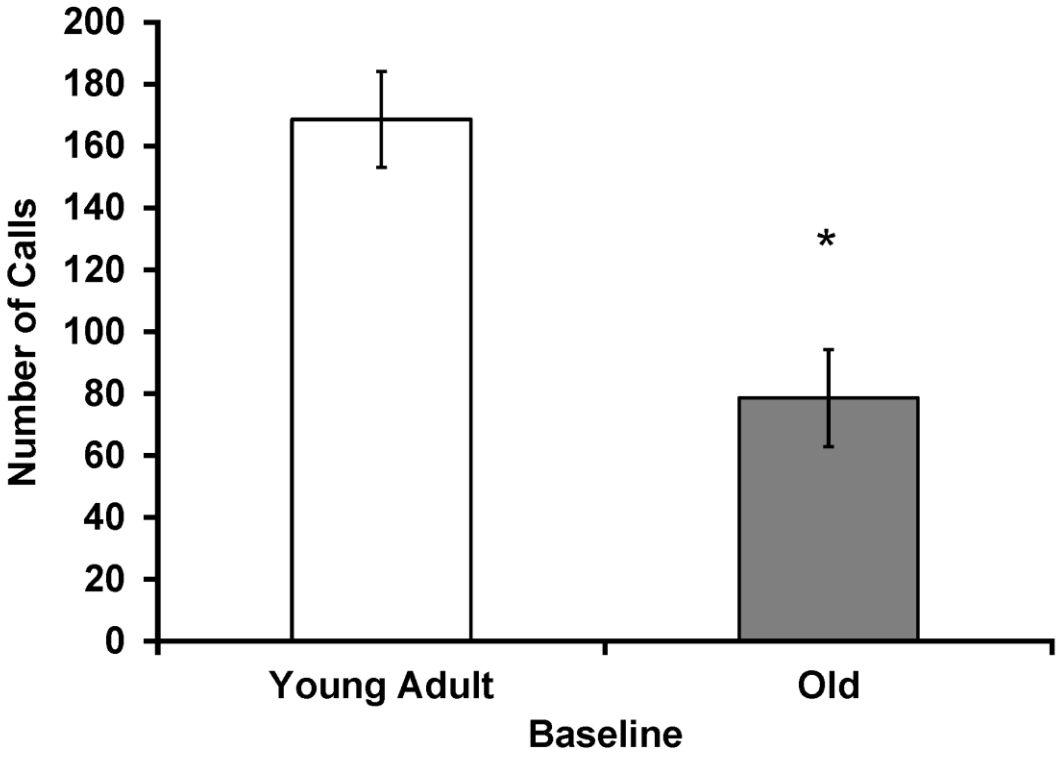
Age-related changes in vocalizations were found in a number of USV variables at baseline. Significant main effects of age were found for number of calls, with a reduced number of calls in the old group (Fig. 10A, $F_{1, 73}=16.68$, $p < 0.001$); average call duration, with an increased call duration in the old group (Fig. 10B, $F_{1, 63}=5.05$, $p=0.03$); and average peak frequency, with a reduced peak frequency in the old group (Fig. 10D, $F_{1, 63}=13.87$, $p < 0.001$). However, there was not a significant main effect of age on average bandwidth (Fig. 10E, $F_{1, 63}=0.31$, $p=0.58$). In addition, a significant interaction effect (age vs condition) was found for average call intensity ($F_{1, 63}=10.32$, $p=0.002$). Post-hoc testing revealed reduced average intensity in the old exercise group (Fig. 10C, $p < 0.001$), along with decreased average intensity in the old exercise group compared with the old control group (Fig. 10C, $p=0.007$). These findings indicate that rats in the old exercise group had the quietest calls at baseline.

4.3.2.2 Effect of Exercise on USVs

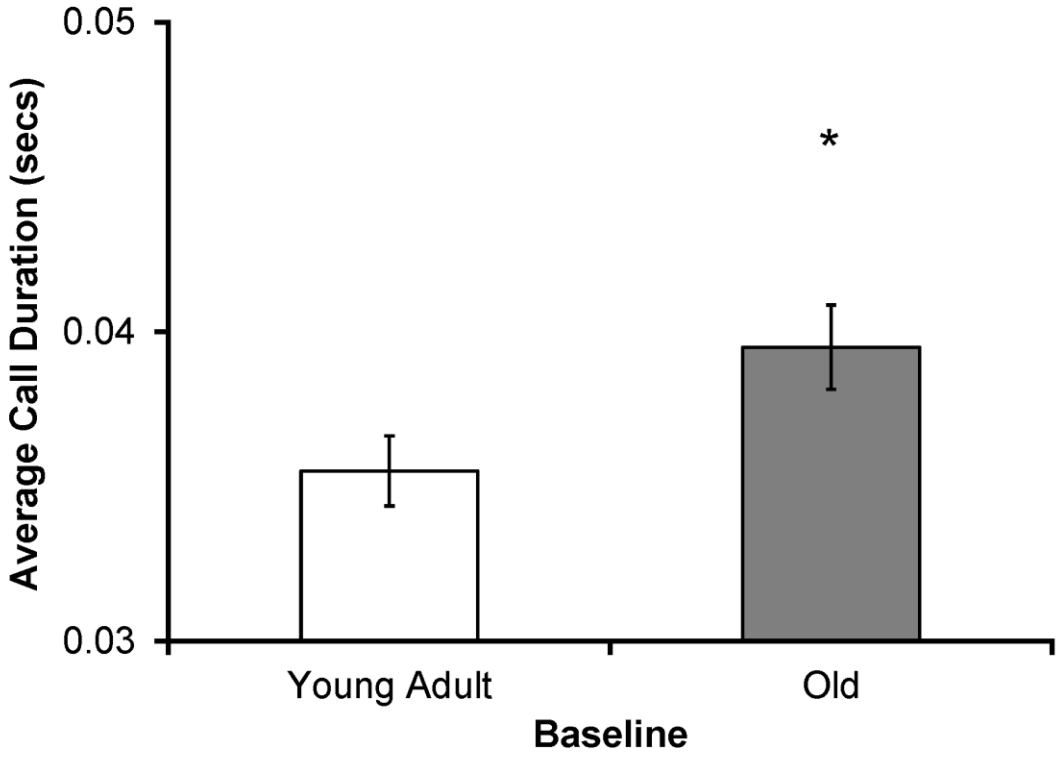
The change in USV characteristics (Δ unit) from baseline to the post exercise time point showed no consistent differences based on condition. Specifically, there were not significant main effects of condition for number of calls (Fig. 11A, $F_{1, 73}=1.12$, $p=0.29$), average call duration (Fig. 11B, $F_{1, 54}=1.05$, $p=0.31$), average peak frequency (Fig. 11D, $F_{1, 54}=0.27$, $p=0.60$) and average bandwidth (Fig. 11E, $F_{1, 54}=0.87$, $p=0.36$). There was a significant main effect of condition on average intensity (Fig. 11C, $F_{1, 54}=4.63$, $p=0.04$), with less reductions in average intensity following tongue exercise, indicating that rats in the exercise group maintained the intensity of their calls over the 8-week training period, while rats in the control group got quieter.

Fig. 10 (A-E). Age-related changes in USV characteristics (A-E) at baseline. (A) Number of calls: Significantly lower ($p < 0.001$) number of calls in the old group. (B) Average call duration: Significantly greater ($p=0.03$) average duration of calls in the old group. (C) Average call intensity: Significantly reduced ($p < 0.001$) average intensity of calls in the old exercise group, and a decreased average intensity in the old exercise group compared with the old control group ($p=0.007$). (D) Average Peak Frequency: Significantly lower ($p < 0.001$) peak frequency of calls in the old group. (E) Average bandwidth: No significant age-related differences ($p=0.58$). * Denotes significant values; error bars represent standard error of the mean.

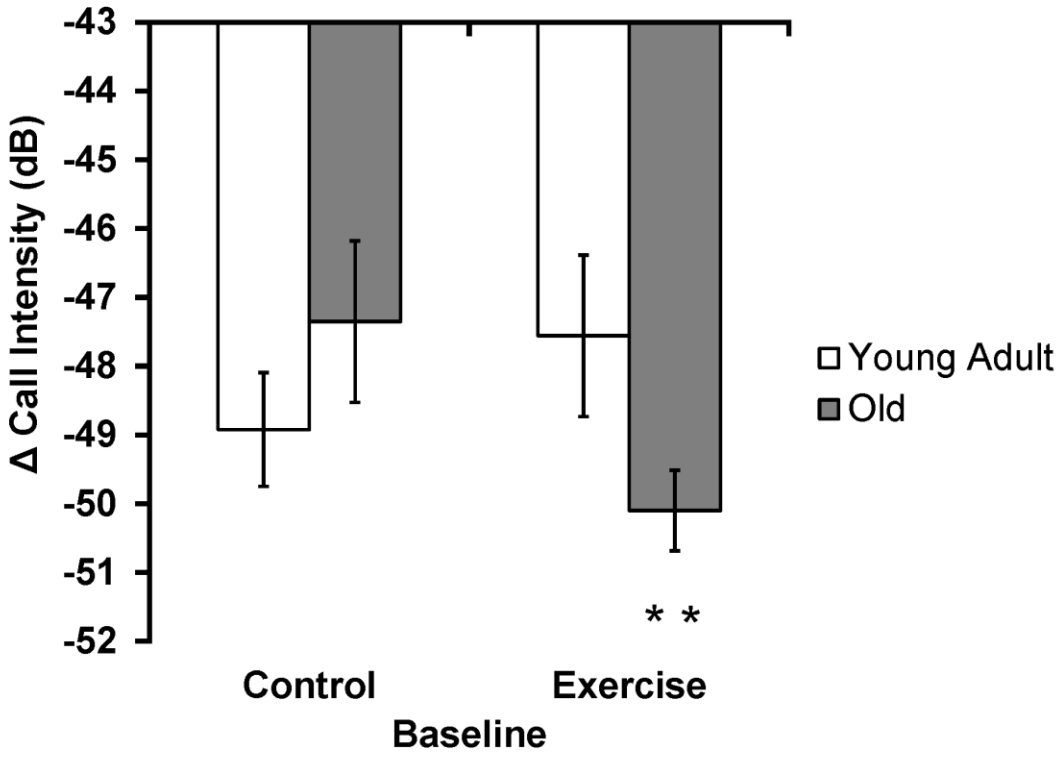
10 (A): Number of calls



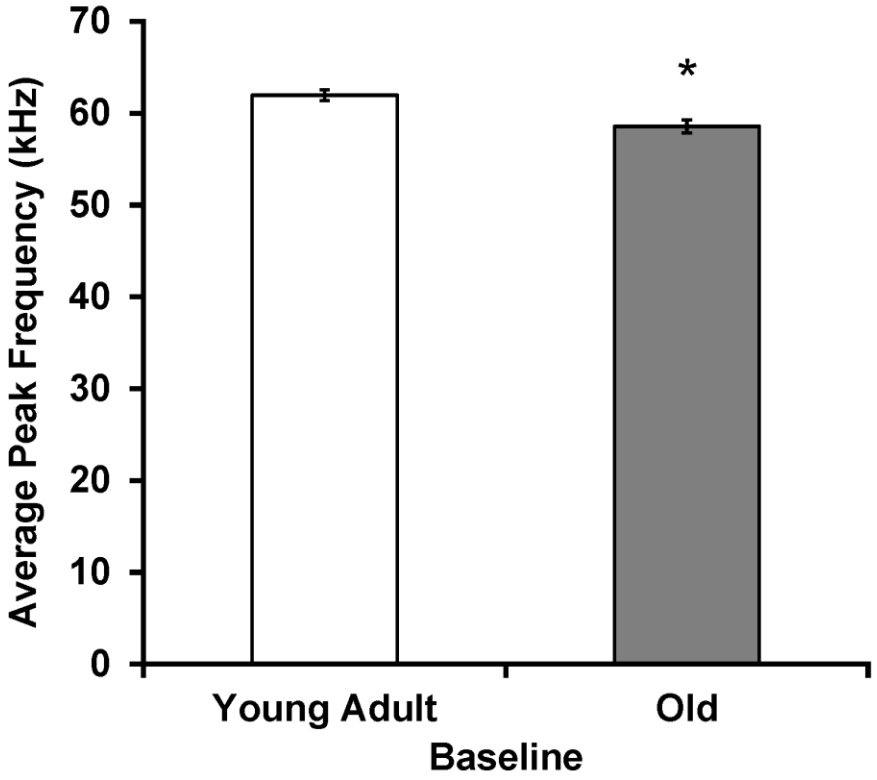
10 (B): Average call duration



10 (C): Average call intensity. Y axis represents normalized intensity (dB), with smaller numbers representing decreased intensity levels relative to maximum.



10 (D): Average peak frequency



10 (E): Average call bandwidth

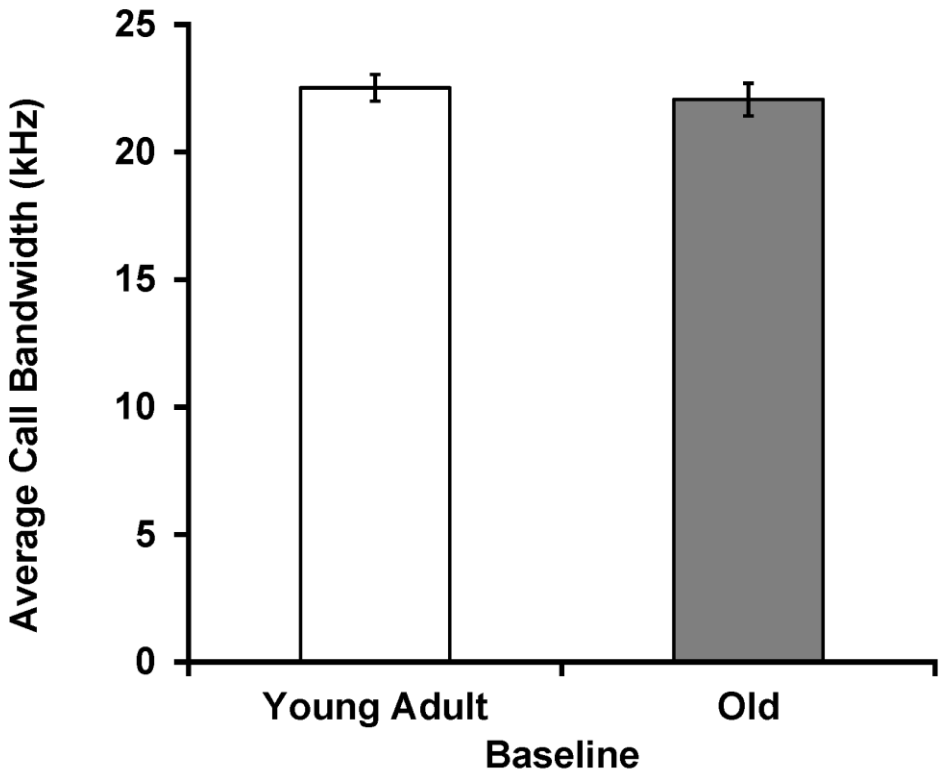
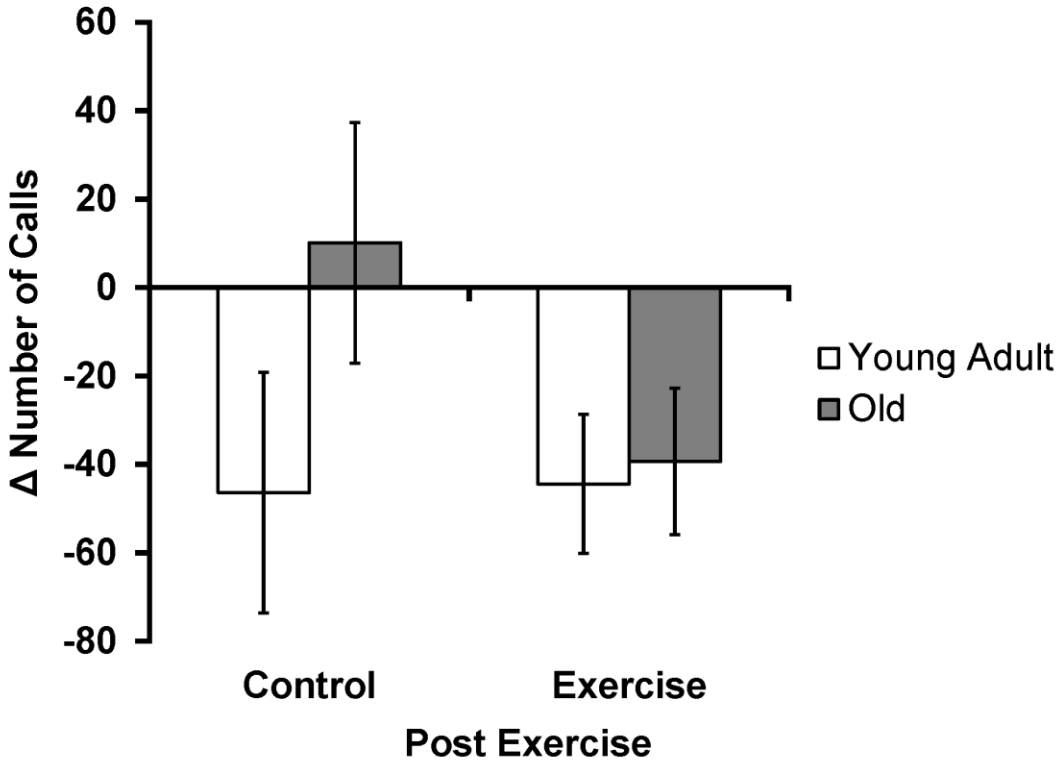
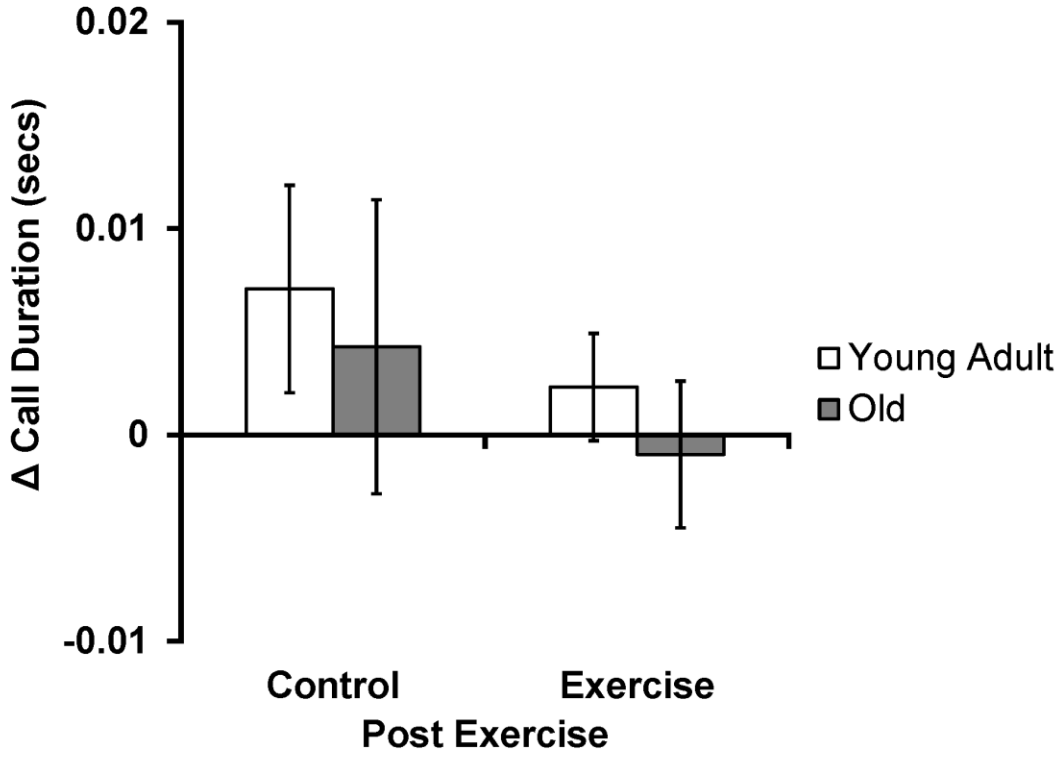


Fig. 11 (A-E). Effect of tongue exercise on USV characteristics. Maintenance of call intensity was found with tongue exercise at the post exercise time point (C). (A) Number of calls: No significant difference in number of calls ($p=0.29$) between control and exercise group. (B) Average call duration: No significant difference in average call duration ($p=0.31$) between control and exercise group. (C) Average call intensity: Significantly less ($p=0.04$) change in intensity of calls in the exercise than the control group. (D) Average Peak Frequency: No significant difference in average peak frequency ($p=0.60$) between control and exercise groups. (E) Average bandwidth: No significant difference in average bandwidth ($p=0.36$) between control and exercise groups. * Denotes significant values; error bars represent standard error of the mean.

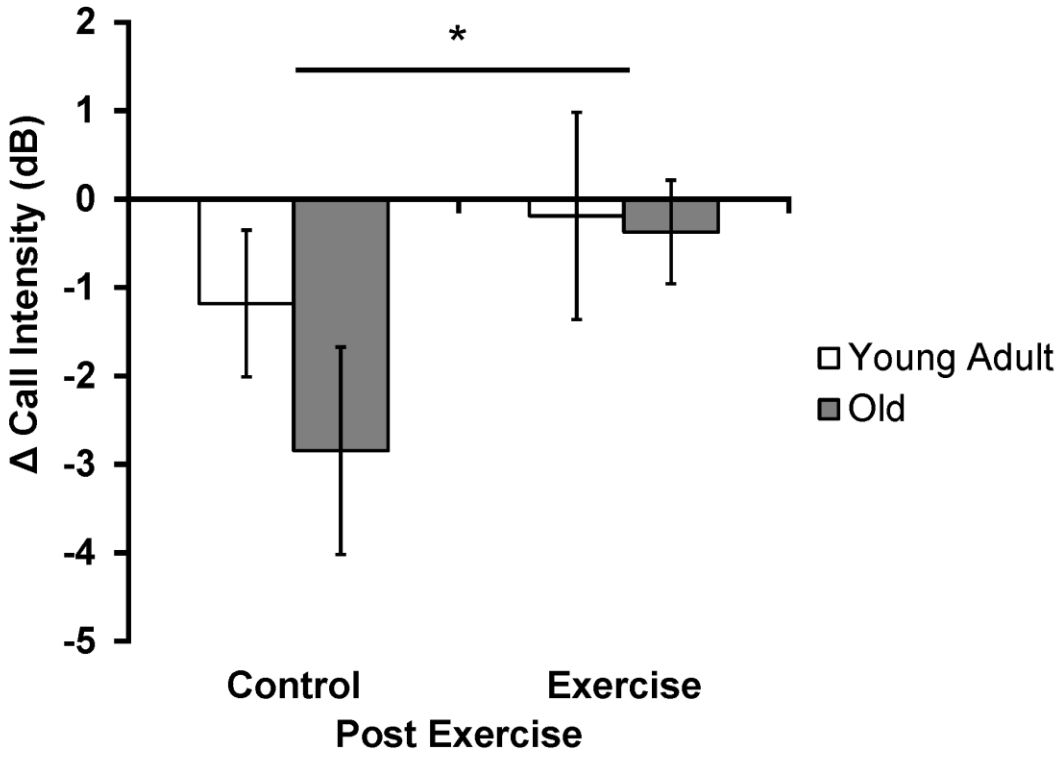
11 (A): Δ Number of calls



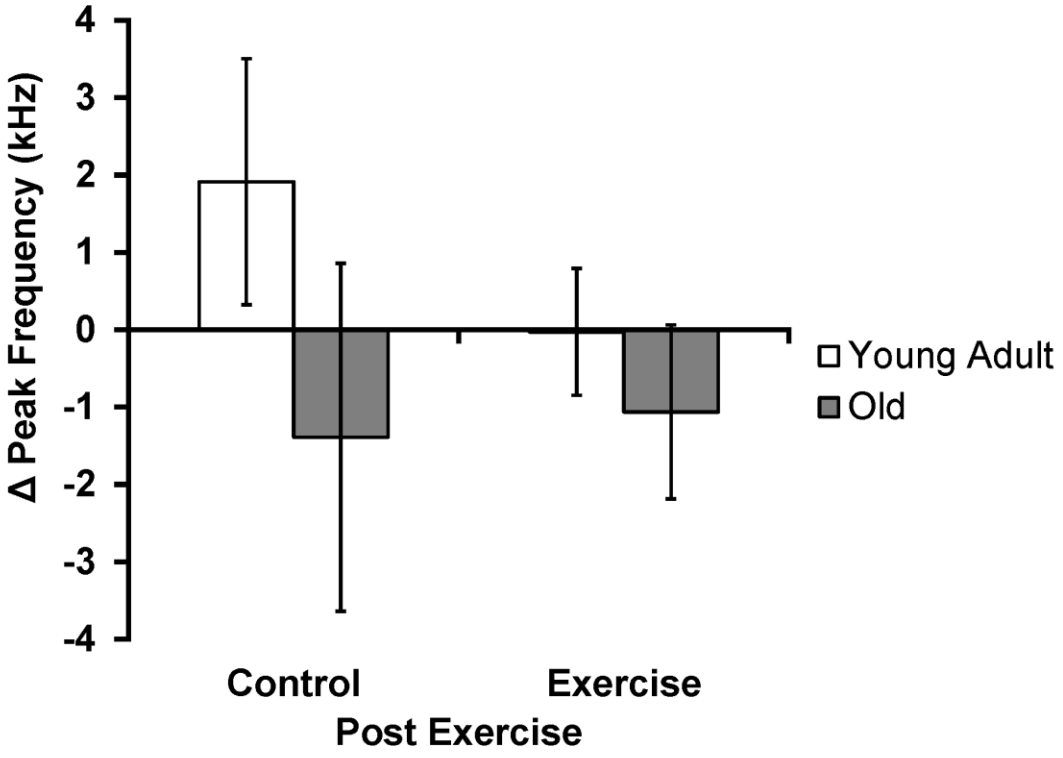
11 (B): Δ Call duration



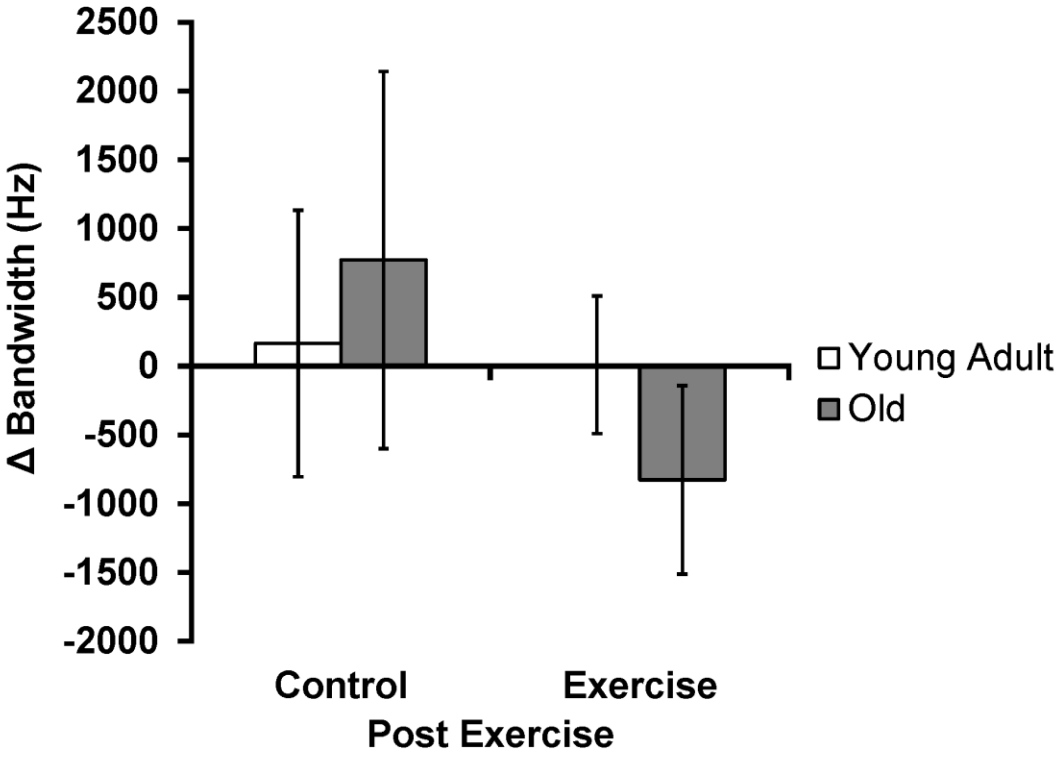
11 (C): Δ Call intensity. Y axis represents change in normalized intensity (dB).



11 (D): Δ Peak frequency



11 (E): Δ Call bandwidth

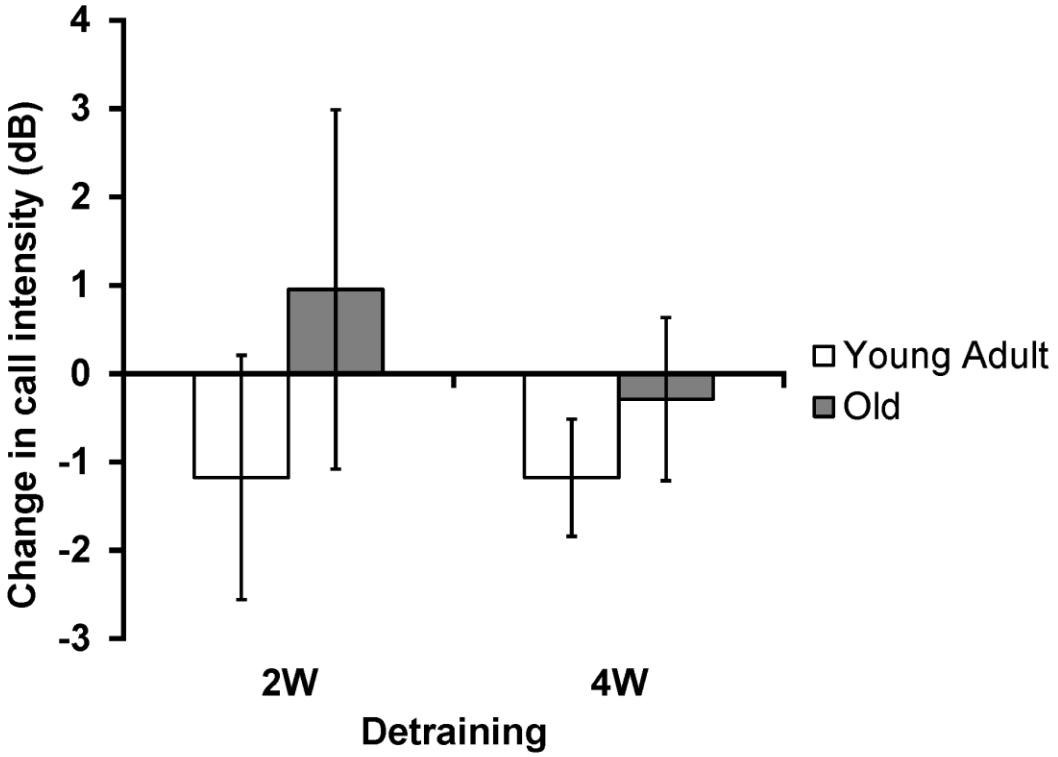


4.3.2.3 No Change in Intensity with Detraining

There was no significant change in intensity (Δ dB) from the post exercise time point to the 2- and 4-week detraining times points. (Fig. 12, p=1.0).

As a result of the lack of significance found after 8 weeks of tongue exercise paired with a water swallow on all other USV characteristics, changes in all USV characteristic following detraining, except for average intensity (above), are not reported in this manuscript.

Fig. 12. No significant difference in intensity following detraining. No difference (Old 2-week [2W]: p=1.0, Young Adult 2W: p=0.28, Old 4-week [4W]: p=0.84, Young Adult 4W: p=0.25) was found in intensity following detraining in either detraining or age group. * Denotes significant values; error bars represent standard error of the mean.



4.3.3 Neurotrophins

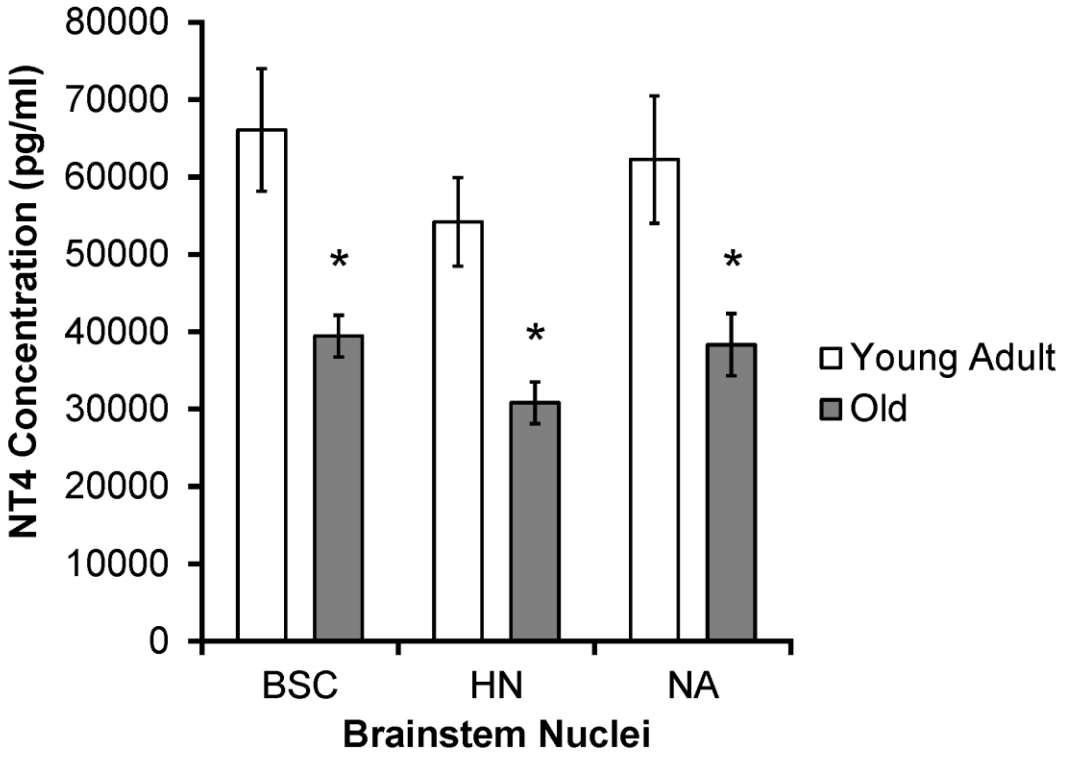
4.3.3.1 Age-related Changes in Neurotrophins

The concentration of NT4 (pg/ml) was significantly lower in the three brainstem nuclei in the old group than in the young adult group. Specifically, the old group had a significantly lower concentration of NT4 in the HN (Fig. 13A, $F_{1, 68} = 13.40$, $p < 0.0001$), NA (Fig. 13A, $F_{1, 34} = 6.45$, $p=0.02$), and BSC (Fig. 13A, $F_{1, 68} = 9.66$, $p=0.003$). There was not a significant difference in NT4 concentration in old group versus the young adult group in the GG (Fig. 13B, $F_{1, 68} = 0.54$, $p=0.47$) or EDL (Fig. 13B, $F_{1, 68} = 2.63$, $p=0.11$). There was a significant increase in the concentration of NT4 in the TA of the old versus young adult group (Fig. 13B, $F_{1, 68} = 5.29$, $p=0.03$).

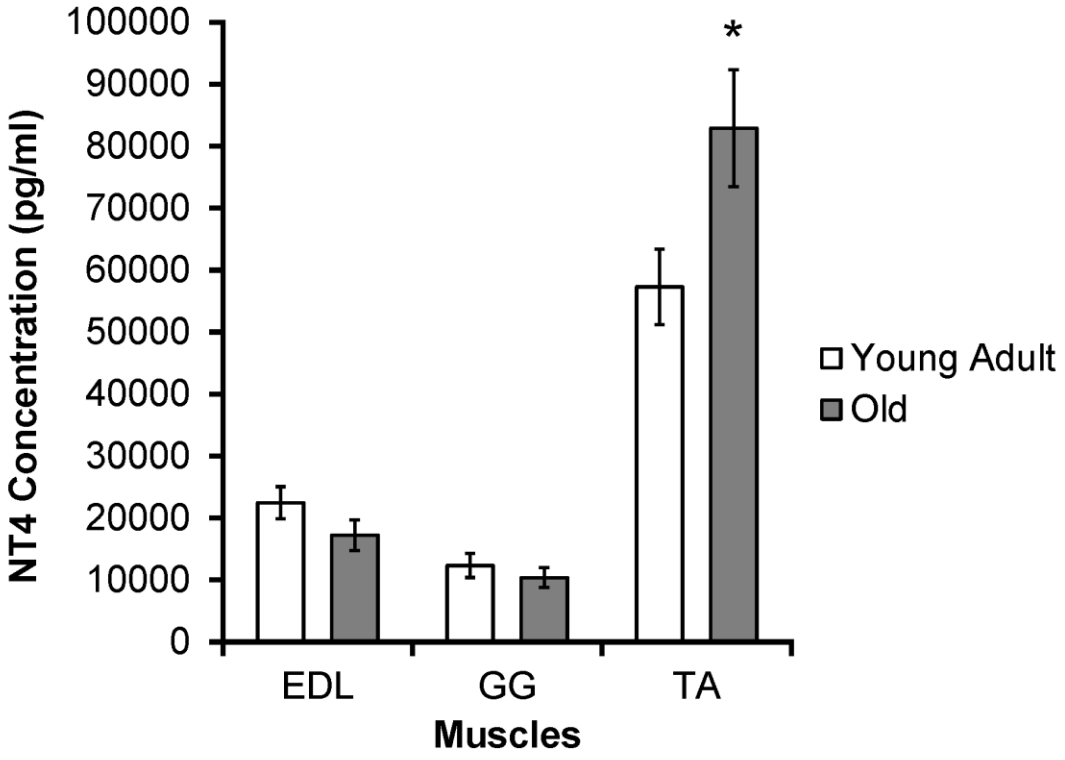
There was a significant interaction effect for age and training group for the concentration of BDNF (pg/ml) in the TA (Fig. 13C, $F_{3,28} = 4.70$, $p=0.04$). Post-hoc testing revealed a significantly lower concentration of BDNF in the TA in the old no-exercise control group than in the young adult no-exercise control group ($p=0.03$). The concentration of BDNF and TrkB did not differ significantly between old and young adult groups in any of the other brainstem nuclei or muscles of interest in this study. See table in the appendix for raw data (means and SEMs).

Fig. 13 (A-C). Age-related differences in neurotrophins. NT4 differences in (A) brainstem nuclei and (B) muscles of interest, (C) BDNF differences in TA. (A) Concentration of NT4 was lower in old groups in all brainstem nuclei: Significantly lower concentration of NT4 in all brainstem nuclei of interest (BSC: $p=0.003$, HN: $p < 0.0001$, NA: $p=0.02$) in the old group than the young adult group. (B) Increased concentration of NT4 in the TA muscle with age: Significantly greater ($p=0.03$) concentration of NT4 in the TA of the old group compared to young adult group. No significant difference in NT4 concentration with age in the EDL ($p=0.11$) or GG ($p=0.47$). (C) Significantly lower ($p=0.03$) concentration of BDNF in the TA of old no-exercise control group vs young no-exercise control group. * Denotes significant values; error bars represent standard error of the mean.

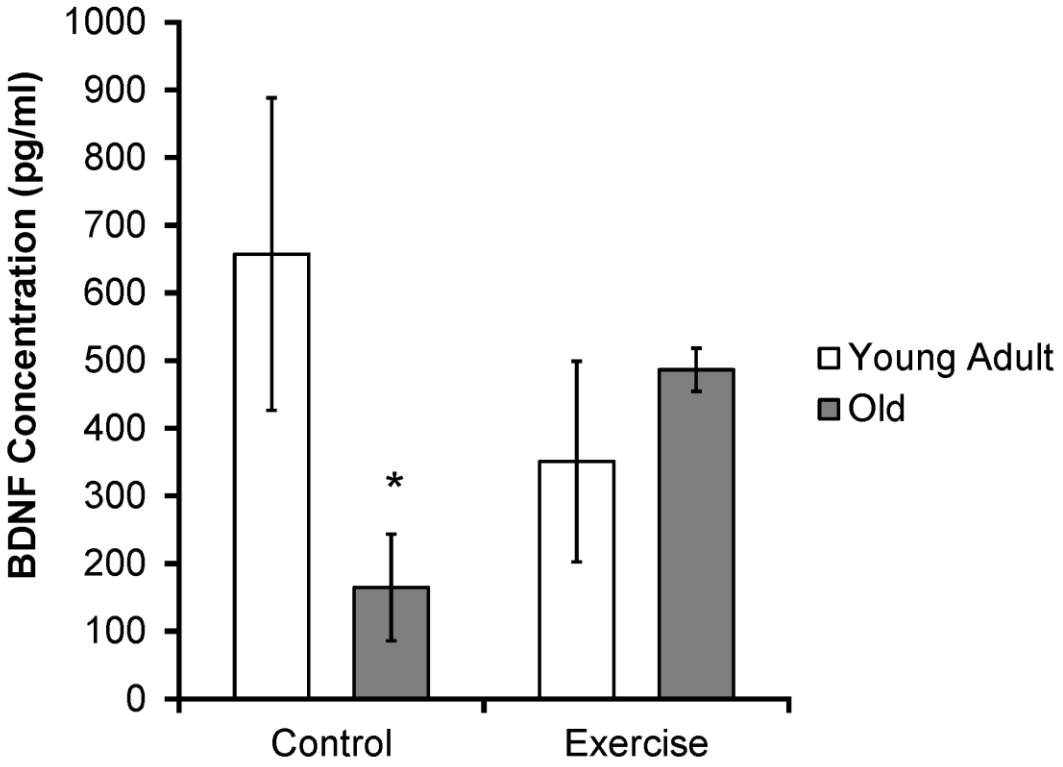
13 (A): Brainstem nuclei



13 (B): Muscles


100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
NT4 Concentration (pg/ml)
*
EDL
GG
TA
Muscles
Young Adult
Old

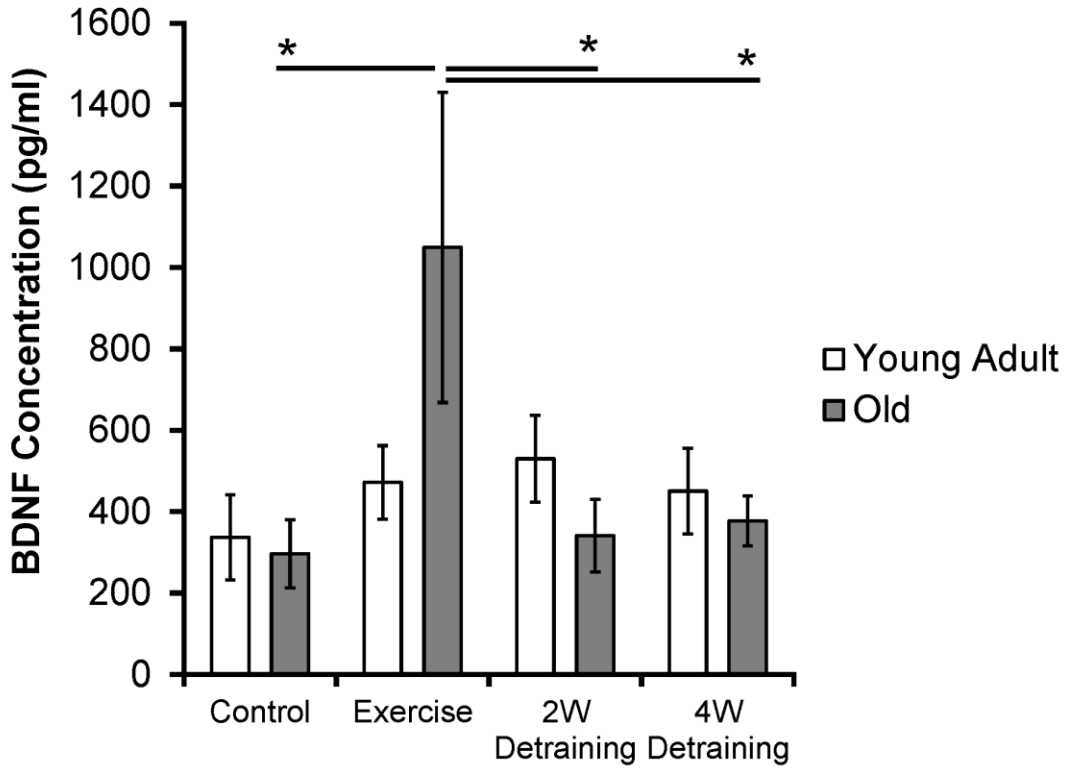
13 (C): BDNF in the TA



4.3.3.2 Differences in BDNF with Level of Training in the HN in Old Group Alone

There was a significant interaction effect for age and training group for the concentration of BDNF (pg/ml) in the HN (Fig. 14A, $F_{3, 62}$ =3.06, p=0.04). Post-hoc testing revealed that the old exercise group had a significantly higher concentration of BDNF in the HN compared to the old no-exercise control group ($p < 0.001$). In addition, both the old 2-week ($p < 0.001$) and old 4-week (p=0.002) detraining groups had a significantly lower concentration of BDNF than the old exercise group. However, there was no significant difference between the young adult exercise and no-exercise control groups (p=0.5) or young adult 2-week and 4-week detraining groups, respectively (p=0.92, p=0.44). These data suggest that a training effect was observed for BDNF concentration in the HN in only the old group.

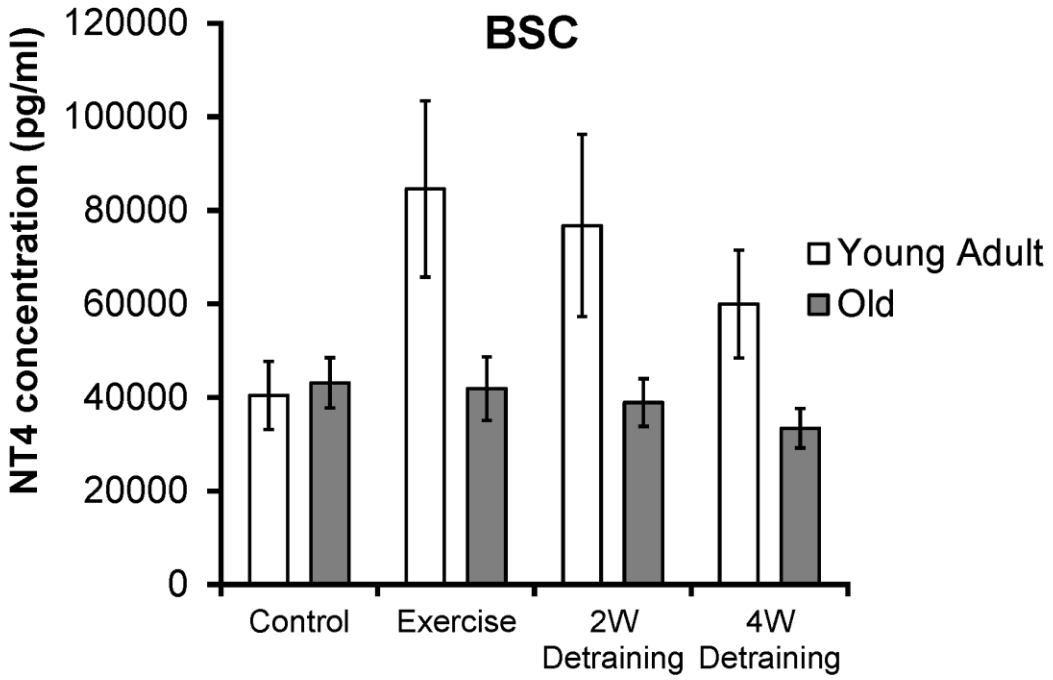
Fig. 14A. Age and exercise interaction effects in the HN. A significant increase ($p < 0.001$) in BDNF concentration was found in the old exercise group compared with the old control group, and a significant decrease ($p < 0.001$, $p=002$) in BDNF concentration was observed between the old exercise group and old 2W and 4W groups, respectively. There was no significant difference between the exercise group and the other training groups (control: $p=0.5$, 2W: $p=0.92$, 4W: $p=0.44$) for the young adult group. * denotes significant values; error bars represent standard error of the mean.



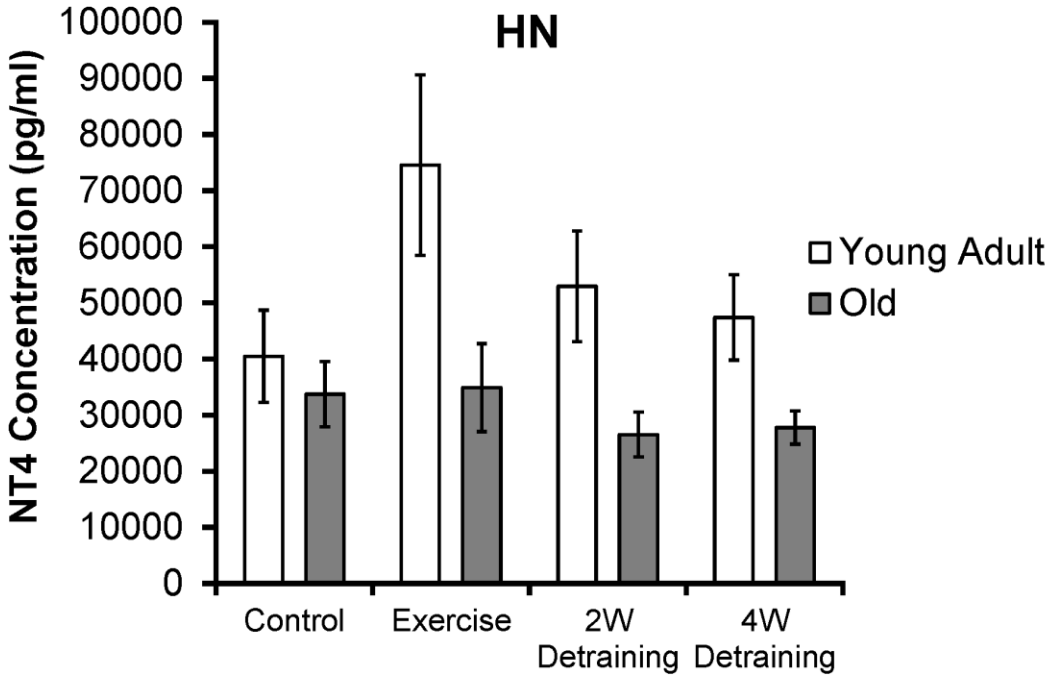
The concentration of TrkB and NT4 did not differ significantly with exercise or detraining in any of the brainstem nuclei or muscles in the old or young adult groups. A pattern similar to the exercise related differences in the HN in the old group was visible in 3 locations from the young adult NT4 data based on means alone (Fig 14B). However due to increased variability these data were not significant (Fig. 14B; BSC: $F_{3, 68}$ =1.47, p=0.23, HN: $F_{3, 68}$ =1.18 p=0.32, EDL: $F_{3, 68}$ =1.90, p=0.14). To review all neurotrophin raw data see table in the appendix.

Fig. 14B. NT4 differences in the young adult group showed a similar pattern of exercise effects as BDNF in the old group in HN, without reaching significance. (1) No significant interaction effect ($F_{3, 68}$ =1.47, p=0.23) in NT4 concentration in BSC. (2) No significant interaction effect ($F_{3, 68}$ =1.18, p=0.32) in NT4 concentration in HN. (3) No significant interaction effect ($F_{3, 68}$ =1.90, p=0.14) in NT4 concentration in EDL. * Denotes significant values; error bars represent standard error of the mean.

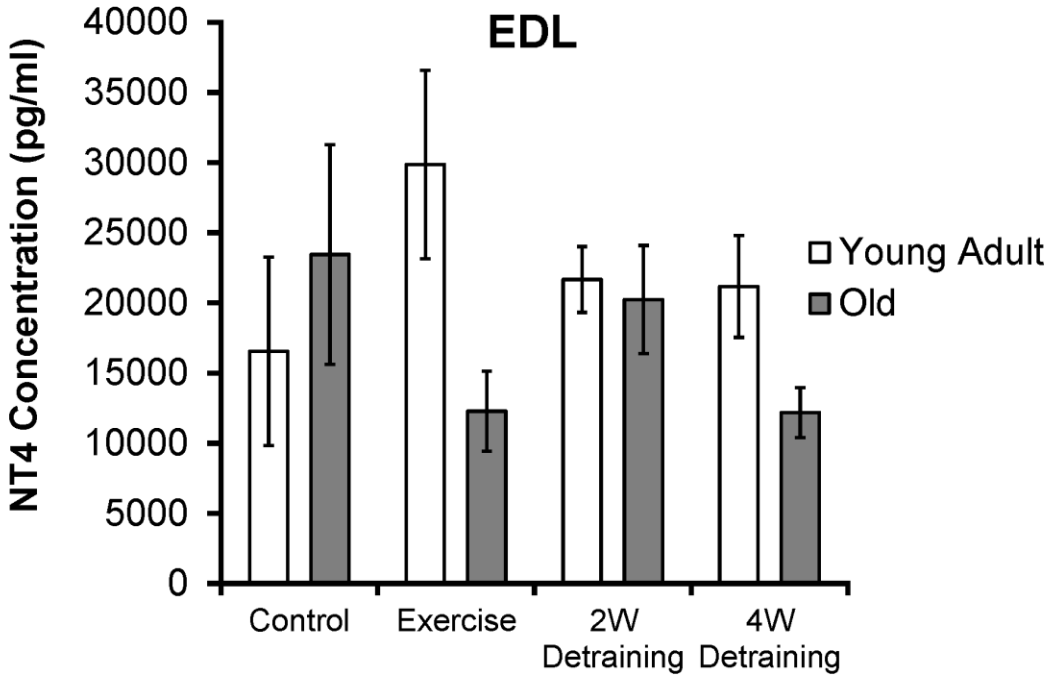
14B1: BSC



14 (B2): HN


HN
NT4 Concentration (pg/ml)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Young Adult
Old
Control
Exercise
2W Detraining
4W Detraining

14 (B3): EDL


EDL
NT4 Concentration (pg/ml)
40000
35000
30000
25000
20000
15000
10000
5000
0
Control
Exercise
2W Detraining
4W Detraining
Young Adult
Old

4.3.4 Correlations

4.3.4.1a Participation was Positively Correlated with Tongue Force, but not USV characteristics, regardless of Age

For the old and young adult groups, average participation (i.e., average number of licks above the individual force threshold set during training [licks/session]) was moderately correlated with maximum tongue force in the old group and strongly correlated in the young adult group (Fig. 15, (A); Old: $\rho=0.62$, $p < 0.0001$ (B) Young Adult: $\rho=0.78$, $p < 0.0001$). However, there were no significant correlations between average participation and any of the USV characteristics in this study ($p > 0.05$). These significant correlations suggest that increases in behavioral tongue force were directly related to participation in the tongue exercise protocol and training condition.

4.3.4.1b Change in Tongue Force with Tongue Exercise was Positively Correlated with Change in Intensity.

For all rats, the change in tongue force from baseline to the post-exercise time point (Δ mN) was moderately correlated with the change in intensity from baseline to the post-exercise time point (Δ dB) ($\rho=0.32$, $p=0.01$). This correlation suggests that the significant difference in intensity between the exercise and control groups at the post exercise time point (Fig. 11C) is related to level of improvement in tongue force with tongue exercise.

4.3.4.2 BDNF Concentration in the HN and Participation

In the old group, BDNF concentration in the HN approached a positive, but weak, correlation with average participation (Fig. 16 (A), Old: $\rho=0.34$, $p=0.05$). Other measures of tongue function in the old group were not significantly correlated with BDNF concentration in the HN, including Δ maximum tongue force (Fig. 16 (B), Old: $\rho=0.06$, $p=0.73$) and maximum tongue force (Fig. 16 (C), Old: $\rho=-0.09$, $p=0.60$). In the young adult group, correlations for all measures of tongue function were not significantly

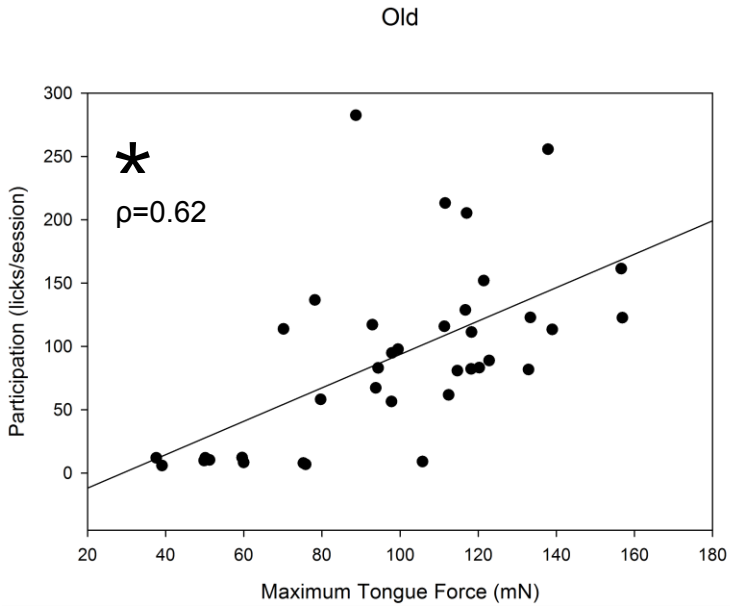
correlated with BDNF concentration in the HN (Fig. 17 (A-C); (A), Average participation: ρ=0.08, p=0.62, (B), Δ Maximum tongue force: ρ=0.22, p=0.20, (C), Maximum tongue force ρ=0.11, p=0.50). These data indicated that while BDNF concentration and participation in tongue exercise may be weakly associated in the old, other mechanisms may be related to the behavioral improvement in tongue force in the young adult group.

4.3.4.3 Neurotrophin Concentrations in the GG were linked to Skill Maintenance

BDNF and NT4 concentrations in the GG were strongly correlated with average participation in the young adult control group, and not correlated with average participation in the old control group (Fig 18 (A), BDNF Young Adult: ρ=0.74, p=0.04; BDNF Old: ρ=0.64, p=0.07; Fig 18 (B), NT4 Young Adult: ρ=0.70, p=0.03; NT4 Old: ρ=0.55, p=0.09). There was also a strong positive correlation between NT4 concentration in the GG and BDNF and TrkB concentrations in the GG in the old control group (Fig 18 (C&D); (C), NT4 vs. BDNF: ρ=0.86, p=0.002, (D), NT4vsTrkB: ρ=0.74, p=0.03). This was not correlated in the young adult group (Fig 18 (C&D); (C), NT4 vs. BDNF: ρ=0.63, p=0.1, (D), NT4 vs. TrkB: ρ=0.53, p=0.12). A possible explanation for these findings is that BDNF and TrkB increase with NT4 to improve skill learning in the old group, and to maintain the skill during exercise because the correlation between NT4 concentration and BDNF concentration in the GG in present in the old exercise group as well (ρ=0.79, p=0.03).

Fig. 15 (A&B). Participation was positively correlated with tongue force regardless of age. Tongue force and participation were moderately correlated in the old (A) and strongly correlated in the young adult (B) groups, respectively (Old: $\rho=0.62$, Young Adult: $\rho=0.78$, Both: $p < 0.0001$). * Denotes significant values; ρ value represented by diagonal line.

15 (A):


Old
*
ρ=0.62
Participation (licks/session)
Maximum Tongue Force (mN)
300
250
200
150
100
50
0
20
40
60
80
100
120
140
160
180

15 (B):

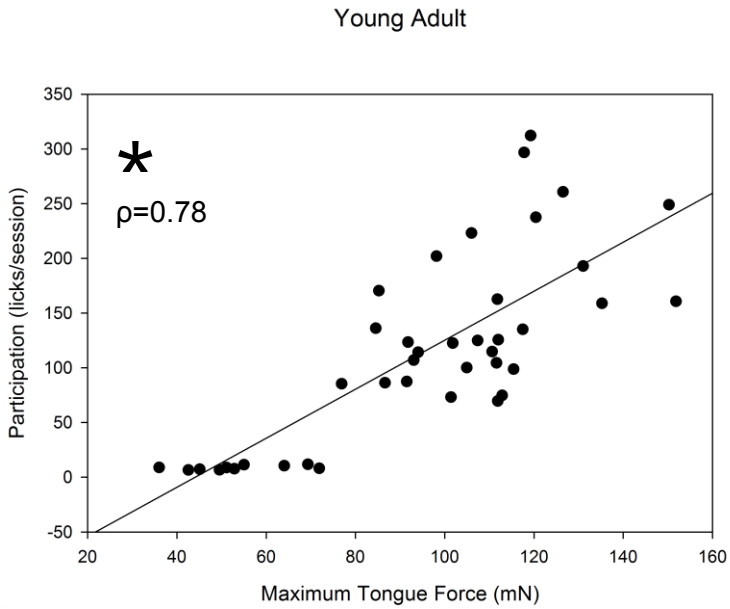
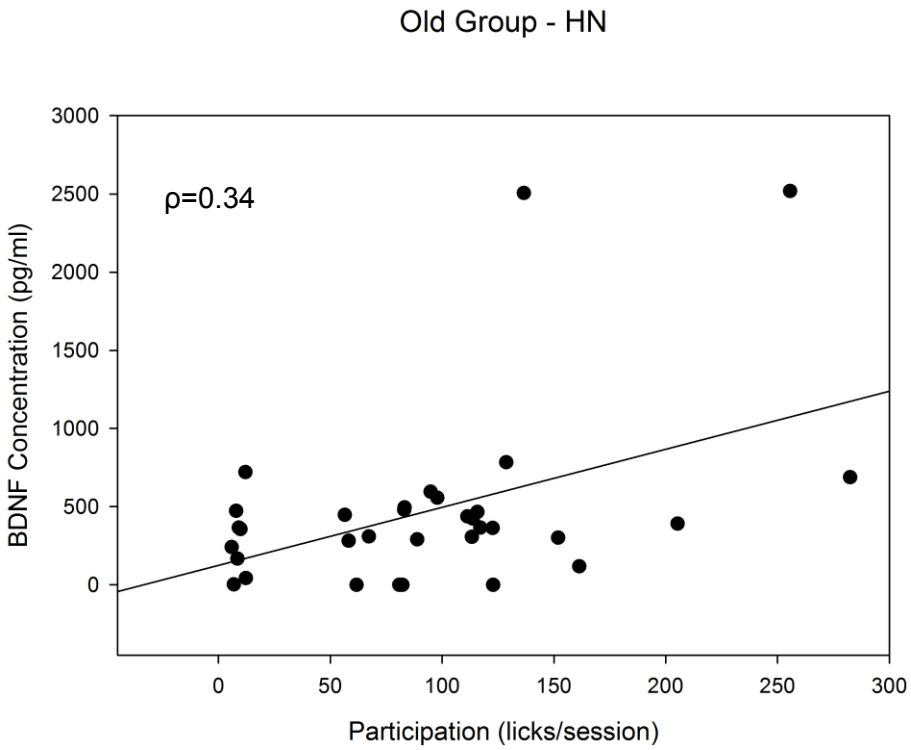
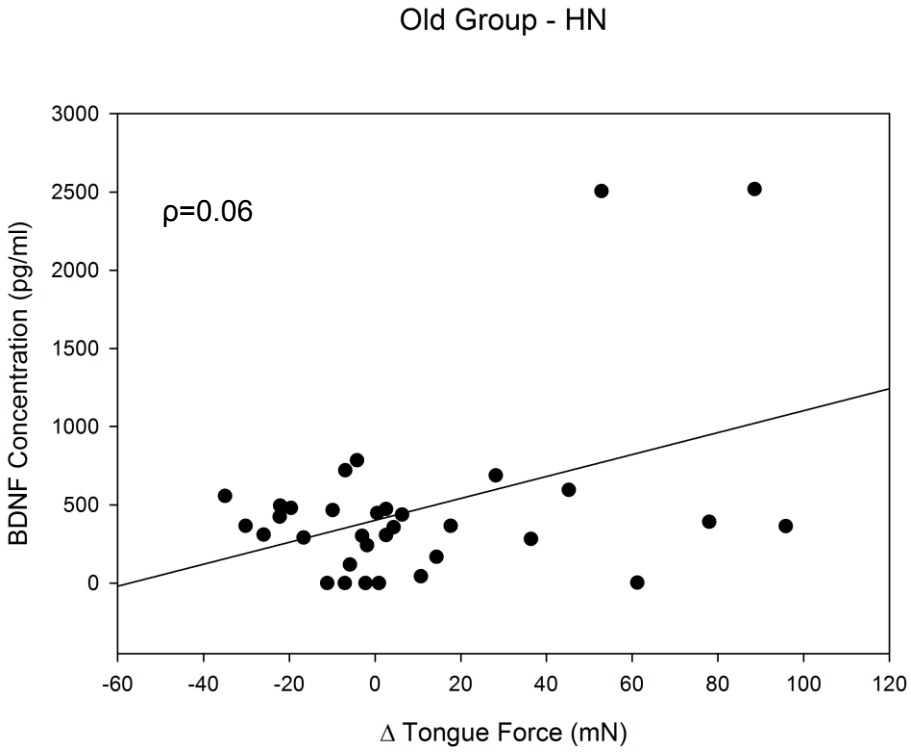

Young Adult
*
ρ=0.78
Participation (licks/session)
Maximum Tongue Force (mN)
350
300
250
200
150
100
50
0
-50
20
40
60
80
100
120
140
160

Fig. 16 (A-C). BDNF concentration in the HN approached a significant positive correlation with participation in the old. BDNF concentration in the HN in the old approached a positive correlated with average participation (A), but was not correlated with Δ maximum tongue force (B), or maximum tongue force (C), respectively (Average participation: ρ=0.34, p=0.05, Δ Maximum tongue force: ρ=0.06, p=0.73, Maximum tongue force: ρ=0.-0.09, p=0.60).* Denotes significant values; ρ value represented by diagonal line.

16 (A):


Old Group - HN
ρ=0.34
BDNF Concentration (pg/ml)
3000
2500
2000
1500
1000
500
0
0
50
100
150
200
250
300
Participation (licks/session)

16 (B):


Old Group - HN
ρ=0.06
BDNF Concentration (pg/ml)
3000
2500
2000
1500
1000
500
0
-60
-40
-20
0
20
40
60
80
100
120
Δ Tongue Force (mN)

16 (C):

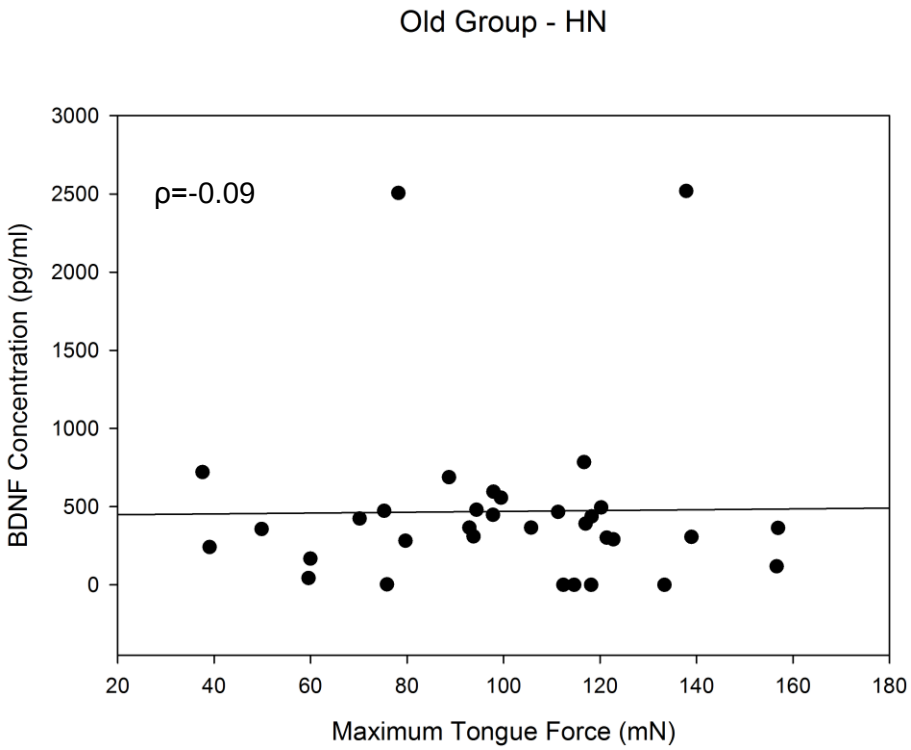
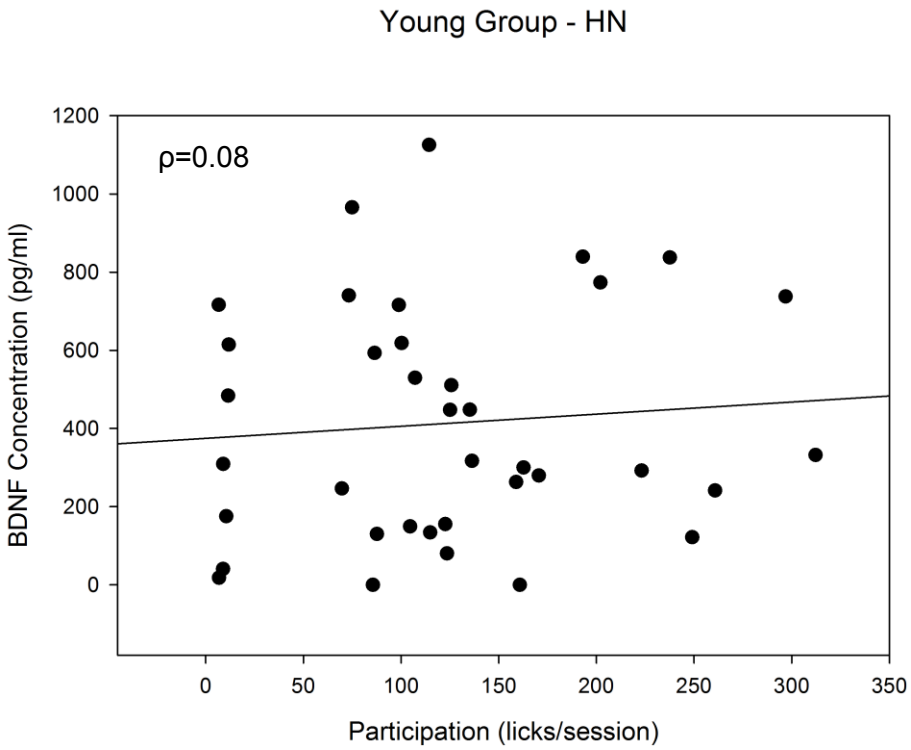
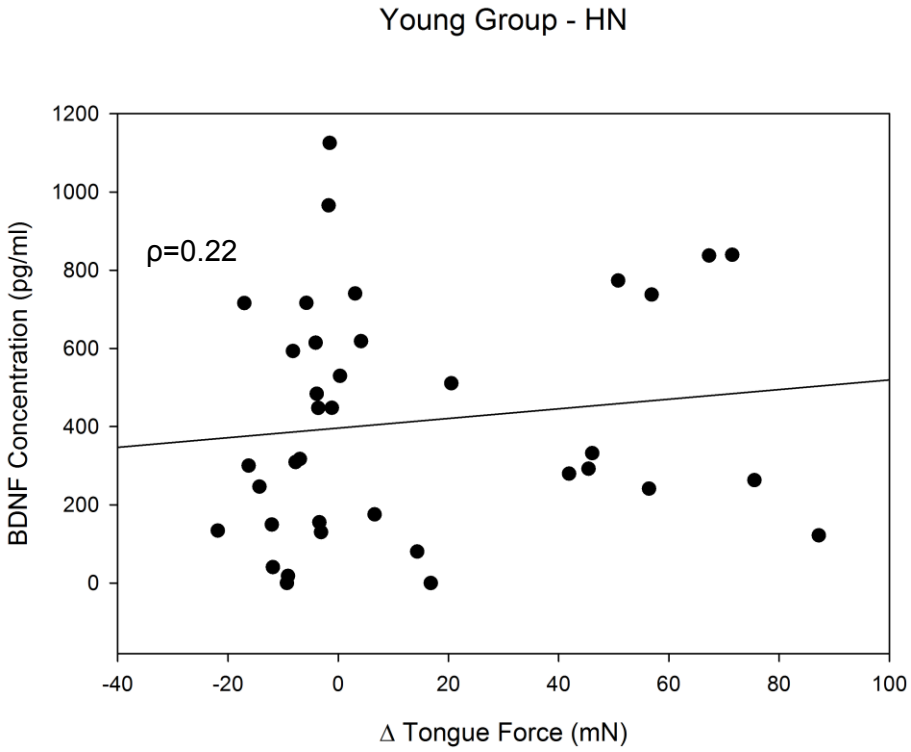

Old Group - HN
ρ=-0.09
BDNF Concentration (pg/ml)
3000
2500
2000
1500
1000
500
0
20
40
60
80
100
120
140
160
180
Maximum Tongue Force (mN)

Fig. 17 (A-C). BDNF concentration was not correlated with tongue measures in the young. Average participation (A) Δ maximum tongue force (B) and maximum tongue force (C) were all weakly correlated with BDNF concentration in the young. (Average participation: $\rho=0.08$, $p=0.62$, Δ Maximum tongue force: $\rho=0.22$, $p=0.20$, Maximum tongue force: $\rho=0.11$, $p=0.50$).* Denotes significant values; ρ value represented by diagonal line.

17 (A):


Young Group - HN
ρ=0.08
BDNF Concentration (pg/ml)
Participation (licks/session)
0
200
400
600
800
1000
1200
0
50
100
150
200
250
300
350

17 (B):


Young Group - HN
ρ=0.22
BDNF Concentration (pg/ml)
Δ Tongue Force (mN)
0
200
400
600
800
1000
1200
-40
-20
0
20
40
60
80
100

17 (C):

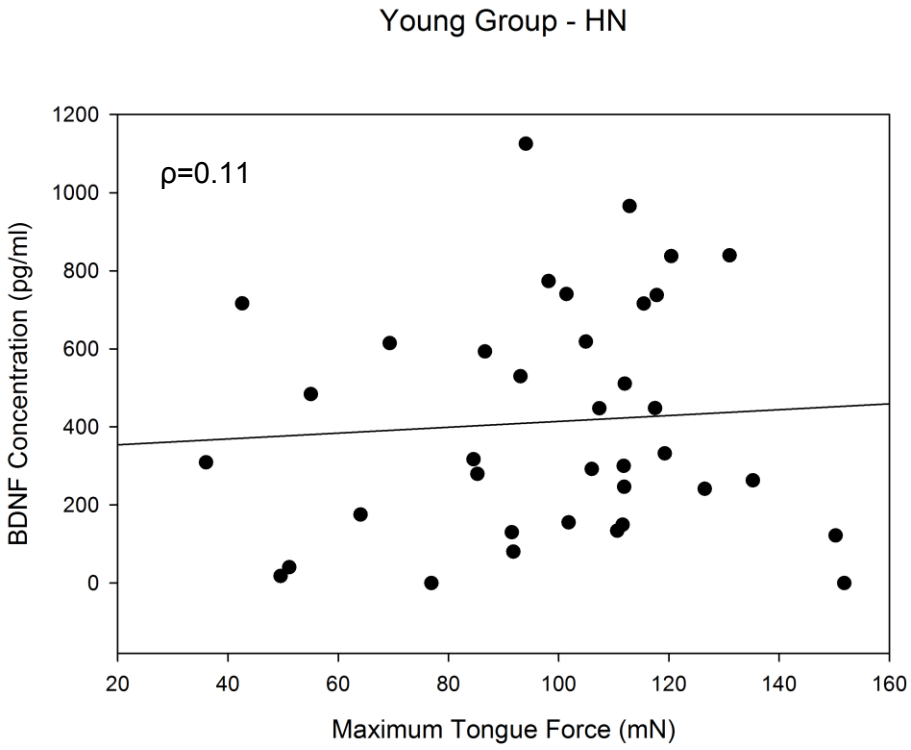
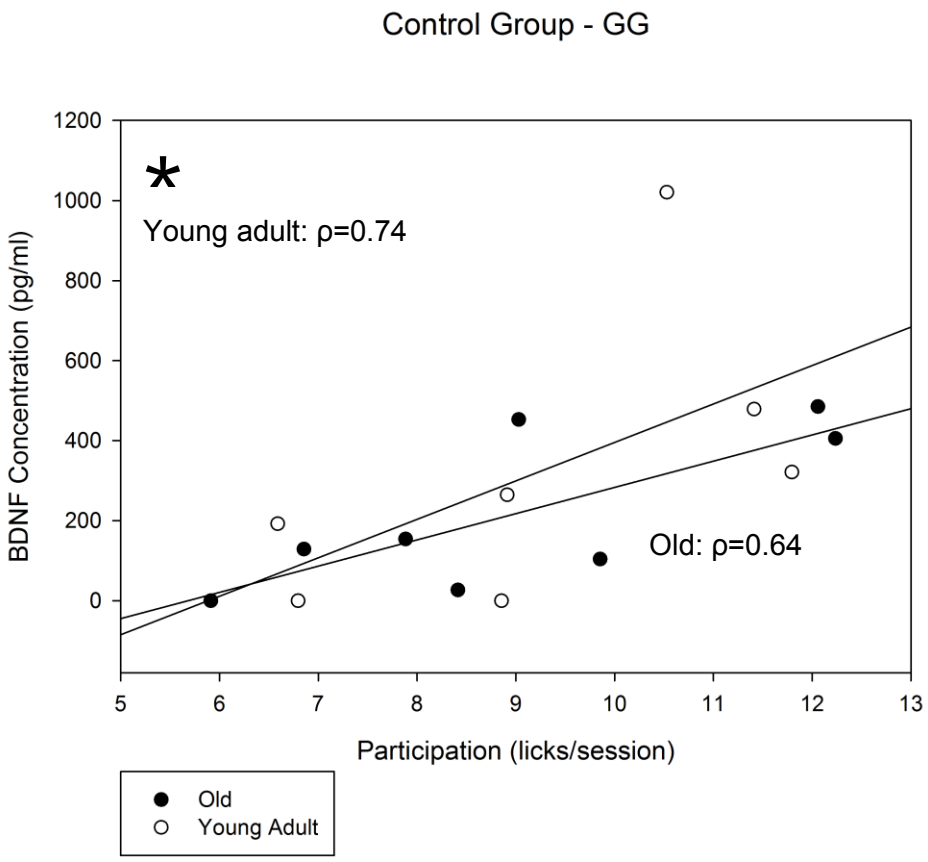
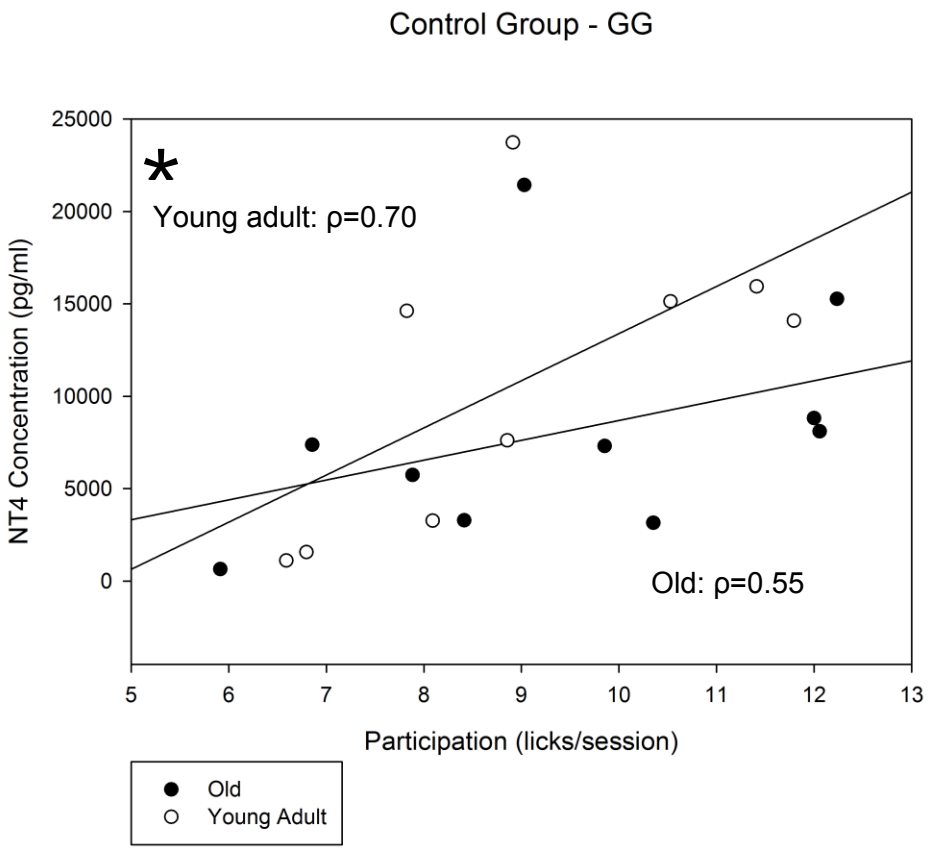

Young Group - HN
ρ=0.11
BDNF Concentration (pg/ml)
1200
1000
800
600
400
200
0
20
40
60
80
100
120
140
160
Maximum Tongue Force (mN)

Fig. 18 (A-D). Neurotrophin concentration and skill maintenance. (A): Average participation and BDNF concentration in the GG were strongly correlated in the young adult control group (ρ=0.74, p=0.04), but not correlated in the old control group (ρ=0.64, p=0.07). (B): Average participation and NT4 concentration in the GG were strongly correlated in the young adult control group (ρ=0.70, p=0.03), but not correlated in the old control group (ρ=0.55, p=0.09). (C): NT4 and BDNF concentration in the GG were strongly correlated in the old control group (ρ=0.88, p=0.002), and but not correlated in the young adult control group (ρ=0.63, p=0.09). (D) NT4 and TrkB concentration in the GG were strongly correlated in the old control group (ρ=0.74, p=0.03), and but not correlated in the young adult control group (ρ=0.53, p=0.12). * Denotes significant values; ρ value represented by diagonal line.

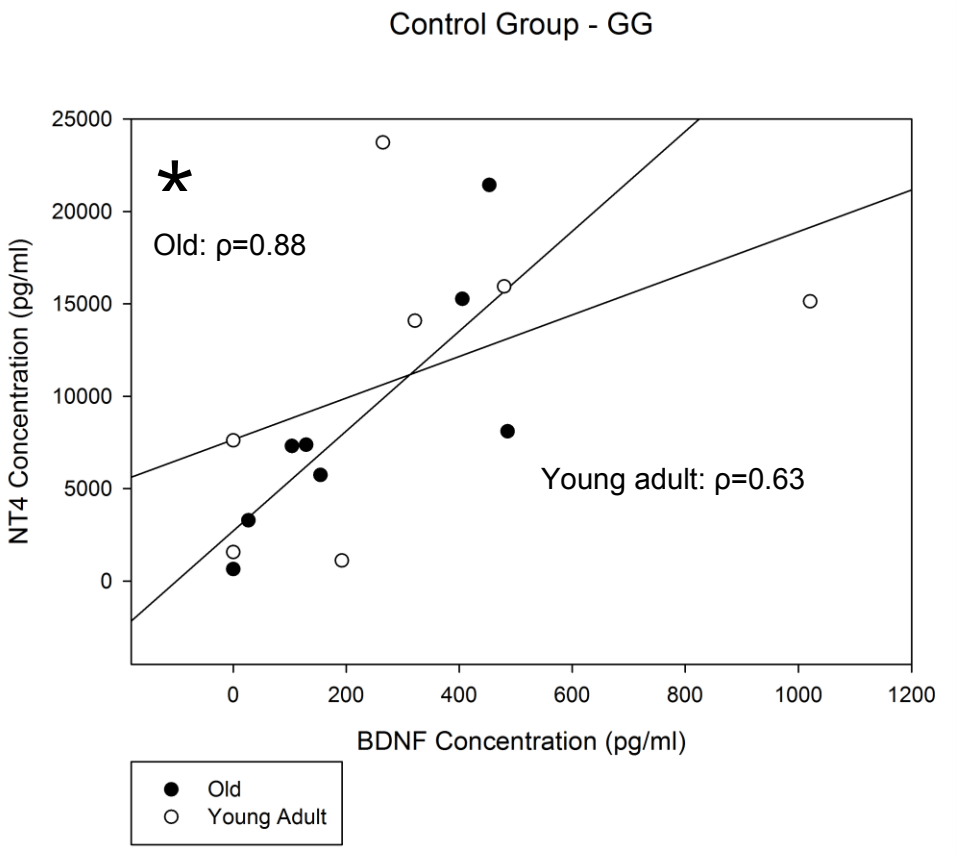
18 (A):


Control Group - GG
*
Young adult: ρ=0.74
Old: ρ=0.64
BDNF Concentration (pg/ml)
Participation (licks/session)
1200
1000
800
600
400
200
0
5
6
7
8
9
10
11
12
13
Old
Young Adult

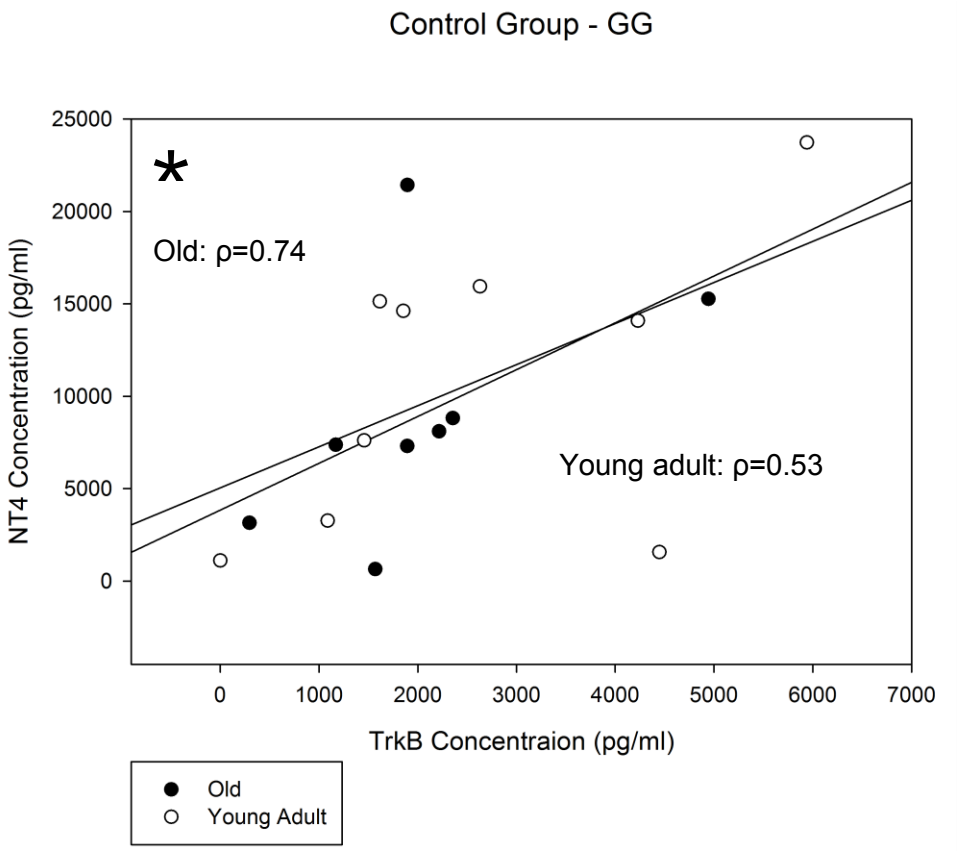
18 (B):


Control Group - GG
*
Young adult: ρ=0.70
Old: ρ=0.55
NT4 Concentration (pg/ml)
Participation (licks/session)
Old
Young Adult

18 (C):


Control Group - GG
*
Old: ρ=0.88
Young adult: ρ=0.63
NT4 Concentration (pg/ml)
BDNF Concentration (pg/ml)
25000
20000
15000
10000
5000
0
0
200
400
600
800
1000
1200
Old
Young Adult

18 (D):


Control Group - GG
*
Old: ρ=0.74
Young adult: ρ=0.53
NT4 Concentration (pg/ml)
TrkB Concentraion (pg/ml)
25000
20000
15000
10000
5000
0
0
1000
2000
3000
4000
5000
6000
7000
Old
Young Adult

4.4 Discussion

The hypotheses of this study were two-fold. First, it was hypothesized that tongue exercise paired with a water swallow would result in behavioral improvements and an increase in neurotrophin concentration, with greater benefits in lingual vs. laryngeal structures. Second, we hypothesized that improvements following tongue exercise paired with a water swallow would be diminished after detraining. The results of this study support our first hypothesis and partially support our second.

Tongue exercise resulted in increased tongue force, regardless of age, compared to skill maintenance alone (Fig. 8B). In addition, BDNF concentration was increased in the old exercise group in the HN compared with the old no-exercise controls (Fig. 14A). For the laryngeal behavioral task, ultrasonic vocalizations, there was only one behavioral difference shown, maintenance of call intensity in the exercise group compared with a reduction in call intensity in the no-exercise control group (Fig. 11C). However, there was no significant difference in neurotrophin concentration in the NA with exercise in either age group (Fig. 14B). These data support the hypothesis that stronger findings would be observed in lingual versus laryngeal structures. That is, tongue exercise, which is focused on strengthening the tongue as its main target, resulted in global improvements in maximum tongue force, but was only associated with maintenance of one measure of laryngeal function, intensity. In addition, the HN, which is the brainstem nucleus that contains the motor neurons that specifically innervate the tongue, was the only brainstem nucleus to have increased BDNF concentration. As such, our results show that tongue exercise paired with a water swallow leads to specific, neuroplastic benefits in lingual brainstem motor control structures.

The hypothesis that improvement gained following tongue exercise paired with a water swallow would be diminished after detraining was supported only in the old group. We found that the change in tongue force from the post-exercise time point to the 4-week time point was significantly diminished only in the old 4-week detraining group (Fig. 9A). In addition, BDNF concentration in the HN was

significantly lower in the old 2-and 4-week detraining groups than the old exercise group (Fig. 14A). However, tongue force was maintained for up to 4 weeks of detraining in the young adult group, and there were no differences in neurotrophin concentration in the young adult group with detraining (Fig. 14B). These data demonstrate that while young rats may maintain performance levels up to 4 weeks following exercise, this is not true for older rats. These data also provide initial evidence for the necessity of exercise maintenance programs following tongue exercise protocols in older populations. Interestingly, the only detraining study conducted in a human clinical population found a decrease in tongue force following detraining in a little as 2 weeks in healthy adults (Clark et al., 2009).

It could be argued that maintenance of USV intensity following tongue exercise paired with a water swallow is evidence for the cross-activation potential of exercise in the cranial sensorimotor system. This argument has already been introduced in the voice and swallowing literature based on clinical findings (Easterling, 2008; El Sharkawi et al., 2002). However, it is important to consider the goal and activation elements of an exercise prior to assuming the cross-activation benefits. We hypothesized that there would be some changes within the larynx following tongue exercise paired with a water swallow because the TA, a muscle within the larynx important for voice, is activated during swallowing (Perlman et al., 1999; Van Daele et al., 2005). The maintenance of USV intensity found with tongue exercise paired with a water swallow can be explained from a cross-activation perspective considering that vocalization intensity is a function of respiratory capacity and glottal closure (Riede, 2011), which are two elements that were finely controlled during the exercise used in this study (Fig. 6). In both the rat and the human, respiratory timing and glottal closure must be precisely coordinated during vocalization and during the swallow to allow materials to safely enter the esophagus and avoid aspiration (Perlman et al., 1999; Riede, 2011). The tongue exercise intervention used here was paired with a swallow, which therefore activated and perhaps strengthened glottal closure, and may have contributed to the observed post exercise maintenance of call intensity in the exercise group. However, the goal of a particular exercise protocol may be improvement in a specific function and not solely maintenance. In this

work, there were no improvements in USVs found after tongue exercise paired with a water swallow. Therefore, cross-activation of the larynx during a swallowing task alone does not appear to be sufficient or specific enough to improve vocal function. As such, based on the results of this initial study, future clinical studies could explore the role of cross-activation for maintenance of function. However, if improvement in a specific function is the goal of therapy, specific exercise appears to be necessary and should be used. Nevertheless, results of this initial study do not rule of the possibility that tongue exercise paired with a water swallow could be used in future clinical studies to optimize both swallow and vocal function in individuals at risk for both disorders, such as elderly individuals and people with Parkinson disease, especially in the early stages of the disease where maintenance or slowing of the disease process is the goal.

We observed age-related changes in tongue force (Fig. 7) and NT4 concentration (Fig. 13). Specifically, we found a lower tongue force in the old group at baseline than in the young adult group. However, reduced tongue force has not been a universal finding in previously reported animal studies (Schaser et al., 2012). This speaks to the relevance of this animal model to human clinical populations where decreased tongue force, or tongue weakness, is not consistently reported (Steele, 2013). This study did not include a direct measure of functional swallowing. As a result, we are unable to comment on specific age-related changes to the swallow in this cohort of animals. However, previous work from our laboratory has shown that older rats demonstrate functional changes to the swallow as a result of normal aging, including reduced bolus transport speed, detected through video fluoroscopic swallowing examination (Russell et al., 2013). Therefore, it can be hypothesized that age-related changes to the functional swallow were also present in the animals in this study, but it is unknown if the increase in tongue force and BDNF concentration in the HN found with tongue exercise in this study has a direct benefit to the functional swallow, such as improved bolus transport speed. Current and future work in our laboratory will explore this hypothesis. We also found a decrease in NT4 concentration in all brainstem nuclei, including our brainstem control nuclei (BSC) and the laryngeal brainstem nucleus of interest

(NA). These findings suggest a putative age-related decrease in NT4 concentration in motor and sensory neurons in the brainstem, which has yet to be shown in previous research.

Previous work from our laboratory and others has shown that TrkB is decreased with age in multiple systems (Greising et al., 2015; Johnson et al., 1996; Schaser et al., 2012; Tong et al., 2015). However, age-related differences in TrkB were not found in this study. There are many methodological differences in this study from our previous study (Schaser et al., 2012) that could account for the lack of change seen in TrkB with age. For example, our previous study used a staining technique to specifically target motor neurons in the ventral middle hypoglossal nucleus. In this study, a gross-dissection through the entire HN was performed and protein concentration was obtained through ELISA methods, versus measures of relative fluorescent intensity. As a result, the specific age-related changes found for TrkB in the ventral middle HN could have been "washed out" due to our analysis of the entire HN. Accordingly, the age-related decrease in TrkB found in our first study could be specific to the motor neurons innervating the muscles within only that area of the HN.

Differences were sparse in neurotrophin concentration as a function of age or exercise in the TA muscle. Only two significant age-related findings were observed: (1) Increased NT4 concentration in the old group versus the young adult group at baseline (Fig. 13B), and (2) Reduced BDNF concentration in the old control group versus the young adult control group (Fig. 13C). The significantly greater NT4 concentration in the old group versus the young adult group was in opposition to our hypothesis and previous reports of reduced neurotrophin concentration with age (Greising et al., 2015; Johnson et al., 1996). However, decreased BDNF concentration was consistent with the hypothesis that neurotrophins are decreased with age in the neuromuscular system (Greising et al., 2015; Kalinkovich and Livshits, 2015). The unique innervation and sustained and frequent activity of the TA (Kuna et al., 1988) may partially explain our findings. The TA is active during the critical life functions of respiration, airway protection, swallowing, and also during vocalization in the rat (Nagai et al., 2005; Riede, 2011) and the human (Hillel et al., 1997; Jafari et al., 2003; Maronian et al., 2003). Because NT4 is specifically

upregulated in an activity-dependent manner in muscle (Funakoshi et al., 1995), it might be expected that greater NT4 levels would be found in muscles with sustained or frequent activity. Support for this view is the seemingly greater NT4 levels in the TA than in the other muscles studied (Fig. 14B). The increased NT4 in the old group may be a compensatory strategy or a sign of growth and remodeling in this muscle (Funakoshi et al., 1995). Increased NT4 in the old control group may be compensatory for reduced BDNF given that both NT4 and BDNF are the ligand for the TrkB receptor (Barbacid, 1995).

In addition to the age-related neurotrophin changes that were found in the NA and TA, we also found age-related changes in USVs. Our findings were similar to the age-related changes reported in previous studies examining aging rat vocalizations (Basken et al., 2012; Johnson et al., 2013), including reduced number of calls, increased duration, and decreased intensity in old versus young adult groups. In the Basken study, these age-related behavioral changes were correlated with significant motor neuron loss in the NA (Basken et al., 2012). While motor neuron count in the NA was not examined in our study, it is possible that loss of NA motor neurons may have contributed to the age-related behavioral decrements in USVs we observed to a greater extent than neurotrophin concentration. However, neurotrophin concentration may have a greater role in tongue actions, as we found increased BDNF concentration with exercise. This interpretation is consistent with the results of a prior study showing that motor neuron number in the HN is preserved with age (Schwarz et al., 2009).

No other significant differences were found in the muscles investigated in this study. However, we found significant correlations in the GG in the control group. Specifically, we found that BDNF concentration (Fig. 18A) and NT4 concentration (Fig. 18B) were strongly correlated with exercise participation in the young adult control group, but not in the old control group. Instead, the old control group showed strong correlations between NT4 concentration and BDNF concentration (Fig. 18C) and TrkB concentration (Fig. 18D) in the GG, and the strong correlation between NT4 concentration and BDNF concentration was also present in the old exercise group. These findings may highlight the importance of and differences in the regulation of these neurotrophins in skill maintenance and learning in

the young adult and old control groups. The control group rats learned the licking task, and maintained the skills needed to complete the task over the 8-week training period, but did not participate in the progressive resistance exercise paradigm. Thus, this control group gives us particular insight into the role of neurotrophins in learning versus activity-dependent exercise mechanisms. It appears from our results that BDNF and NT4 concentrations are linked to skill learning in young adult animals, because the concentrations of these neurotrophins in the GG muscle was highly correlated with participation in the control group but not in the exercise group. However, these high correlations were not found in the old group. Instead, the neurotrophins themselves were correlated in the old control group in the GG, and remained correlated in the old exercise group in the GG. This finding in the old groups highlights the importance of both the ligand and receptor working together to produce downstream signaling and task maintenance in the old (Greising et al., 2015; Mantilla et al., 2004).

The other two correlations performed in this study again highlight the importance of including a no-exercise control group in this study. Due to the inclusion of this group and the detraining groups we found relationships between participation and tongue force in both the old and young adult groups (Fig. 15). This finding is proof of principle that it is participation in the progressive resistance tongue exercise program that results in increased tongue force and not just learning or improvement in the licking task over time. Participation at or over the individual force threshold set for each individual animal was needed to improve tongue force and decreased participation during the detraining time periods led to decreased tongue force (Fig. 15). The final correlation examined was the relationship between BDNF concentration in the HN and measures of tongue task performance (Fig. 16 &17). Results did not identify any significant relationships for any age or condition. However, BDNF concentration and participation approached a significant correlation in the old group (Fig. 16), but not the young adults (Fig. 17). This finding suggests that participation in the progressive resistance tongue exercise protocol is partially responsible for the change in BDNF concentration in the HN seen based on training condition in the old (Fig. 14). It may be hypothesized that BDNF concentration in the HN is increased in the old due to the

increased motor neuron activation during exercise. This hypothesis cannot be confirmed in this study, because specific activation levels of the motor neurons in the HN during exercise were not recorded. However, future research can examine this hypothesis.

The question remaining in this study concerns the underlying changes responsible for increased behavioral tongue force in the young adult group. BDNF concentration did not increase in the young adult group with exercise. However, there appeared to be a pattern of increased NT4 concentration in the young adult group (Fig. 14B). Therefore, it is possible that NT4 may lead to improved neuromuscular transmission in the young adult group in combination with other factors not explored in this study. For example, other neurotrophins and their receptors, such as NT3 and TrkC have been shown to be activity-dependent in nature and upregulated with exercise in the spinal sensorimotor system (Gomez-Pinilla et al., 2001). Future research can examine this potential contribution and others.

In summary, results from this study support both of the hypotheses established for this work. Specifically, tongue exercise paired with a water swallow resulted in specific gains in a behavioral measure of tongue force as well as an underlying change in neurotrophin concentration, specifically an increase in BDNF concentration in the HN with exercise in the old group. These findings highlight the neuroplastic potential of tongue exercise paired with a water swallow in the cranial sensorimotor system. However, this work did not examine whether an increase in tongue force and BDNF concentration in the HN with exercise resulted in an improvement to the functional swallow in aged rats. It is hypothesized that an increase in tongue strength will result in improved bolus transport, but this must be confirmed in future studies. This work also demonstrates the importance of continued examination of the current exercise protocols used in clinical populations with voice and swallowing disorders. While the cross-activation benefits found in this study were minimal and were tied specifically to behavioral functions that were directly activated during our exercise task, maintenance of vocalization intensity following tongue exercise may be beneficial in some clinical contexts, as long as tongue exercise is paired with a swallowing activity. In addition, our second hypothesis, that detraining would result in loss of tongue

force, was supported by data in the old group. This finding has clinical significance in voice and swallowing populations because it stresses the need for continued maintenance after a therapeutic protocol has been completed, especially for elderly people.

Thus, the results of this study provide critical foundational evidence for future clinical research. Human clinical studies, as well as future animal studies, should continue to deconstruct our current exercise based therapy protocols to determine the true cause of improvement, at both the behavioral and underlying level. These types of studies will increase the efficacy and efficiency of our current exercise protocols and provide clinicians with the evidence they need to decide which therapy protocol or exercise to use based on the presentation and specific diagnosis of the individual clients they treat.

Acknowledgements

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V. Summative Discussion and Clinical Implications

This dissertation represents the first comprehensive examination of neurotrophins as mediators of neuroplasticity in the cranial sensorimotor system. Findings from both Study 1 and Study 2 provide a mechanistic rationale for exploring the use of tongue exercise paired with a water swallow as an exercise-based treatment for swallowing and/or voice disorders in clinical populations. In these studies, this treatment was associated with global changes in behavioral aspects of swallowing and limited changes in behavioral aspects of voice in addition to concentrations of neurotrophins known to be important mechanisms underlying neuromuscular efficiency. Furthermore, this work supports the further exploration of tongue exercise paired with a swallowing task in clinical populations to elicit cross-activation and cross-system benefits in the cranial sensorimotor system as a whole. However, this dissertation also highlights that it is important to maintain a clear and realistic understanding of the underlying physiological goals and activation pathways of an exercise when theorizing cross-activation effects. Clinicians and researchers exploring the cross-activation potential of this form of tongue exercise should note that behavioral differences in vocalizations following our exercise protocol were limited to maintenance of only one vocalization measure in this study, intensity, a measure that specifically benefits from the coordination of respiratory and laryngeal muscle activation employed during this exercise task (Riede, 2011).

Overall, this work is significant because it examined the neuroplastic potential of exercise in the cranial sensorimotor system in muscles and the central nervous system, along with the enduring effects of exercise (detraining) with the long term and future goal of using our results to guide current therapy timelines and protocols used to treat people with voice and swallowing problems.

5.1 Limitations

There were some limitations to the work reported within this dissertation, including some methodological concerns that may have led to variation in our findings.

First, the method used to determine neurotrophin protein concentration and/or the specific area of the HN that was analyzed may have introduced some variation or error, between the two studies in this dissertation. The specificity of the TrkB staining in Study 1 was definitive and showed age-related relative fluorescent intensity differences in the rostral area of the HN that was targeted for our analysis. However, the BDNF staining was more diffuse and may not have captured age and exercise related differences with the same level of specificity. To control for staining differences in the neurotrophins of interest, we used an ELISA method in Study 2 to measure protein concentration. However, because gross dissection of the nuclei of interest was performed it is possible that the brainstem samples analyzed were not specific to our nuclei of interest alone. To guard against this possibility, brainstem tissue (BSC) was carefully selected to be directly caudal and rostral to known locations of the particular nuclei of interest, based on previous anatomical studies in our lab (Basken et al., 2012; Behan et al., 2012; Schaser et al., 2012) and published anatomical references (Palkovits and Brownstein, 1988).

A second methodological concern was the use of a mating paradigm to elicit calls from both the old and young adult rats. The old rats demonstrated a significant decrease in number of calls and it cannot be ruled out that this reduction in number of calls was due to lack of interest in mating or altered responsiveness from the females during the acclimation periods and testing sessions that in turn affected vocalization characteristics in the males. A recent vocalization playback study demonstrated that female rats were differentially responsive to normal rat vocalizations versus those from parkinsonian rats (Pultorak et al., 2015). It is possible that this phenomenon may extend to aged rats and may have affected our findings. However, we attempted to control for this issue by having no-exercise control groups in each age group. In addition, we attempted to control for reduced mounting behavior in the old group by not allowing the young adult group to mount the females during the testing sessions.

Our results showed that tongue exercise paired with a water swallow was linked with behavioral tongue force increases and increased BDNF concentration in the HN of old rats. However, this study has not shown that these behavioral and neurotrophic effects resulted in improvements in swallowing behaviors in the rat. As a result, the direct implication of this work on human clinical populations is somewhat limited. However, previous work from our laboratory has shown that older rats demonstrate functional changes to the swallow as a result of normal aging, including reduced bolus transport speed, detected through videofluoroscopic swallowing examinations (Russell et al., 2013). Therefore, it can be hypothesized that age-related changes to the functional swallow were also present in the animals in this study, but it is unknown if the increase in tongue force found with exercise resulted in benefits to the functional swallow, such as improved bolus transport speed. Current and future work in our laboratory will explore this hypothesis. Thus, this study is a first step in the research pipeline and provides the foundational evidence needed to support future animal and clinical studies.

5.2 Clinical Implications

Future clinical studies should strive to obtain increased levels of control, similar to that obtained in animal studies, by including appropriate control groups and examining both behavioral measures and to the extent possible, underlying mechanisms responsible for behavioral change. For example, serum level analyses of BDNF and other neurotrophins can be completed in human clinical populations, and could serve as potential biomarkers for age-related declines, including sarcopenia (Kalinkovich and Livshits, 2015). In addition, a genetic variant of BDNF, the Val66Met polymorphism, exists in the normal human clinical population, and has been hypothesized to be the cause of reduced BDNF in serum levels (Elfving et al., 2012). This polymorphism has been related to cognitive declines, learning disabilities, and a lack of behavioral response to exercise and rehabilitation in elderly populations and people recovering from stroke (Mang et al., 2013). Based on the results of this dissertation, future clinical studies examining the effect of exercise on voice and swallow function could consider including a genetic screening or serum

level analysis of BDNF to rule out possible factors that could lead to increased variability in an individual's response to exercise.

5.3 Future Studies

The results of this dissertation point to the sufficiency of increases in BDNF concentration in the HN for improvements in tongue force in old rats. However, this study cannot be used to determine necessity. A future study or line of inquiry could use genetic BDNF knockout or knockdown rats to determine if increases in BDNF concentration in the HN are necessary to have a functional improvement in tongue force in old rats, or if other redundant mechanisms are available or at work. Currently, our lab has tissue from BDNF knockdown rats from a collaborating laboratory on campus, and it would be very interesting to examine NMJ morphology in these animals in our muscles of interest to determine if reduced BDNF results in morphological changes in the NMJ similar to or different from those seen with normal aging in the GG and TA (Johnson and Connor, 2011; Johnson et al., 2013).

Additional studies should also examine the impact of tongue exercise-based increases in tongue force on measures of functional swallowing, a project which is already ongoing on our laboratory, using videofluoroscopic swallowing measures (Russell et al., 2013).

Finally, future studies should also examine the effect of exercise in different patient populations, including Parkinson disease (PD), as previous research from our laboratory has shown that tongue function is affected in PD animal models (Ciucci et al., 2011) and can be improved with tongue exercise, through mechanisms other than striatal dopamine sparing (Ciucci et al., 2013). As a result, future studies should examine additional brainstem nuclei of interest in PD related to the cranial sensorimotor system, both as early biomarkers of PD and in response to exercise in genetic animal models. Additionally, other exercise protocols that target coordination and effort, as opposed to strength alone, should be examined in the PD population, because previous work from our lab has shown that maximally stimulated forces in the

GG muscle in a PD animal model are not significantly reduced, indicating a central control mechanism and not a peripheral mechanism of behavioral force declines in this population (Ciucci et al., 2011).

References

Abramoff, M.D., Magelhaes, P.J., Ram, S.J., 2004. Image Processing with ImageJ. Biophotonics International. 11**,** 36-42.

Baken, R.J., 2005. The aged voice: a new hypothesis. J Voice. 19**,** 317-25.

Baker-Herman, T.L., et al., 2004. BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nature neuroscience. 7**,** 48-55.

.

Barbacid, M., 1995. Neurotrophic factors and their receptors. Current opinion in cell biology. 7**,** 148-155.

Barde, Y.A., 1990. The nerve growth factor family. Progress in growth factor research. 2**,** 237-248.

Basken, J.N., Connor, N.P., Ciucci, M.R., 2012. Effect of aging on ultrasonic vocalizations and laryngeal sensorimotor neurons in rats. Exp Brain Res. 219**,** 351-61.

Becker, B.J., Russell, J.A., Connor, N.P., 2015. Effects of aging on evoked retrusive tongue actions. Arch Oral Biol. 60**,** 966-971.

Behan, M., et al., 2012. The effect of tongue exercise on serotonergic input to the hypoglossal nucleus in young and old rats. Journal of speech, language, and hearing research : JSLHR. 55**,** 919-929.

Behrman, A., et al., 2008. Vocal hygiene education, voice production therapy, and the role of patient adherence: a treatment effectiveness study in women with phonotrauma. Journal of speech, language, and hearing research : JSLHR. 51**,** 350-366.

Bergman, E., et al., 1996. Neuropeptides and neurotrophin receptor mRNAs in primary sensory neurons of aged rats. The Journal of comparative neurology. 375**,** 303-319.

Berlucchi, G., Buchtel, H.A., 2009. Neuronal plasticity: historical roots and evolution of meaning. Experimental brain research.Experimentelle Hirnforschung.Experimentation cerebrale. 192**,** 307-319.

Bialy, M., Rydz, M., Kaczmarek, L., 2000. Precontact 50-kHz vocalizations in male rats during acquisition of sexual experience. Behavioral neuroscience. 114**,** 983-990.

Blanchard, R.J., et al., 1992. Sex differences in the incidence and sonographic characteristics of antipredator ultrasonic cries in the laboratory rat (Rattus norvegicus). Journal of comparative psychology (Washington, D.C.: 1983). 106**,** 270-277.

Bliss, T., Collingridge, G., Morris, R., 2004. Long-term Potentiation: Enhancing Neuroscience for 30 Years, Vol. 1, Oxford University Press, Oxford.

Bliss, T.V., Lomo, T., 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol. 232**,** 331-56.

Bothwell, M., 1995. Functional interactions of neurotrophins and neurotrophin receptors. Annual Review of Neuroscience. 18**,** 223-253.

Bottinelli, R., et al., 1994. Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle. Journal of muscle research and cell motility. 15**,** 413-419.

Bottinelli, R., Reggiani, C., 2000. Human skeletal muscle fibres: molecular and functional diversity. Prog Biophys Mol Biol. 73**,** 195-262.

Brooks, S.V., Faulkner, J.A., 1994. Skeletal muscle weakness in old age: underlying mechanisms. Medicine and science in sports and exercise. 26**,** 432-439.

Brudzynski, S.M., 2005. Principles of rat communication: quantitative parameters of ultrasonic calls in rats. Behavior genetics. 35**,** 85-92.

Burkhead, L.M., Sapienza, C.M., Rosenbek, J.C., 2007. Strength-training exercise in dysphagia rehabilitation: principles, procedures, and directions for future research. Dysphagia. 22**,** 251-265.

Campbell, M.J., McComas, A.J., Petito, F., 1973. Physiological changes in ageing muscles. Journal of neurology, neurosurgery, and psychiatry. 36**,** 174-182.

Carnaby, G.D., Harenberg, L., 2013. What is "usual care" in dysphagia rehabilitation: a survey of USA dysphagia practice patterns. Dysphagia. 28**,** 567-74.

Carrasco, D.I., English, A.W., 2003. Neurotrophin 4/5 is required for the normal development of the slow muscle fiber phenotype in the rat soleus. The Journal of experimental biology. 206**,** 2191-2200.

Chao, M.V., 1992. Neurotrophin receptors: a window into neuronal differentiation. Neuron. 9**,** 583-93.

Chen, P.H., et al., 2009. Prevalence of perceived dysphagia and quality-of-life impairment in a geriatric population. Dysphagia. 24**,** 1-6.

Ciucci, M.R., et al., 2007. Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol: a preliminary study. Behavioural brain research. 182**,** 284-289.

Ciucci, M.R., et al., 2008. Limb use and complex ultrasonic vocalization in a rat model of Parkinson's disease: deficit-targeted training. Parkinsonism & related disorders. 14 Suppl 2**,** S172-5.

Ciucci, M.R., et al., 2009. Reduction of dopamine synaptic activity: degradation of 50-kHz ultrasonic vocalization in rats. Behavioral neuroscience. 123**,** 328-336.

Ciucci, M.R., Connor, N.P., 2009. Dopaminergic influence on rat tongue function and limb movement initiation. Experimental brain research.Experimentelle Hirnforschung.Experimentation cerebrale. 194**,** 587-596.

Ciucci, M.R., et al., 2010. A translational approach to vocalization deficits and neural recovery after behavioral treatment in Parkinson disease. Journal of communication disorders. 43**,** 319-326.

Ciucci, M.R., et al., 2011. Tongue force and timing deficits in a rat model of Parkinson disease. Behav Brain Res. 222**,** 315-20.

Ciucci, M.R., Schaser, A.J., Russell, J.A., 2013. Exercise-induced rescue of tongue function without striatal dopamine sparing in a rat neurotoxin model of Parkinson disease. Behav Brain Res. 252**,** 239-45.

Clark, H.M., et al., 2009. Effects of directional exercise on lingual strength. Journal of speech, language, and hearing research : JSLHR. 52**,** 1034-1047.

Cohen, S.M., et al., 2012a. Direct health care costs of laryngeal diseases and disorders. The Laryngoscope. 122**,** 1582-1588.

Cohen, S.M., et al., 2012b. Prevalence and causes of dysphonia in a large treatment-seeking population. The Laryngoscope. 122**,** 343-348.

Connor, N.P., et al., 2008. Differences in age-related alterations in muscle contraction properties in rat tongue and hindlimb. Journal of speech, language, and hearing research : JSLHR. 51**,** 818-827.

Connor, N.P., et al., 2009. Effect of tongue exercise on protrusive force and muscle fiber area in aging rats. Journal of speech, language, and hearing research : JSLHR. 52**,** 732-744.

Cook, I.J., et al., 1994. Influence of aging on oral-pharyngeal bolus transit and clearance during swallowing: scintigraphic study. The American Journal of Physiology. 266**,** G972-7.

Cooke, S.F., Bliss, T.V., 2006. Plasticity in the human central nervous system. Brain. 129**,** 1659-73.

Costa, H.O., Matias, C., 2005. Vocal impact on quality of life of elderly female subjects. Braz J Otorhinolaryngol. 71**,** 172-8.

Cramer, S.C., et al., 2011. Harnessing neuroplasticity for clinical applications. Brain : a journal of neurology. 134**,** 1591-1609.

Davids, T., Klein, A.M., Johns, M.M., 3rd, 2012. Current dysphonia trends in patients over the age of 65: is vocal atrophy becoming more prevalent? The Laryngoscope. 122**,** 332-335.

Dechant, G., Neumann, H., 2002. Neurotrophins. Adv Exp Med Biol. 513**,** 303-34.

Ding, Q., Ying, Z., Gomez-Pinilla, F., 2011. Exercise influences hippocampal plasticity by modulating brain-derived neurotrophic factor processing. Neuroscience. 192**,** 773-780.

Dishman, R.K., et al., 2006. Neurobiology of exercise. Obesity (Silver Spring, Md.). 14**,** 345-356.

Doherty, T.J., 2003. Invited review: Aging and sarcopenia. Journal of applied physiology (Bethesda, Md.: 1985). 95**,** 1717-1727.

Dutta, C., 1997. Significance of sarcopenia in the elderly. The Journal of nutrition. 127**,** 992S-993S.

Easterling, C., 2008. Does an exercise aimed at improving swallow function have an effect on vocal function in the healthy elderly? Dysphagia. 23**,** 317-26.

Ebadi, M., et al., 1997. Neurotrophins and their receptors in nerve injury and repair. Neurochemistry international. 30**,** 347-374.

Edstrom, L., Grimby, L., 1986. Effect of exercise on the motor unit. Muscle & nerve. 9**,** 104-126.

El Sharkawi, A., et al., 2002. Swallowing and voice effects of Lee Silverman Voice Treatment (LSVT): a pilot study. Journal of neurology, neurosurgery, and psychiatry. 72**,** 31-36.

Elfving, B., et al., 2012. Depression, the Val66Met polymorphism, age, and gender influence the serum BDNF level. J Psychiatr Res. 46**,** 1118-25.

Fink, B.R., 1974. Folding mechanism of the human larynx. Acta Oto-Laryngologica. 78**,** 124-128.

Fluck, M., 2006. Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. The Journal of experimental biology. 209**,** 2239-2248.

Fox, C.M., et al., 2006. The science and practice of LSVT/LOUD: neural plasticity-principled approach to treating individuals with Parkinson disease and other neurological disorders. Seminars in speech and language. 27**,** 283-299.

Fregosi, R.F., Fuller, D.D., 1997. Respiratory-related control of extrinsic tongue muscle activity. Respiration physiology. 110**,** 295-306.

Friedman, B., et al., 1995. BDNF and NT-4/5 exert neurotrophic influences on injured adult spinal motor neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 15**,** 1044-1056.

Funakoshi, H., et al., 1995. Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. Science (New York, N.Y.). 268**,** 1495-1499.

Garcia, N., et al., 2010. Localization of brain-derived neurotrophic factor, neurotrophin-4, tropomyosin-related kinase b receptor, and p75 NTR receptor by high-resolution immunohistochemistry on the adult mouse neuromuscular junction. J Peripher Nerv Syst. 15**,** 40-9.

Gay, T., Rendell, J.K., Spiro, J., 1994. Oral and laryngeal muscle coordination during swallowing. The Laryngoscope. 104**,** 341-349.

Gilliam, E.E., Goldberg, S.J., 1995. Contractile properties of the tongue muscles: effects of hypoglossal nerve and extracellular motoneuron stimulation in rat. Journal of neurophysiology. 74**,** 547-555.

Golub, J.S., et al., 2006. Prevalence of perceived dysphonia in a geriatric population. Journal of the American Geriatrics Society. 54**,** 1736-1739.

Gomez-Pinilla, F., et al., 2001. Differential regulation by exercise of BDNF and NT-3 in rat spinal cord and skeletal muscle. The European journal of neuroscience. 13**,** 1078-1084.

Gomez-Pinilla, F., et al., 2002. Voluntary exercise induces a BDNF-mediated mechanism that promotes neuroplasticity. Journal of neurophysiology. 88**,** 2187-2195.

Gomez-Pinilla, F., et al., 2011. Exercise impacts brain-derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. The European journal of neuroscience. 33**,** 383-390.

Green, C.S., Bavelier, D., 2008. Exercising your brain: a review of human brain plasticity and training-induced learning. Psychol Aging. 23**,** 692-701.

Greising, S.M., et al., 2015. Ageing and neurotrophic signalling effects on diaphragm neuromuscular function. J Physiol. 593**,** 431-40.

Grondard, C., et al., 2005. Regular exercise prolongs survival in a type 2 spinal muscular atrophy model mouse. J Neurosci. 25**,** 7615-22.

Hennigan, A., O'Callaghan, R.M., Kelly, A.M., 2007. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. Biochemical Society transactions. 35**,** 424-427.

Hewitt, A., et al., 2008. Standardized instrument for lingual pressure measurement. Dysphagia. 23**,** 16-25.

Hillel, A.D., Robinson, L.R., Waugh, P., 1997. Laryngeal electromyography for the diagnosis and management of swallowing disorders. Otolaryngol Head Neck Surg. 116**,** 344-8.

Hodges, S.H., Anderson, A.L., Connor, N.P., 2004. Remodeling of Neuromuscular Junctions in Aged Rat Genioglossus Muscle. Annals of Otology, Rhinology & Laryngology. 113**,** 175-179.

Hopkins, M.E., Nitecki, R., Bucci, D.J., 2011. Physical exercise during adolescence versus adulthood: differential effects on object recognition memory and brain-derived neurotrophic factor levels. Neuroscience. 194**,** 84-94.

Ingalls, C.P., 2004. Nature vs. nurture: can exercise really alter fiber type composition in human skeletal muscle? Journal of applied physiology (Bethesda, Md.: 1985). 97**,** 1591-1592.

Intlekofer, K.A., Cotman, C.W., 2013. Exercise counteracts declining hippocampal function in aging and Alzheimer's disease. Neurobiol Dis. 57**,** 47-55.

Irles Rocamora, J.A., et al., 2009. [A prevalence study of dysphagia and intervention with dietary counselling in nursing home from Seville]. Nutr Hosp. 24**,** 498-503.

Jafari, S., et al., 2003. Sensory regulation of swallowing and airway protection: a role for the internal superior laryngeal nerve in humans. J Physiol. 550**,** 287-304.

Jakeman, L.B., et al., 1998. Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury. Experimental neurology. 154**,** 170-184.

Janssen, I., 2010. Evolution of sarcopenia research. Appl Physiol Nutr Metab. 35**,** 707-12.

Jaradeh, S., 1994. Neurophysiology of swallowing in the aged. Dysphagia. 9**,** 218-220.

Johnson, A.M., et al., 2010. Ultrasonic output from the excised rat larynx. The Journal of the Acoustical Society of America. 128**,** EL75-9.

Johnson, A.M., Connor, N.P., 2011. Effects of electrical stimulation on neuromuscular junction morphology in the aging rat tongue. Muscle & nerve. 43**,** 203-211.

Johnson, A.M., et al., 2011. Targeted training of ultrasonic vocalizations in aged and Parkinsonian rats. J Vis Exp.

Johnson, A.M., Ciucci, M.R., Connor, N.P., 2013. Vocal training mitigates age-related changes within the vocal mechanism in old rats. J Gerontol A Biol Sci Med Sci. 68**,** 1458-68.

Johnson, H., Hokfelt, T., Ulfhake, B., 1996. Decreased expression of TrkB and TrkC mRNAs in spinal motoneurons of aged rats. The European journal of neuroscience. 8**,** 494-499.

Johnson, H., Hokfelt, T., Ulfhake, B., 1999. Expression of p75(NTR), trkB and trkC in nonmanipulated and axotomized motoneurons of aged rats. Brain research.Molecular brain research. 69**,** 21-34.

Kalinkovich, A., Livshits, G., 2015. Sarcopenia - The search for emerging biomarkers. Ageing Res Rev. 22**,** 58-71.

Kays, S., Robbins, J., 2006. Effects of sensorimotor exercise on swallowing outcomes relative to age and age-related disease. Seminars in speech and language. 27**,** 245-259.

Kempermann, G., van Praag, H., Gage, F.H., 2000. Activity-dependent regulation of neuronal plasticity and self repair. Progress in brain research. 127**,** 35-48.

Kendall, K., 2007. Presbyphonia: a review. Curr Opin Otolaryngol Head Neck Surg. 15**,** 137-40.

Kirkendall, D.T., Garrett, W.E., Jr., 1998. The effects of aging and training on skeletal muscle. The American Journal of Sports Medicine. 26**,** 598-602.

Kleim, J.A., Jones, T.A., 2008. Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. Journal of speech, language, and hearing research : JSLHR. 51**,** S225-39.

Knaepen, K., et al., 2010. Neuroplasticity - exercise-induced response of peripheral brain-derived neurotrophic factor: a systematic review of experimental studies in human subjects. Sports medicine (Auckland, N.Z.). 40**,** 765-801.

Kocdor, P., et al., 2015. Characteristics of dysphagia in older patients evaluated at a tertiary center. Laryngoscope. 125**,** 400-5.

Kraemer, W.J., et al., 2002. American College of Sports Medicine position stand. Progression models in resistance training for healthy adults. Medicine and science in sports and exercise. 34**,** 364-380.

Kuna, S.T., Insalaco, G., Woodson, G.E., 1988. Thyroarytenoid muscle activity during wakefulness and sleep in normal adults. J Appl Physiol (1985). 65**,** 1332-9.

Lang, T., et al., 2010. Sarcopenia: etiology, clinical consequences, intervention, and assessment. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 21**,** 543-559.

Larsson, L., 1995. Motor units: remodeling in aged animals. J Gerontol A Biol Sci Med Sci. 50 Spec No**,** 91-5.

Leslie, J.H., Nedivi, E., 2011. Activity-regulated genes as mediators of neural circuit plasticity. Prog Neurobiol. 94**,** 223-37.

Lexell, J., 1995. Human aging, muscle mass, and fiber type composition. The journals of gerontology.Series A, Biological sciences and medical sciences. 50 Spec No**,** 11-16.

Li, Y., Lee, Y., Thompson, W.J., 2011. Changes in aging mouse neuromuscular junctions are explained by degeneration and regeneration of muscle fiber segments at the synapse. J Neurosci. 31**,** 14910-9.

Linden, D.J., 1994. Long-term synaptic depression in the mammalian brain. Neuron. 12**,** 457-72.

Lindgren, S., Janzon, L., 1991. Prevalence of swallowing complaints and clinical findings among 50-79-year-old men and women in an urban population. Dysphagia. 6**,** 187-192.

Lindsay, R.M., 1996. Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview. Philosophical transactions of the Royal Society of London.Series B, Biological sciences. 351**,** 365-373.

Linville, S.E., 2002. Source characteristics of aged voice assessed from long-term average spectra. J Voice. 16**,** 472-9.

Liu, C.J., Latham, N.K., 2009. Progressive resistance strength training for improving physical function in older adults. Cochrane database of systematic reviews (Online). (3)**,** CD002759.

Liu, W., et al., 2014a. Calcineurin-NFAT Signaling and Neurotrophins Control Transformation of Myosin Heavy Chain Isoforms in Rat Soleus Muscle in Response to Aerobic Treadmill Training. J Sports Sci Med. 13**,** 934-44.

Liu, Y., Grumbles, R.M., Thomas, C.K., 2014b. Electrical stimulation of transplanted motoneurons improves motor unit formation. J Neurophysiol. 112**,** 660-70.

Loeb, D.M., et al., 1992. NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140prototrk. Neuron. 9**,** 1053-65.

Loeb, J.A., et al., 2002. Neuregulin expression at neuromuscular synapses is modulated by synaptic activity and neurotrophic factors. J Neurosci. 22**,** 2206-14.

Logemann, J.A., 1990. Effects of aging on the swallowing mechanism. Otolaryngologic clinics of North America. 23**,** 1045-1056.

Logemann, J.A., et al., 1992. Closure mechanisms of laryngeal vestibule during swallow. The American Journal of Physiology. 262**,** G338-44.

Logemann, J.A., et al., 2000. Temporal and biomechanical characteristics of oropharyngeal swallow in younger and older men. J Speech Lang Hear Res. 43**,** 1264-74.

Logemann, J.A., 2005. The role of exercise programs for dysphagia patients. Dysphagia. 20**,** 139-140.

Lomo, T., 2003. The discovery of long-term potentiation. Philos Trans R Soc Lond B Biol Sci. 358**,** 617-20.

Lu, B., 2003. BDNF and activity-dependent synaptic modulation. Learning & memory (Cold Spring Harbor, N.Y.). 10**,** 86-98.

Ludlow, C.L., et al., 2008. Translating principles of neural plasticity into research on speech motor control recovery and rehabilitation. Journal of speech, language, and hearing research : JSLHR. 51**,** S240-58.

Maeda, K., Akagi, J., 2015. Decreased Tongue Pressure is Associated with Sarcopenia and Sarcopenic Dysphagia in the Elderly. Dysphagia. 30**,** 80-7.

Mahne, A., et al., 2007. Assessment of age-related morphological and functional changes of selected structures of the head and neck by computed tomography, magnetic resonance imaging, and positron emission tomography. Semin Nucl Med. 37**,** 88-102.

Mang, C.S., et al., 2013. Promoting neuroplasticity for motor rehabilitation after stroke: considering the effects of aerobic exercise and genetic variation on brain-derived neurotrophic factor. Phys Ther. 93**,** 1707-16.

Mantilla, C.B., Zhan, W.Z., Sieck, G.C., 2004. Neurotrophins improve neuromuscular transmission in the adult rat diaphragm. Muscle & nerve. 29**,** 381-386.

Mantilla, C.B., et al., 2014. TrkB kinase activity maintains synaptic function and structural integrity at adult neuromuscular junctions. J Appl Physiol (1985). 117**,** 910-20.

Marais, L., Stein, D.J., Daniels, W.M., 2009. Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. Metabolic brain disease. 24**,** 587-597.

Marcell, T.J., 2003. Sarcopenia: causes, consequences, and preventions. The journals of gerontology.Series A, Biological sciences and medical sciences. 58**,** M911-6.

Marik, P.E., Kaplan, D., 2003. Aspiration Pneumonia and Dysphagia in the Elderly. Chest. 124**,** 328.

Maronian, N., et al., 2003. Electromyographic findings in recurrent laryngeal nerve reinnervation. The Annals of Otology, Rhinology, and Laryngology. 112**,** 314-323.

Matochik, J.A., White, N.R., Barfield, R.J., 1992. Variations in scent marking and ultrasonic vocalizations by Long-Evans rats across the estrous cycle. Physiology & Behavior. 51**,** 783-786.

Mattson, M.P., 2000. Neuroprotective signaling and the aging brain: take away my food and let me run. Brain research. 886**,** 47-53.

McCulloch, T.M., et al., 1996. Laryngeal activity during swallow, phonation, and the Valsalva maneuver: an electromyographic analysis. The Laryngoscope. 106**,** 1351-1358.

McFarland, D.H., Tremblay, P., 2006. Clinical implications of cross-system interactions. Seminars in speech and language. 27**,** 300-309.

McFarland, D.H., Paydarfar, D., 2011. Proceedings of the Integrative Neural Systems Underlying Vital Aerodigestive Tract Functions Conference, June 17-19, 2010: work group summary and call to action. Head & neck. 33 Suppl 1**,** S54-7.

McGinnis, M.Y., Vakulenko, M., 2003. Characterization of 50-kHz ultrasonic vocalizations in male and female rats. Physiology & Behavior. 80**,** 81-88.

McMullen, C.A., Andrade, F.H., 2006. Contractile dysfunction and altered metabolic profile of the aging rat thyroarytenoid muscle. Journal of applied physiology (Bethesda, Md.: 1985). 100**,** 602-608.

Merrill, R.M., Anderson, A.E., Sloan, A., 2011. Quality of life indicators according to voice disorders and voice-related conditions. The Laryngoscope. 121**,** 2004-2010.

Miller, A.J., 2002. Oral and pharyngeal reflexes in the mammalian nervous system: their diverse range in complexity and the pivotal role of the tongue. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 13**,** 409-425.

Miller, J.L., Watkin, K.L., Chen, M.F., 2002. Muscle, adipose, and connective tissue variations in intrinsic musculature of the adult human tongue. Journal of speech, language, and hearing research : JSLHR. 45**,** 51-65.

Mujika, I., Padilla, S., 2000a. Detraining: loss of training-induced physiological and performance adaptations. Part II: Long term insufficient training stimulus. Sports medicine (Auckland, N.Z.). 30**,** 145-154.

Mujika, I., Padilla, S., 2000b. Detraining: loss of training-induced physiological and performance adaptations. Part I: short term insufficient training stimulus. Sports medicine (Auckland, N.Z.). 30**,** 79-87.

Nagai, H., et al., 2005. Discoordination of laryngeal and respiratory movements in aged rats. Am J Otolaryngol. 26**,** 377-82.

Nagai, H., et al., 2008. Effect of aging on tongue protrusion forces in rats. Dysphagia. 23**,** 116-121.

National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory, A., 2011. The National Academies Collection: Reports funded by National Institutes of Health. In: Guide for the Care and Use of Laboratory Animals. Vol., ed.^eds. National Academies Press (US)

National Academy of Sciences, Washington (DC).

Ney, D.M., et al., 2009. Senescent swallowing: impact, strategies, and interventions. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 24**,** 395-413.

Nicosia, M.A., et al., 2000. Age effects on the temporal evolution of isometric and swallowing pressure. The journals of gerontology.Series A, Biological sciences and medical sciences. 55**,** M634-40.

Niederman, M.S., et al., 1998. The cost of treating community-acquired pneumonia. Clin Ther. 20**,** 820-37.

Nishida, N., et al., 2013. Age-related changes in rat intrinsic laryngeal muscles: analysis of muscle fibers, muscle fiber proteins, and subneural apparatuses. Eur Arch Otorhinolaryngol. 270**,** 975-84.

Nishio, M., Niimi, S., 2004. Relationship between speech and swallowing disorders in patients with neuromuscular disease. Folia phoniatrica et logopaedica : official organ of the International Association of Logopedics and Phoniatrics (IALP). 56**,** 291-304.

Nowinski, W.L., et al., 2012. Three-dimensional interactive and stereotactic atlas of the cranial nerves and their nuclei correlated with surface neuroanatomy, vasculature and magnetic resonance imaging. Journal of neuroscience methods. 206**,** 205-216.

Ohnishi, A., et al., 1992. [Quantitative evaluation of tongue atrophy on midsagittal magnetic resonance images (MRIs)]. Rinsho Shinkeigaku. 32**,** 317-20.

Ota, F., Connor, N.P., Konopacki, R., 2005. Alterations in Contractile Properties of Tongue Muscles in Old Rats. Annals of Otology, Rhinology & Laryngology. 114**,** 799-803.

Palkovits, M., Brownstein, M.J., 1988. Maps and guide to mircodissection of the rat brain, Vol., Elsevier Science Publishing Co., Inc., New York, NY.

Palmer, P.M., et al., 2008. Quantitative contributions of the muscles of the tongue, floor-of-mouth, jaw, and velum to tongue-to-palate pressure generation. Journal of speech, language, and hearing research : JSLHR. 51**,** 828-835.

Patapoutian, A., Reichardt, L.F., 2001. Trk receptors: mediators of neurotrophin action. Current opinion in neurobiology. 11**,** 272-280.

Pedersen, B.K., et al., 2009. Role of exercise-induced brain-derived neurotrophic factor production in the regulation of energy homeostasis in mammals. Exp Physiol. 94**,** 1153-60.

Perlman, A.L., et al., 1999. Electromyographic activity from human laryngeal, pharyngeal, and submental muscles during swallowing. Journal of applied physiology (Bethesda, Md.: 1985). 86**,** 1663-1669.

Peterson, M.D., Sen, A., Gordon, P.M., 2011. Influence of resistance exercise on lean body mass in aging adults: a meta-analysis. Medicine and science in sports and exercise. 43**,** 249-258.

Pette, D., Staron, R.S., 1990. Cellular and molecular diversities of mammalian skeletal muscle fibers. Rev Physiol Biochem Pharmacol. 116**,** 1-76.

Pette, D., Staron, R.S., 2000. Myosin isoforms, muscle fiber types, and transitions. Microscopy research and technique. 50**,** 500-509.

Pette, D., Staron, R.S., 2001. Transitions of muscle fiber phenotypic profiles. Histochemistry and cell biology. 115**,** 359-372.

Ploughman, M., 2008. Exercise is brain food: the effects of physical activity on cognitive function. Developmental neurorehabilitation. 11**,** 236-240.

Pontes, P., Brasolotto, A., Behlau, M., 2005. Glottic characteristics and voice complaint in the elderly. J Voice. 19**,** 84-94.

Pontes, P., Yamasaki, R., Behlau, M., 2006. Morphological and functional aspects of the senile larynx. Folia phoniatrica et logopaedica : official organ of the International Association of Logopedics and Phoniatrics (IALP). 58**,** 151-158.

Poo, M.M., 2001. Neurotrophins as synaptic modulators. Nature reviews.Neuroscience. 2**,** 24-32.

Pressman, J.J., 1941. SPhincter action of the larynx. Vol. 33, ed.^eds., pp. 351-377.

Pultorak, J., et al., 2015. Decreased approach behavior and nucleus accumbens immediate early gene expression in response to Parkinsonian ultrasonic vocalizations in rats. Social Neuroscience.

Rage, F., et al., 2007. Effect of aging on the expression of BDNF and TrkB isoforms in rat pituitary. Neurobiology of aging. 28**,** 1088-1098.

Riede, T., 2011. Subglottal pressure, tracheal airflow, and intrinsic laryngeal muscle activity during rat ultrasound vocalization. Journal of neurophysiology. 106**,** 2580-2592.

Riede, T., 2014. Rat ultrasonic vocalization shows features of a modular behavior. J Neurosci. 34**,** 6874-8.

Robbins, J., et al., 1992. Oropharyngeal swallowing in normal adults of different ages. Gastroenterology. 103**,** 823-829.

Robbins, J., et al., 1995. Age effects on lingual pressure generation as a risk factor for dysphagia. J Gerontol A Biol Sci Med Sci. 50**,** M257-62.

Robbins, J., et al., 2005. The Effects of Lingual Exercise on Swallowing in Older Adults. Journal of the American Geriatrics Society. 53**,** 1483-1489.

Robbins, J., et al., 2007. The effects of lingual exercise in stroke patients with dysphagia. Archives of Physical Medicine and Rehabilitation. 88**,** 150-158.

Robbins, J., et al., 2008. Swallowing and dysphagia rehabilitation: translating principles of neural plasticity into clinically oriented evidence. Journal of speech, language, and hearing research : JSLHR. 51**,** S276-300.

Rosenberg, I.H., 1997. Sarcopenia: origins and clinical relevance. The Journal of nutrition. 127**,** 990S-991S.

Rother, P., et al., 2002. Morphometrically observable aging changes in the human tongue. Ann Anat. 184**,** 159-64.

Roubenoff, R., 2001. Origins and clinical relevance of sarcopenia. Canadian journal of applied physiology = Revue canadienne de physiologie appliquee. 26**,** 78-89.

Roy, N., et al., 2003. Three treatments for teachers with voice disorders: a randomized clinical trial. Journal of speech, language, and hearing research : JSLHR. 46**,** 670-688.

Roy, N., et al., 2007. Epidemiology of voice disorders in the elderly: preliminary findings. The Laryngoscope. 117**,** 628-633.

Russell, J.A., et al., 2010. Targeted exercise therapy for voice and swallow in persons with Parkinson's disease. Brain research. 1341**,** 3-11.

Russell, J.A., et al., 2013. Videofluorographic assessment of deglutitive behaviors in a rat model of aging and Parkinson disease. Dysphagia. 28**,** 95-104.

Sauerland, E.K., Mitchell, S.P., 1975. Electromyographic activity of intrinsic and extrinsic muscles of the human tongue. Texas reports on biology and medicine. 33**,** 444-455.

Schaser, A.J., et al., 2011. Biochemistry of the anterior, medial, and posterior genioglossus in the aged rat. Dysphagia. 26**,** 256-263.

Schaser, A.J., et al., 2012. The effect of age and tongue exercise on BDNF and TrkB in the hypoglossal nucleus of rats. Behavioural brain research. 226**,** 235-241.

Schinder, A.F., Poo, M., 2000. The neurotrophin hypothesis for synaptic plasticity. Trends in neurosciences. 23**,** 639-645.

Schindler, J.S., Kelly, J.H., 2002. Swallowing disorders in the elderly. The Laryngoscope. 112**,** 589-602.

Schwarz, E.C., et al., 2009. The effects of aging on hypoglossal motoneurons in rats. Dysphagia. 24**,** 40-48.

Shaker, R., et al., 2002. Rehabilitation of swallowing by exercise in tube-fed patients with pharyngeal dysphagia secondary to abnormal UES opening. Gastroenterology. 122**,** 1314-21.

Siegelbaum, S.A., Kandel, E.R., 1991. Learning-related synaptic plasticity: LTP and LTD. Current opinion in neurobiology. 1**,** 113-120.

Skaper, S.D., 2008. The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS & neurological disorders drug targets. 7**,** 46-62.

Smith, A.D., Zigmond, M.J., 2003. Can the brain be protected through exercise? Lessons from an animal model of parkinsonism. Experimental neurology. 184**,** 31-39.

Sonies, B.C., et al., 1988. Durational aspects of the oral-pharyngeal phase of swallow in normal adults. Dysphagia. 3**,** 1-10.

Stathopoulos, E., Felson Duchan, J., 2006. History and principles of exercise-based therapy: how they inform our current treatment. Seminars in speech and language. 27**,** 227-235.

Steele, C.M., 2012. Exercise-based approaches to dysphagia rehabilitation. In: Nestle Nutr Inst Workshop Ser. Vol. 72, ed.^eds. Basel., Switzerland, pp. 109-17.

Steele, C.M., 2013. Optimal approaches for measuring tongue-pressure functional reserve. J Aging Res. 2013**,** 542909.

Stemple, J.C., et al., 1994. Efficacy of vocal function exercises as a method of improving voice production. J Voice. 8**,** 271-8.

Story, B.H., Titze, I.R., 1995. Voice simulation with a body-cover model of the vocal folds. The Journal of the Acoustical Society of America. 97**,** 1249-1260.

Stuart, T.P.A., 1891. On the Mechanism of the Closure of the Larynx. A Preliminary Communication. Proceedings of the Royal Society of London. 50**,** 323-339.

Suzuki, T., et al., 2002. Age-Related Alterations in Myosin Heavy Chain Isoforms in Rat Intrinsic Laryngeal Muscles. Annals of Otology, Rhinology & Laryngology. 111**,** 962.

Takeda, N., Thomas, G.R., Ludlow, C.L., 2000. Aging effects on motor units in the human thyroarytenoid muscle. The Laryngoscope. 110**,** 1018-1025.

Tay, E.Y., Phyland, D.J., Oates, J., 2012. The effect of vocal function exercises on the voices of aging community choral singers. J Voice. 26**,** 672.e19-27.

Thomas, D.R., 2010. Sarcopenia. Clinics in geriatric medicine. 26**,** 331-346.

Tian, H., et al., 2013. Health care utilization and costs among patients with AD with and without dysphagia. Alzheimer Dis Assoc Disord. 27**,** 138-44.

Tibbling, L., Gustafsson, B., 1991. Dysphagia and its consequences in the elderly. Dysphagia. 6**,** 200-2.

Tong, C.W., et al., 2015. Effects of senescence on the expression of BDNF and TrkB receptor in the lateral geniculate nucleus of cats. Dongwuxue Yanjiu. 36**,** 48-53.

Toth, L.A., Gardiner, T.W., 2000. Food and water restriction protocols: physiological and behavioral considerations. Contemporary topics in laboratory animal science / American Association for Laboratory Animal Science. 39**,** 9-17.

Tracy, J.F., et al., 1989. Preliminary observations on the effects of age on oropharyngeal deglutition. Dysphagia. 4**,** 90-4.

Turley, R., Cohen, S., 2009. Impact of voice and swallowing problems in the elderly. Otolaryngol Head Neck Surg. 140**,** 33-6.

Turturro, A., et al., 1999. Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. The journals of gerontology.Series A, Biological sciences and medical sciences. 54**,** B492-501.

Valdez, G., et al., 2010. Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci U S A. 107**,** 14863-8.

Van Daele, D.J., et al., 2005. Timing of Glottic Closure During Swallowing: A Combined Electromyographic and Endoscopic Analysis. Annals of Otology, Rhinology & Laryngology. 114**,** 478-487.

Vaynman, S., Ying, Z., Gomez-Pinilla, F., 2003. Interplay between brain-derived neurotrophic factor and signal transduction modulators in the regulation of the effects of exercise on synaptic-plasticity. Neuroscience. 122**,** 647-657.

Vaynman, S., Gomez-Pinilla, F., 2005. License to run: exercise impacts functional plasticity in the intact and injured central nervous system by using neurotrophins. Neurorehabilitation and neural repair. 19**,** 283-295.

Verdonck-de Leeuw, I.M., Mahieu, H.F., 2004. Vocal aging and the impact on daily life: a longitudinal study. J Voice. 18**,** 193-202.

Warraich, Z., Kleim, J.A., 2010. Neural plasticity: the biological substrate for neurorehabilitation. Pm r. 2**,** S208-19.

Waters, D.L., et al., 2010. Advantages of dietary, exercise-related, and therapeutic interventions to prevent and treat sarcopenia in adult patients: an update. Clinical interventions in aging. 5**,** 259-270.

Widmer, H.R., et al., 1993. Rapid phosphorylation of phospholipase C gamma 1 by brain-derived neurotrophic factor and neurotrophin-3 in cultures of embryonic rat cortical neurons. J Neurochem. 60**,** 2111-23.

Wilkerson, J.E., Mitchell, G.S., 2009. Daily intermittent hypoxia augments spinal BDNF levels, ERK phosphorylation and respiratory long-term facilitation. Experimental neurology. 217**,** 116-123.

Wohr, M., et al., 2008. Effects of experience and context on 50-kHz vocalizations in rats. Physiology & Behavior. 93**,** 766-776.

Yeates, E.M., Molfenter, S.M., Steele, C.M., 2008. Improvements in tongue strength and pressure-generation precision following a tongue-pressure training protocol in older individuals with dysphagia: three case reports. Clinical interventions in aging. 3**,** 735-747.

Ying, Z., et al., 2003. Voluntary exercise increases neurotrophin-3 and its receptor TrkC in the spinal cord. Brain research. 987**,** 93-99.

Youmans, S.R., Youmans, G.L., Stierwalt, J.A., 2009. Differences in tongue strength across age and gender: is there a diminished strength reserve? Dysphagia. 24**,** 57-65.

Yuen, E.C., Mobley, W.C., 1995. Therapeutic applications of neurotrophic factors in disorders of motor neurons and peripheral nerves. Molecular medicine today. 1**,** 278-286.

Yuen, E.C., 2001. The role of neurotrophic factors in disorders of peripheral nerves and motor neurons. Physical Medicine and Rehabilitation Clinics of North America. 12**,** 293-306, viii.

Zhan, W.Z., Mantilla, C.B., Sieck, G.C., 2003. Regulation of neuromuscular transmission by neurotrophins. Sheng Li Xue Bao. 55**,** 617-24.

Zoladz, J.A., Pilc, A., 2010. The effect of physical activity on the brain derived neurotrophic factor: from animal to human studies. J Physiol Pharmacol. 61**,** 533-41.

Appendix

Table 2: Means and SEM. Values are neurotrophin (NT) concentration (pg/ml), YA= Young Adult. Open cells represent detraining data from the laryngeal brainstem nuclei (NA) and muscle (TA). These data were not analyzed due to the lack of an initial exercise effect on the laryngeal tissue.

NT	Location		Control		Exercise		2W		4W	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NT4	BSC	YA	40447	7278	84578	18838	76744	19468	59983	11523
		Old	43113	5390	41886	6800	38904	5112	33402	4206
	HN	YA	40482	8228	74547	16076	52964	9872	47394	7602
		Old	33745	5836	34885	7833	26533	3989	27782	2946
	NA	YA	54954	11099	68808	12173				
		Old	38618	6404	37893	5072				
	EDL	YA	16542	6722	29858	6707	21663	2343	21168	3635
		Old	22575	7825	12279	2848	20235	3860	12181	1770
	GG	YA	10783	2593	7357	1830	17618	4653	13375	5102
		Old	8111	1943	9797	1849	14536	5790	9257	1610
	TA	YA	46989	3635	66573	10547				
		Old	82788	8724	83013	18156				
BDNF	BSC	YA	168	62	133	43	252	80	163	36
		Old	85	28	376	205	137	40	170	34
	HN	YA	337	105	471	91	530	107	450	105
		Old	296	83	1049	381	341	89	377	61
	NA	YA	107	35	206	77				
		Old	144	23	555	308				
	EDL	YA	401	180	584	236	188	62	338	178
		Old	228	152	586	279	129	36	351	306
	GG	YA	325	133	129	43	311	47	328	90
		Old	220	70	375	122	282	98	192	33
	TA	YA	657	230	351	148				
		Old	165	79	486	32				
TrkB	BSC	YA	645	191	1277	567	709	262	483	159
		Old	775	324	733	190	771	237	1379	844
	HN	YA	834	323	1354	612	956	419	792	267
		Old	974	414	1059	352	1058	280	960	492
	NA	YA	920	360	436	203				
		Old	506	238	787	279				
	EDL	YA	3882	613	5278	834	3620	413	2785	427
		Old	4268	773	3193	889	3490	788	5069	1116
	GG	YA	2584	636	2815	692	3210	302	2184	482
		Old	2041	475	2575	433	2496	420	3145	637
	TA	YA	4635	1151	8568	2204				
		Old	6826	1851	9894	4433				