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The Effects of Aging on Hypoglossal Motoneurons in Rats

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Abstract

Aging can result in a loss of neuronal cell bodies and a decrease in neuronal size in some regions of the brain and spinal cord. Motoneuron loss in the spinal cord is thought to contribute to the progressive decline in muscle mass and strength that occurs with age (sarcopenia). Swallowing disorders represent a large clinical problem in elderly persons; however, age-related alterations in cranial motoneurons that innervate muscles involved in swallowing have been understudied. We aimed to determine if age-related alterations occurred in the hypoglossal nucleus in the brainstem. If present, these changes might help explain alterations at the neuromuscular junction and changes in the contractile properties of tongue muscle that have been reported in older rats. We hypothesized that with increasing age, there would be a loss of motoneurons and a reduction in neuronal size and the number of primary dendrites associated with each hypoglossal motoneuron. Neurons in the hypoglossal nucleus were visualized with the neuronal marker NeuN in young (9–10 months), middle-aged (24–25 months), and old (32–33 months) male F344/BN rats. Hypoglossal motoneurons were retrograde labeled with injections of Cholera Toxin β into the genioglossus muscle of the tongue and visualized using immunocytochemistry. Results indicated that the number of primary dendrites of hypoglossal motoneurons decreased significantly with age, while no age-associated changes were found in the number or size of hypoglossal motoneurons. Loss of primary dendrites could reduce the number of synaptic inputs and thereby impair function.

Keywords

deglutition; deglutition disorders; Sarcopenia; cranial motoneurons; genioglossus muscle; tongue; aging

Introduction

The tongue, which is largely composed of fast-contracting, fatigue-resistant muscles [1–5], is innervated by motoneurons in the hypoglossal nucleus in the caudal brainstem [6–9]. The tongue plays an essential role in speech, swallowing, and maintaining upper-airway patency [8,10–17].

Aging can result in impairments in voice, speech, and swallowing [18–23], and alterations in the neuromuscular properties of tongue may contribute to this functional decline [24–26]. For example, Crow and Ship [27] found a significant decrease in tongue strength with age in both men and women. Further, human radiographic swallow studies [19,22] have demonstrated altered temporal patterns in the oral phase of swallowing in healthy, elderly human participants. Because the tongue principally mediates the oral preparatory and oral phases of swallowing,

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these data suggest that alterations in lingual neuromuscular integrity may be manifested in elderly persons.

While considerable data are available concerning age-related changes in spinal motoneurons and the muscles they innervate, less information is available on the effects of age on hypoglossal motoneurons and their associated muscles. For example, aging can result in a loss of spinal motor neurons and a decrease in neuronal size in humans and rodents [28–33]. These changes in motoneurons are thought to contribute to a progressive decline in muscle mass and strength, a condition known as sarcopenia [34–36]. It is not clear if similar manifestations of aging are present within hypoglossal motoneurons. Studies in mice and rats have reported a reduction in the number of neurons in some cranial motor nuclei but not in others [37–44]. Accordingly, data are inconsistent with regard to age-related motoneuron decline within the cranial motor system.

There is good reason to suspect that the number and size of hypoglossal motoneurons may be affected by aging. For instance, other parameters of hypoglossal motoneurons appear to change with age, such as serotonergic input to hypoglossal nuclei and serotonin receptors [45–47]. Aging also impairs plasticity in hypoglossal motor output in response to intermittent hypoxia [48]. In tongue muscles, changes in the contractile properties have been reported in aged rats [24–26], in addition to an age-associated loss of cholinergic receptors at the neuromuscular junction [49]. As such, loss of motoneuron number and size may be a potential mechanism for the changes in muscle contractile properties and neuromuscular junction morphology found in aging tongue muscles.

Unlike spinal motor neurons, some of which show distinct age-associated changes in morphology, it is unclear whether hypoglossal motoneuron morphology is altered with aging. We hypothesized that, with increasing age, there would be a decrease in the number and size of hypoglossal motoneurons in rats and a reduction in the number of primary dendrites associated with each motoneuron. Neurons in the hypoglossal nucleus were identified with the neuronal marker NeuN, and motoneurons were retrograde labeled by injection of the neuroanatomical tracer Cholera Toxin β into the genioglossus muscle of the tongue.

Methods

All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin School of Veterinary Medicine and School of Medicine and Public Health. Three age groups of rats were studied: young (9–10 months), middle-aged (24–25 months), and old (32–33 months). A total of 15 (5 young, 5 middle-aged, 5 old) male Fischer 344/Brown Norway (F344/BN) rats were obtained from the National Institute of Aging colony. Every effort was made to minimize the number of animals used and their suffering.

Retrograde labeling of motoneurons

Rats were anesthetized with isoflurane in an induction chamber and maintained via a nose cone (2% isoflurane in 30% O_2). Their tongues were extended with a forceps. A Hamilton microliter syringe with a 30-gauge needle was used to inject 5 μl of Cholera Toxin β (CTβ; List Biological Laboratories, Campbell, CA, USA) into the genioglossus muscle of the tongue at 2 different injections sites. Rats survived for 4 days prior to perfusion.

Perfusion

Rats were anesthetized with isoflurane followed by sodium pentobarbital (120 mg/kg I.P.). Anesthetized rats were transcardially perfused with 200 ml 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 0.1 M phosphate buffer. Sections were cut coronally (50 μm) and stored in 0.1 M PB containing 0.02% sodium azide at 4° C. Each hypoglossal nucleus yielded approximately 45 sections. These sections were divided equally to represent rostral, middle, and caudal regions of the nucleus.

Immunocytochemistry

Fifteen equally spaced sections (5 rostral, 5 middle, 5 caudal) through the hypoglossal nucleus from each animal were reacted for the presence of the neuronal marker NeuN. Sections were washed, blocked with 10% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS) for 1 hour, and incubated in primary antibody overnight (mouse MAB377, Chemicon, Temecula, CA, USA; 1:10,000) with 0.3% Tx-100, 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in 0.01 M PBS at 4º C. Negative controls consisted of the omission of primary antibody. Sections were washed and incubated with secondary antibody Alexafluor 568 goat anti-rabbit (Molecular Probes; 1:300) with 1% NGS, 0.75% Tx-100 (Tx-100; Sigma, St. Louis, MO, USA) in 0.01 M PBS for 90 minutes. Sections were washed and mounted on slides with Vectashield Mounting media (Vector Laboratories, Burlingame, CA, USA). Slides were stored in the dark at 4° C.

Fifteen additional equally spaced sections (5 rostral, 5 middle, 5 caudal) through the hypoglossal nucleus from each animal were reacted for CTβ immunoreactivity. Tissue sections were incubated in 1% hydrogen peroxide in 0.1 M PBS for 30 minutes. Sections were washed, blocked with 20% normal rabbit serum (NRS) in 0.3% Tx-100, and 2% BSA in 0.1 M PBS for 45 minutes, and incubated in primary antibody overnight (goat anti-CTβ; List Biological Laboratories; 1:20,000) with 1% NRS, 0.3% Tx-100, and 2% BSA in 0.1 M PBS at room temperature. Negative controls consisted of the omission of primary antibody. Sections were washed, incubated in biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; 1:300) with 0.3% Tx-100, 2% BSA in 0.1 M PBS for 3 hours. Sections were washed again and incubated in Vectastain ABC (Vector Elite Kit; Vector Laboratories, Burlingame, CA, USA) at the recommended dilution for 1 hour. Sections were washed and incubated in 0.04% 3, 3′-diaminobenzidine (DAB) in 0.1 M PB with 0.004% hydrogen peroxide. Sections were washed, mounted on slides, dried overnight, and cover-slipped with Eukitt (Calibrated Instruments, Hawthorne, NY, USA).

Analysis

Fluorescence microscopy was used to quantify NeuN-stained motoneurons in sections through the hypoglossal nucleus (Nikon Eclipse E600; filter 535/50). Digital images of left and right hypoglossal nuclei were visualized on a computer screen (SPOT, Diagnostic Instruments) at 20x magnification, and the location of each neuron recorded on a transparent sheet affixed to the computer monitor. By focusing through the 50 μm section, we could mark and count all NeuN-stained neurons without duplication. In order to distinguish motoneurons from interneurons, only cells >20 μm in diameter were counted as motoneurons because interneurons are significantly smaller (10–18 μm) than motoneurons (25–50 μm) (Boones and Aldes, 1984).

The mean diameter of motoneurons was determined from sections through the hypoglossal nucleus that contained retrograde-labeled neurons. Photomicrographs of CTβ-immunoreactive neurons in the hypoglossal nucleus ipsilateral to the tongue injection were captured using a 60x objective lens. The mean diameter of each clearly labeled neuron was measured using Image-Pro Plus software (Media Cybernetics) from these digital images. Prior to photography, by focusing through the entire depth of the 50 μm section at 60x magnification, we could count

all primary dendrites associated with each retrograde-labeled neuron. Numbers were averaged within each region (5 sections/region), within each rat (3 regions/rat), and within each age group (5 rats/group). The microscopist was blinded to the identity of each rat.

For each of the three variables measured (number of neurons, number of dendrites, and neuron diameter), values were averaged across all sections for an animal within a region (caudal, medial, rostral) to obtain a mean. The regional means for each animal were analyzed in a mixed two-way analysis of variance (ANOVA) model with region, age, and their interaction as fixed effects and the rat by region interaction as a random effect; this is equivalent to a split-plot design with age as a whole-plot factor and region as a sub-plot factor. P-values were obtained for each of the three fixed effects, and, if deemed significant ($P < 0.05$, two-sided) pair-wise comparisons between the level factors were obtained (Fisher's LSD). The plausibility of the assumptions for ANOVA were first examined via exploratory analysis and residual plots and found to be adequate for use of this statistical method. Statistical computations were done with Proc Mixed in SAS version 9.1.3 for Windows (SAS Institute, Cary, NC).

Results

Number of Neurons

NeuN-stained neurons were found throughout the rostrocaudal extent of the hypoglossal nucleus (Figure 1). Two-way analysis of variance revealed that the number of neurons did not differ among the age groups ($F[2,24] = .69$, $p = .51$). However, as the nucleus tapers at each end, significant differences were noted among regions ($F[2,24] = 35.9$, $p < .0001$). Posthoc pairwise comparisons found that there were more neurons located in the middle than in rostral or caudal regions ($p < 0.001$; Table 1). There was not a significant interaction between age and region ($F[2,24] = .02$, $p = .999$).

Diameter of Neurons

Neurons that were retrogradely labeled from the genioglossus muscle of the tongue were located primarily in the ventral half of the ipsilateral hypoglossal nucleus (Figure 2A). Labeled neurons had a multipolar morphology typical of motoneurons, with from one to seven primary dendrites (Figure 2B, C). The mean diameter of retrograde-labeled hypoglossal motoneurons is shown in Table 2. Two-way analysis of variance revealed that the mean diameter of hypoglossal motoneurons did not differ among the age groups ($F[2,24] = .70$, $p = .51$). Significant differences were noted among regions ($F[2,24] = 43.89$, $p = .0001$), with larger neurons located rostrally and smaller neurons located caudally. Posthoc pairwise comparisons found that neuronal size differed between caudal and middle regions ($p < 0.001$), between caudal and rostral regions ($p < .0001$) and between middle and rostral regions ($p = .0002$). There was not a significant interaction between age and region ($F[2,24] = 1.12$, $p = .37$). The size distribution of retrogradely labeled neurons is shown in Figure 3. There appears to be a shift to the right in the distribution of large neurons in old rats with a cut-off at 22.5 μm. However, when neurons >22.5 μm in diameter were analyzed in the three age groups, no significant differences were detected among the age groups ($F[2,7] = .03$, $p = .97$). Pairwise posthoc comparisons of neurons >22.5 μm in diameter found that neuronal size differed between caudal and rostral regions ($p < .002$) and between middle and rostral regions ($p = .009$).

Number of primary dendrites

Two-way analysis of variance revealed that the number of primary dendrites was significantly different among the age groups ($F[2,24] = 4.12$, $p = .03$). Posthoc pairwise comparisons found that middle-aged rats had more primary dendrites associated with hypoglossal motoneurons than young ($p = .03$) or old ($p = .03$). No significant differences were noted among regions (F

[2,24] = 1.61, p=.22).. There was not a significant interaction between age and region (F[2,24] = .34, p=.85).

Discussion

We hypothesized that there would be a decrease in the number of hypoglossal motoneurons and a reduction in neuronal size and the number of primary dendrites with increasing age. However, we found that while neuronal number and size remained stable throughout life in F344/BN rats, the number of primary dendrites was significantly reduced in the old animals. These data suggest that, unlike spinal motoneurons which show age-associated changes in neuronal number and soma size, hypoglossal motoneurons appear to be less vulnerable to these morphological changes. Nonetheless, fewer primary dendrites reduces the surface area available for synaptic input, and could lead to changes in function.

Our data are consistent with those of Sturrock [42] who found no age-associated change in the number of hypoglossal motoneurons or motoneuron size in mice. Similarly, in a study of human patients with Parkinson disease, the control population (aged 61–88 years) did not show any loss of hypoglossal motoneurons [50]. Sturrock [42] hypothesized that the fine movements in which the tongue is involved on a daily basis during respiration, speech, and swallowing could account for the stable number of hypoglossal motoneurons. Sturrock also noted that the number of motoneurons in nuclei innervating the extra-ocular muscles, which are also activated continuously, do not decline with age [39]. Sustained neuromuscular activity may upregulate neurotrophins that are involved in motoneuron survival. In the spinal cord, for example, neuromuscular activity can upregulate neurotrophin expression in motoneurons as well as in the muscles they innervate, and it has been suggested that the exercise-induced neurotrophins synthesized in muscle are transported in retrograde to spinal motoneurons [51]. Consequently, a reduction in spontaneous locomotor activity that is frequently associated with aging may deprive spinal motoneurons of critical trophic support for survival, whereas tonic activity in select cranial motoneurons may enhance their survival.

The tongue is critically involved in breathing, and genioglossal motoneurons discharge rhythmically during each inspiratory phase of respiration [10–13]. Thus, hypoglossal motoneurons may have unique properties that enable them to function optimally throughout life and resist the affects of aging and disease. In the SOD1 G93A transgenic rat model of amyotrophic lateral sclerosis (ALS), at a disease stage where there was considerable loss of spinal and phrenic motoneurons, hypoglossal motoneuron cell counts were relatively normal [52,53]. In human patients with ALS, loss of phrenic motoneurons innervating the diaphragm with muscle atrophy and subsequent respiratory failure is the usual cause of death [54].

Previous studies of lumbar motoneuron size in F344 rats showed a size increase with age, and the authors suggested that this effect may be an adaptive mechanism to accommodate the increased amount of lipofuscin that accumulates in neurons as they age [33]. Our data suggest that larger motoneurons (>22.5 μm diameter) in the hypoglossal nucleus tend to increase slightly in size with age, although this was not statistically significant (Fig. 3). Our data also show that more large neurons are located in the rostrally than in the middle or caudal parts of the hypoglossal nucleus, which may reflect the topographic organization of neurons that innervate specific muscle of the tongue [6,7,8,9].

It is possible that additional age-related changes occur in the hypoglossal nucleus, but that the experimental design we used was unable to detect them. Tongue injections of CTβ selectively labeled motoneurons innervating the primary protrudor muscle of the tongue, which is critical to respiration, but not retractor muscle motoneurons, which are involved in swallowing [55]. Both protrudor and retractor muscles are involved in speech, mastication, and licking [55]. As

protrudor and retractor neuronal populations are approximately equal, any loss of neurons innervating retractor tongue muscles would have been detected. However, changes in neuronal cell size associated with retractor neurons would not have been detected in this study.

Although no age-associated changes in the number of genioglossal motoneurons were detected, it is possible that interneurons in the hypoglossal nucleus are selectively affected by aging [31,56,57]. Interneurons were included in our neuronal counts, but they were excluded in measurements of neuronal size and dendrite number. Approximately 13% of neurons in the hypoglossal nucleus are interneurons [42], thus it is unlikely that we would have detected any morphologic changes in this small population.

The number of primary dendrites associated with retrogradely-labeled genioglossal motoneurons increased significantly from young to middle-age and decreased from middle-age to old in the present study. Interestingly, the number of dendrites associated with genioglossal motoneurons in cats was reported to remain constant during development [58]. Loss of primary dendrites in hypoglossal motoneurons with increasing age may contribute to the changes in tongue function that have been reported in older human and rats [48]. Hypoglossal motoneurons receive synaptic inputs from premotor neurons in several brainstem nuclei [55]. Premotor neurons help to orchestrate the complex interactions between concurrent orofacial behaviors, and it is likely that a reduction in their synaptic input to hypoglossal motoneurons, particularly if there are fewer primary dendrites, contributes to alterations in speech, swallowing, and upper-airway control seen in the elderly. Age-related synaptic loss has been described in a number of brain regions [62] as well as in spinal motoneurons in rats [63]. Neuromodulatory systems, including acetylcholine, norepinephrine, and serotonin, are also affected by aging, and previous studies in our laboratory have shown an age-associated decrease in serotonergic synaptic input to the hypoglossal nucleus in the F344 rat strain [45, 46]. In addition to the reduction in the number of primary dendrites from middle-aged to old in rats, it is possible that the morphology of more distal dendrites also changes with age. Studies in primates and rodents have described pruning of dendritic arbors with age in pyramidal neurons in the cerebral cortex (for a review, see [59]), although proliferation of dendritic branches has been described in motoneurons supplying the intrinsic muscles of the foot in old cats [60].

Despite no changes in the number or diameter of hypoglossal motoneurons, there remains the possibility that axons of hypoglossal nerves degenerate or demyelinate with increasing age, which could result in decreased tongue strength and impaired function [24,27]. However, in a recent study of human hypoglossal nerve, the total number and diameter of myelinated fibers did not change when adult (<60 yrs) and elderly (>60 yrs) individuals were compared [61]. Receptors for select neurotransmitters and neuromodulators on hypoglossal motoneurons may also show age-associated changes that could contribute to altered function [47,59]. Age-associated changes in neurotrophic factors could impact the efficacy of synaptic transmission in hypoglossal neurons, as has been seen in the hippocampus [64]. Johnson et al. [43] described a decreased expression of neurotrophin receptors in spinal motoneurons of aged rats. Several lines of evidence suggest that oxidative stress and mitochondrial dysfunction play a major role in sarcopenia [65], and in a recent study of human skeletal muscle, the transcriptome profile for many mitochondrial genes was shown to be significantly altered with aging [66]. In summary, cranial neuromuscular dysfunction may result from a combination of factors that involves both the central and peripheral nervous system, the neuromuscular junction, and muscle cells.

Recently, progressive resistance training of the tongue for 8 weeks has been shown to increase maximal isometric pressure as well as swallowing pressures in the elderly [67], suggesting that a decline in cranial neuromuscular function can be partially reversed. Similar positive effects

of exercise on skeletal neuromuscular function in the elderly have been reported, although the underlying mechanism(s) are not clear [68–70]. Muscle activity has been shown to enhance the expression of muscle-derived neurotrophins at the neuromuscular junction [71,72], alter the regulation of central serotonergic activity [73], and increase nerve terminal branching at the neuromuscular junction [74]. Exercise can enhance the expression of anti-apoptotic markers in aging muscle (for a review, see [75]). Additionally, the signature mitochondrial transcriptome profile of aged vs. young humans is substantially reversed following 6 months of resistance-exercise training in skeletal muscle [66], suggesting that some of the beneficial effects of exercise are associated with energy balance within the muscle fiber itself. Thus, interventions aimed at reversing the effects of aging on orofacial behaviors, including speech, swallowing, and breathing, may need to be targeted to multiple sites in the cranial neuromuscular system.

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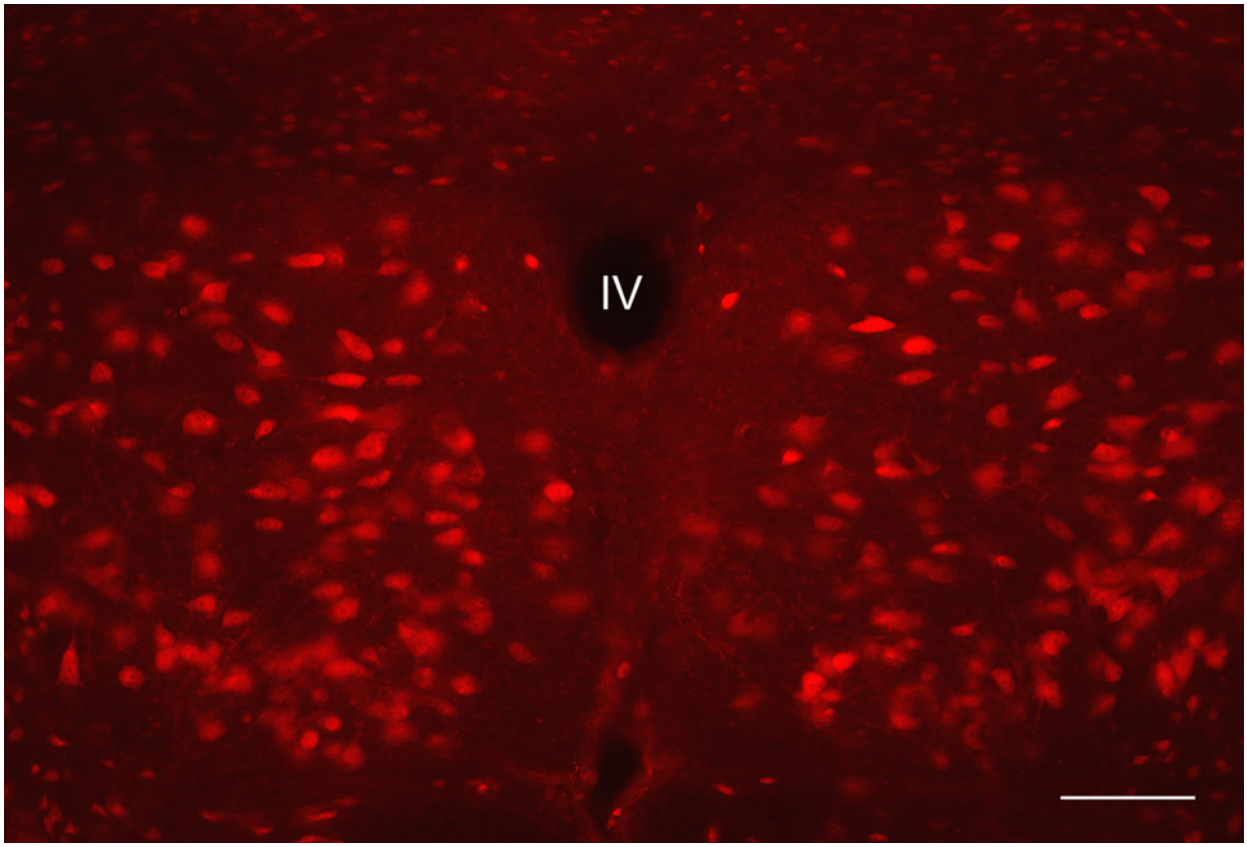

Figure 1.
Photomicrograph of NeuN-stained neurons in the paired hypoglossal nuclei of a middle-aged rat. The hypoglossal nuclei are located ventral and lateral to the fourth ventricle (IV). Scale bar = 150 μm.

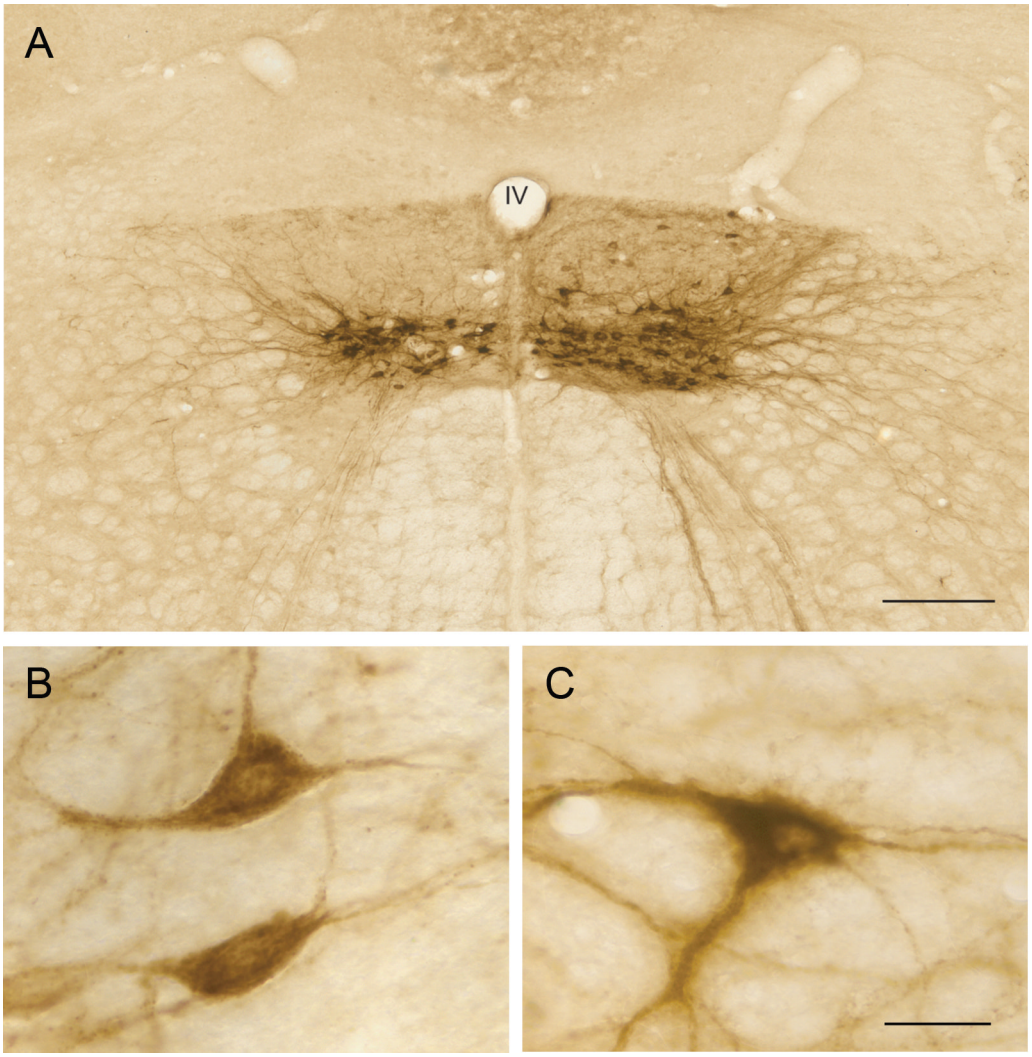

Figure 2.
A. Photomicrograph of the paired hypoglossal nuclei containing retrograde-labeled neurons from bilateral injections of Cholera toxin-β into the genioglossus muscle of the tongue. Note, the majority of labeled neurons are located in the ventral half of the nucleus. B, C. Examples of retrograde-labeled hypoglossal motoneurons. IV, fourth ventricle. Scale bar: A = 250 μm; B, C = 25 μm.

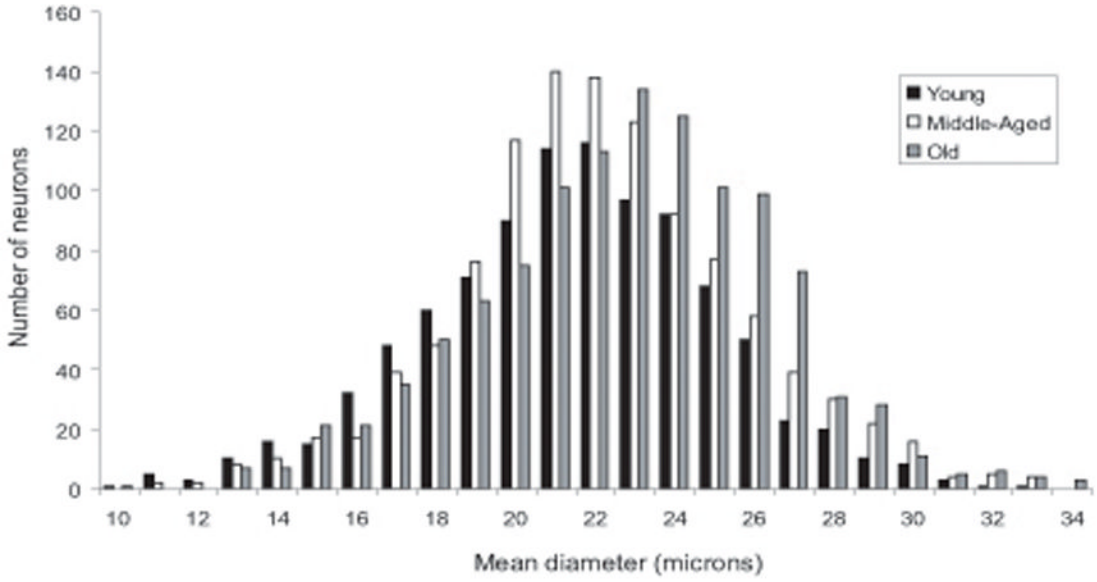

Figure 3.
Histogram of the mean diameter (μm) of retrograde-labeled hypoglossal motoneurons in young, middle-aged, and old rats.

Table 1

Number of neurons in the hypoglossal nucleus

	Hypoglossal nucleus	Rostral region	Middle region	Caudal region
Young	86 ± 7.75*	75 ± 15.55	97 ± 7.33	56 ± 7.51
Middle-aged	77 ± 5.19	57 ± 9.02	93 ± 10.96	53 ± 7.54
Old	73 ± 9.22	65 ± 9.19	102 ± 13.63	50 ± 4.61

* mean number of neurons per 50 μm section ± standard error; n = number of sections.

Table 2

Diameter of neurons in the hypoglossal nucleus

	Hypoglossal nucleus	Rostral region	Middle region	Caudal region
Young	21.6μm ± 0.98*	22.6 μm ± 0.91 (n = 281)	21.4 μm ± 1.16 (n = 367)	19.6 μm ± 1.40 (n =188)
Middle-aged	22.7μm ± 0.68	24.1 μm ± 0.79 (n =277)	22.5 μm ± 0.62 (n =567)	21.9 μm ± 0.75 (n =234)
Old	23.4μm ± 0.86	24.1 μm ± 0.84 (n =362)	23.48 μm ± 0.93 (n =522)	22.2 μm ± 0.84 (n = 180)

* mean diameter of neurons ± standard error; n = number of neurons measured.

Table 3

Number of primary dendrites/neuron in the hypoglossal nucleus

	Hypoglossal nucleus	Rostral region	Middle region	Caudal region
Young	2.9* ± 0.25	2.6 ± 0.12 (n = 281)	2.5 ± 0.06 (n = 367)	2.6 ± 0.17 (n = 188)
Middle-aged	2.9 ± 0.13	2.8 ± 0.11 (n = 277)	2.9 ± 0.12 (n = 567)	3.0 ± 0.24 (n = 234)
Old	2.5 ± 0.12	2.4 ± 0.15 (n = 362)	2.5 ± 0.16 (n = 522)	2.7 ± 0.12 (n = 180)

* mean number of dendrites per neuron ± standard error; n = number of neurons analyzed.