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Absence of morphological and molecular correlates of sarcopenia in the macaque tongue muscle styloglossus

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Abstract

Introduction—Equivocal decline of tongue muscle performance with age is compatible with resistance of the tongue to sarcopenia, the loss of muscle volume and function that typically occurs with aging. To test this possibility we characterized anatomical and molecular indices of sarcopenia in the macaque tongue muscle styloglossus (SG).

Methods—We quantified myosin heavy chain (MHC), muscle fiber MHC phenotype and size and total and phosphorylated growth- and atrophy-related proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot and immunohistochemistry (IHC) in the SG in twenty-four macaque monkeys (*Macaca rhesus,* age range 9 months to 31 years) categorized into Young (<8 years of age), Middle-aged (15–21 years of age) and Old (> 22 years of age) groups.

Results—In Young, Middle and Old age groups, by SDS-PAGE MHCI comprised ~1/3 and MHCII ~2/3 of total MHC. MHCI relative frequency was lower and MHCII higher in Middle versus Young (p=0.0099) and Middle versus Old (p=0.052). Relative frequencies of MHC fiber phenotype were not different by age but were different by phenotype (rates 233, 641 and 111 per 1000 fibers for MHCI, MHCII and MHCI-II respectively, P = 0.03). Few or no fibers were positive for developmental MHC. Mean cross-sectional area (CSA) was not different among the three age groups for MHCII and MHCI-II; however MHCI fibers tended to be larger in Middle versus Old and Young (mean = 2257 um^2, 1917 um^2 (p=0.05) and 1704 um^2 (p=0.06), respectively). For each age group, mean CSA increased across MHC phenotype (lowest mean CSA for MHCI and highest mean CSA for MHCII). Spearman analysis demonstrated age-related increases in total p70 ribosomal protein S6 kinase (P70), phosphorylated $P70^{421/424}$, phosphorylated P38 mitogen-activated protein kinase and muscle atrophy F-Box, a trend to age-related decrease in total

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extracellular signal-regulated kinase (ERK), and no age-related change in total protein kinase B (Akt/PKB), phosphorylated Akt, phosphorylated ERK, phosphorylated c-Jun N-terminal kinase (JNK46) and phosphorylated P70[389].

Conclusion—Common anatomical and molecular indices of sarcopenia are absent in our sample of macaque SG. Relative frequencies of MHCII protein and phenotype are preserved with age. Although MAFbx expression increases with age, this is not associated with fiber atrophy, perhaps reflecting compensatory growth signaling by p70. The resistant nature of the styloglossus muscle to sarcopenia may be related to routine activation of tongue muscles in respiration and swallowing and the preservation of hypoglossal motoneuron number with age.

Keywords

Tongue; aging; sarcopenia; swallowing; myosin heavy chain; human; muscle

1.1. INTRODUCTION

Swallowing dysfunction (dysphagia) affects >15% of the aging population with negative impact on quality of life including malnutrition and aspiration (Ney et al., 2009). Many muscles lose mass and function with age, i.e. undergo sarcopenia (Mitchell et al., 2012), and sarcopenia of head and neck muscles has been suggested to contribute to age-related swallowing dysfunction (Robbins et al., 2005). Because the tongue generates pressures critical for swallowing, sarcopenia of the tongue might contribute to age-related dysphagia. A reported decrease with age in tongue “functional reserve”, i.e., the ability to produce tongue force beyond routine requirement for swallowing, is compatible with tongue sarcopenia (e.g., Robbins et al. 2005). However, normal and effortful swallow pressures do not decline with age in women (Yeates et al., 2010) and by some measures tongue functional reserve is maintained with age (Steele, 2013) suggesting tongue muscles may be resistant to sarcopenia and that age-related decline in tongue motor performance may be non-myogenic in origin.

Morphological and molecular features of aging differ by muscle, fiber type, species and gender (e.g., Ciciliot et al., 2013; Deschenes et al., 2013), and it is unclear whether tongue muscles are susceptible to sarcopenia. Features common to sarcopenic appendicular muscle, i.e., a shift to slower myosin heavy chain (MHC) isoforms, Type II muscle fiber atrophy and neuromuscular junction dysmorphology, are absent or minimal in rat tongue muscles (Connor et al., 2009; Oliven et al., 2001; Hodges et al., 2004; Rahnert et al., 2011). In humans, tongue muscle fiber size has been reported to increase (Nakayama et al., 1991) or decrease (Rother et al., 2002) with age. Previously we noted stability of MHCI and MHCIIX relative frequency and a trend to decreased MHCIIA relative frequency in the human tongue muscle genioglossus with age (Daugherty et al, 2012) but failed to find appreciable developmental MHC, indicative of regenerating sarcopenic muscle (Snow et al., 2005), even in very old individuals (Sokoloff et al., 2007a; Sokoloff et al., 2010; Daugherty et al., 2012).

Muscle mass reflects the balance between anabolic and catabolic processes, and perturbation of either with age may lead to sarcopenia (Arthur and Cooley, 2012). Studies primarily report stability of atrophy-related signaling and changes in growth-related signaling in

muscle with age (i.e., Gaugler et al. 2011; Foletta et al., 2011) but also indicate variability by muscle, fiber type, species and gender (e.g., Foletta et al., 2011; Parkington, 2004). Our previous study in the rat demonstrated that, compared to the biceps brachii, pERK and p70S6k T421/S424 was preserved with age in head and neck muscles including the tongue muscle styloglossus. Whether growth-related signaling is maintained with age in human tongue muscles is not known. Rapid post-mortem degradation of protein phosphorylation status (e.g., Li et al., 2003) and comorbidities of human aging which affect muscle mass (e.g., chronic obstructive pulmonary disease, cancer, Ciciliot et al., 2013) complicate study of signaling pathways with age in human tongue muscles. Therefore we tested for anatomical and molecular indices of sarcopenia in the tongue muscle styloglossus of the macaque, a primate with relatively long life-span and tongue MHC composition similar to humans (Sokoloff et al., 2007a). Our findings indicate minimal changes in measures typically associated with sarcopenia and suggest that primate styloglossus is resistant to common features of muscle aging.

1.2. MATERIALS AND METHODS

Whole tongues including extrinsic tongue muscles were removed immediately post-mortem from 12 male and 12 female macaques (*Macaca mulatta*) ranging in age from 0.9 months to 31 years (Table 1), frozen in liquid nitrogen and stored at −80 degrees C. Tissue was briefly thawed and the left or right styloglossus was removed immediately proximal to its entry into the tongue body to enable comparable sampling across subjects. SG tissue was mounted on tongue depressors with Tissue-Tek O.C.T. Compound (Sakura, Finetek), frozen in isopentane supercooled with liquid nitrogen and stored at −80°C. Tissue was provided by the California National Regional Primate Center or the Yerkes Regional Primate Research. The study involved only post-mortem tissue and is Institutional Animal Care and Use Committee-exempt. Human tissue was obtained from a single, 80-year old cadaver through the Emory University School of Medicine Body Donor Program and is IRB-exempt.

1.2.1. Tissue Preparation and SDS-PAGE-Coomassie of Myosin Heavy Chain

Approximately 40–50 mg of muscle tissue was cut from frozen SG tissue blocks, homogenized in 200μl of 0.1M potassium phosphate (PBS) buffer (pH 7.3) and 5% protease inhibitor cocktail (Sigma, Aldrich) following Kohn and Myburgh (2006) with a tissue homogenizer (Fisher Scientific, PowerGen 500) in an ice bath, followed by centrifugation at 10,000g (4°C) for 10 minutes and re-suspended in 0.1M PBS buffer (pH 7.3) and 5% protease inhibitor cocktail for extraction of the myosin fraction. Total protein content was assayed by bicinchoninic acid assay according to manufacturer specifications (Synergy HT multimode microplate reader, Biotek Instruments, Inc., Pierce® BCA protein assay, Thermo Fisher Scientific Inc). Samples were stored at −80°C. The separation gel electrophoresis protocol is described in Daugherty et al. (2012). Briefly, protein samples were mixed with Laemmli sample buffer (Bio-Rad Laboratories) at 1:1, and equivalent amounts of sample protein (approximately 1.0 μg/lane) were loaded. A 45–200 kDa molecular weight standard (Bio-Rad SDS-PAGE molecular weight standards, High range, Bio-Rad Laboratories, Hercules, CA) was loaded in the initial lane for reference. SDS-PAGE gels were run at 140 V for 22 hours at 4°C and on ice. SDS-PAGE gels were rinsed with water for 3 X 5 minutes,

stained with Imperial™ Protein Stain (Thermo Fisher Scientific Inc.) at room temperature for 1 hour, de-stained with water for 1.5 hours and scanned at 2400 DPI resolution (Epson Perfection V33).

1.2.2. Myosin Heavy Chain Western

Previously by immunohistochemistry (IHC) we identified primarily MHCIIA and MHCI fibers in the macaque SG, a smaller population expressing MHCIIX and limited or no developmental MHC (Sokoloff et al., 2007a). Here by separation SDS-PAGE-Coomassie we distinguished two MHC bands (see also Kischel et al., 2001), a fast band that co-migrated with human MHCI and a slow band that migrated between human MHCIIA and MHCIIX (Figure 1). Western blotting revealed the slower migrating band to be positive for both MHCIIA and MHCIIX. We interpret this to indicate co-migration of macaque MHCIIA and MHCIIX in our hands, and a preponderance of MHCIIA>MHCIIX in the macaque SG (compatible with findings by IHC, see Sokoloff et al., 2007a). Because of weak MHCIIX expression and MHCIIX/MHCIIA co-migration on gels, we do not distinguish between MHCIIA and MHCIIX in this paper.

1.2.3. Myosin Heavy Chain Gel Analysis

Coomassie-stained MHCI and MHCII in SG were quantified in IMAGE J software (publicly available from the National Institutes of Health at http://rsbweb.nih.gov/ij/). The outcome variables were the percent MHCI and percent MHCII for each animal. These response variables sum to 100% for each animal. The response profile between the three age groups were compared by ony-way multivariate analysis of variance (MANOVA) implemented using SAS Proc GLM (version 9.4).

1.2.4. Tissue Preparation and SDS-PAGE-Western of Growth and Atrophy related Signaling Proteins

Samples of SG were cut from tissue blocks and placed in a low salt detergent buffer (50 mM Tris, pH 7.5; 30 mM NaCl; 5 mM EDTA, 1% Triton X-100 plus NaF, NaVO3 and protease inhibitors) to minimize myo lament extraction and pelleted at 15,000 × g (see Rahnert et al., 2011). Soluble protein (15ug) was separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by Western blot. Primary antibody dilutions were: p-ERK$^{T202/T204}$ 1:3000; p-JNK, 1:500; p-P38$^{T180/Y182}$ 1:2000; p-Akt^{S473} 1:2000; p-P70S6k$^{T421/S424}$ 1:2000; p-p70S6k^{T389} 1:2500; total ERK2 1:2500; total p38 1:2000; total Akt 1:2000; pan p70 1:2000 and total MAFbx. All antibodies are purchased from Cell Signaling except JNK and MAFbx from Santa Cruz. Bands were visualized by enhanced chemiluminescence and quanti ed by scanning densitometry. Muscle protein from four Young animals (<8 years of age) was run with samples from older animals on a gel and data from each gel was normalized to the average of the four Young muscles to allow comparisons across age groups.

1.2.5. Analysis of Growth and Atrophy related Signaling Proteins

The Spearmen rank correlation coefficient was used to explore possible monotonic relationships between signaling proteins and age (SAS Proc Corr version 9).

1.2.6. Immunohistochemical Methods

Serial 12-μm thick cross-sections of SG for IHC were cut on a cryostat at −23°C, mounted on gelatin-subbed and stored at −80°C. The following antibodies were used on serial sections for MHC characterization by IHC: Ab MY-32 (Sigma, anti-MHCII, anti-MHCextraocular and anti-MHCneonatal, Daugherty et al., 2012) at dilution of 1:400; Ab NCL-MHCd (Clone WB-MHCd, Vector Laboratories, Burlingame, CA, likely specific for MHCembryonic, Brueckner et al 1996, Daugherty et al. 2012); Ab NCL-MHCn (Clone WB-MHCn, Vector Laboratories, Burlingame, CA specific for MHCneonatal or MHCneonatal and MHCembryonic by Western, Daugherty et al., 2012) at dilution of 1:10; and Ab A4.84 (anti-MHCI, The Developmental Studies Hybridoma Bank, reported to be specific for MHCIβcardiac or MHCIβcardiac and MHCIαcardiac by Western, Daugherty et al., 2012) at dilution of 1:5. Tissue was sometimes co-reacted with the anti-laminin Ab D-18 (The Developmental Studies Hybridoma Bank; 1:50–1:100) to facilitate identification of muscle fiber boundaries.

Tissue was reacted as described in Daugherty et al. (2012). Briefly, tissue sections were incubated in a blocking solution composed of 2% normal goat serum, 0.03% Triton-X, and 0.1M Tris-HCl (T-NGS) at room temperature for 1 hour, followed by incubation overnight with primary antibody in blocking solution in a humid chamber at 4 ºC. Tissue was then washed in Tris-HCl buffer and incubated with the appropriate secondary antibody (peroxidase-conjugated goat IgM fraction or goat IgG fraction to mouse immunoglobulins at dilution 1:100) for 1 hour at room temperature. A standard DAB reaction was used to visualize label (0.5 mg DAB/mL, 0.1 M PBS, 0.03% H_2O_2). Slides were then washed with water for 5 minutes ×2, dehydrated, and coverslipped in Permount Mounting Medium (Fisher Scientific).

Tissue sections were viewed on an Olympus BX51 microscope, images collected with Neurolucida software (Microbrightfield, Burlington, VT) and a MicroFire digital microscope camera (Optronics, Goleta, CA) and stored onto computer (Dell Optiplex GX270, 1280 × 1024 pixel resolution). To quantify expression of developmental myosin, the number of fibers positive for Abs NCL-MHCn and NCL-MHCd, were counted, expressed per mm2 tissue and values compared for Young versus Old age Groups (Exact Wilcoxon Two-Sample Test).

1.2.7. Muscle Fiber Phenotype and Morphometry

Given the absence of unconventional MHC and limited expression of MHCIIX and developmental MHC by Western and IHC (see below and also Sokoloff et al., 2007a) three muscle fiber phenotypes were categorized by reaction profiles to MHC antibodies: MHCI (exclusively positive for Ab A4.84), MHCII (exclusively positive for Ab MY-32), and MHCI-MHCII (positive for both Abs A4.84 and MY-32). Perimeters of 192–200 fibers from each subject were traced and fiber cross-sectional area, perimeter and diameter were calculated in Neurolucida software. Relative frequency of phenotypes MHCI, MHCII and MHCI-II by three age groups (Young,<8 years of age (< 96 mo); Middle, 15–21 years of age (180–252 mo); and Old, > 22 years of age (> 264 mo); see Table 1) were estimated and compared by performing a generalized estimating equation (GEE) Poisson regression

analysis of the fiber counts, with age as a continuous covariate, implemented using SAS Proc Genmod, using an exchangeable correlation structure for the phenotype fiber counts within animal. Results are summarized using the estimated counts per 1000 fibers plus the 95% confidence interval for MHCI, MHCII and MHCI-II for each age group. Cross-sectional area was analyzed using a mixed linear model fit with SAS Proc Mixed (version 9) providing separate estimates of the cross-sectional area means by MHC phenotype (MHCI, MHCII and MHCI-II) and age group (Young, Middle, Old). A compound-symmetric variance-covariance form in the measurements was assumed for cross-sectional area and robust estimates of the standard errors of parameters were used to perform statistical tests and construct 95% confidence intervals. T-tests were used to compare the differences between the model-based age means (least-squares means) at each level of phenotype within the framework of the mixed effects linear model. All pairwise statistical tests were two-sided and unadjusted for multiple comparisons. Statistical significance was $p < 0.05$.

1.3. RESULTS

1.3.1. Quantification of MHC by SDS-PAGE/Coomassie

Two bands corresponding to MHCI and MHCII were visible by SDS-Page-Coomassie (Figure 2). MHCI was ~one third and MHCII ~two thirds of total MHC (Table 2). Mean MHCI% and MHCII% is statistically different between the three age groups (p=0.03). Mean MHCI% is 33.2% for Young, 22.5% for Middle and 36.1% for Old animals. MHCI% is significantly lower and MHCII% is significantly higher in the Middle compared to the Young group ($p = 0.0099$) and MHCI% is nearly significantly lower and MHCII% is nearly significantly higher in the Middle compared to the Old group ($p = 0.052$). The MHCI% and MHCII% is similar for the Young and Old groups ($p = 0.52$).

1.3.2. Relative frequency and Morphometry of MHC Phenotype with Age

MHCI, MHCII and MHCI-II muscle fiber phenotypes were distinguished with antibodies to MHCI and MHCII (Figure 3A, 3B, 3E, 3F). Fibers counts per 1000 were highest for MHCII. The estimated relative frequency pooled across age groups were 233/1000, 641/1000 and 112/1000 for MHCI, MHCII and MHCI-II respectively. Table 3 provides relative frequency alence estimates by MHC phenotype and age. Relative frequency did not differ with age ($p = 0.20$ for age categorical and $p = 0.18$ for age continuous) but fiber counts were different by MHC phenotype ($p = 0.03$).

Cross-sectional area (CSA) in the 3 age groups differed in significantly different ways across MHC phenotype ($p = 0.03$, test for interaction between age group and MHC phenotype). Figure 4 provides the estimated CSA mean and the 95% confidence interval for phenotypes MHCI, MHCII and MHCI-II by age group. Mean CSA was significantly different within the MHCI phenotype (p=0.045), with the middle age group larger than old ($p = 0.05$) and trending larger than young (p=0.06). Cross-sectional area of MHCI-II fibers did not differ with age (p=0.91). For each age group, mean CSA increased across MHC phenotype (lowest mean CSA for MHCI and highest mean CSA for MHCII).

1.3.3. Immunohistochemical Identification of Developmental MHC

Antibodies to MHCneonatal and MHCembryonic labeled very few fibers in the macaque SG (Figure 3C, 3D, 3G, 3H; Table 4). The number of MHCneonatal-positive fibers in Young animals compared to Old animals were statistically similar (median = 19 and 6 respectively, p=0.11; the Exact Wilcoxon Two-Sample Test). Only one young animal (47 months) had notable MHCneonatal which may reflect persistence of a developmental myogenic program or sampling at fascicle/fiber terminations which can express MHCneonatal in the tongue (Sokoloff et al., 2010).

1.3.4. Growth and Atrophy Related Signaling Molecules

By Spearman's rank correlation coefficient analysis, four of the ten signaling molecules and phosphorylation states we studied demonstrated age-specific correlations (at $p<0.05$): MAFbx, pP38, p70S6K and $p70S6K^{421/423}$ increased with age with ERK trending toward decrease (p=0.06; Figure 5; Table 5).

1.4. DISCUSSION

Common features of sarcopenic muscle are absent in the macaque tongue muscle styloglossus. MHCI and MHCII percentage is similar in young and old animals, although there was a decrease in MHCI and increase in MHCII in middle age animals, MHC phenotype relative frequency and developmental MHC expression does not differ with age. At the signaling level, MAFbx expression tended to increase with age, while it tended to decrease in appendicular muscle (Alton et al., 2010), while both expression and S421 phosphorylation of p70S6K increased. These findings suggest the styloglossus is resistant to sarcopenia.

1.4.1. Myosin composition and fiber morphology of appendicular muscle with aging

Conventional changes in fiber type and morphology of appendicular muscles with age include decreased relative frequency and size of "fast" muscle fibers, increased hybridization of different MHC in single fibers and expression of developmental MHC (Andersen, 2003; D'Antona et al., 2003; Snow et al., 2005). Recent studies however indicate variation in these features by muscle, gender and study. For example, whereas MHCI relative frequency and fiber size is stable with age in the rat soleus, extensor digitorum longus (EDL) and plantaris (PL) muscles, individual MHCII isoform relative frequency and size varies by muscle (Deschenes et al, 2013). In the human deltoideus, fiber type relative frequency (by ATPase histochemistry) and size are stable with age with the exception of decreased type IIB relative frequency in males and atrophy of type II fibers in females (Fayet et al., 2001). In human females, size of type I and type II fibers are stable with age in the biceps brachii (Mattiello-Sverzut et al., 2009) but decrease in the vastus lateralis (Grimby et al., 1982). In the vastus lateralis (VL) reports also document decrease in type I relative frequency with no difference in type I and type II fiber size (Frontera et al., 2000) and decrease in size of type I but not type IIA fibers (D'Antona et al., 2003), divergent findings that may relate to differences by study in methodology, age, gender and comorbidity. Decrease in type II fiber size thus describes some (e.g. Nilwik et al., 2013) but not all muscle aging. Relevant data on appendicular muscle aging in non-human primates are limited. In the female vervet monkey

VL, relative frequency of type I versus type II fibers is stable with age with decreased area of type I, type IIX/IIB and type IIA fibers (by ATPase) and decreased fiber hybridization (by separation SDS-PAGE-silver staining of single fibers, Feng et al, 2012). In contrast to the results in appendicular muscles, in the present study we noted stability of MHC composition and MHC phenotype relative frequency and muscle fiber size with age in the macaque tongue muscle, styloglossus.

1.4.2. Myosin composition and fiber morphology of head and neck muscle with aging

Studies of fiber type and morphology in aged head and neck muscles demonstrate variable response by muscle. Fiber type is stable with age in the rat masseter (Norton et al., 2001) as is MHC isoform composition (Rahnert et al., 2011). Fast-glycolytic fiber relative frequency increases and fast-oxidative-glycolytic fiber relative frequency decreases in the rat geniohyoid and sternohyoid with age (van Lunteren et al., 1995). Changes in MHC composition of human jaw and suprahyoid muscles with age also differ by muscle, with stability in lateral pterygoid and anterior digastric and decreased I–IIA hybridization in lateral pterygoid (Monemi et al., 2000). In a reverse of the classic appendicular scheme, proportional area of MHCI decreases with age in the human masseter in association with decreased MHCneonatal (Cvetko, et al., 2012, but see Monemi et al., 1999).

Studies of aging of tongue muscles are few but do not support a conventional model of muscle aging: a shift to slower MHC isoforms, type II muscle fiber atrophy and neuromuscular junction dysmorphology are absent or minimal in rat tongue muscles (Connor et al., 2009; Oliven et al., 2001; Hodges et al., 2004; Rahnert et al., 2011). Studies in humans have reported an increase (Nakayama, 1991) or decrease (Rother et al., 2002) in tongue muscle fiber size with age. Previously we reported a trend to decreased MHCIIA but stability of MHCI and MHCIIX in the human genioglossus (GG) with age (Daugherty et al., 2012). In prior studies of aged GG, hyoglossus and SG we never observed more than occasional fibers positive for developmental MHC (Sokoloff et al., 2007a; Sokoloff et al., 2010; Daugherty et al., 2012). The present study extends prior findings by confirming stability of conventional MHC composition and fiber size and no increase in developmental MHC in the macaque SG with age.

1.4.3. Growth and Atrophy Signaling

Akt-p70 Signaling cascade—Changes in growth and atrophy-signaling with age also differ by muscle and study. Parkington et al (2004) reported stability of total p70S6K with age in rat PL and tibialis anterior (TA) and increased phosphorylation of $p70S6K^{T389}$ in TA only. In contrast, Kinnard et al (2005) reported decreased p70S6K and increased $p70S6K^{T389}$ with age in the rat EDL and soleus. We previously noted decline in total p70S6K and $p70S6K^{421/424}$ but no change in $p70S6K^{T389}$ in the rat biceps brachii with age and stability of all in the rat SG (Rahnert et al., 2011) suggesting difference in p70S6K signaling with age in rat versus macaque tongue muscle. Stability of Akt signaling with age in the macaque SG mirrors findings in rat head and neck muscles including the SG (Rahnert et al., 2011). In contrast, decrease in pAkt in rat TA (Clavel et al., 2006), in total and pAkt in rat soleus and epitrochlearis (Gupte et al., 2008) and mouse gastrocnemius (Kovacheva et

al., 2010) and increase in total Akt but not pAkt in the human VL (Leger et al., 2008) may point to a dysregulation of Akt signaling with age in hindlimb muscles.

MAP kinase signaling cascade—Phosphorylation of c-Jun NH(2)-terminal kinase (JNK) and p38 map-kinase in mouse gastrocnemius (Kovacheva et al., 2010) and of JNK in rat soleus and epitrochlearis (Gupte et al., 2008) increases with age. In the rat we noted increase with age of pP38 in branchial muscles but not in somitic muscles including the SG which differs from our finding of increased pP38 n the macaque SG. In the present study total ERK decreased with age in the macaque SG without decrease in pERK, indicating maintenance of ERK activation. In the human VL pERK was elevated (Williamson et al, 2003) or unchanged (Drummond et al., 2008) with age.

MAFbx/Atrogin-1 Signaling—MAFbx is one of the major ubiquitin ligases regulating active atrophy. Sarcopenia is associated with accelerated proteasome activity (Altun et al 2010), but the specific role of MAFbx is less clear, with diverse reports showing no change (Gaugler et al., 2011, Haddad and Adams, 2006), decrease (Altun et al., 2010, Edstrom et al., 2006), or increase (Clavel et al., 2006) in rodent models. Repeated investigation of the human VL have failed to find any change in MAFbx expression with age (Dalbo et al., 2011, Leger et al., 2008, Raue et al., 2007, Whitman et al., 2005). Our finding of increased MAFbx in the aged macaque SG thus differs from the human VL. One of the canonical repressors of MAFbx is Akt, via FOXO3 transcription factor. Akt also canonically activates the mTOR/p70S6K module, so the complimentary decrease in MAFbx and increase in p70 observed here is consistent with increased Akt signaling, even though we were unable to resolve this.

1.4.4. Limitations of Study

We recognize several limitations in the present study. (1) With one exception our sample did not include animals of very old age (e.g. > 30 years). In some muscles sarcopenia may be especially pronounced in the very old age (e.g., Snow et al., 2005). Our sample however compared animals before and after the age of onset of sarcopenia in macaque appendicular muscles (14.1±2.8 years in males and 15.8±2.5 in females) and included many individuals of age at which loss of mass in appendicular muscles is marked (i.e., 20% loss of muscle mass at 23.2 years in males and 24.5 years in females, Colman et al., 2005). Common features of sarcopenia were present in the VL of the cercopithecoid vervet monkey with 21 year demarcation of old age (Feng et al., 2012). (2) We combined males and females in our analysis which would mask differences by gender. (3) We were unable to quantify MHCIIA and MHCIIX by separation-SDS-PAGE and thus did not evaluate a possible shift from MHCIIX to MHCIIA with age; however we note minimal MHCIIX by Western in any age. (4) Protein phosphorylation is unstable post-mortem (e.g., Li et al., 2003). Although we treated tissue of all ages equally we cannot rule out a contribution of post-mortem degradation to our measures. Such instability would not however impact basal protein measures. (5) We did not contrast tongue to appendicular muscle to control for individual differences in sarcopenia expression.

1.4.5. Possible bases for resistance of tongue muscle to sarcopenia

Our findings suggest the macaque SG is resistant to common features of sarcopenia, a finding similar to prior studies in tongue muscle of rats and humans (Nakayama et al., 1991; Oliven et al., 2001; Hodges et al., 2004; Connor et al., 2009; Rahnert et al., 2011). Bases for stability of the SG and other tongue muscles with age are not known. Tongue muscles differ from appendicular muscles in several features that can promote muscle growth and thus may protect tongue muscles from sarcopenia. In contrast to appendicular muscles, tongue muscles experience a high duty cycle (i.e., during respiration, mastication and swallowing) that undergoes minimal or no change with age (Kays et al., 2010; King et al., 1986; Peng and Kang, 1984; Zhang et al., 2008). Additionally, some tongue motor units are constitutively active (Tsuiki et al., 2000; Saboisky et al., 2006). In humans, high levels of chronic exercise may prevent the loss of muscle mass typical of elderly individuals (Wroblewski et al., 2011). Tongue muscle fibers undergo routine forceful contraction during swallowing, similar in intensity to resistance training regimes that promote muscle hypertrophy (Taaffe et al., 1996), and only a few such contractions are required to maintain mass in denervated muscle (Dow et al., 2004). Further, co-activation of orthogonally-aligned tongue muscles during some tongue movements (e.g., Miyawaki et al., 1974) indicates that tongue muscles undergo eccentric contraction. In rodents eccentric training promotes hypertrophy and growth-related signaling (Ochi et al., 2011; Tsumiyama, et al., 2014; Heinermeier et al., 2007). Finally, hypoglossal motoneuron number is preserved with age in mouse, rat and human (Gai et al., 1992; Sturrock, 1991; Schwarz et al., 2009), protecting tongue muscles from motoneuron-loss-induced denervation/reinnervation remodeling that typifies some appendicular muscles and is thought to promote type II-to-I conversion, MHC hybridization and fiber atrophy (Drey et al., 2014). Hypoglossal motoneurons also receive inputs from numerous central and peripheral sources, and it is possible that this rich synaptic milieu supports hypoglossal motoneurons during dysfunction in any one projection system.

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Abbreviations

Akt/PKB	Protein kinase B
DAB	Diaminobenzidine
EDL	extensor digitorum longus
ERK	Extracellular signal-regulated kinase
GG	genioglossus
IHC	immunohistochemistry

JNK	c-Jun N-terminal kinase
MAFbx	Muscle atrophy F-Box
MHC	Myosin heavy chain
P38	P38 mitogen-activated protein kinase
P70	p70 ribosomal protein S6 kinase
PL	plantaris
SG	styloglossus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TA	tibialis anterior
VL	vastus lateralis

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Highlights

- Common markers of sarcopenia were studied in macaque tongue muscle styloglossus.
- Relative frequency of slow and fast myosin isoforms was similar in young and old age groups.
- Size and relative frequency of fast myosin muscle fibers were preserved with old age.
- Findings suggest the macaque styloglossus is resistant to sarcopenia.
- Activation of styloglossus in oromotor behaviors may protect against sarcopenia.

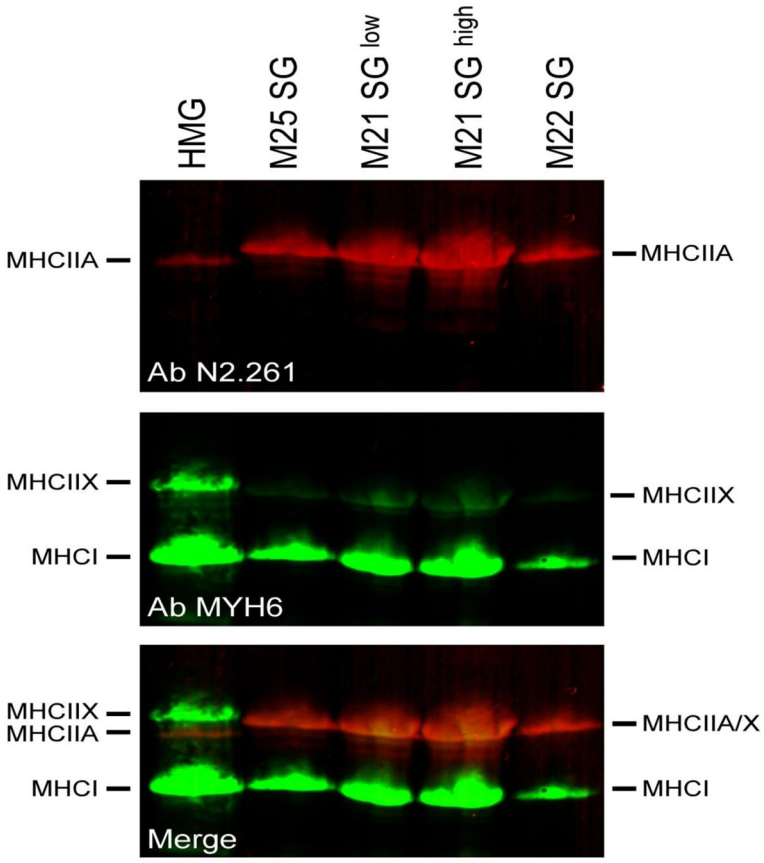

Figure 1.

Separation SDS-PAGE/Western of the human medial gastrocnemius (HMG) and the macaque styloglossus (SG, M21 at low and high concentrations, M22, M25) showing identification of myosin heavy chain (MHC) I, IIA and IIX. Top panel. Antibody (Ab) N2.261 labels MHCIIA in human and macaque tissue (red). Note faster migration of MHCIIA in the HMG. Middle Panel. Ab MyH6 labels MHCI and MHCIIX in human and macaque tissue (green). Note slower migration of MHCIIX in the HMG. Bottom panel. Merge of top and middle panels showing overlap of MHCIIA and MHCIIX (yellow) in the macaque by Western.

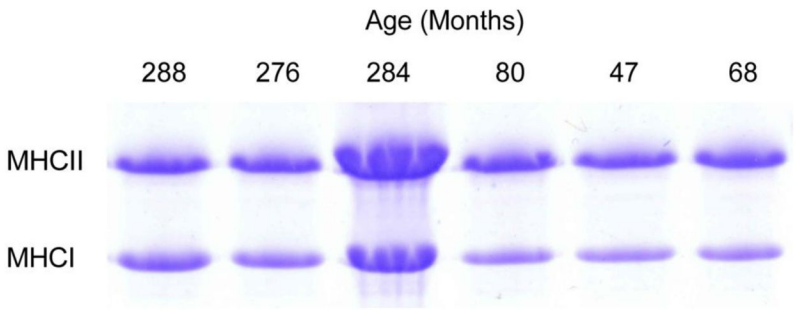

Figure 2.
Representative separation SDS-PAGE/coomassie showing relative MHCI and MHCII in the macaque styloglossus (age in months).

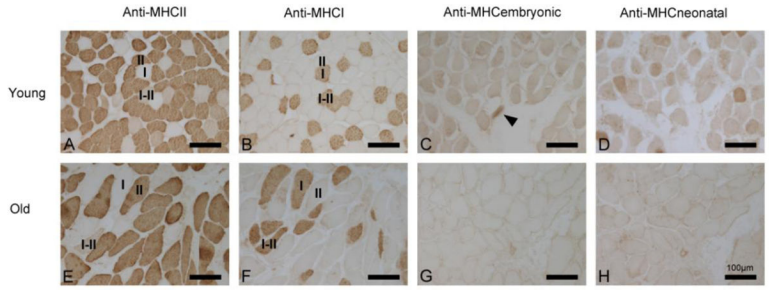

Figure 3.

Identification of myosin heavy chain (MHC) in the young and old macaque styloglossus with antibodies to MHCII (Ab MY-32, Anti-MHCII), MHCI (Ab A4.84, Anti-MHCI), MHCembryonic (NCL-MHCd, Anti-MHCembryonic) and MHCneonatal (NCL-MHCd, Anti MHC-Neonatal). A,B. Serial sections showing fiber of phenotype MHCI, MHCII and MHCI-II in a young subject (M10). C,D. Positive reaction to Anti-MHCembryonic (arrow) and to Anti-MHCneonatal in a young subject (M6). E,F. Serial sections showing fibers of phenotype MHCI, MHCII and MHCI-II in an old subject (M29). G,H. Absence of reaction to Anti-MHCembryonic and Anti-MHCneonatal in an old subject (M29). Calibration bar = 100µm.

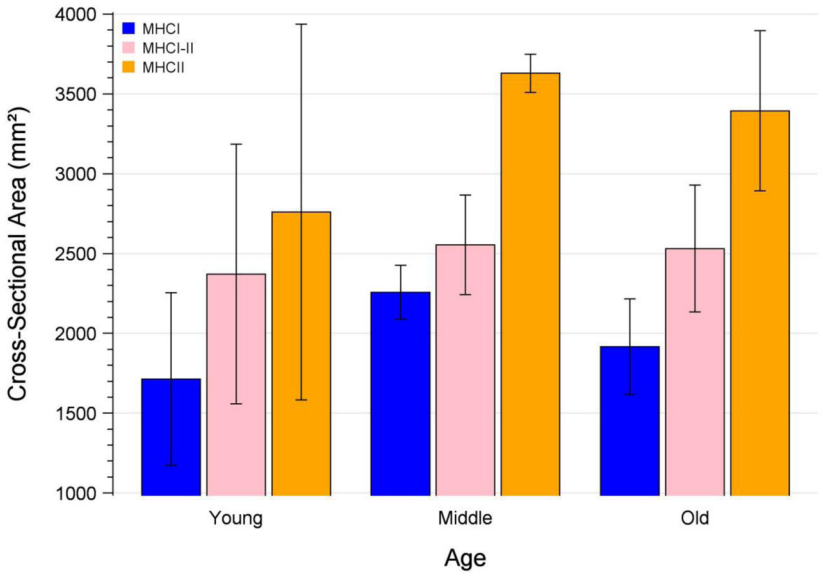

Figure 4.

Mean cross-sectional area for phenotypes MHCI, MHCII and MHCI-II by age group [Young (< 8 years), Middle (15–21 years) and Old (>22 years)]. The vertical bars indicate the 95% confidence intervals for the means.

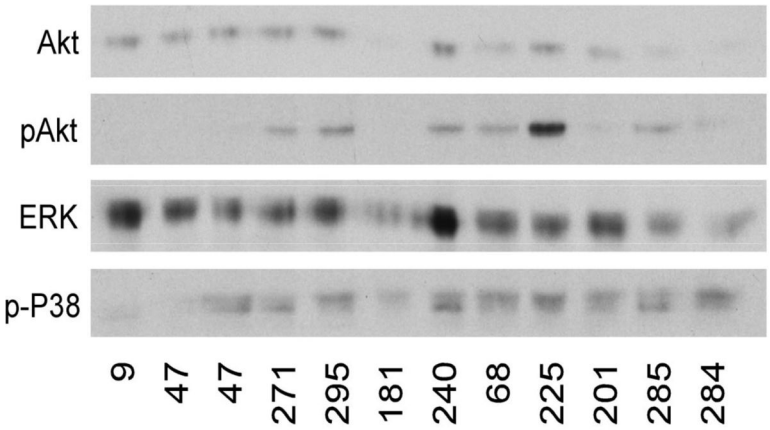

Figure 5.

Immunoblot of macaque SG with antibodies to Akt, pAkt, ERK and p-P38. Age of monkeys in months indicated below gel. Muscle protein from four Young animals (<8 years of age) was run with samples from middle and old animals on a gel and data from each gel was normalized to the average of the four Young muscles to allow comparisons across age groups.

Table 1

Subject Information and Experimental Tests

Subject Information				Molecular Tests			
Su Subject ID	**Age Months**	**Age Category**	**Sex**	**Growth and Atrophy Signaling**	**MHC Dev[#]**	**MHC Coomassie**	**MHC Phenotype, Morphometry**
M1	9	Y	Male	+			
M4	47	Y	Male	+	+	+	
M6	47	Y	Male	+	+	+	+
M21	47	Y	Female	+	+	+	
M15	68	Y	Male	+	+	+	+
M16	72	Y	Male	+	+	+	+
M10	80	Y	Female	+	+	+	+
M26	89	Y	Male	+	+	+	+
M9	181	M	Male	+		+	+
M19	201	M	Female	+		+	
M11	204	M	Female	+		+	
M17	225	M	Female	+		+	+
M13	240	M	Male	+		+	
M33	249	M	Female	+		+	
M32	265	O	Male	+		+	+
M7	271	O	Female	+		+	+
M29	276	O	Male	+	+	+	+
M31	276	O	Male	+	+	+	+
M22	279	O	Female	+	+	+	+
M25	284	O	Male	+	+	+	+
M24	285	O	Female	+	+	+	+
M30	288	O	Female	+	+	+	+
M8	295	O	Female	+	+	+	+
M23	372	O	Female	+	+	+	+

[#]Developmental myosin heavy chain (MHC), MHCembryonic and MHCneonatal

Table 2

Percent MHCI and MHCII in Young, Middle-Aged and Old Macaque Styloglossus Muscle

Age Category	Number	Age (Average)[#]	Percent MHCI[*] (Mean±SD)	Percent MHCII[*] (Mean±SD)
Young	N=7	47–89 (64)	33.2±8.3	66.8±8.3
Middle	N=6	181–249 (217)	22.5±14.4	77.5±14.4
Old	N=10	279–372 (289)	36.1±5.5	63.9±5.5

[#]Age in Months

[*]Percent relative to total MHC by SDS-PAGE Coomassie

SD = Standard Deviation

Table 3

Relative frequency of MHC Phenotype by Age per 1000 Fibers

MHC Phenotype	Age Category (#Subject/#Fibers)			
	All Ages (17/3391)	Young (5/992)	Middle (2/399)	Old (10/2000)
MHCI	233/1000 [95% CI:192–283]	201/1000 [95% CI:130–309]	195/1000 [95% CI:137–278]	324/1000 [95% CI:276–380]
MHCII	641/1000 [95% CI:614–669]	662/1000 [95% CI:594–739]	652/1000 [95%CI:663–670]	610/1000 [95% CI:572–650]
MHCI-II	111/1000 [95% CI:80–155]	137/1000 [95% CI:66–286]	153/1000 [95% CI:86–271]	66/1000 [95% CI:46–94]

CI: 95% confidence interval

Table 4

Number of Muscle Fibers Reacting with Antibodies to Developmental Myosin Heavy Chain

Age (Mnths)	Age Category	Sex	MHCembryonic-Positive Fibers	MHCneonatal-Positive Fibers	MHCneonatal Positive Fibers/mm2
47	Y	M	0	405	10.8
47	Y	M	1	50	1.1
47	Y	F	7	53	2.1
68	Y	M	0	19	0.8
72	Y	M	0	1	0.0
80	Y	F	0	6	0.5
89	Y	M	0	1	0.0
276	O	M	0	3	0.1
276	O	M	0	0	0.0
279	O	F	0	11	0.8
284	O	M	0	0	0.0
285	O	F	0	8	0.3
288	O	F	0	8	0.3
295	O	F	0	0	0.0
372	O	F	0	15	0.4

Table 5

Descriptive statistics and the association between proteins with age.

Protein	Sample Size	rs with Age*	P value
Phos ERK	24	0.20	0.36
ERK	22	−0.41	0.06
Rel ERK	22	0.33	0.14
pP38	22	**0.45**	**0.03**
pJNK46	19	−0.04	0.88
pAkt	22	0.35	0.12
Akt	24	0.02	0.93
Rel Akt	22	0.39	0.08
pp70 389	24	0.29	0.18
pp70 421/424	24	**0.55**	**0.005**
p70	22	**0.51**	**0.02**
Rel 389	22	−0.29	0.18
Rel 421	22	−0.11	0.63
MAFbx	24	**0.55**	**0.006**

*indicates the Spearman rank correlation coefficient.