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Immunohistochemical Characterization of Slow and Fast Myosin Heavy Chain Composition of Muscle Fibres in the Styloglossus Muscle of the Human and Macaque (*M. rhesus*)

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Abstract

Objective—Muscle fibre contractile diversity is thought to be increased by the hybridization of multiple myosin heavy chain (MHC) isoforms in single muscle fibres. Reports of hybrid fibres composed of MHCI and MHCII isoforms in human, but not macaque, tongue muscles, suggest a human adaptation for increased tongue muscle contractile diversity. Here we test whether hybrid fibres composed of MHCI and MHCII are unique to human tongue muscles or are present as well in the macaque.

Methods—MHC composition of the macaque and human styloglossus was characterized with antibodies that allowed identification of three muscle fibre phenotypes, a slow phenotype composed of MHCI, a fast phenotype composed of MHCII and a hybrid phenotype composed of MHCI and MHCII.

Results—The fast phenotype constitutes 68.5% of fibres in the macaque and 43.4% of fibres in the human ($P < 0.001$). The slow phenotype constitutes 20.2% of fibres in the macaque and 39.3% of fibres in the human ($P < 0.001$). The hybrid phenotype constitutes 11.2% of fibres in the macaque and 17.3% of fibres in the human ($P = 0.002$). Macaques and humans do not differ in fiber size (cross-sectional area, diameter). However, measures of fibre size differ by phenotype such that fast > hybrid > slow ($P < 0.05$).

Conclusion—These data demonstrate differences in the relative percent of muscle fibre phenotypes in the macaque and human styloglossus but also demonstrate that all three phenotypes are present in both species. These data suggest a similar range of mechanical properties in styloglossus muscle fibres of the macaque and human.

Keywords

Tongue; Muscle; Myosin; Fibre Type; Macaque

INTRODUCTION

The tongue of mammals participates in many oromotor behaviors, including swallowing, respiration, oral transport, and, in humans, speech production. The extent to which these

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behaviors involve the selective activation of different tongue motor units (MU = a motoneuron and the muscle fibre it innervates) is not known, however, the demonstration of multiple, functionally distinct populations of MUs in the human genioglossus^{1, 2} is compatible with the selective activation of tongue MUs in at least some oromotor behaviors.

The consequences of selective activation of different tongue MUs for tongue movement will in part depend upon the contractile properties of MU muscle fibres. Contractile properties of muscle fibres are in large measure related to myosin heavy chain (MHC) composition^{3,4} and it has been hypothesized that muscles with multiple kinematic functions will have complex patterns of MHC protein expression⁵. Contractile properties of muscle fibres may be modified by two strategies of MHC protein expression. In one strategy, heterogeneity of muscle fibre contractile properties is increased through the expression of MHC isoforms with specific mechanical properties. This strategy appears to be in operation in some human cranial muscles which differ from extrafusal fibres of post-cranial muscles in the expression of MHC neonatal, MHC embryonic, MHC extraocular (MHC laryngeal), MHC slow tonic and/or MHC cardiac isoforms⁶⁻⁸. In another strategy, heterogeneity of muscle fibre contractile properties is increased through the hybridization of different MHC isoforms in single muscle fibres. In such hybrids, contractile properties are usually related to the type and amount of each constituent isoform^{7,9,10}. This strategy also appears to be in operation in cranial muscles, many of which contain a large complement of fibres composed of both slow MHC and fast MHC isoforms^{6-8,11,12} (but see Monemi et al.¹³). In contrast, in most post-cranial muscles thus far studied, slow MHC-fast MHC hybrid fibres are generally less prevalent^{14,15} (but see^{16,17}).

The extent to which hybridization of slow MHC and fast MHC isoforms occurs in muscles of the primate tongue is not known. Among the human intrinsic tongue muscles transversus, verticalis and superior longitudinalis (muscles with both origin and insertion within the tongue body) histochemical and immunohistochemical data show that, on average, from 5%-15% of muscle fibres are composed of both MHCI and fast MHCII isoforms¹⁸. In contrast, in the intrinsic tongue muscles in the macaque, only a small number of fibres that likely express both slow MHC and fast MHC isoforms (Type IIC or Type IM fibres) have been described (on average <0.72%¹⁹). No slow MHC-fast MHC hybrid fibres were noted in a recent study of extrinsic muscles of the macaque tongue²⁰. These differences in the percent of slow MHC-fast MHC hybrid fibres may represent species differences in the MHC composition of tongue muscles, but also may reflect differences in study methodologies. We thus characterized MHC isoforms in the extrinsic tongue muscle styloglossus (SG) in the macaque and human to compare the prevalence of slow MHC-fast MHC hybrid fibres in these species. Our results indicate species differences in the proportion of slow MHC, fast MHC and slow MHC-fast MHC hybrid fibres but the presence of a large population (i.e., >10%) of slow MHC-fast MHC hybrid fibres in both the macaque and human SG. These findings indicate that the co-expression of both slow MHC and fast MHC isoforms in tongue muscle fibres is not a unique adaptation for speech.

MATERIALS AND METHODS

Subjects and Tissue Preparation

Tissue from the left or right styloglossus (SG) was obtained within 8 hours post-mortem from one juvenile and five adult monkeys (*Macaca rhesus*, Yerkes Regional Primate Center) and from five adult humans (Emory University School of Medicine Body Donor Program) (for specific ages and genders see Table 1). Tissue was taken from the SG immediately proximal to its entry into the tongue body to avoid the inclusion of fibres of other tongue muscles which interdigitate with the SG in the posterior tongue body (e.g., hyoglossus, transversus^{21,22}). Tissue samples were mounted onto tongue depressors with OCT tissue tek and quick-frozen in isopentane cooled by liquid nitrogen, and stored at -80°C. Control tissue for the

immunohistochemical investigation of MHCembryonic and MHCneonatal isoforms was obtained from the tongue body of two human fetal tongues (gestational ages 22–40 weeks, frozen < 12 hours post-mortem, National Disease Research Interchange). Control tissue for the immunohistochemical investigation of the MHCIIb isoform was obtained from the triceps surae of an adult mouse (muscle unspecified, frozen < 1 hour post-mortem). Control tissue for immunoblot studies was obtained from the cat soleus and the rat lateral gastrocnemius (superficial region) and frozen < 1 hours post-mortem.

Immunohistochemical Study of SG MHC Isoforms

Tests of Antibody Specificity—Tissue samples of the SG were cut into 12 μ m thick cross-sections on a cryostat at -23°C and mounted onto gelatin-subbed slides and semi-serial sections were reacted with various antibodies (Abs) to MHC isoforms (Table 2). Specificity of these Abs has been reported in mice, rats and cats, but has not been clearly established in primates (Table 2). Our primary objective was to determine whether muscle fibres composed of both slow MHCI and fast MHCII isoforms were present in the human and macaque SG. Because of differences in reported specificities of Abs to MHC isoforms in primates, we first conducted three control experiments to (1) determine which Ab provides the most reliable labeling of the MHCI isoform, (2) determine which Ab provides the most reliable labeling of MHCII isoforms and (3) ensure that Ab MY-32 label is not due to reactivity with a developmental MHC isoform. First, in two macaques and two humans, we compared muscle fibre label in tissue sections of the SG that were reacted with different Abs to the MHCI isoform, one section with Ab A4.84 and the other with Ab BA-D5. The Ab A4.84 (Developmental Studies Hybridoma Bank, DSHB) recognizes the MHCI(cardiac β) isoform in humans^{23,24}. The Ab BA-D5 (American Type Culture Collection, ATCC) recognizes the MHCI isoform in humans²⁵ and, in the rabbit masseter, has been shown to provide the most complete staining of the MHCI isoform²⁶. Second, in four macaques and two humans we compared muscle fibre label in serial tissue sections that were each reacted with one of the following Abs to MHCII isoforms: MY-32, BF-F3, A4.74, and SC-71. The Ab MY-32 (Sigma-Aldrich) is reported to label all fast MHC isoforms and the MHCneonatal isoform in the rat²⁷ and all MHCII isoforms in humans^{7,13,28–30}. The Ab BF-F3 (ATCC) is specific for the MHCIIb isoform in the rat and mouse³¹, has been used to identify the MHCIIb isoform in the macaque²⁰ and does not label extrafusal fibers in the human^{28,32}. The Abs A4.74 (DSHB) and SC-71 (ATCC) are thought either to be specific for the MHCIIa isoform^{23,28,33} or to label multiple MHCII isoforms^{15,18,20,34} in primates. To enable complete characterization of these muscle fibers, additional sections were reacted with the anti-slow Abs A4.84 and/or BA-D5. Third, in all subjects we reacted tissue with Abs to MHCneonatal (Ab NCL-MHCn, Vector)³⁵ and to MHCembryonic (Ab F1.652, DSHB)³⁶ to control for the possibility that MY-32 label was due to reaction with MHCneonatal or MHCembryonic isoforms. Tissue reacted with Abs NCL-MHCn and F1.652 was also reacted with an Ab to the laminin β 2 chain (Ab D-18, DSHB, working dilution 1:10) to assist in muscle fibre identification¹⁸. The control experiments (see RESULTS) identified the following Ab specificities in both the macaque and human SG: Ab A4.84 specific for MHCI, Ab BA-D5 specific for MHCI, Ab SC-71 specific for a subset of MHCII, here categorized as MHCIIa and Ab MY-32 specific for MHCII (Table 3).

Classification of MHCI, MHCII and MHCI-MHCII Muscle Fibres—Although control experiments demonstrated that accurate classification of fibres into MHCI, MHCIIa, MHCIIx phenotypes and their hybrids could be made based on labeling with Abs A4.84, SC-71 and MY-32, we are not able to exclude the co-expression of MHCIIx from fibres positive for Ab SC-71. For this reason we used a more generalized classification of muscle fibres into MHCI, MHCII and MHCI-II phenotypes based on labeling with Abs A4.84 and MY-32 alone (Table 3) to determine whether muscle fibres composed of both slow MHCI and fast MHCII isoforms were present in the macaque and human SG. A total of 1033 muscle fibres from the SG of five

monkeys and 1181 fibres from the SG of five humans were analyzed from serial sections reacted with the Abs A4.84 and MY-32 to determine the prevalence and morphometry of three principal MHC phenotypes, a slow phenotype (MHCI), a fast phenotype (MHCII) and a slow-fast hybrid phenotype (MHCI-II).

Immunohistochemical Methods—Tissue was reacted following the protocol of Eason et al.³⁷ 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 one hour, followed by incubation overnight with primary antibody in blocking solution in a humid chamber at -4 degrees C (see Table 2 for Abs and dilutions used). Tissue was then washed in Tris-HCl buffer and incubated with secondary antibody (peroxidase-conjugated goat IGG fraction to mouse immunoglobulins, Capel; 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% H₂O₂). Slides were then washed with H₂O for 1 minute, dehydrated and coverslipped in permount.

Tissue sections were viewed on an Olympus BX51 microscope at 100x, 200x and 400x magnification. Images were collected with Neurolucida software (Microbrightfield, Burlington, VT) and a MicroFire Digital Microscope Camera (Optronics, Goleta, CA) and stored onto computer (Dell Optiplex GX270, 1280 x 1024 pixel resolution). Most fibres had either strong label or no/very weak label following immunoreaction; however, in some fibres a moderate amount of label was present. We classified as positive those fibres with moderate label. Classification of fibres with moderate label as negative would not have changed the categorization of MHC phenotypes in either macaques or humans, but would have resulted in a decreased prevalence of hybrid fibres composed of both MHCI and MHCII isoforms.

Morphometric Analysis and Statistics—Perimeters of individual muscle fibres classified as phenotype MHCI (Ab A4.84 positive), phenotype MHCII (Ab MY-32 positive) and phenotype MHCI-II (Abs A4.84 and MY-32 positive) were traced at 400x magnification in Neurolucida software (Microbrightfield, Vermont). Measurements of fibre cross-sectional area and diameter were calculated by the program. The diameter measurement used is defined as the feret max of the fibre, which is calculated as the maximum distance between two parallel lines that enclose the muscle fibre (Neurolucida).

Graphs of the distribution of MHC phenotypes and fibre measurements were generated in Statistica (ver 6, Statsoft) and SAS Proc Mixed (version 8, SAS Institute, Cary NC). Fibre counts per 1000 were estimated and compared using exact methods based on the Poisson distribution. Fibre counts were summarized by type of fibre and by species using ninety-five percent confidence intervals (CI). Repeated-measures analyses using mixed linear models were performed for perimeter, diameter and area utilizing SAS Proc Mixed thereby providing separate estimates of the means by type of fibre and species. A compound symmetry variance-covariance form in repeated measurements was assumed for each outcome, and robust estimates of the standard errors of parameters were used to do statistical tests and construct 95% confidence intervals. The model-based means are unbiased with unbalanced and missing data so long as the missing data were non-informative (missing at random). Statistical tests were 2-sided. A P-value less than 0.05 was considered statistically significant.

Electrophoretic Immunoblotting—Tissue for gel electrophoresis was obtained from the same tissue blocks used for immunohistochemical study in M3, M5, M6, H3, H4 and H5. Control tissue was obtained from a cat soleus muscle which is almost exclusively composed of the MHCI isoform³⁸ and the superficial region of the rat lateral gastrocnemius muscle which is exclusively composed of MHCII isoforms³⁹. Approximately 50 milligrams of muscle tissue was placed in 2 ml of non-denaturing lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA,

1% Triton X-100, with 0.02% NaN₃ and 0.1% protease inhibitors (Sigma) and homogenized for 30 seconds with a Tissuemiser at 15,000 rpm in an ice bath and cleared by centrifugation at 6,000 xg for 5 minutes. Protein content was assayed by bicinchronic acid assay (BCA, Pierce) according to the manufacturer's instructions. Protein (0.5-2.0 µgrams/lane) was separated by SDS-page through a 7.5% acrylamide resolving gel at 25 mA for 90 minutes. Gels were transferred to a nitrocellulose membrane using a wet transfer system. Membranes were blocked for 60 minutes in 5% non-fat dry milk in TTBS, prior to incubation with primary antibodies to slow MHC (A4.84, DSHB, 1:500 dilution) and fast MHC (MY-32, Sigma, Ab 1:10,000,000 dilution). Well rinsed membranes were conjugated with secondary HRP-conjugated goat anti-mouse IgG (1:20,000 dilution), detected by enhanced chemiluminescence (ECL, Amersham), and visualized by exposure to radiographic film followed by scanning densitometry.

RESULTS

Immunohistochemical Comparison of Myosin Abs in the Macaque and Human SG

In a subset of subjects, we tested a panel of Abs to determine which provided the most reliable label of MHCI and MHCII isoforms in the primate SG and, thus, which Abs would enable the most accurate categorization of MHCI-MHCII hybrid muscle fibers.

Abs A4.84 and BA-D5—Comparison of label with Ab A4.84 and Ab BA-D5 was made for two monkeys and two humans (data not shown). Both Abs provided effectively equivalent label of the MHCI isoform (same response to both Abs in 99.4% of fibres in macaque and 100% of fibres in human).

Abs BF-F3, MY-32, A4.74, SC-71—Comparison of reaction to Abs BF-F3, MY-32, A4.74, SC-71 was made for four macaques and two humans. Antibody BF-F3 labeled fibres in mouse triceps surae that were also labeled with Ab MY-32 but not with Abs A4.74, SC-71 and A4.84 (Figure 1). We classify these fibres as phenotype MHCIIb. In the macaque SG, however, the Ab BF-F3 either did not label any fibres (M1, M2) or weakly labeled fibres with a slow phenotype (M5, M6). In the human SG the Ab BF-F3 labeled all fibres (Figure 1). We conclude that Ab BF-F3 does not specifically label a MHCII isoform in the macaque and human SG.

In the macaque and human all fibres labeled with Abs A4.74 or SC-71 were also labeled with Ab MY-32; however, many fibres labeled with Ab MY-32 were not labeled with Abs A4.74 and SC-71 (3.5% of fibres in the human, 7.8% of fibres in the macaque; Figure 2; Table 4). We conclude that Ab MY-32 provides the most reliable label of MHCII isoforms in the macaque and human SG and that in both species fibres expressing MHCIIx but not MHCIIa are present. In the macaque, most fibres had identical reaction with Abs A4.74 and SC-71 suggesting the specificity of both Abs for the MHCIIa isoform. In the human, some fibres were positive for Ab A4.74 but negative for Ab SC-71, suggesting the greater specificity of Ab SC-71 for the MHCIIa isoform in the human (Table 4).

Abs NCL-MHCn and F1.652—Reaction to Abs NCL-MHCn and F1.652 was determined for all subjects. These Abs labeled all fibres in human fetal tongue tissue (data not shown) but only a few fibres in the macaque and human SG (Figure 3). Among fibres used for determination of MHCI, MHCII and MHCI-II phenotype prevalence (see below), only five were positive for Ab NCL-MHCn and only one was positive for Ab F1.652. We conclude that Ab MY-32 label in the adult macaque and human SG is not due to reaction with MHCneonatal or MHCembryonic isoforms.

Prevalence of Fast, Slow and Hybrid Muscle Fibre Phenotypes

Control studies demonstrated equivalent label of the MHCI isoform with Abs A4.84 and BA-D5 and most reliable label of MHCII isoforms with Ab MY-32. A total of 1033 muscle fibres from the SG of five monkeys and 1181 fibres from the SG of five humans were thus analyzed from serial sections reacted with the Abs A4.84 and MY-32 to determine the prevalence and morphometry of three principal MHC phenotypes, a slow phenotype composed of MHCI, a fast phenotype composed of MHCII (specific isoforms unknown) and a hybrid phenotype composed of both MHCI and MHCII isoforms (specific MHCII isoforms unknown).

All three phenotypes were present in each species (Figure 4), however, the percent of each phenotype differed between species. Prevalence of the fast phenotype was greater in monkeys (685 per 1000 fibers; 95% CI: 636 to 738) compared to humans (434 per 1000 fibers; 95% CI: 398 to 474; $P < 0.0001$). However, the slow phenotype was less frequent in monkeys (202 per 1000 fibers; 95% CI: 176 to 232) compared to humans (393 per 1000 human fibers; 95% CI: 358 to 430; $P < 0.0001$). The hybrid phenotype was also less frequent in monkeys (112 per 1000 monkey fibers; 95% CI: 93 to 135) compared to humans (173 per 1000 human fibers; 95% CI: 150 to 198; $P = 0.0002$).

There was little difference in the relative prevalence of fibre phenotypes in individual macaques (Figure 4). The fast phenotype was the most prevalent in all five subjects. The slow phenotype was the second-most prevalent in four of the subjects but was less prevalent than the hybrid phenotype in one subject (M5). Thus relative prevalence was either fast>slow>hybrid (4 monkeys) or fast>hybrid>slow (M5). In contrast, in humans, phenotype prevalence was more variable with rankings of fast>slow>hybrid, fast>hybrid>slow, slow>fast>hybrid or slow>hybrid>fast among the five subjects.

Muscle Fibre Size and Phenotype Classification

The relationship between muscle fibre size and phenotypes MHCI, MHCII and MHCI-II was evaluated across and within species (Figure 5). No differences were identified between humans and macaques for diameter and area. However the mean diameter was larger for 'fast' compared to 'hybrid' phenotype ($P = 0.0047$), for 'fast' compared to 'slow' ($P < 0.0001$) and for 'hybrid' compared to 'slow' ($P = 0.017$). The mean area was larger for 'fast' compared to 'hybrid' ($P = 0.01$) and for 'fast' compared to 'slow' ($P = 0.0003$) but not for 'hybrid' compared to 'slow' ($P = 0.23$).

Immunoblots with anti-MHCI and anti-MHCII Abs

Both MHCI and MHCII isoforms were demonstrated in immunoblots of the human and macaque SG with the Ab A4.84 (anti-MHCI) and the Ab MY-32 (anti-MHCII) (Figure 6).

DISCUSSION

Summary of Data

The primary findings of this study of the styloglossus of the macaque and human are that (1) slow, fast and hybrid slow-fast muscle fibre phenotypes are described by antibodies to MHCI and MHCII isoforms in both species, (2) macaques and humans differ in the relative prevalence of fibre MHC phenotypes (the fast phenotype has a greater prevalence in the macaque whereas the slow and slow-fast hybrid phenotypes have greater prevalence in the human), (3) in both species fibres of the fast phenotype are the largest for measures of muscle fibre size and fibres of the slow phenotype are the smallest, (4) in both species fibres with an Ab profile indicative of phenotype MHCI-IIx are present, and (5) in neither species do a significant number of muscle fibres express MHCneonatal or MHCembryonic isoforms. To our knowledge this is the first

report of the MHC isoform composition of an “extrinsic” muscle of the human tongue and the first report of MHCembryonic and MHCneonatal isoforms in primate tongue muscles.

Relation to Other Studies of Primate Tongue

The findings of the current study are generally similar to reports of muscle fibre type composition of intrinsic tongue muscles in the human. Stal et al.¹⁸ reported fibre types by ATPase activity of 39-62% fast, 33-50% slow and 5-15% fast-slow hybrids among verticalis, transversus and superior longitudinalis. Fibre diameter was similar to ranges described here for the human SG (especially for posterior intrinsic muscles, 41-64 µm). Our results are also comparable with another human extrinsic tongue muscle, genioglossus; Saigusa et al.⁴⁰ noted regional differences in fibre type, also by ATPase activity, with Type II fibres decreasing from 68% anterior to 50% posterior, without recognition of fast-slow hybrids.

Results of the present study are also similar to previous reports of the dominance of Type II fibres, by ATPase histochemistry, in macaque intrinsic tongue muscles¹⁹ and the dominance of fibres expressing MHCII isoforms in macaque extrinsic tongue muscles²⁰. In a recent study of the MHC composition of the macaque SG, hyoglossus and genioglossus muscles, Smith et al.²⁰ reported that in the SG, fibres composed of MHCIIa-IIx were the most prevalent (64%) followed by fibres composed of MHCI (29%). Remaining fibres were composed of MHCIIb (6.0%). Hybrid fibres composed of both slow and fast MHC isoforms were not identified. In the present study we found a similar prevalence of fibres with a fast (MHCII) phenotype (69% versus 71% - sum of MHCIIa-IIx and MHCIIb prevalences in Smith et al.²⁰). In contrast to Smith et al.²⁰ we found (1) evidence for expression of the MHCIIx isoform, (2) no evidence for expression of the MHCIIb isoform, (3) a lower prevalence of fibres with MHCI phenotype (20% versus 29%), and (4) a population of fibres (11%) that expressed both slow and fast MHC isoforms.

Several factors may account for differences in the findings of these studies. We used the Ab MY-32 to characterize MHCII isoforms. In mammals, Ab MY-32 is reported to label MHCIIa, MHCIIb, MHCIIx and MHCneonatal isoforms²⁷ or all MHCII isoforms²⁹ and thus provides a “broad spectrum” labeling of MHCII isoforms. In contrast, many studies report that Ab A4.74, used by Smith et al.²⁰ to identify “MHC type MHCIIa-IIx” fibres, is specific for the MHCIIa isoform in humans, with minimal reactivity to the MHCIIx isoform^{23, 41} (the MHCIIb isoform is not present in humans²⁸). In our tests of Ab specificity, 88 of 1138 fibres in the macaque SG reacted with Ab MY-32 but did not react with Ab A4.74 (Table 4; Figure 2). These findings suggest that Ab A4.74, in our hands, is specific for the MHCIIa isoform in the macaque SG and we propose that fibres in the macaque SG with profile MY-32+/A4.74- contain MHCIIx but not MHCIIa isoforms. The presence of MHCIIx isoforms in these fibres would not have been detected by the methods of Smith et al.²⁰ and fibres of phenotype MHCI-IIx would have been classified as phenotype MHCI.

In the present study we also identified fibres positive for Ab A4.84, Ab MY-32 and Ab A4.74. These fibres, classified as MHCI-IIa, would have been identified with the methods employed by Smith et al.²⁰ and thus their absence in that study requires explanation. It is possible that Smith et al.²⁰ did not directly search for hybrid fibres. It is also possible that there are species differences in the presence of slow-fast hybrid fibres, i.e., slow-fast hybrid fibres are present in the SG of *M. rhesus* (present study) but are absent in the SG of *M. cynomolgus* studied by Smith et al.²⁰. Type IM/IIC fibres were rare or absent in intrinsic tongue muscles of young adult *M. fascicularis*¹⁹. Additionally, there may be a gender and age specific expression of slow-fast hybrid fibres in the macaque SG. Slow-fast hybrid fibres were present in the SG of young/adult male macaques and old female macaques (M1, M3, M4, M5, M6 in the present study), but were rare or absent in young/adult female macaques (M2 in the present study and all three monkeys studied by Smith et al.²⁰). In our human sample, the single adult female

(H3) also had the fewest slow-fast hybrid fibres (Figure 4). An increase in the prevalence of slow-fast hybrid muscle fibres with aging occurs in some human muscles^{14,42,43}. Further study of age and gender differences in prevalence of slow MHC, fast MHC and slow-fast MHC phenotypes in primate tongue muscles is required to address these possibilities.

In adult mammals, expression of MHCembryonic and MHCneonatal isoforms is thought to indicate muscle fiber remodeling, denervation or reinnervation and expression of these isoforms with aging is associated with sarcopenia^{44,45}. Previous studies of primate tongue muscles did not investigate the presence of MHCneonatal or MHCembryonic isoforms. In the present study, only occasional fibres were labeled with Abs to MHCneonatal and/or MHCembryonic, even in old subjects (macaques > 22 years of age and humans > 79 years of age). Thus the SG is similar to most other cranial muscles in which MHCneonatal and/or MHCembryonic isoforms are absent or rare (e.g., digastric, interarytenoid, lateral pterygoid, palatopharyngeus^{30,42,46}) and appears to lack a robust expression of developmental isoforms found in some aging muscle.

Functional Consequences of MHC Phenotypes

Our data demonstrate >10% prevalence of slow-fast hybrid muscle fibres in both the human and macaque SG. In this respect, the primate SG is similar to many human cranial muscles (e.g., masseter, mylohyoid, temporalis, and extraocular muscles^{7,8,11,12}) and some human post-cranial muscles (e.g. vastus lateralis, medial gastrocnemius^{16,17}).

Explanations for the co-expression of multiple MHC isoforms in single muscle fibres include response to injury⁴⁷ and the transitional stage of a fibre in the process of changing between stable, homogeneous expression⁴⁸. These explanations would not, however, appear to account for the large and stable populations of hybrid fibres in some cranial muscles^{6-8,11,12} and post-cranial muscles⁴⁹⁻⁵². Muscle fibre phenotype is strongly influenced by its pattern of activation (for review see Pette⁵³) and it has been suggested that MHC hybridization is a consequence of the recruitment of a MU to “perform different tasks at different times”⁵. An additional possibility is that MHC hybridization is a consequence of the selective recruitment of a MU for a specific task (or tasks) that entails an intermediate, but homogeneous, pattern of neuronal activation (e.g., Rome et al.⁵⁴). It is not known whether MUs in the primate SG have multiple or single patterns of activation. In the human genioglossus, however, MUs may be discriminated into six populations based on differing activity patterns². Whether these MUs populations also differ with respect to MHC phenotype is not known.

The SG of mammals is active during many oromotor behaviors including, swallowing and respiration⁵⁵⁻⁵⁶. Compromise of these behaviors with aging and/or disease is thought to be related to alterations in tongue muscle function⁵⁷⁻⁵⁹. The similar classification of muscle fiber MHC phenotypes in the macaque and human SG suggests the macaque as an ideal model for investigation of muscular correlates of SG dysfunction in humans. In the rat SG, <1% of muscle fibers are MHCI phenotype, and no fast-slow hybrids have been reported⁶⁰.

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Figure 1.

Serial sections from the mouse triceps surae (A-D), adult macaque SG (E-H, subject M6) and adult human SG (I-L, subject H5) reacted with Ab MY-32 thought to be specific for MHCII isoforms and MHCneonatal (A, E, I), Ab A4.74 thought to be specific for MHCIIa and possibly MHCIIx in monkeys and humans (B, F, J), Ab BF-F3 specific for MHCIIb in rat and mouse and of uncertain specificity in primates, and Ab A4.84 specific for the MHC I isoform in humans. In the mouse triceps surae Ab BF-F3 labels a subset of muscle fibres of MHCII phenotype (i.e., phenotype MHCIIb). In the macaque SG, Ab BF-F3 weakly labels fibres with a slow (A4.84-positive) phenotype. In the human SG, Ab BF-F3 is not specific for either slow or fast MHC phenotypes. For clarity of illustration, fibres positive for both Ab MY-32 and Ab A4.74 are here classified as phenotype MHCIIa, but the possible inclusion of MHCIIx isoforms in these fibres cannot be ruled out. Calibration bar = 50 μ m.

Figure 2.

Serial sections of the macaque SG (A-D) and human SG (E-H) showing the five primary reaction profiles described in muscle fibres with Abs MY-32 (A, E), SC-71 (B, F), A4.74 (C, G) and BA-D5 (D, H) (see Table 2 and Materials and Methods for reported Ab specificities). The symbol “*” denotes fibres with strong/moderate reaction for all Abs to MHCII isoforms but weak/absent reaction for Ab BA-D5 (anti MHCI isoform). The symbol “o” denotes fibres with strong/moderate reaction for Ab BA-D5 but weak/absent reaction for Abs to MHCII isoforms. The symbol “++” denotes fibres with strong/moderate reaction for all Abs to MHCII isoforms and Ab BA-D5. The symbol “+” denotes fibres with strong/moderate reaction for Ab BA-D5 and for Ab MY-32 but weak/absent reaction for Abs A4.74 and SC-71. The symbol “x” denotes fibres with strong/moderate reaction for Abs BA-D5, MY-32 and A4.74 but weak/absent reaction for Ab SC-71. This reaction profile was only found among fibres in the human. Calibration bar = 50 μ m

Figure 3.

Serial sections from the adult monkey SG (A-C, subject M4) and adult human SG (D-F, subject H5) reacted with Ab F1.652 specific for MHCembryonic (A, D), Ab NCL-MHCn specific for MHCneonatal (B, E) and Ab MY-32 specific for MHCII isoforms and possibly MHCneonatal (C, F). Note the presence of two fibres labeled with both Ab NCL-MHCn and Ab MY-32 in the human tissue (E-F, arrows). Fibres with MHCembryonic or MHCneonatal isoforms were rare or absent in human and primate subjects. Symbols “o”, “*” and “+” identify the same fibre in each section. Calibration bar = 100 μ m.

Figure 4.

Pie charts showing the prevalence of fibres of MHCII phenotype, MHCII-MHCI phenotype and MHCII-MHCI-MHCI phenotype in five macaques and five humans. For each species, pie charts are arranged by increasing prevalence of MHCII-MHCI-MHCI phenotype.

Figure 5.

Diameter of slow (MHCI), fast (MHCII) and slow-fast (MHCI-II) hybrid muscle fibres from the macaque SG (○) and human SG (●). The mean diameter was larger for 'fast' compared to 'hybrid' phenotype ($P = 0.0047$), for 'fast' compared to 'slow' ($P < 0.0001$) and for 'hybrid' compared to 'slow' ($P=0.017$).

Figure 6.

Immunoblots of rat superficial lateral gastrocnemius (lane 1), cat soleus (lane 2), human SG (lane 3) and macaque SG (lane 4) demonstrating specificity of Ab A4.84 for MHCI, specificity of Ab MY-32 for MHCII and immunoreaction of protein from human SG and macaque SG with Abs A4.84 and MY-32.

Table 1

Age and Sex of Specimens

Species	Specimen	Sex	Age
Monkey	M1	M	9 mos
Monkey	M2	F	3 yrs 11 mos
Monkey	M3	M	3 yrs 11 mos
Monkey	M4	M	3 yrs 11 mos
Monkey	M5	F	22 yrs 7 mos
Monkey	M6	F	24 yrs 7 mos
Human	H1	M	63 yrs
Human	H2	M	80 yrs
Human	H3	F	66 yrs
Human	H4	M	56 yrs
Human	H5	F	86 yrs

Table 2
Reported Specificities of Antibodies to Myosin Heavy Chain (MHC) Isoforms in Primates^a

Antibody Name	Source	MHCI	MHCIIa	MHCIIb	MHCIIx	MHC neonatal	MHC embryonic	Working Solution ^b
A4.74 ^c	DSHB	-	+	-	+/-	-	-	1:5
A4.84 ^d	DSHB	+	-	-	-	-	-	1:5
BA-D5 ^g	ATCC	+	-	-	-	-	-	1:30
BF-F3, ^e g	ATCC	-	-	+/-	-	-	-	1:5
FL625	DSHB	-	-	-	-	-	+	1:5
MY-32, ^{df}	Sigma-Aldrich	-	+	+	+	-	-	1:400
NCL-MHCn	Vector	-	-	-	-	+	-	1:5
SC-71, ^c g	ATCC	-	+	-	+/-	-	-	1:5

^a References in Materials and Methods

^b Working solution for immunohistochemistry

^c Reported to be specific either for MHCIIa or for MHCIIa and MHCIIx

^d Ab used in present study of MHCII, MHCII and MHCII-II phenotypes

^e Reported to identify MHCIIb in macaques but not in humans

^f Reported to react with MHCneonatal in rats but not humans

^g Supernatant gift of Dr. Arthur English

Table 3

Classification Scheme of Antibody Label and Muscle Fibre Phenotype in the Macaque and Human SG

Fibre Phenotype	A4.84	Antibodies MY-32	SC-71
MHCI ^a	+	-	
MHCII ^a	-	+	
MHCI-II ^a	+	+	
MHCIIa ^b	-	+	+
MHCIIx	-	+	-
MHCI-IIa ^b	+	+	+
MHCI-IIx	+	+	-

^a Reaction with Abs A4.84 and MY-32 used to define MHCI, MHCII and MHCI-II phenotypes^b Cannot rule out presence of MHCIIx isoform

Table 4
Prevalence of SG Fibre MHC Phenotypes Categorized with Abs A4.84, MY-32, A4.74 and SC-71

Muscle Fibre MHC Phenotype	M		H		Muscle Fibre Antibody Profiles and Prevalence (%) (Average±SD)			
	M		M		A4.84	MY-32	A4.74	SC-71
MHCI	20.0 (5.7)		47.5 (5.7)		+	-	-	-
MHCIIa ^d	66.3 (3.6)		34.6 (3.4)		-	+	+	+
MHCIIb ^e	0.2 (0.2)		0.3 (0.4)		-	+	+	-
MHCII-IIIa ^d	5.0 (3.6)		5.4 (2.3)		+	+	+	+
MHCII-III ^c	8.5 (5.3)		13.0 (11.0)		+	+	-	-

^a Cannot rule out presence of MHCIIx isoform.
^b Categorization of MHCIIx based on absence of reaction with Ab SC-71. Because of specificity of Ab A4.74 in the macaque SG, the fibres with profile MY-32+/A4.74+ in the macaque may be phenotype MHCIIa.
^c Categorization of MHCIIx based on absence of reaction with Ab SC-71. Because of specificity of Ab A4.74 in the macaque SG, the fibres with profile A4.84+/MY-32+/A4.74+ in the macaque may be phenotype MHCII-IIIa.