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Myosin heavy chain composition of tongue muscle in microphthalmic (*mi/mi*) mice before and after weaning

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running title: MyHC in tongue muscle of *mi/mi* mice

Summary

To elucidate the effects of teeth on muscle fibers in tongue during the development process, we examined the expression of muscle contractile proteins and the genes for those proteins in normal mice and microphthalmic (*mi/mi*) mice with impaired tooth eruption. The mice were observed during the growth period, including weaning, which is when feeding movements undergo major changes. Expression of the myosin heavy chain (MyHC)-2a protein, whose contraction speed is relatively slow, disappeared after weaning in normal mice, while it remained in high concentrations even after weaning in *mi/mi* mice. The presence of MyHC-2a after weaning in mice with no tooth eruption was attributed to a compensation for lack of proper masticatory function and sucking-like movements, as MyHC-2a is necessary for these movements.

Key words

tongue muscle, microphthalmic mice, myosin heavy chain, muscle fiber properties

Introduction

Myosin is one of the muscle contractile proteins that constitute myofibrils. The contractile protein with the greatest mass is myosin heavy chain (MyHC), which is known to directly reflect the properties of muscle (Pette and Starton, 1990). Recently a variety of isoforms of MyHC have been identified, and it was found that these could be classified according to speed of contraction (Brueckner et al., 1996). Bottinelli et al. reported that the 3 fast MyHC isoforms include the MyHC-2b isoform which has the fastest contraction and the MyHC-2a isoform which has the slowest contraction (Bottinelli et al., 1991). It has also been observed that the composition ratios of 3 fast isoforms can determine the properties of muscle (Wydro et al., 1983; Parker-Thornburg et al., 1992; Schiaffino and Reggiani, 1996). MyHC isoforms have been reported to undergo reversible change (Sartorius et al., 1998). Moreover, this change has been shown to be affected by extrinsic factors, such as masticatory movements, and intrinsic factors, such as the presence of teeth (Gojo et al., 2002; Doi et al., 2003). Consequently, an examination of MyHC isoforms is important in obtaining an understanding of their function.

Previous investigations have shown that tongue muscle consists of fast MyHC

isoforms (Dalrymple et al., 1999, 2000; Shuler and Dalrymple, 2001). Agbulut et al. determined MyHC composition ratios in mouse tongue muscle and reported that while MyHC-2b increases from birth onward, MyHC-2a is expressed transiently, for only a few weeks postpartum (Agbulut et al., 2003). Moreover, tongue consists of both intrinsic and extrinsic muscles, and differences have been reported in the properties of fibers among the intrinsic muscles (Abe et al., 2002). However, these differences were observed where teeth were present. It is believed that masticatory movements, and particularly tongue movements, are affected by the presence or absence of teeth. Therefore, to obtain a deeper understanding of the function of the tongue, it is also important to investigate MyHC isoforms in tongue muscle under conditions where teeth are absent.

In osteopetrotic mice, due to a resorption failure of the alveolar bone, teeth do not erupt, even in adulthood, resulting in these mice having no teeth (Tiffée et al., 1999). Four types of congenital osteopetrotic mice have been reported (Felix et al., 1996), and microphthalmic (*mi/mi*) mice exhibit abnormalities of osteoclasts, pigment and mast cells (Ebi et al., 1990; Steingrímsson et al., 2003).

To clarify how the presence of teeth affects the properties of fibers in tongue muscle and tongue function during development, we investigated levels of MyHC-2b and

MyHC-2a, which are the constituent proteins of tongue, by immunohistochemical analysis and gel electrophoresis. In addition, we examined to expression of the genes that encode those MyHC proteins at the transcription level using *mi/mi* mice and normal mice.

Materials and Methods

1. Materials

Male and female mice with B6C3Fe *a/a Mitf^{Mi}/+* genotypes (Jackson Laboratory, ME, USA) were mated. Offspring of the *a/a Mitf^{Mi}/Mitf^{Mi}* genotype were used as the *mi/mi* mice, and offspring of the *a/a +/+* genotype were used as controls. All the mice then selected for use in the present study were males. The average time for weaning has been reported to be approximately 3 weeks postpartum (Gojo et al., 2002). Consequently, the mice in this study were separated from their parents from 3 weeks postpartum and given powdered feed (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) after weaning. The mice were observed immediately before weaning (2 weeks of age), immediately after weaning (4 weeks), as adults (at 9 weeks), and at 16 weeks to monitor subsequent

changes. For each genotype of mouse, 5 of each age were used for immunohistochemical examination, 3 of each age for protein detection by gel electrophoresis, and 5 of each age for examination for mRNA expression. This means a total of 104 mice were used in the study.

The mice were deeply anesthetized with ether and pentobarbital and then sacrificed, in accordance with the experimental animal guidelines of Tokyo Dental College. The portion of the tongue from the anterior to the sulcus terminalis was excised according to the method of Maejima et al (Maejima et al., 2005). A 5.0 mm-long area extending 2.5 mm anterior and 2.5 mm posterior to the midpoint between the sulcus terminalis and apex was harvested. The muscles observed were the superior longitudinal muscle and transverse muscle located on the superolateral margin of the tongue (Fig. 1). The samples were frozen in liquid nitrogen immediately after excision and stored in -80 °C isopentane until testing.

2. Immunohistochemical staining

Using a cryostat (Leica Instruments GmbH, Nussloch, Germany), contiguous frozen 8- μ m-thick sections orthogonal to the sagittal plane were prepared. The sections were

stained according to the following procedure: monoclonal antibodies extracted from hybridoma cells were used as the primary antibodies, namely, BF-F3 (anti-MyHC-2b: American Type Tissue Culture, P.O. Box 1549 Manassas, VA 20108, USA) and SC-71 (anti-MyHC-2a: American Type Tissue Culture, P.O. Box 1549 Manassas, VA 20108, USA) (Schiaffino et al., 1989; Eason et al., 2000). The hybridoma cells were cultured in DMEM (10% FBS) medium at 37 °C in 5% CO for 72 hr. The cells were then separated by centrifugation and the supernatant used for the antibodies. RITC goat anti-mouse IgM antibody against BF-F3 and FITC goat anti-mouse IgG antibody (Novocastora Laboratories, Newcastle, UK) against SC-71 were used as secondary antibodies. Observations were performed using a confocal laser microscope (MRC-1024/2P; Nippon Bio-Rad Lab. Tokyo, Japan). Muscle fibers labeled with FITC were considered MyHC-2a positive, and those labeled with RITC were considered MyHC-2b positive.

3. Protein fraction by sodium dodecyl sulphate (SDS) – polyacrylamide-gel electrophoresis (PAGE)

To assay MyHC protein content, the constituent proteins of the muscle were separated by gel electrophoresis as described by Talmadge et al. and Agbulut et al

(Talmadge and Roy, 1993; Agbulut et al., 1996). First, the protein level in each sample was adjusted to 0.125 mg/ml using the Bio-Rad Protein Assay (Nippon Bio-Rad Lab., Tokyo, Japan) and GeneQuant*pro* spectrophotometer (Biochrom Ltd., Cambridge, UK). Electrophoresis was then performed for 24 hr in a cold laboratory environment (4 °C). The gel was stained with Coomassie Brilliant Blue R-250 and an analysis performed using the Lane Analyzer for Macintosh (ATTO, Tokyo, Japan).

4. Analysis of level of mRNA expression using LightCycler™ system

Quantitative assays of mRNA for MyHC-2a and MyHC-2b were performed for each stage of development using the LightCycler™ (Roche Diagnostics, Mannheim, Germany) system, which enables polymerase chain reaction (PCR) amplification to be monitored in real time. The mRNA at each stage was extracted using the QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK), and complementary DNA was then prepared using the Ready-To-Go™ kit (Amersham Biosciences, Buckinghamshire, UK).

After optimal conditions were established for all primers, previously prepared LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim,

Germany) was used as the Hot Start PCR reaction solution for the LightCycler™.

A dilution series was prepared from 4.0 ng/μl stock solutions of PCR products of each isoform, with dilutions of 10⁶, 10⁷, 10⁸, and 10⁹, and used to prepare a calibration curve. PCR mixtures (20 μl) for each dilution included 2.4 μl of MgCl₂ (25 mM), 2 μl of LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.6 μl each of the forward and reverse primers prepared using Oligo 5 primer design (10 pmol/μl; Biogene, Ltd., Kimbolton, UK), 5 μl of the dilution series PCR products, and 9.4 μl of sterilized water. Two primer sets for MyHC-2b and MyHC-2a were designed to amplify specific regions from full-sequence DNA for each protein: MyHC-2b (forward: 5'-ACAGACTAAAGTGAAAGCC-3'; reverse: 5'-CTCTCAACAGAAAGATGGAT-3'; accession number: XM_126119) and MyHC-2a, forward: 5'-CGATGATCTTGCCAGTAATG-3', reverse: 5'-TGATAACTGAGATACCAGCG-3'; accession number: NM_144961.

PCR mixtures (20 μl) were prepared by adding 2.4 μl of MgCl₂ (25 mM), 2 μl of LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany), 0.6 μl each of the forward and reverse primers (10 pmol/μl), and 1 μl of the target DNA to 13.4 μl of sterilized water. The full volumes (20 μl) of each of the PCR mixtures (MyHC-2b and MyHC-2a) prepared in this manner were placed in the glass portion of a

capillary tube. After template denaturing for 10 min at 95 °C, 40 cycles of 10 sec at 95 °C, 10 sec at 62 °C, and 7 sec at 72 °C were carried out. To calculate the level of mRNA expression, the level of each MyHC isoform determined by this method was divided by the level determined for GAPDH, which was used as the housekeeping gene. The GAPDH primers (accession number, NM_008084) used for amplification were as follows: forward, 5'- TGAACGGGAAGCTCACTGG-3' and reverse, 5'- TCCACCACCCTGTTGCTGTA-3'.

Differences in levels of mRNA expression between the two groups (MyHC-2b and MyHC-2a) were tested statistically using a Student's t-test. Differences among ages within the same MyHC group were tested using Tukey's HSD test. The analysis was performed using the Windows version of the SPSS base system (version 12.0, SPSS Inc.). The levels used to examine the significant differences between means were $p < 0.005$ for the Student's t-test and $p < 0.01$ for Tukey's HSD test.

Results

1. Immunohistochemical staining

In the samples from before weaning (2 weeks old) that were stained with anti-MyHC-2b antibody, some fibers in the longitudinal muscles were positive for both the microphthalmic (*mi/mi*) mice and the normal mice. Immediately after weaning (4 weeks old), an increase in MyHC-2b-positive fibers was observed in the longitudinal muscle in both types of mouse. At 9 weeks, almost no MyHC-2b-positive fibers were seen in the transverse muscle in the *mi/mi* mice, while some fibers in the transverse muscle of normal mice showed positive staining. At 16 weeks of age, a trend similar to that observed at 9 weeks was seen in the *mi/mi* mice, and nearly all of the transverse muscle fibers of the normal mice stained positive for the anti-MyHC-2b antibody (Fig. 2).

In the samples from the 2-week-old mice that were stained with anti-MyHC-2a antibody, nearly all of the fibers of the transverse muscle and many of the fibers of the longitudinal muscles in both types of mouse showed positive staining. At 4 weeks, the number of the longitudinal muscle fibers that stained positively for anti-MyHC-2a antibody showed a decrease compared to with at 2 weeks. However, the number of positively stained fibers was greater for *mi/mi* mice than for normal mice. At 9 weeks of age, nearly all of the transverse muscle fibers, and only a small number of the longitudinal muscle fibers, in the *mi/mi* mice showed positive staining for

anti-MyHC-2a antibody. In contrast, only some of the transverse muscle fibers, and none of the longitudinal muscle fibers, in the normal mice stained positively. At 16 weeks of age, a trend similar to that observed at 9 weeks was seen in the *mi/mi* mice. However, almost no positively stained muscle fibers were observed in the normal mice (Fig. 3).

2. MyHC protein determination by gel electrophoresis

The electrophoretic profile for total protein extracted from each sample is shown in Fig. 4. The gel shows the presence of MyHC proteins in the tongue at each age. Densitometry results are shown in Table 1. The proportion of each MyHC isoform was expressed as relative percentage against the total amount of MyHC present in the muscles studied.

MyHC-2b protein was showed its lowest level at 2 weeks of age in both *mi/mi* and normal mice. In both types of mouse, its expression increased markedly between 2 and 4 weeks of age. MyHC-2b protein expression continued to increase through the 9- and 16-week stages. MyHC-2a protein was showed its highest levels at 2 weeks of age, and decreased at 4 weeks in both *mi/mi* and normal mice. However, the rate of decrease was

smaller in the *mi/mi* mice than in the normal mice. The level of expression was also higher in the *mi/mi* mice than in the normal mice at 9 and 16 weeks (Table 1).

3 . Analysis of messenger RNA (mRNA) expression level using LightCycler™

Messenger RNA for MyHC-2b was expressed at its lowest level at 2 weeks of age in both *mi/mi* and normal mice. In both types of mouse, its expression increased markedly between 2 and 4 weeks of age. MyHC-2b mRNA expression continued to increase through the 9- and 16-week stages. However, no significant difference was seen between the two types of mouse at any age (Fig. 5).

MyHC-2a mRNA was expressed at its highest levels at 2 weeks of age in both *mi/mi* and normal mice, with no significant difference seen between the two types of mouse. Expression decreased at 4 weeks in both types of mouse. However, the rate of decrease was smaller in the *mi/mi* mice than in the normal mice. The level of expression was also higher in the *mi/mi* mice than in the normal mice at 9 and 16 weeks (Fig. 6).

Discussion

Since it was first shown that the muscle fibers of mouse tongue consisted entirely of fast isoforms of MyHC (Dalrymple et al., 1999, 2000; Shuler and Dalrymple, 2001), the composition ratio for each isoform has been studied in detail using gel electrophoresis (Agbulut et al., 2003). These studies have indicated that although MyHC-2b continues to increase after birth, MyHC-2a is expressed only transiently for several weeks postpartum. It has also been shown that the MyHC isoforms undergo reversible changes (Sartorius et al., 1998). Moreover, these reversible changes occur according to changes in oral motor function (Gojo et al., 2002; Doi et al., 2003; Shida et al., 2005; Maejima et al., 2005). This indicates that oral motor function and composition ratios for the isoforms are closely related. It was, therefore, surmised that the presence of teeth might affect masticatory movement, resulting in changes in the function of the tongue muscle and changes in the isoform composition of the muscle fibers that constitute the tongue.

Morphologically, tongue muscle consists of intertwined muscles oriented in various directions. Consequently, when examined in cross-section in immunohistological observations, the proportion of MyHC antibody-positive fibers per unit area is not necessarily consistent with the structural proportion for each isoform. However, it is possible to observe general trends. In this study, we found that some MyHC-2b-positive fibers and some MyHC-2a-positive fibers were present in the longitudinal muscles of

the tongue at 2 weeks of age, i.e., before weaning, in both microphthalmic mice and normal mice. On the other hand, few MyHC-2b-positive fiber and many MyHC-2a-positive fibers were observed in the transverse tongue muscles of microphthalmic mice and normal mice. At 4 weeks of age, immediately after weaning, large differences were seen in the characteristics of the muscle fibers. As compared with before weaning, a rapid increase in MyHC-2b-positive fibers of the longitudinal muscles and a decrease in MyHC-2a of both the longitudinal and the transverse muscles were seen in both types of mouse. However, the rate of decrease in MyHC-2a-positive fibers in the longitudinal and transverse muscles was smaller in the *mi/mi* mice than in the normal mice. MyHC-2a-positive fibers were also more numerous in the *mi/mi* mice than in the normal mice at subsequent stages of development, and remained at particularly high levels in the transverse muscles.

These results suggest that the abundant presence of MyHC-2a-positive muscle fibers in transverse and longitudinal muscles, particularly before weaning, may be attributable to the intake of mother's milk requiring a wave-like movement of the tongue, and the tongue being required to change shape in response to this need. The finding that MyHC-2b-positive fibers accounted for most of the longitudinal muscles in both types of mouse after weaning may be attributable to the fact that the tongue must move

rapidly forward and backward during eating, regardless of whether tooth eruption has occurred or not. That is, as in the masseter muscle, the muscle fibers of the tongue grow and develop to accommodate the changes in function from sucking to mastication. The difference between the *mi/mi* mice and the normal mice with respect to the proportion of MyHC-2b-positive muscle fibers was not great. However, the proportional decrease in MyHC-2a-positive muscle fibers after weaning was smaller in the *mi/mi* mice than in the normal mice, and MyHC-2a was also subsequently expressed in the *mi/mi* mice. This indicates that the post-weaning development of the muscle fibers that would have been prompted by the start of mastication did not occur normally. That is, this effect occurred due to the absence of tooth eruption, which prevented the function of the tongue muscle from undergoing the marked changes that would otherwise naturally occur.

The concentrations measured by gel electrophoresis and the results obtained with the LightCycler™ system also showed a large increase in MyHC-2b and a decrease in MyHC-2a immediately after weaning. No differences were seen between the two types of mouse with respect to MyHC-2b. However, the rate of decrease in MyHC-2a was smaller in the *mi/mi* mice than in the normal mice. The levels of protein and mRNA expression observed in this study also indicated change from sucking to masticatory

movements. The absence of tooth eruption prevented the major change in muscle function that would otherwise naturally occur. As a result, after weaning, there was only a small change in the composition of the isoforms that constituted the muscle fibers.

There is a paper which suggests that microphthalmic mice have the peculiar genes for coding the type of mast cells, which are different from the ones of normal mice (Funaba et al., 2003). Even though they did not study about the skeletal muscles, there is a possibility that the genes coding the skeletal muscles in microphthalmic mice are different from the ones in normal mice. Then, we had studied the composition of MyHC isoforms of the tibialis anterior (TA), one of skeletal muscles, in both microphthalmic mice and normal mice. In the result, we could not find any significant difference between microphthalmic mice and normal mice. Therefore, it is thought that if microphthalmic mice have the peculiar genes coding the skeletal muscles, it is not always expressed as the protein. However, the composition of MyHC isoforms of masseter muscle and tongue muscle were significantly different between microphthalmic mice and normal mice (Yanagisawa et al., 2004). These results suggest that the muscles relating to masticatory movements largely receive the effects in the composition of the isoforms.

This study showed that the isoforms of the tongue muscle changed at weaning,

regardless of whether teeth were present or not. In the absence of tooth eruption, however, the tongue muscle had to compensate for lack of masticatory function with sucking-like movements. Consequently, the change in the proportion of the MyHC-2a isoform, which provides the shape needed for these movements, was small. These results strongly suggest that changes in MyHC isoforms are an adaptation to changes in tongue movement.

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		Fast isoforms of MyHC			Slow isoforms of MyHC
		2a	2d	2b	1
<i>mi/mi</i> mice	2 w	20.1 (4.4)	59.2 (9.4)	20.7 (5.1)	0 (0.0)
	4 w	10.5 (4.6)	46.8 (13.8)	42.7 (10.6)	0 (0.0)
	9 w	7.5 (3.8)	37.0 (13.9)	55.5 (12.0)	0 (0.0)
	16 w	7.2 (3.5)	32.9 (12.6)	59.9 (10.0)	0 (0.0)
normal mice	2 w	18.9 (4.2)	59.9 (8.9)	21.2 (4.7)	0 (0.0)
	4 w	5.1 (3.2)	45.8 (8.8)	49.1 (11.9)	0 (0.0)
	9 w	3.5 (2.3)	38.0 (13.9)	58.5 (12.3)	0 (0.0)
	16 w	3.2 (2.4)	33.2 (10.3)	63.6 (10.3)	0 (0.0)

Table 1. MyHC Isoform Proportions in Tongue

(Values are presented as means (SD), n = 3; w: weeks after birth)

Relative expression of each MyHC was determined by quantitative densitometry of gel. The proportions of MyHC isoforms were expressed as relative percentages of the total amount of MyHC present in the muscles studied. MyHC-2a protein in *mi/mi* mice was at its highest level at 2 weeks of age and remained at high levels.

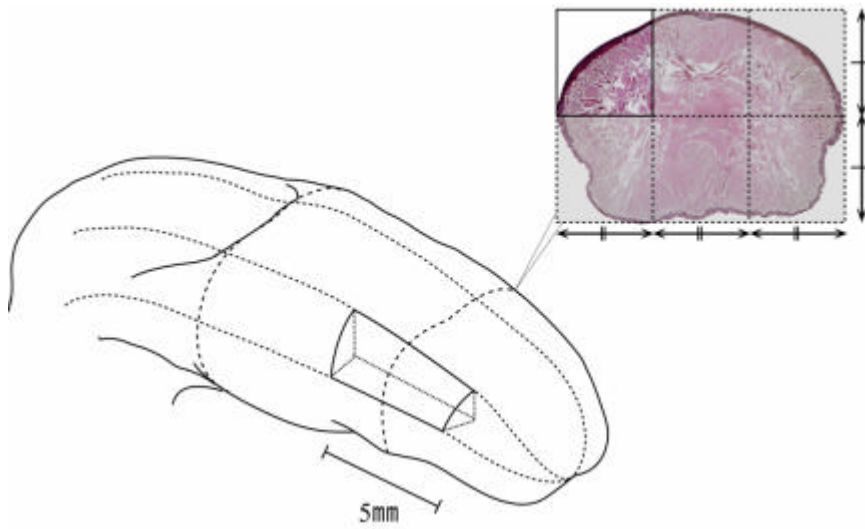


Figure 1. Region of Tongue Observed

Frontal section of area at midpoint between sulcus terminalis and apex. This portion was divided equally into superior and inferior regions, and these were further divided into 3 equal portions from left to right. Superolateral margin was used as sample. This site consists mainly of superior longitudinal and transverse muscles.

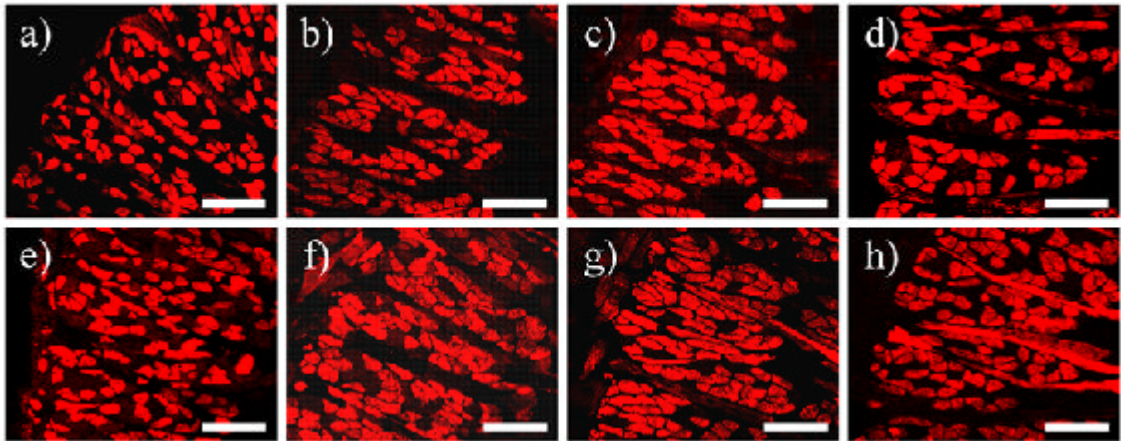


Figure 2. Immunohistochemical Staining of Tongue Muscle Fibers with MyHC-2b

Antibody

a), b), c) and d), *mi/mi* mice; e), f), g) and h), normal mice; a) and e), 2 weeks old; b) and f), 4 weeks old; c) and g), 9 weeks old; d) and h), 16 weeks old. Bars: 100 ? .

MyHC-2b protein increased over time in both types of mouse. BF-F3 (anti-MyHC-2b) monoclonal antibody was used to stain muscle fibers.

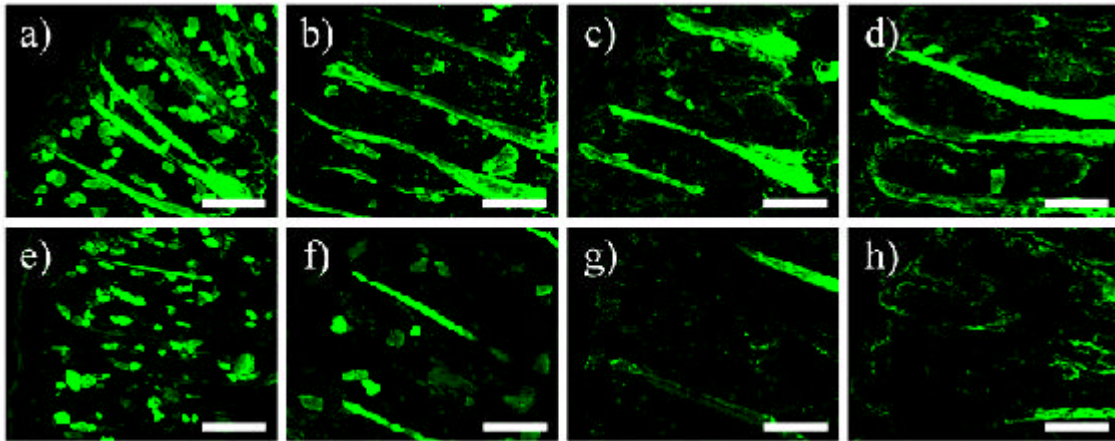


Figure 3. Immunohistochemical Staining of Tongue Muscle Fibers with MyHC-2a

Antibody

a), b), c) and d), *mi/mi* mice; e), f), g) and h), normal mice; a) and e), 2 weeks old; b) and f), 4 weeks old; c) and g), 9 weeks old; d) and h), 16 weeks old. Bars: 100 μ m.

Extensive staining indicating presence of MyHC-2a protein was seen in longitudinal and transverse muscle fibers in *mi/mi* mice at 2 weeks of age. Large number of positive fibers subsequently remained in transverse muscle fibers. SC-71 (anti-MyHC-2a) monoclonal antibody was used to stain muscle fibers.

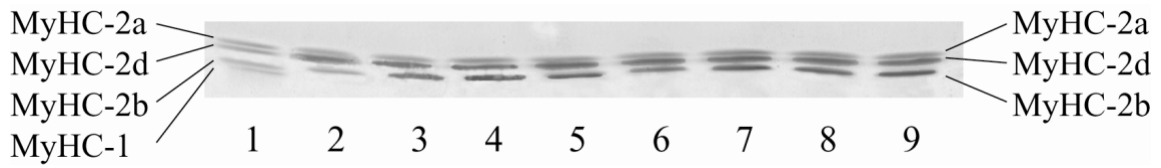


Figure 4. MyHC Isoforms of Tongue Muscle Assayed by SDS-PAGE

Lane 1: marker containing all four MyHC isoforms; lanes 2, 3, 4 and 5: *mi/mi* mice; lanes 6, 7, 8 and 9: normal mice. Lanes 2 and 6: 2 weeks old; lanes 3 and 7: 4 weeks old; lanes 4 and 8: 9 weeks old; lanes 5 and 9: 16 weeks old.

Gel was stained with Coomassie Blue R-250. Presence of MyHC in mouse tongue was seen at each stage of development.

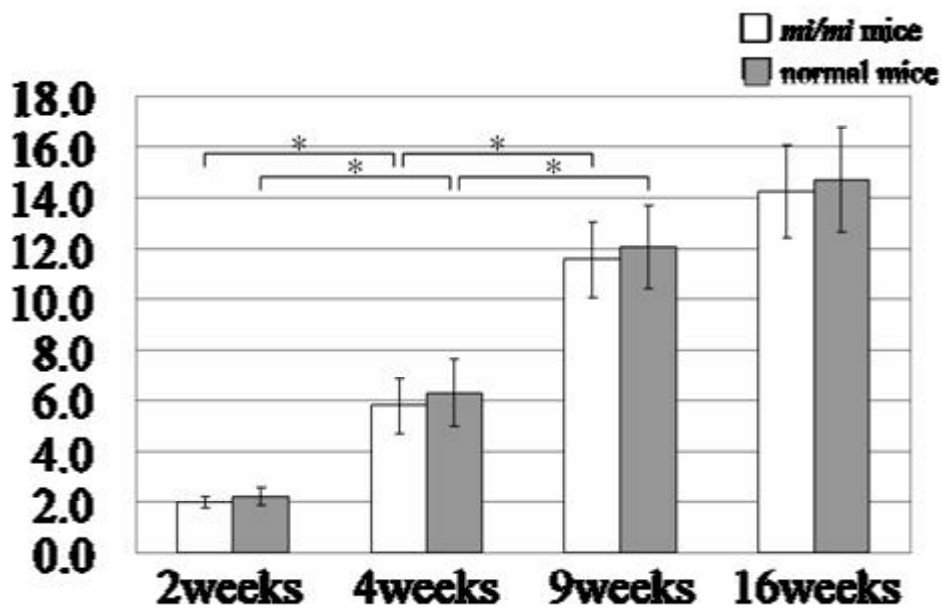


Figure 5. Level of Expression of mRNA for MyHC-2b (LightCycler™, I: ± SD)

Statistical analysis: Tukey's HSD test and Student's t-test

Significant difference: * $p < 0.01$ (Tukey's HSD test), ** $p < 0.005$ (Student's t-test)

Level of MyHC-2b mRNA expression was determined at each age stage using LightCycler™ system (Roche Diagnostics, Mannheim, Germany). Level of MyHC-2b mRNA expression increased over time in both types of mouse.

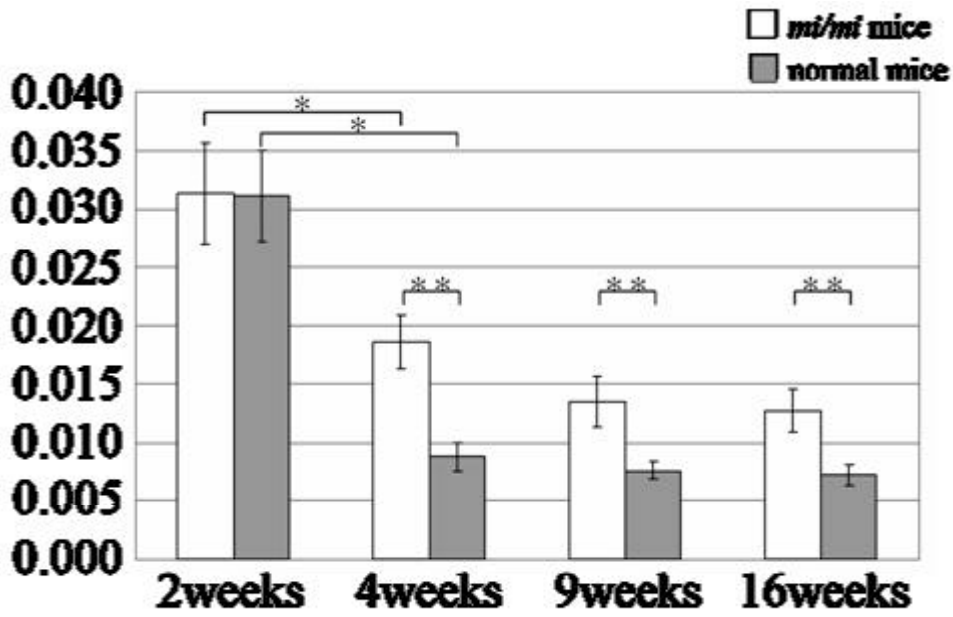


Figure 6. Level of Expression of mRNA for MyHC-2a (LightCycler™, I: ± SD)

Statistical analysis: Tukey's HSD test and Student's t-test

Significant difference: *p < 0.01 (Tukey's HSD test), ** p < 0.005 (Student's t-test)

Level of MyHC-2a mRNA expression was determined at each age stage using LightCycler™ system (Roche Diagnostics, Mannheim, Germany). Level of MyHC-2a mRNA expression in *mi/mi* mice was highest at 2 weeks of age and remained at high levels.