Changes in Myosin Heavy Chain Isoforms Along the Length of Orbital Fibers in Rabbit Extraocular Muscle

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PURPOSE. Extraocular muscles express 10 myosin heavy chain (MyHC) isoforms that cater for a wide range of contractile speeds. We aim to characterize the variations in MyHC expression along the length of singly innervated fibers (SIFs) and multiply innervated fibers (MIFs) in the orbital layer of rabbit superior rectus muscle.

METHODS. Monospecific antibodies to nine MyHCs, including an anti–slow-tonic antibody characterized here were used to immunohistochemically map variations in MyHC distribution in serial sections along the muscle’s full length.

RESULTS. The fastest MyHC, EO, is expressed at the endplate zone (EPZ) of SIFs, flanked proximally and distally by segments expressing the slower 2A, with or without embryonic MyHC. MIFs with constant diameter express α-cardiac MyHC at the EPZ, flanked by segments co-expressing α-cardiac/embryonic and possibly slow-tonic MyHCs. MIFs with varying diameter also express α-cardiac MyHC at the EPZ in their thin, central region, flanked by thin segments co-expressing α-cardiac/embryonic MyHCs, with long proximal and distal extensions of larger diameter that co-express embryonic/slow-tonic and α-cardiac or β/slow MyHCs.

CONCLUSIONS. Orbital fiber types express multiple MyHCs, with faster ones in SIFs, slower ones in MIFs, but all have fast EPZs and slower end segments. We hypothesize that these unique MyHC distributions enable these fibers to relax in two kinetically distinct phases while acting in an antagonistic manner during a saccade: the fast phases facilitate acceleration of eyeball rotation during agonist contraction, while the slow phases help its deceleration toward the visual target, thereby linearizing the saccade. These properties also facilitate pulse movements to implement Listing’s law.

Keywords: extraocular muscle, myosin immunocytochemistry, saccadic eye movements

Extraocular muscles (EOMs) are organized into two layers, a thin orbital layer facing the orbit and a more substantial global layer facing the globe. These two layers differ in fiber type composition and patterns of gene expression, and are widely believed to play functionally distinct roles during eye movements, the global layer moving the eyeball while the orbital layer moving pulleys, which modulate the direction of movements, the global layer moving the eyeball while the widely believed to have functionally distinct roles during eye movements.

Fibers found in amphibian limb muscles. A remarkable feature of the orbital SIFs (oSIFs) and orbital MIFs (oMIFs) is that unlike their global counterparts, they show marked systematic variations along their lengths in structural and functional features, such as fiber diameter, myofibrillar size, mitochondrial volume, sarcomplasmic reticulum volume, and expression of myosin heavy chain (MyHC) genes.

Myosin isoforms control the intrinsic contractile properties of muscle fibers. Mechanical analyses of single EOM fiber segments have revealed that the dynamic range of contractile parameters, such as stiffness minimum frequency that reflects the rate of cross-bridge cycling and the maximal speed of shortening, is considerably wider than that of limb muscle fibers. This is correlated with the fact that EOMs express 10 MyHC isoforms whereas adult limb muscles only express up to four isoforms. The 10 MyHCs expressed in EOMs encompass all members of the mammalian sarcomeric MyHC gene family except MYH 16 that encodes jaw-specific masticatory MyHC. These 10 MyHCs include the six found in developing and adult limb fast muscles, namely, embryonic (MYH3), neonatal (MYH8), slow or β-cardiac (MYH7; hereafter referred to as β/slow), and the fast 2A (MYH2), 2X (MYH1), 2B (MYH4), together with α-cardiac (MYH6), and EO (MYH13), slow-tonic (14/7B), and the recently described slow B (MYH15). MyHC expression in EOM fibers has been described in several species, including rabbit, mouse, dog, and human. It is extremely complex, the dominant features being the co-expression of multiple MyHCs in single fibers and variations in MyHC expression along the fiber length. Early work on rabbit EOMs showed that oSIF reacted with an antibody specific against all fast MyHCs, while oMIF expressed α-cardiac MyHC. The percentages of EOM fibers reacting with several anti–MyHC antibodies change significantly along the length, with more fibers expressing fast MyHC in the middle of the muscle and more slow MyHC.
expressing fibers toward the end. A similar segmental difference in MyHC distribution based on SDS-PAGE analysis of whole EOM segments has also been reported. An anti-EO antibody was first shown to stain fibers in the global layer of rabbit EOM. In contrast, it was subsequently reported that the difference in MyHC distribution based on SDS-PAGE analysis of orbital and global fibers. A more detailed analysis of rabbit superior rectus (SR) revealed that anti-EO stained the orbital fibers only where they increase in diameter around the EPZ, but elsewhere these fibers express embryonic MyHC, while the smaller diameter oMIFs at the EPZ expressed α-cardiac MyHC. The anti-EO did not stain orbital fibers away from the EPZ, which explains the earlier result showing that this antibody only stained global fibers.

The above studies on rabbit EOMs were based on very limited use of monospecific anti-MyHC antibodies, and provided incomplete and imprecise information about how the expression of the 10 MyHCs varied along EOM fiber types. The function significance of these longitudinal variations in MyHC expression in the context of ocular motility has so far been elusive. In this study, we use a definitive set of antibodies (in parentheses) to be used hereafter, and the references documenting their specificities in the rabbit. The nine primary antibodies used, their specificities against the nine MyHCs expressed in EOMs are listed in Table 1. Eight of these were monoclonal antibodies (mabs) and one was a polyclonal antibody. They were: (1) 10A10 with specificity against rabbit EO MyHC, (2) 10F5 against 2B MyHC, (3) 6H1 against 2X MyHC (10F5 and 6H1, developed in our laboratory), are available from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), (4) SC-71 against 2A MyHC in the rat, but specific for 2A/2X in rabbit, (5) BA-G5 (from the American Type Culture Collection, Manassas, VA, USA) raised against cow atrium, but specific for slow MyHC in mammalian limb and EOM, (7) NCL-MHCn (Novacastra Laboratories, Ltd., Newcastle-Upon-Tyne, UK) against rabbit neonatal MyHC, and (9) a polyclonal antibody specific against slow-tonic MyHC described in the Supplementary Data. Table 1 also gives the simplified names of the antibodies (in parentheses) to be used hereafter, and the references documenting their specificities in the rabbit.

### Abbreviations

- SC-71: An essential antibody for studying rabbit orbital fibers, which stain the 2A/2X MyHC. It is used to stain the orbital fibers away from the EPZ.
- BA-G5: A polyclonal antibody specific for slow MyHC in mammalian limb and EOM.
- NCL-MHCn: A monoclonal antibody specific for rabbit neonatal MyHC.
- 10A10, 10F5, 6H1: Monoclonal antibodies specific for 2A, 2B, and 2X MyHCs, respectively.

### Tissue Preparation

Three adult New Zealand white rabbits (each weighing 2 kg, ~2-months old) were euthanized with an overdose of sodium pentobarbital, and the heart, extracocular, and some limb muscles were removed for biochemical and immunohistochemical analyses. The SR muscles for immunohistochemistry were dissected intact from origin to insertion. One newborn rabbit was also euthanized and some limb muscles were removed. The animals were maintained and used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and complies with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Animal Ethics Review Committee of the School of Medical Sciences at the University of Sydney approved the study. The muscles for immunohistochemistry were mounted on cork with Tissue-Tek (Miles Scientific, Elkhart, IN, USA), frozen in isopentane cooled in liquid nitrogen, and 10- to 15-μm thick sections were cut at −20°C. Three SR muscles were sectioned along the entire length. Beginning from the proximal end, the number of sections cut was recorded using the counter on the cryostat so that the location of the section along the muscle length could be determined and expressed on a proximal to distal (P-D) scale in millimeters. The salient immunohistochemical features of all three SR muscles were very similar; the photomicrographs presented were all derived from the same SR.

### Results

#### Immunochemistry Survey of MyHC Expression in Orbital Fibers of the Superior Rectus

Serial sections at specific sites along the length of the SR were analyzed by indirect immunohistochemistry using peroxidase-labeled anti-mouse or anti-sheep immunoglobulin secondary antibodies (Dako, Dakopatts, Denmark) as previously described. The nine primary antibodies used, their specificities against the nine MyHCs expressed in EOMs are listed in Table 1. Eight of these were monoclonal antibodies (mabs) and one was a polyclonal antibody. They were: (1) 10A10 with specificity against rabbit EO MyHC, (2) 10F5 against 2B MyHC, (3) 6H1 against 2X MyHC, (4) SC-71 against 2A MyHC in the rat, but specific for 2A/2X in rabbit, (5) BA-G5 (from the American Type Culture Collection, Manassas, VA, USA) raised against cow atrium, but specific for slow MyHC in mammalian limb and EOM, (6) NCL-MHCn (Novocastra Laboratories, Ltd., Newcastle-Upon-Tyne, UK) against rabbit neonatal MyHC, and (9) a polyclonal antibody specific against slow-tonic MyHC described in the Supplementary Data. Table 1 also gives the simplified names of the antibodies (in parentheses) to be used hereafter, and the references documenting their specificities in the rabbit.
(oSIFs), where they also stain with anti-EO (data not shown). Anti-neo does not stain fibers in the OO layer, and only weakly stains a few scattered fibers along the entire length of the IO layer, consistent with our previous study.15 The above results were confirmed for all three serially sectioned SR muscles. Therefore, in the rest of this study, we focus on the staining patterns along the length of the rabbit SR orbital layer, using antibodies against six MyHCs, viz, anti–slow-tonic, anti-emb, anti-β/slow, anti-α, anti-2A, and anti-EO.

Expression of MyHCs Proximal to the Endplate Region

The descriptions of the topography and variations in fiber diameter along the length of various types of OO layer fibers of the rabbit SR by Davidowitz et al.10 provided very useful landmarks for correlating their variations in MyHC immunohistochemistry. These authors have shown that the SR OO layer has three major fiber types: (1) 65% of oSIFs, approximately 10 μm in diameter, which swell to 15 to 20 μm at the EPZ, (2) 10% of orbital MIFs (oMIFs) with constant diameter (coMIFs) of approximately 10 μm, and (3) 25% of oMIFs which vary in diameter (voMIFs), from 5 μm along a short central segment which encompasses the EPZ, to approximately 15 to 20 μm in their proximal and distal ends. The voMIFs are the longest fibers, typically extending from the muscle origin to well into the distal third, whereas the oSIFs and coMIFs are short, typically beginning somewhat distal to the muscle origin, terminating throughout its middle third. In the first 2.5 mm of the muscle, referred to here as P-D 0.0 to 2.5, fibers in the OO layer are mostly voMIFs, which are at their maximal diameter (15–20 μm), while there are few oSIFs and coMIFs here, most of these fibers in the OO layer begin to appear beyond P-D 2.5.10 To correlate with their data, we have used rabbits of the same body weight as those used by Davidowitz et al.10

Figure 1 shows the staining pattern of the six antibodies at approximately P-D 1.4, where most fibers in the OO layer are voMIFs.10 These fibers strongly costain with anti-emb (Fig. 1A) and anti–slow-tonic (Fig. 1B), but are not stained by anti-α (Fig. 1C) except for a few weakly staining fibers deep in the OO layer. Anti-2A (Fig. 1E) does not stain fibers in the OO layer, but does stain most fibers in the IO layer. The differential staining of OO and IO layers indicates that oSIFs emerge in the IO layer more proximally than those in the OO layer. Most of these fibers are apparently not stained by anti-emb (Fig. 1A). Anti-α (Fig. 1C) strongly stains scattered oMIFs in the IO layer, which also co-express embryonic (Fig. 1A) and slow-tonic (Fig. 1B) MyHCs. Anti-EO (Fig. 1F) does not stain fibers in the OO layer, but weakly stains only a few oSIFs deep in the IO layer, which costain with anti-2A (Fig. 1E) but not anti-emb (Fig. 1A). Anti-β/slow (Fig. 1D) does not stain fibers in the orbital layer.

Figure 2 shows the MyHC staining transitions in oMIFs at P-D 3.5 (Figs. 2A–D) and P-D 5.0 (Figs. 2E–H), using anti-emb (Figs. 2A, 2E), anti–slow-tonic (Figs. 2B, 2F), anti-α (Figs. 2C, 2G), and anti-2A (Figs. 2D, 2H) antibodies, while Figure 3 shows the staining transitions in oSIFs at P-D 3.5 (Figs. 3A–C) and P-D 5.0 (Figs. 3D–F), using anti-emb (Figs. 3A, 3D), anti-2A (Figs. 3B, 3E), and anti-EO (Figs. 3C, 3F). These locations are interesting because around P-D 3.5, oSIFs and coMIFs are beginning to emerge in the OO layer, while around P-D 5.0 voMIFs narrow down to 5 μm, and all these fibers get close to their EPZ localized in the endplate region between P-D 5.0 and P-D 7.5.10

In the OO layer at P-D 3.5, the large voMIFs seen in Figure 1 continue to stain with anti-emb (Fig. 2A) and anti–slow-tonic (Fig. 2B), but now acquire staining with anti-α (Fig. 2C), a triple staining pattern seen in the IO layer at P-D 1.4 (Fig. 1). Only few oSIFs have started to appear in the OO layer, as indicated by the sparse fibers costaining with anti-2A (Fig. 2D) and anti-emb (Fig. 2A). In the IO layer at P-D 3.5, however, the majority of fibers are oSIF of various sizes that stain strongly with anti-
**FIGURE 2.** Immunoperoxidase staining of semi-serial sections of the rabbit SR at approximately P-D 3.5 (A–D) and P-D 5.0 (E–H) to illustrate the transitions in staining of oMIFs using anti-emb (A, E), anti-slow-tonic (B, F), anti-α (C, G), and anti-2A (D, H). Note the complementary staining of fibers by anti-α (oMIFs) and anti-2A (oSIFs) in (C, D) and (G, H), respectively. *Arrowheads* in (A–D) point to triple stained oMIFs that are not stained by anti-2A, which stain the few oSIFs here. *Arrows* in (E–H) point to oMIFs staining only with anti-α.

**FIGURE 3.** Immunoperoxidase staining of semiserial sections of the rabbit SR at approximately P-D 3.5 (A–C) and P-D 5.0 (D–F) to illustrate the transitions in staining of oSIFs using anti-emb (A, D), anti-2A (B, E), and anti-EO (C, F). *Arrows* in (D–F) point to largest fibers in the OO layer staining with anti-2A acquiring staining with anti-EO but losing staining with anti-emb.
2A (Fig. 2D), but not stained with anti-emb (Fig. 2A). Here, oMIFs are large and sparse, and show triple staining, strongly with anti-α (Fig. 2C), and more weakly with anti-emb (Fig. 2A) and anti-slow-tonic (Fig. 2B).

In the OO layer at P-D 5.0, anti-α (Fig. 2G) intensely stains oMIFs in a manner complementary to the oSIFs stained by anti-2A (Fig. 2H), but now staining weaker with anti–slow-tonic (Fig. 2F) and anti-emb (Fig. 2E). Many of these oMIFs in the OO layer stained by anti-α (Fig. 2G) appear to show a reduction in fiber diameter compared with those seen in Figure 2C at P-D 3.5.

In the IO layer at P-D 5.0, oMIFs identified by staining with anti-α (Fig. 2G) appear less abundant than in the OO layer, and also appear to show some variation in diameter and lose staining with anti–slow-tonic (Fig. 2F) and anti-emb (Fig. 2E), only an occasional fiber staining weakly with these antibodies.

In the OO layer at P-D 5.0, we see the emergence of oSIF, indicated by the appearance of scattered fibers staining with anti-2A (Fig. 3B) that costain with anti-emb (Fig. 3A), in contrast to the lack of fibers staining with anti-2A in the OO layer at P-D 1.4 (Fig. 1E). These fibers show no staining with anti-EO (Fig. 3C) at this level. In the IO layer at P-D 3.5, anti-2A staining (Fig. 3B) identified numerous oSIFs of various diameters, the largest of which no longer stain with anti-emb (Fig. 3A), but are still unstained by anti-EO (Fig. 3C).

The OO layer at P-D 5.0 begins to thicken due to the increase in number of oSIFs, indicated by the increase in proportion of fibers staining with anti-2A (Figs. 3E, 2D). These fibers show size heterogeneity and staining pattern similar to those in IO layer at P-D 3.5 (Fig. 3B): smaller fibers costain with anti-emb (Fig. 3D) while the largest ones no longer stain with anti-emb, but now acquire weak staining with anti-EO (Fig. 3F).

The IO layer at P-D 5.0 also thickens due mainly to the increase in diameter of oSIFs to approximately 20 μm as they approach the EPZ. These fibers with apparently larger diameter strongly stain with anti-EO (Fig. 3F), but have lost staining with anti-emb (Fig. 3D) and anti-2A (Fig. 3E). Anti-2A continues to react with a population of oSIFs of different diameters, only the smaller of which stain with anti-emb (Fig. 3D) at this level. Anti-β/slow failed to stain fibers along the entire proximal region in both the OO and IO layer (data not shown for P-D 5.0, but see Fig. 1D at P-D 1.4).

Expression of MyHCs at the Endplate Region

Davidowitz et al.10 reported that the OO layer is thickest (150–200 μm) at the endplate region (P-D 5.0–7.5), where oSIFs reach their maximal diameter of 15 to 20 μm, while the coMIFs and voMIFs are smaller in diameter, 10 and 5 μm, respectively.10 Figure 4 shows the staining patterns at approximately P-D 6.5 using antibodies against the six MyHCs expressed in orbital fibers. A striking feature at this level is that oMIFs stained by anti-α (Fig. 4C) have completely lost triple staining seen in more proximal sections, now no longer staining with anti-emb (Fig. 4A) and anti–slow-tonic (Fig. 4B), except for a few fibers staining with anti-emb at the very surface of the OO layer. The smaller oMIFs are more abundant in the OO layer while the larger fibers are more abundant in the IO layer.

The oSIFs with enlarged diameter at their EPZ in both OO and IO layers stain with anti-EO (Fig. 4F), but no longer with anti-2A (Fig. 4E) seen at P-D 5.0 (Fig. 2H), nor with anti-emb (Fig. 4A), seen more proximally (Fig. 2A). Anti-2A (Fig. 4E) stains scattered smaller oSIFs in both OO and IO layers, which are not stained by anti-emb (Fig. 4A). Anti-β/slow (Fig. 4D) does not stain any fiber in the OO or IO layer at P-D 6.5.

Expression of MyHCs Distal to the Endplate Region

Figure 5 shows the transitions in staining pattern in the region just distal to the endplate region at P-D 7.8 (Figs. 5A–E) and more distally at P-D 11.0 (Figs. 5F–J), using anti-emb (Figs. 5A, 5F), anti-slow-tonic (Figs. 5B, 5G), anti-α (Figs. 5C, 5H), anti-2A (Figs. 5D, 5I), and anti-EO (Figs. 5E, 5J). The OO layer at P-D 7.8 decreases in width as the bulging of oSIFs at the endplate...
region subsides. This is accompanied by changes in MyHC expression similar to those at the other end of the EPZ at P-D 5.0 (Figs. 3D–F): most oSIFs (those not stained by anti-slow-tonic or anti-α) have lost staining with anti-EO (Fig. 5E) but now stain with anti-2A (Fig. 5D), and some with anti-emb (Fig. 5A, see examples indicated between arrows in these figures). A few large oSIFs are still seen, and stain exclusively with anti-EO (Fig. 5E) or anti-EO and anti-2A. The IO layer at P-D 7.8 retains its thickness, as most of the oSIFs are still of large diameter, and stain exclusively with anti-EO (Fig. 5E), while scattered smaller fibers stain with anti-2A (Fig. 5D) and not anti-emb (Fig. 5A), as seen at P-D 6.5 (Figs. 4E and 4A, respectively).

The oMIFs at P-D 7.8 stain with anti-α (Fig. 5C) showing fiber size heterogeneity as in the endplate region (Fig. 4C), with abundant small fibers in the OO layer, and mostly larger fibers in the IO layer. Most of the small, anti-α staining fibers (Fig. 5C) in the OO layer now acquire costaining with anti-emb (Fig. 5A), which stains nearly all fibers in the OO layer, and some also with anti-slow-tonic (Fig. 5B). In the IO layer, the larger diameter fibers staining with anti-α (Fig. 5C) do not stain with anti-emb (Fig. 5A), nor with anti-slow-tonic (Fig. 5B), the same as seen at the EPZ.

At P-D 11.0 (Figs. 5F–J), the OO layer further decreases in thickness as the oSIFs progressively terminate, indicated by the reduced proportion of fibers in this layer staining with anti-2A (Fig. 5I) compared with that at P-D 7.8 (Fig. 5D). These oSIFs continue to be stained by anti-emb (Fig. 5F), which stains all fibers in the OO layer. The IO layer at P-D 11.0 also decreases in width as the bulging anti-EO staining oSIFs seen at P-D 7.8 (Fig. 5E) return to smaller diameter after passing through their EPZ. Here, oSIFs no longer stain with anti-EO (Fig. 5I), but with anti-2A (Fig. 5I), while a smaller subpopulation begins to costain with anti-emb (Fig. 5F).

In the OO layer at P-D 11.0, the oMIFs stain intensely with anti-slow-tonic (Fig. 5G) and anti-emb (Fig. 5F) but staining with anti-α (Fig. 5H) has become weaker. In the IO layer, the majority of oMIFs show triple staining with anti-emb (Fig. 5F), anti-slow-tonic (Fig. 5G), and anti-α (Fig. 5H).

Davidowitz et al.10 reported that in the distal third of the SR (beyond approximately P-D 16.0), the oSIFs and coMIFs have completely terminated, and that voMIFs have reached their maximal diameter (15–20 μm). They observed that toward the middle of the muscle’s distal third, orbital fibers located in the central zone along the width of the muscle terminate early, and are surpassed by the underlying global fibers, resulting in a thin layer of pseudo-orbital MIFs originating from the global layer. Along the lateral edge of the muscle, the opposite occurs: orbital fibers surpass the terminating global layer fibers, resulting in an isolated lateral arm of the orbital layer, which accounts for approximately a quarter of the muscle’s distal width, and these fibers carry on distally to insert onto the eyeball. Our findings are consistent with these observations. We observe that anti-2A, anti-2B, and anti-EO failed to stain any fiber in the lateral arm or the pseudo-orbital layer at P-D 17.0 (data not shown), consistent with the absence of oSIFs.

Figure 6 shows the staining pattern of voMIFs at approximately P-D 17.0 with anti-emb, anti-slow-tonic, anti-α, and anti-β/slow. Anti-emb (Fig. 6A) and anti-slow-tonic (Fig. 6B) co-stain almost all fibers in the OO layer, including the lateral arm and the pseudo-orbital MIFs in the midregion, while anti-α (Fig. 6C) continues to stain these fibers in varying intensities. Anti-β/slow (Fig. 6D), which fails to stain fibers in both the OO and IO layers throughout the entire length of the proximal two-thirds of the SR, does react with the majority of MIFs in the OO layer and the lateral arm in a roughly complementary fashion with anti-α (Fig. 6C).
Figure 7 shows the staining pattern of the isolated lateral arm at approximately P-D 18.0 using anti-emb (A), anti–slow-tonic (B), anti-α (C), and anti-β/slow (D). Most voMIFs here co-stain with anti-emb (Fig. 7A), anti–slow-tonic (Fig. 7B), and anti-β/slow (Fig. 7D) while anti-α (Fig. 7C) no longer shows staining.

Table 2 summarizes MyHC expression at various distances along the fibers of the major populations of oMIFs and oSIFs in the OO and IO layers of the rabbit SR muscle. In the OO layer, the oSIFs are short, first appearing at P-D 3.5 and terminating around P-D 11.0. They express EO MyHC exclusively at their EPZ around P-D 6.5 where they increase in diameter. For a short distance on either side of the EPZ, OO oSIFs express 2A MyHC exclusively, but they coexpress 2A and embryonic MyHCs in their proximal and distal segments. In the IO layer, oSIFs are already present at P-D 1.4, terminating beyond P-D 7.8. They also increase in diameter and express EO and 2B MyHCs at their EPZs, which are spread over a region extending from P-D 5.0 to P-D 7.8. Proximal and distal to the EPZ, they express 2A MyHC, with very little, if any, embryonic MyHC, except at their distal end.

The oMIFs in both layers express α-cardiac MyHC exclusively at the endplate region around P-D 6.5. In the IO oMIFs, this exclusive expression of α-cardiac MyHC is extended to P-D 7.8. On either side of the endplate region, voMIFs co-express embryonic, slow-tonic, and α-cardiac or β/slow MyHcs. We can only distinguish the thin (5 μm) voMIFs from coMIFs (10 μm) between P-D 5.0 and P-D 7.8, but this difference becomes blurred more proximally and distally as the voMIFs enlarge in diameter. This makes it difficult to ascertain whether coMIFs coexpress embryonic/α-cardiac/slow-tonic MyHCS or only α-cardiac/embryonic MyHCS.

DISCUSSION

Variations in MyHC Expression Along the Length of Orbital Layer Fibers of Rabbit Superior Rectus

In this study, we serially sectioned rabbit SR muscles along its entire length and used a set of nine monospecific antibodies to immunohistochemically analyze in detail the MyHC distribution of orbital fibers at critical points along the length. The points chosen for detailed analysis were guided by the descriptions of changes in diameters along the length of oSIFs and oMIFs in the OO layer.10 We included the IO layer in this study, and showed that it contained oSIFs and oMIFs with similar MyHC expression and changes in fiber diameter to those in the OO layer. However, oSIFs in the IO layer differ by coexpressing 2B and EO MyHCs at their EPZs, which are more spread out, and they do not express embryonic MyHC except at the distal end.

We confirm earlier reports that the expression of EO MyHC is restricted to the large diameter region of oSIF at the EPZ of rabbit14,15 and rat.13 The expression of 2A MyHC in oSIFs refines an earlier work showing that these fibers reacted with an antibody against all fast MyHCS,27 and we rule out...
expression of 2B MyHC in oSIFs except in the IO layer, where it is coexpressed with EO MyHC at the EPZ.

Our finding that all rabbit oMIFs express α-cardiac MyHC virtually along the entire length confirms the report of Rushbrook et al.27 The expression of embryonic MyHC in the proximal and distal segments of oMIFs but sparing the EPZ is consistent with earlier reports of the staining of rat MIFs with an antibody reactive against embryonic13 or embryonic/neonatal MyHC.12 While expression of slow-tonic MyHC in polynodraulically innervated orbital fibers has been reported in human19 and in rat orbital fibers,20 we show that in the rabbit, this isoform is restricted to the long, large diameter proximal and distal extensions of the thin segments of oMIFs but absent in the thin segments themselves. We show that slow-tonic MyHC is here coexpressed with α-cardiac and embryonic MyHCs, and in the case of the distal end segment, with β/slow MyHC as well.

The 10 MyHCs expressed in EOMs functionally encompass the full spectrum of striated muscle speeds. The maximal speeds of contraction of mammalian EOMs exceed those of their fast-twitch limb muscles.37,48 The ALR of EOMs can be attributed to their unique content of EO MyHC, thus showing that EO-MyHC is the fastest isoform. Of the isoforms found in limb muscles, 2B, 2X, and 2A, and β/slow are in descending order of speeds.37,49 MyHC α-cardiac is 2- to 3-fold faster than β/slow MyHC,50,51 placing its kinetic properties close to that of 2A MyHC. Neonatal and embryonic MyHCs are as slow or slower than β/slow MyHC,52 while the slow contractures of toad slow-tonic fibers,53 which expresses slow-tonic MyHC,54 suggest that it is the slowest isoform. The MyHC isoforms listed in Table 1 are in their approximate descending order of speeds.

The distribution of slow B MyHC was not investigated in this work. It is known to be expressed in oSIFs and oMIFs of the rat, but absent in global fibers.55 This isoform is also expressed in some human EOM samples,21 and may be a common feature in mammalian orbital fibers. We described two isoforms of rabbit embryonic MyHC, based on SDS-PAGE and immunoreactivity to embryonic MyHC-specific monoclonal antibodies.15 One of these isoforms is EOM-specific, expressed only during postnatal life, and is largely confined to oSIFs and oMIFs, properties that characterize the expression of slow B MyHC.56 Thus, it seems very likely that this isoform is in fact the rabbit slow B MyHC that share antigenic properties with rabbit embryonic MyHC. Further work is needed to verify this suggestion. It should be noted that this isoform is absent in the central segment of oMIFs.15 Although the kinetic properties of slow B MyHC have not been determined, it is not likely to resemble those of slow-tonic MyHC, as fast and slow twitch motor units have been recorded from the orbital layer of cat EOM.54

The MyHC compositions of the four types of orbital fibers suggest that their overall speeds are as listed in descending order in Table 2. These differences in overall speeds reflect the continuum of mechanical properties of single EOM fibers,17,20 but give little insight into their role in ocular kinetics. Importantly, our results demonstrate clearly that all these fiber types express their fastest MyHC in their central segment, with slower MyHCs in both end segments.

### Correlation of MyHC Expression With Longitudinal Variations in Structural and Functional Features

Changes in MyHC isoform expression along the length of orbital fibers are associated with variations in ultrastructural and functional features that match the contractile kinetics of the MyHC. At the EPZ of oSIFs, there is a localized decrease in myofilibrillar size,53 an increase in sarcoplasmic reticulum and mitochondrial volumes,60 and the expression of the fast isoform of the sarcoplasmic reticulum Ca2+ pump (SERCA1).51 These features promote rapid diffusion of activating Ca2+ and its reuptake, and thus rapid contraction and relaxation. Rapid relaxation of the EPZ may be further facilitated by the local presence of parvalbumin, a cytoplasmic Ca2+ buffer that strips Ca2+ from troponin-C, pending its reuptake by the sarcoplasmic reticulum,60,55 whose concentration is correlated with the relaxation rate of a variety of fast muscle fibers.56 Although
parvalbumin is present in some rat global fibers,\(^{59}\) its local expression at the EPZ of oSIFs has not been excluded, and should now be explored.

At the central segment of oMIFs, there is also a localized decrease in myofibrillar size\(^{55}\) and increase in mitochondrial volumes\(^{11}\) relative to the rest of the fiber, which shows tonic-like ultrastructural features. In the rat, the oMIFs receive an en plaque endplate here in addition to their distributed en gruppe innervation,\(^{60}\) and the central segment can generate spike potentials.\(^{51}\) Consistent with this, stimulating nerve fibers innervating oMIFs generates slow-twitch responses in cat\(^{2,63}\) and sheep EOM.\(^{64}\) En plaque endplates are also found in rabbit oMIFs.\(^{33}\) It seems probable that the central segments of rabbit oMIFs can likewise generate twitch contractions.

In the proximal and distal segments of voMIFs, fiber morphology in rabbit\(^{66}\) and electrical properties in rat\(^{61}\) resemble those of slow-tonic GMIFs. Contraction and relaxation kinetics in these segments would be slower than at the central segment, but faster than GMIFs because rabbit oMIFs, but not GMIFs, express SERCA1\(^{31}\) and probably slow B MyHC.

In summary, the oSIFs, coMIFs and voMIFs all have a fast central segment flanked by slower end segments due to their MyHC profile, electrical properties, and other features affecting Ca\(^{2+}\) release and reuptake, posing questions as to their functional significance.

### Functional Characteristics of Orbital Fibers

Mechanical analyses of isolated single fibers\(^{17,20}\) can only reveal their overall contractile characteristics, but not the nuances introduced by variations in MyHC and other features along their length. However, the following scenario can be envisaged from first principles. Following a burst of neural impulses, the fast central segments of orbital fibers would contract first, stretching the slower end segments just before they start to contract. This would have the effect of enhancing the rate of rise of tetanic tension in the slower segments\(^{65}\) due to the conversion of low-force to high-force cross bridges.\(^{66}\) Upon cessation of stimulation, each type of orbital fiber would relax in two phases, a fast phase due to the specializations at the central segments, and a slow phase due to the slower proximal and distal segments. Few EOM motor unit studies have focused on their relaxation characteristics. However, a biphasic relaxation following a tetanus in a slow-twitch motor unit in the cat lateral rectus has been recorded by Goldberg et al.\(^{67}\): a rapid phase lasting approximately 20 msec, followed by a slow phase lasting 50 msec.

Variations in fiber diameter along the length of orbital fibers introduce mechanical problems. Assuming tetanic tension is proportional to fiber cross-sectional area, the 2- to 4-fold variation in diameter of oSIF and oMIFs would generate a 4- to 16-fold variation in tetanic tension. Such fibers are likely to break if active tension is transmitted exclusively along the sarcomeres within the fiber. It is highly likely that tensions generated in the large diameter EPZ of oSIFs and end segments of voMIFs are transmitted laterally,\(^{68-70}\) to neighboring fibers. This topic will be further discussed below.

### Functional Roles of Orbital Fibers in Oculomotor Kinetics

Orbital fibers have long been known to participate in fixation\(^{71,72}\) and in rotating the eyeball in all types of eye movements,\(^{21-23}\) However, recent analysis of orbital tissues purports to show that the short orbital fibers insert onto soft tissue “pulleys,” whereas global fibers insert onto the eyeball. This led to the active pulley hypothesis, according to which contraction of orbital fibers moves only the pulleys, while contraction of the global fibers only rotates the eyeball.\(^{4,74}\) Pulleys inflect the direction of action of EOMs, and movements of pulleys alter the direction of rotation of the eyeball in such a way as to implement Listing's law, which specifies ocular torsion for any given eye position.\(^{4,74-75}\) The existence of pulleys greatly simplifies the neural control of smooth compensatory ocular rotation in three-dimensional space,\(^{76-77}\) but what drives them is still controversial.\(^{5}\)

There is considerable anatomic and physiologic evidence that orbital and global fibers can move both pulley and eyeball. Anatomically, some global fibers in human and monkey EOMs inserts onto their pulleys.\(^{78}\) We confirm that a subpopulation of global fibers at the lateral edge of the rabbit SR terminates early,\(^{10,41}\) and probably insert onto the pulley, while some orbital fibers (voMIFs) continue on to form the lateral arm that inserts directly onto the eyeball. Other recti may also have similar features. Physiologically, contraction of cat orbital motor units can be recorded at the tendon of insertion.\(^{2,5}\) A high proportion of motor units in the cat lateral rectus are “bilayer units” with fibers localized in both orbital and global layers.\(^{2,5}\) Further, it is well established that force developed by a given muscle fiber can be transmitted through the sarcolemma and perimysium to the tendon of insertion.\(^{68-70}\) This would allow orbital fibers to move the eyeball and global fibers to move the pulley. Collins\(^{72}\) has shown that only orbital fibers are recruited during fixation at eye positions up to 20° beyond the field of action of the EOM, implying that only orbital fibers are holding the eyeball in these positions. In light of the above, we will discuss the functional roles of orbital fibers in moving the eyeball and pulleys separately below.

### Neural and Mechanical Activities of EOMs During Fixation and Ocular Rotation

The functional significance of the longitudinal variations in dynamic characteristics of orbital fibers can best be appreciated when we consider in some detail the neural and mechanical events before, during, and after the saccade. During fixation at various eye positions, neurons are recruited at specific eye positions (the thresholds), with position sensitivities increasing linearly with eye position in the direction of action of the muscle.\(^{79-81}\) Before a saccade, the eye is held in position by motor units located in the orbital layer, have low-firing thresholds, low-position sensitivities, and high-fatigue resistance.\(^{71-73}\)

At the onset of the saccade, agonist motor units whose thresholds lie between the current eye position and the target position will be recruited, each delivering a burst of impulses of high frequency with a duration proportional to the saccade magnitude. This is followed by a post-saccadic “slide” in discharge frequency that levels off to a “step” appropriate for the new eye position.\(^{79,80,82-84}\) Most antagonist units active before the saccade are abruptly silenced, but at the end of the saccade, antagonist units whose firing thresholds remain exceeded at the new eye position will recommence firing.\(^{79,80,82-84}\)

The agonist during the saccade needs to overcome the changing viscoelastic load imposed by the relaxing antagonist and the mainly elastic load of the orbital tissues to reach the new eye position.\(^{85,86}\) Mechanical recordings of medial and lateral recti tensions and eye positions during saccades in the monkey have shown that the agonist tension rapidly rises and reaches a peak as the eyeball attains its target, then tension falls without eye movement roughly over 100 msec to a new steady level appropriate for the new eye position, in response to the “pulse-slide” step of saccadic innervation.\(^{87}\) The antagonist tension decreases during the saccade, and then increases with a waveform inversely matching that of the
agonist. These changes generate an initial sharp acceleration of the eyeball position, followed by a deceleration, resulting in an almost linear eyeball rotation, which constitutes the saccade.\(^{79,87,88}\)

**Proposed Functional Significance of Longitudinal Variations in Dynamic Characteristics of Orbital Fibers**

We propose that the variations in dynamic characteristics along the length of oSIFs and oMIFs are instrumental in linearizing the saccade. At the beginning of a saccade, the rapid relaxation rates of the central segments of the antagonist oSIFs and oMIFs would, in successive steps, rapidly yet smoothly reduce the viscous load on the contracting agonist units, thus facilitating the initial eyeball acceleration. A similar mechanism has been proposed for permitting rapid change of gaze in oSIFs.\(^{13,15}\) Much of this load in relaxing antagonist units resides in the attached cross bridges. It is therefore of considerable interest that kinetic analysis of recombinant subfragments-1 of human MyHCs has shown that cross-bridge detachment rates of MyHCs expressed in the central segments of oSIFs and oMIFs, namely, EO and \(\alpha\)-cardiac, exceed those of 2A, 2X, and 2B MyHCs.\(^{80}\) Certain structural features of orbital fibers also facilitate eyeball acceleration. The doubling of oSIF diameter at the EPZ would enhance fiber force and relaxation effectiveness 4-fold. Orbital fibers are more elastic than global fibers due to structural specializations in the M-band of their sarcomeres.\(^{90}\)

Rapid relaxation of oMIFs may also be promoted by the expression of a special isoform of the essential myosin light chain or MLC1, which interacts with actin during the crossbridge cycle. Canine oMIFs express isoform MLC1\(_{c/e}\) which is found in embryonic skeletal muscle and cardiac atrium, whereas gMIFs express MLC1\(_{a}\) found in adult slow skeletal fibers. The MLC1\(_{c/e}\) isoform has a weaker interaction with actin compared with MLC1\(_{a}\), and thus is associated with increased speeds of contraction, relaxation,\(^{92,93}\) and cross-bridge cycling.\(^{94}\) Rabbit EOM may similarly express MLC1\(_{c/e}\) in oMIFs but MLC1\(_{a}\) in gMIFs, promoting faster cross-bridge detachment in oMIFs.

Towards the latter part of the saccade, eye movement decelerates, even though active breaking by the development of antagonist force does not occur.\(^{43,37}\) We propose that deceleration is promoted by the graded slow relaxation rates of the end segments of oSIFs and oMIFs, which present gradually decreasing viscous loads to the agonist. To be effective, the relaxation rate of the slowest end segments should be fast enough to match the duration of the saccade. The expression of SERCA1\(^{51}\) and the probable expression of MLC1\(_{c/e}\) \(^{91}\) and slow B MyHC in oMIFs would help to ensure this. The time course of relaxation of cat slow-switch motor unit after tetanus lasts approximately 70 msec\(^{62}\); this appears reasonable for breaking saccades that, in monkeys, may last for 80 msec.\(^{34}\)

**Role of Global MIFs in Fixation and Saccades**

Global MIFs are included in the slow fibers innervated by tonic motoneurons with low thresholds and low-position sensitivities.\(^{86,87}\) It is thus likely that they are recruited along with orbital fibers during fixation and inhibited in the antagonist during a saccade. These fibers express \(\alpha\)-cardiac and \(\beta\)-slow MyHCs at their central segment (Figs. 6C, 6D), confirming an earlier report,\(^{37}\) and are thus slower than the central segment of voMIFs, which express \(\alpha\)-cardiac MyHC only. We show further that they express \(\alpha\)-cardiac MyHC (Figs. 2C, 2G, 5C, 5H), and probably also \(\beta\)-Slow MyHC, with little or no slow-tonic and embryonic MyHCs for a considerable distance on either side of the endplate region (Figs. 2, 5), and that most fibers at both end segments of gMIFs coexpress \(\alpha\)-cardiac, slow-tonic and embryonic MyHCs, with or without \(\beta\)-slow MyHC (Figs. 1, 6). Thus, gMIFs may generally also have a fast central segment flanked by slower segments, and thus may contribute to the smoothing of the saccade. The gMIFs in rabbit and rat do not express SERCA1\(^{51}\) and in the dog they express the slow MLC1\(_{c/e}\) rather than MLC1\(_{c/e}\).\(^{91}\) The end segments of gMIFs are expected to be slower than the corresponding segments of voMIFs. Mechanical responses of global slow-tonic units in the cat relax over a time course lasting approximately 150 msec,\(^{95}\) which would be well beyond the end of most saccades. These units would thus provide antagonist EOMs with some muscle tone even at the end of the saccade.

**Role of Orbital Fibers Following the Saccade**

Orbital fibers also play significant roles following the saccade. At the end of the saccade, the antagonist is stretched to a new eye position, and will only reactivate units with low thresholds and low position sensitivities, being largely orbital fibers and gMIFs. They will generate the slowly rising phase of the postsaccadic antagonist muscle tension,\(^{87}\) which is balanced by the postsaccadic agonist tension with a matching slow time course to maintain fixation. It has been shown that the motoneuron slide signal is a discharge characteristic that controls muscle tension during the postsaccadic phase, and that tonic motoneurons innervating MIFs have the most prominent slide signals.\(^{89}\) This ensures a slowly decaying agonist tension to match the slow time course of postsaccadic antagonist tension.

**Orbital Fibers and the Active Pulley Hypothesis**

Miller\(^{7}\) critically reviewed the various versions of the theory of pulleys in ocular motility. According to the dominant coordinated pulley hypothesis, pulleys move longitudinally with respect to their EOMs while elastically resisting transverse movement. During the saccade, the agonist must rapidly move its pulley backward, while the antagonist must allow its pulley to move forward rapidly. As discussed above, both orbital and global layer fibers can move pulley as well as eyeball. The contraction of agonist fibers against the elastic load of its pulley attachments to the orbit promotes its backward translocation. The biphasic contractile characteristics of orbital fibers facilitates this pulley movement, the central segments contract first, enhancing the rate of rise of tension as the rest of the fibers contract, as explained above. The participation of some global fibers that insert onto the pulley may also enable the pulleys to move more rapidly than by orbital fibers alone. Forward translocation of the antagonist pulley is effected by the elastic recoil of the orbital attachments stretching the biphasically relaxing fibers holding the pulley in position before the saccade. This allows motor units recruited at the end of the saccade to hold the pulley in a position appropriate for the new fixation point.

In summary, our investigation revealed that all orbital fibers have fast MyHCs in their central segments and slower ones in their end segments. Structural and functional features affecting the speeds of \(\mathrm{Ca}^{2+}\) release and reuptake, and thus their speeds of contraction and relaxation match the segmental differences in speed due to MyHC variations. In view of the presence of bilayer units and lateral force transmission, EOM behaves as a network whereby motor units moving the pulleys may move the eyeball and vice versa. We propose that during a saccade, the biphasic relaxation rates of antagonist orbital fibers linearize ocular rotation by facilitating initial eyeball acceleration and its deceleration toward the end of the saccade.
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