# Human Extraocular Muscles in ALS

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**Purpose.** To investigate the general morphology, fiber type content, and myosin heavy chain (MyHC) composition of extraocular muscles (EOMs) from postmortem donors with amyotrophic lateral sclerosis (ALS) and to evaluate whether EOMs are affected or truly spared in this disease.

**Methods.** EOM and limb muscle samples obtained at autopsy from ALS donors and EOM samples from four control donors were processed for immunohistochemistry with monoclonal antibodies against distinct MyHC isoforms and analyzed by SDS-PAGE. In addition, hematoxylin and eosin staining and nicotinamide tetrazolium reductase (NADH-TR) activity were studied.

**Results.** Wide heterogeneity was observed in the appearance of the different EOMs from each single donor and between donors, irrespective of ALS type or onset. Pathologic morphologic findings in ALS EOMs included presence of atrophic and hypertrophic fibers, either clustered in groups or scattered; increased amounts of connective tissue; and areas of fatty replacement. The population of fibers stained with anti-MyHCslow tonic was smaller than that of MyHCIpositive fibers and was mostly located in the orbital layer in most of the ALS EOM samples, whereas an identical staining pattern for both fiber populations was observed in the control specimens. MyHCembryonic was notably absent from the ALS EOMs.

**Conclusions.** The EOMs showed signs of involvement with altered fiber type composition, contractile protein content, and cellular architecture. However, when compared to the limb muscles, the EOMs were remarkably preserved. EOMs are a useful model for the study of the pathophysiology of ALS. (*Invest Ophthalmol Vis Sci.* 2010;51:3494–3501) DOI:10.1167/iovs.09-5030

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative syndrome affecting primarily the upper and lower motor neurons resulting in progressive muscle paresis, fasciculation, and muscle atrophy. Generalized paresis and inevitable death due to respiratory failure occur usually within 3 to 5 years.[1,2] ALS is sporadic (SALS) in 90% of cases and is of the familial type (FALS) in the remaining cases. The neurodegenerative process has been proposed to be due to several different factors, including glutamate excitotoxicity, impaired axonal transport, mitochondrial abnormalities, and intraneuronal aggregations.[3] Mutations in seven genes have been identified as causing ALS; the most common are mutations in the ubiquitously expressed gene superoxide dismutase type 1 (*SOD1*) that are present in 12% to 23% of FALS cases and 1% to 7% of SALS cases. The collective evidence suggests that mutated *SOD1* causes FALS by a gain of cytotoxic function and not by reduced SOD1 activity.[4–7] Since 1993, more than 120 missense mutations have been reported in the *CuZn-SOD1* gene on chromosome 21. The most common mutation is D90A, which has been found in almost all countries studied.[8]

The clinical manifestations of ALS suggest the involvement not only of motor neurons but also of other neuronal populations and their supporting cells at many levels of the nervous system. Bulbar onset of ALS is seen in approximately one fourth of all patients[1] and includes such symptoms as dysphagia, dysarthria, and/or dysphonia.[9–11] Histopathologic changes in ALS skeletal muscles encompass the presence of atrophic fiber groups separated by groups of preserved muscle fibers, increased subsarcolemmal nuclei, connective tissue proliferation, and fatty replacement.[10] Signs of focally diminished mitochondrial oxidation activity[12] and ultrastructural changes[13] have also been reported in ALS muscles.

Although all skeletal muscles, including the cranially innervated ones responsible for speech and swallowing, are affected in ALS, oculomotor disturbances are not typical features of the disease. However, there are reports of ALS patients who display ocular motility dysfunction,[14–18] some with the coexistence of Parkinsonism.[19] In particular, ALS patients with long-term respiratory support may develop oculomotor disturbances, including ophthalmoparesis.[20] Pathologic changes in ocular motor nuclei have rarely been observed in ALS patients at autopsy.[10,21] Neuronal loss and gliosis in the oculomotor, trochlear, and abducens nuclei were observed in one ALS patient with ophthalmoplegia.[22]

To the best of our knowledge, no studies investigating the extraocular muscles (EOMs) at the cellular and molecular level in ALS patients have been reported so far. The EOMs are considered a unique muscle class, intrinsically distinct from the limb and masticatory muscles.[23,24] Each EOM consists of two layers: a central global layer (GL) facing the globe and a thin orbital layer (OL) facing the walls of the orbit.[25] The heterogeneity in the contractile properties of muscle fibers is predominantly determined by differences in myosin heavy chain (MyHC) expression (reviewed in Ref. 26). The fiber type composition and MyHC content of human EOMs are remarkably complex and include fibers that coexpress several MyHC isoforms.[27,28] Three main fiber types, based on their MyHC content have been described in human EOMs; fast fibers containing MyHCIIa, slow fibers containing MyHCI, and fibers lacking either MyHCIIa or MyHCI but containing MyHC extraocular (MyHCeom). Fibers containing MyHCI often coexpress MyHCslow tonic, and a subgroup of these fibers also express MyHC$\alpha$ cardiac. Several fibers of all three groups contain embryonic MyHC (MyHCemb), and they are more abundant in the OL.[27]

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**TABLE 1.** Characteristics of ALS Subjects and Muscles Examined in Each Subject

| Donor | Age at Death (y) | Sex | Symptomatic Disease Duration (mo) | Diagnosis | Site of 1st Symptom | SOD1 Genotype | Muscles Examined | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | EOM | Limb |
| 1 | 80 | Male | 31 | SALS | Right hand | wt/wt | RS, RL (4), RM, OS | Vastus (2) |
| 2 | 75 | Male | 317 | FALS | Left leg | D90A/D90A | RS, RL (3), RM (2) | Tibialis anterior |
| 3 | 64 | Female | 132 | FALS | Left leg | D90A/D90A | RS, RL (2), RM | Vastus |
| 4 | 80 | Female | 12 | SALS | Pharynx | wt/wt | RL (3), RM, RS (3) | Vastus |
| 5 | 66 | Male | 13 | SALS | Pharynx | wt/wt | RL (4), OS (3) | Biceps |
| 6 | 70 | Male | 144 | FALS | Legs | D90A/D90A | RS, OS, RL | Biceps |
| 7 | 58 | Female | 50 | SALS | Pharynx | wt/wt | RS (2), RL, OS, RM (2) | Vastus |
| 8 | 71 | Male | 21 | SALS | Thorax | wt/wt | RL, RM, OS | Biceps |

The inferior oblique and the inferior rectus muscles were not available due to autopsy procedures. wt, wild type phenotype. The middle part of each EOM listed was examined. In some cases, the distal and proximal parts of the muscle or the middle part of the contralateral muscle were also examined; the total number of samples studied is given in parentheses. A total of 43 EOM samples were investigated. RS, rectus superior; RL, rectus lateralis; RM, rectus medialis; OS, obliquus superior.

The EOMs exhibit distinctive behavior in disease. They are selectively involved in some forms of mitochondrial myopathy as well as in myasthenia gravis and Miller-Fisher syndrome, but are preferentially spared in Duchenne muscular dystrophy and merosin-deficient muscular dystrophy, which affect all other muscles in the body.[29]

We used different morphologic and biochemical methods to investigate whether the EOMs are affected or truly spared in ALS. Our results indicated that the EOMs are not completely spared in ALS, although they are rather well preserved.

## METHODS

### Muscle Specimens

A total of 43 EOM samples were collected at autopsy from eight donors who had a history of clinically and histopathologically diagnosed ALS, with no reported ocular motility abnormalities on clinical neurologic examination (Table 1). Samples from the biceps brachii, vastus lateralis, and tibialis anterior were also collected from both ALS and control donors. Five EOM samples from three aged-matched donors (a female of 72 years and males of 82 and 86 years) and a 26-year-old female with no previously known neuromuscular disease were taken at autopsy and used as control specimens. The study followed the recommendations of the Swedish Transplantation Law, was approved by the Medical Ethical Committee Umeå University, and adhered to the tenets of the Declaration of Helsinki. The muscle samples were divided into three pieces (proximal, middle, distal), mounted on cardboard, immediately frozen in propane chilled with liquid nitrogen, and stored at −80°C until needed.

### Immunohistochemistry

Series of 5-$\mu$m-thick cross-sections were cut from the limb muscle samples and from the middle portion of each EOM sample in a cryostat (Reichert-Jung, Vienna, Austria). Additional serial cross-sections were cut from the distal and proximal parts of some EOM samples. The sections were processed for immunohistochemistry[30] with well-characterized monoclonal antibodies against distinct MyHC isoforms: A4.74 against MyHCIIa[31,32]; A4.951 against MyHCI[33,34]; N2.261 against MyHCI, IIa, extraocular, and $\alpha$-cardiac, herein referred to as anti-MyHCI+IIa+eom[32,33] (all from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); ALD 19 against $MyHC_{slow\ tonic}$[35,36] (gift from Donald A. Fishman, Cornell University, New York, NY); and 2B6 against MyHC embryonic[31,36,37] (gift from Alan Kelly, University of Pennsylvania, Philadelphia, PA). Double-staining with either anti-MyHCI or anti-MyHCIIa and a polyclonal antibody (PAb) against laminin (PC 128; The Binding Site, Birmingham, UK; labels the basal lamina and thereby delineates the muscle fiber contours) was used for measurement of fiber area.

### Histochemistry

Additional 8-$\mu$m-thick cryostat sections were stained for hematoxylin and eosin and nicotinamide tetrazolium reductase (NADH-TR).[38]

### Morphometric Analysis

The muscle sections were viewed and photographed with a microscope (Eclipse, E800; Nikon, Tokyo, Japan) equipped with a digital camera (Spot RT color; Diagnostic Instruments, Sterling Heights, MI). After the overall staining pattern of each section was studied, representative areas of the OL and GL were analyzed in further detail.

In sections double-stained with either anti-MyHCI or anti-MyHCIIa and PAb against laminin, the fibers were typed into three groups (MyHCIpositive, MyHCIIapositive, and MyHCeompositive/MyHCIIanegative/MyHCInegative), and the circumference of each fiber was traced along the periphery of the basement membrane for measurement of the muscle fiber area with image-analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). Care was taken to ensure that the areas chosen were correctly cross-sectioned, and obliquely sectioned fibers were excluded. The fiber area was determined in 25 areas with a clear presence of atrophic and/or hypertrophic fibers in the GL and 13 areas in the OL of six of the ALS samples. In the same samples, the fiber area was measured in 16 additional areas (8 in the OL and 8 in the GL) that were apparently more normal. Twelve areas in the GL and 12 in the OL of the four control specimens were measured. A total of 4047 fibers, 3021 from ALS and 1026 from control EOMs, were measured.

### Statistical Analysis

Data are presented as the mean ± SD. The variability in fiber area (expressed as a percentage) was estimated for each fiber type as the coefficient of variation (CV), according to the formula CV = (SD/mean fiber area) × 100%. A Mann-Whitney U test was used for statistical evaluation of differences in fiber area and statistical analyses, and measurements and graphs were generated (Statview 4.5; SAS Institute Inc., Cary, NC). The significance level chosen was $\alpha = 0.05$.

### Biochemistry

Thick cryostat sections (30–50 $\mu$m) were lysed in Laemmli sample buffer with 5% $\beta$-mercaptoethanol. SDS-PAGE was performed on a gel electrophoresis unit (Hoefer SE-600; Amersham Biosciences, Uppsala, Sweden) at 4°C for 46 hours, at 100 V for the first 3 hours and at 230 V for the remaining time.[39] The gels were subsequently silver stained (Silver Stain Plus; Bio-Rad, Sundbyberg, Sweden) and photographed.

## RESULTS

### General Muscle Pathology

**Limb Muscles.** Typical pathologic changes were seen in all ALS limb muscle samples (Fig. 1A) and included the presence

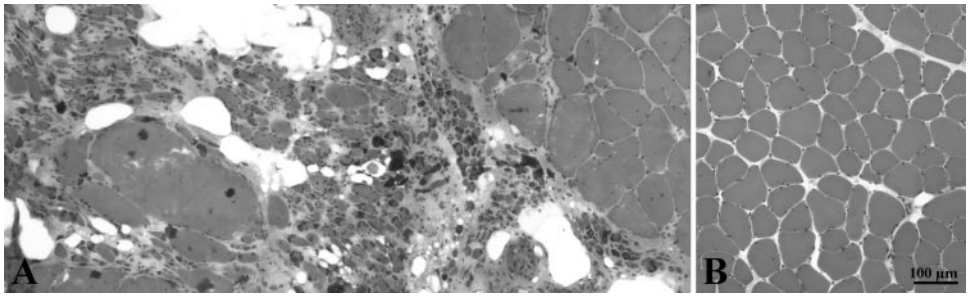


**FIGURE 1.** Cross-sections of tibialis anterior muscles from FALS donor 2 (**A**) and a normal control donor (**B**) stained with hematoxylin and eosin. Note the pathologic changes including fiber atrophy and hypertrophy, fiber splitting, internal nuclei, increased connective tissue, and areas of fatty replacement in the ALS limb muscle (**A**).

of both extremely large and very small fibers, as well as signs of fiber grouping, fiber splitting, necrosis, and regeneration. Increased connective tissue, areas of fatty replacement, and increased cellularity in the connective tissue septa were also noted. Collectively, these findings confirm those of previous studies and the ALS diagnosis in the donors.

**Extraocular Muscles.** Compared to the limb muscle samples, the cytoarchitecture of most of the ALS EOM samples were well preserved. Most of the muscle fibers in the EOMs were small and round, typically loosely arranged in fascicles (Fig. 2). The organization of the muscle fibers into an OL and a GL was generally preserved and identifiable. Larger variation in fiber size than that in the age-matched normal EOMs (Fig. 2A) was noted in 18 of 43 ALS EOMs, and it was present in all donors. Clearly enlarged fibers were either scattered or clustered in groups in these samples (Figs. 2B, 2E, 2G). The paucity of muscle fibers and increased amounts of connective tissue separating the individual muscle fibers and/or some of the muscle fascicles in parts of the EOMs were apparent in 20 ALS samples (Figs. 2E, 2F). In the rectus lateralis (RL) sample from one of the oldest SALS cases (donor 4), there was extensive replacement of muscle fibers by connective tissue (Fig. 2F) whereas the rectus medialis (RM) sample from the same eye of this donor (Fig. 2C) was much less affected. Minor areas of fatty replacement were present in 11 ALS samples from seven donors (Fig. 2G, arrows). In the RL of the male donor with FALS, there was a paucity of muscle fibers in the GL, and the space between muscle fibers and fascicles was remarkably rich in nerve trunks of various sizes, to an extent not seen in normal EOMs of any age (Fig. 2H). Central and/or internal nuclei were noted in less than 1% of fibers in all ALS samples. Cellular reaction, seen as an increased number of nuclei not belonging to muscle fibers, was present in small areas of 3 of the 43 samples (not shown).

## Mitochondrial NADH-TR Activity

The level and pattern of NADH-TR activity varied considerably among the ALS EOM specimens (Fig. 2). The NADH-TR activity tended to be higher in fibers of the OL, and it varied from high to low in GL fibers, resembling the pattern in the normal EOMs. The enlarged muscle fibers in ALS specimens showed low to high levels of NADH activity (Figs. 2B, 2C, 2E), and the smallest fibers had high activity (Figs. 2B, 2D).

## MyHC Composition

SDS-PAGE of whole EOM extracts revealed variation in the relative amounts of the different MyHC isoforms in the ALS samples (Fig. 3).

The control EOMs of older donors displayed similar staining patterns with the antibodies against the different MyHC isoforms as those previously described for the human EOMs.[27] There was wide heterogeneity in the appearance of the different EOMs from each single donor and between donors, irrespective of type of disease onset or whether it was SALS or FALS (Fig. 4). The staining patterns observed with antibodies against MyHCIIa and MyHCI in the ALS EOMs generally resembled those in the control EOMs. In most of the ALS EOM samples, the number of MyHCslow tonic–positive fibers was smaller than that of MyHCIpositive fibers in the same area (Figs. 5A–F), whereas in EOMs from normal elderly donors, practically all fibers that were MyHCIpositive were also MyHC-

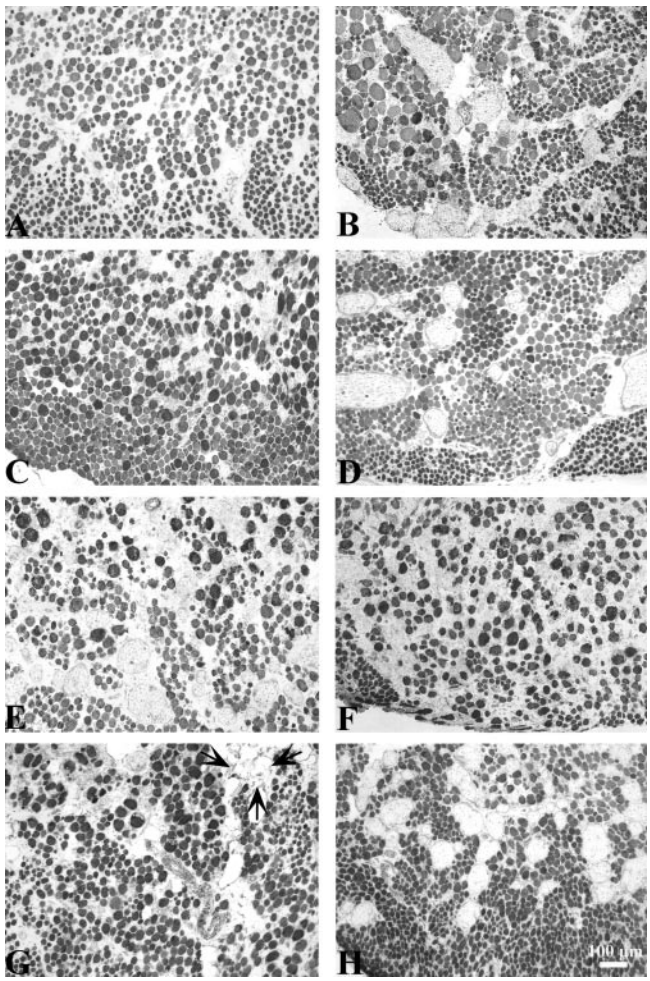


**FIGURE 2.** Cross-sections showing the morphology and NADH activity in a control EOM sample (**A**) and in different ALS EOM samples (**B**: FALS donor 3, RL; **C**, **E**: SALS donor 4, RM; **D**: FALS donor 2, RM; **F**: SALS donor 4, RL; **G**: SALS donor 5, obliquus superior; and **H**: FALS donor 2, RL). The GL is shown in the upper part and the OL in the lower part of the photographs in (**A**), (**B**), (**C**), (**D**), (**F**), and (**H**). (**E**, **G**) Only the GL is shown. (**B**, **E**, **G**) The variation in fiber diameter is shown with increased connective tissue (**E**, **F**). Area with fatty replacement and (**H**) area filled with nerve trunks. There was a wide variation in the level and pattern of NADH activity in ALS EOMs compared with that in the control EOMs. The NADH activity in larger fibers ranged from high to low (**B**, **C**, **E**).

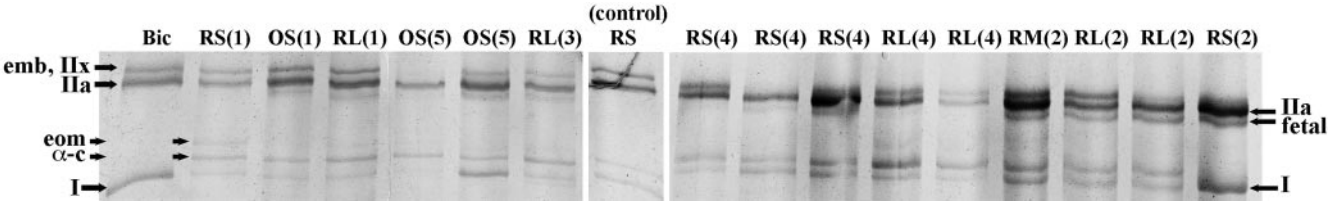


**FIGURE 3.** SDS-PAGE of whole muscle extracts from five ALS donors and biceps (Bic) and rectus superior (RS) from control donors. The extracts from the biceps revealed only three bands: MyHCI (the fastest migrating band), MyHCIIa, and MyHCIIx. Whole EOM extracts from ALS and control donors revealed MyHCI (fastest migrating), MyHC$\alpha$-c, MyHCeom, MyHCfetal, MyHCIIa, MyHCemb, and MyHCIIx. MyHCslow and MyHCIIa were detected in all muscle specimens. The relative amounts of different myosin forms differed markedly from donor to donor and among EOMs. OS, obliquus superior. The numbers in parentheses refer to the donor numbers in Table 1.

slow tonic–positive (Figs. 5G–L). The MyHCslow tonic–positive fibers were mostly located in the OL, whereas MyHCIpositive fibers were more evenly distributed in both the OL and GL (Figs. 5A, 5D).

Strikingly, most of the fibers were unstained by anti-MyHC-emb in practically all ALS muscle samples. Very sporadic fibers (<20 muscle fibers per whole-muscle cross-section examined) that were only weakly stained with anti-MyHCemb were present in the OL of the ALS samples, in clear contrast to the normal pattern in the controls (Fig. 4).

### Variability in Muscle Fiber Area

Fiber area in clearly affected parts of the ALS EOMs varied markedly (Figs. 2B, 2E, 2G). Variation in fiber area was reflected in the higher coefficient of variation (CV) obtained both for the fibers containing MyHCI in affected areas of the OL and the fibers in affected parts of the GL of the ALS EOMs (Table 2). Fiber areas measured in apparently more normal parts of the same samples were smaller than those in the control specimens, both in the OL and the GL. The wide variation in fiber size distribution in clearly affected parts the RL of FALS donor 3 is illustrated in Fig 6.

## DISCUSSION

The present study showed that the EOMs were not completely spared in terminal-phase ALS patients, irrespective of ALS type and site of symptomatic onset. However, when compared to the limb muscles, the EOMs were remarkably well preserved. The major pathologic findings in the ALS EOMs were (1) altered cellular architecture, showing hypertrophic and atrophic fibers, increased connective tissue, and areas with fatty replacement, and (2) altered myosin heavy chain content of

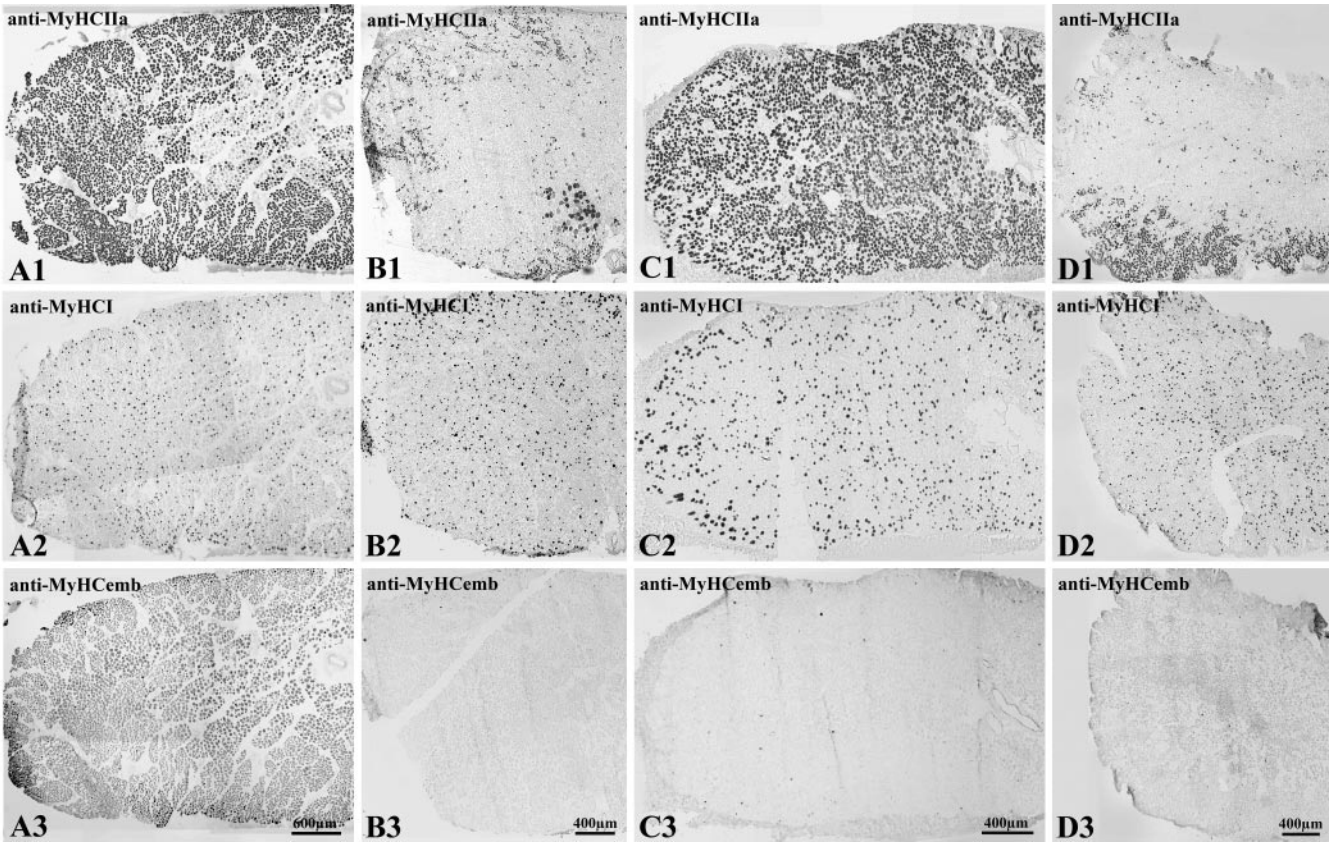


**FIGURE 4.** Cross-sections from normal EOMs (**A**) and ALS EOMs samples (**B**, FALS donor 3, rectus superior; **C**, SALS donor 4, RL; and **D**, FALS donor 2, RL), treated with antibodies against MyHCIIa, MyHCI, and MyHCemb. Note the wide heterogeneity in the appearance of the different ALS EOMs. Only very sporadic fibers were weakly stained with anti-MyHCemb in ALS muscle samples (**B3–D3**).

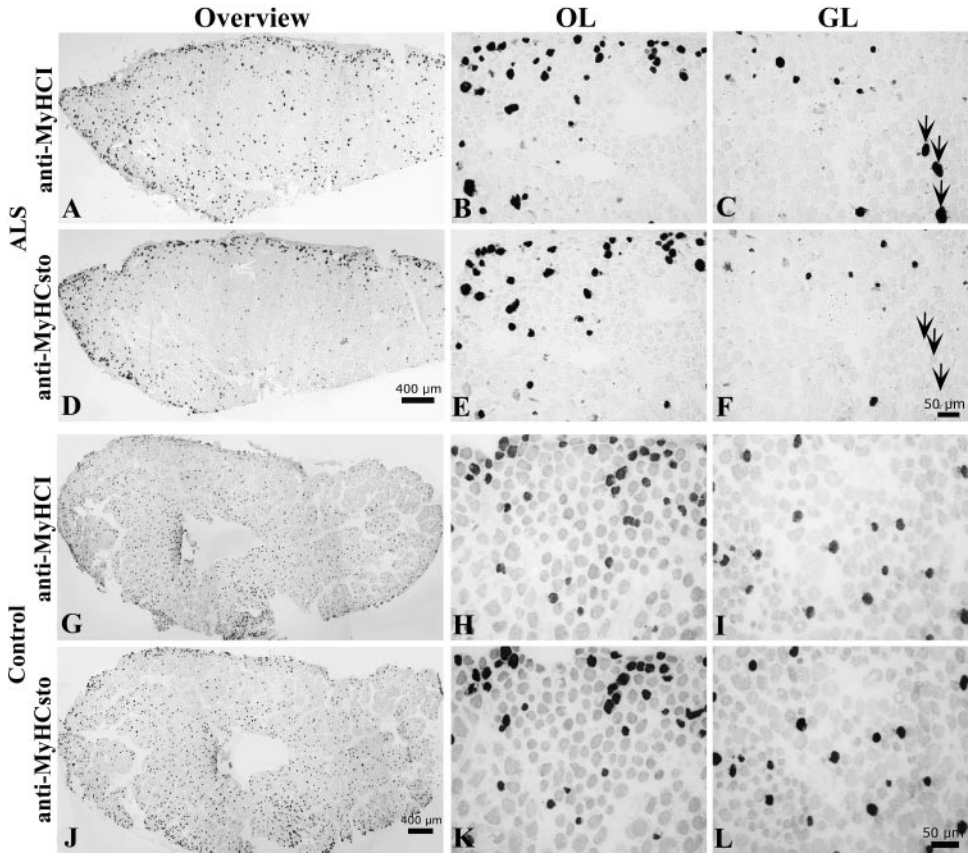


**FIGURE 5.** EOM cross-sections from SALS donor 1, obliquus superior, (**A**–**F**) and a normal donor (**G**–**L**) treated with antibodies against MyHCI and MyHC-slow tonic. The population of fibers stained with anti-MyHCslow tonic was smaller than that of MyHCIpositive fibers in ALS EOM. *Arrows*: fibers labeled with anti-MyHCI but not with anti-MyHCslow tonic in the GL of the ALS case (**C**, **F**). In the normal donor, the fibers labeled with anti-MyHCI were also labeled with MyHCslow tonic.

the EOM fibers, with a lesser population of fibers containing MyHCslow tonic and a general absence of MyHCemb.

Muscle fiber size is determined by innervation, trophic and growth factors, and workload.[40] In adult limb muscle, denervation leads to massive tissue atrophy, with loss of muscle mass up to 80% and atrophy of the individual muscle fibers.[41,42] Cell death has also been shown to be an important contributor to postdenervation muscle atrophy.[43] The EOMs differ fundamentally from limb muscle, and they may therefore be expected to behave differently on denervation. In fact, denervation of primate EOMs leads to no apparent waste of the muscles and no alteration in fiber size or fiber type distribution at 3 months, but at longer time intervals grouping of small slow fibers is present.[44] In another set of oculomotorius nerve section experiments in primates,[45] no gross muscle atrophy or significant fiber loss was reported. The singly innervated fibers in the OL show the most pronounced response to denervation, with atrophy to 54% of normal cross-sectional area and, after reinnervation, hypertrophy to 175% of control fiber area values. The OL multiply innervated fibers also show transient atrophy followed by an earlier recovery, whereas the GL singly innervated fibers are less affected and the GL multiply innervated

**TABLE 2.** Muscle Fiber Area of Fibers Expressing Different MyHC Isoforms

| | OL | | | | | | GL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ALS* | | ALS† | | Control | | ALS* | | ALS† | | Control | |
| | Mean Area | CV (%) | Mean Area | CV (%) | Mean Area | CV (%) | Mean Area | CV (%) | Mean Area | CV (%) | Mean Area | CV (%) |
| MyHCcom$^{pos}$/MyHClla$^{neg}$/ MyHCl$^{neg}$ | 395 ± 198 (160–1246) | 50 | 288 ± 126 (46–613) | 44 | 541 ± 255 (139–1031) | 47 | 592 ± 384 (31–2705) | 65 | 329 ± 131 (47–779) | 40 | 565 ± 254 (36–1315) | 45 |
| MyHClla$^{pos}$ | 220 ± 89 (24–1001) | 40 | 163 ± 56 (40–476) | 34 | 234 ± 105 (59–969) | 45 | 451 ± 498 (30–2739) | 110 | 153 ± 71 (41–536) | 46 | 426 ± 213 (55–1333) | 50 |
| MyHCl$^{pos}$ | 186 ± 123 (25–576) | 66 | 126 ± 73 (24–314) | 58 | 203 ± 110 (63–665) | 54 | 147 ± 131 (17–891) | 89 | 138 ± 84 (19–345) | 60 | 236 ± 173 (36–875) | 73 |
| Mean | 239 ± 15 $n = 5$ | 48 | 169 ± 76 $n = 5$ | 45 | 357 ± 169 $n = 4$ | 38 | 442 ± 96 $n = 6$ | 73 | 227 ± 135 $n = 5$ | 59 | 465 ± 52 $n = 4$ | 65 |

Muscle fiber area data are expressed in square micrometers ± SD (range). $n$ = number of subjects. The variability in fiber area is expressed as the coefficient of variation (CV). A CV higher than 25% in limb muscles suggests abnormal variability in fiber size[41]; a corresponding value for normal human EOM has not been presented earlier.

* Clearly affected areas (including both atrophic and hypotrophic fibers).

† Apparently less affected areas in the same samples.

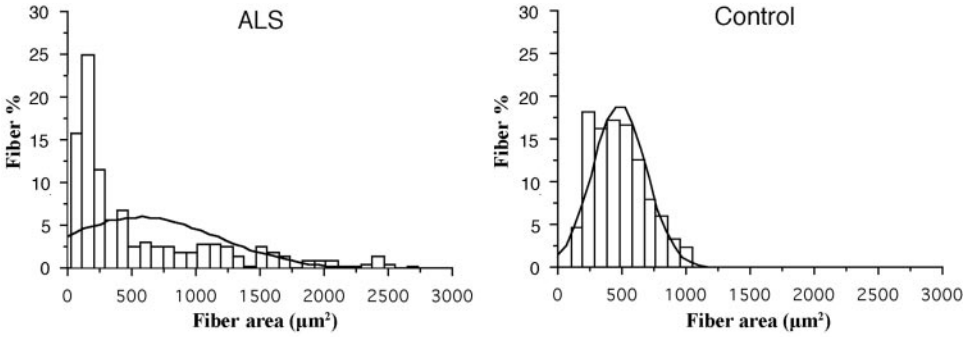


**FIGURE 6.** Fiber area distribution in a clearly affected part of the rectus superior sample from FALS donor 3 and a control subject. Note the large variability in fiber area in the ALS specimen compared with that in the control, indicating the presence of both atrophic and very hypertrophic muscle fibers. The table presents the mean fiber area of fibers expressing different MyHC isoforms. Data are expressed as the mean ± SD.

| | ALS | Control | p value |
|---|---|---|---|
| MyHCeom$^{pos}$/MyHCIIa$^{neg}$/MyHCI$^{neg}$ | 682±569 | 601±195 | N.s. |
| MyHCIIa$^{pos}$ | 622±662 | 471±195 | p=0.005 |
| MyHCI$^{pos}$ | 150±135 | 331±171 | p=<0.001 |
| All fibers | 571±608 | 484±209 | p=0.004 |

fibers remain unaffected.[45] Botulinum toxin–induced paralysis of primate EOMs causes hypertrophy of the OL singly innervated fibers (25% increase in mean fiber cross-sectional area) in the acute phase followed by a 24% decrease in cross-sectional area when compared to normal fibers in the long term, with recovery of neuromuscular activity, whereas the other fibers are apparently unaffected.[46] Although these experiments involving nerve section, nerve crush or botulinum toxin paralysis are not suitable models of ALS, they are a source of information on the response of the EOMs to denervation and reinnervation. In contrast to the primate nerve section experiments, both OL and GL fibers were clearly affected in our study, and there was both general atrophy of all fiber types in both layers in parts of the muscles that were perceived as less affected and atrophy of MyHCI-containing fibers and hypertrophy of fibers containing MyHCIIa in areas of the muscles that were perceived as more severely affected.

In the GL, there was loss of normal expression of MyHC-slow tonic in the slow fiber population. In all ALS EOMs, MyHCemb was not detected in the OL, a feature normally seen in the adult EOMs and certainly present in the old control specimens. It is difficult to speculate on the functional implications of loss of MyHCemb or MyHCslow tonic. However, the latter can be taken as a marker of multiple innervation, and its absence from a subgroup of the fibers may reflect changes in innervation pattern (i.e., reinnervation by a different type of motor neuron or loss of multiple innervations). In fact, on denervation, multiply innervated rat EOM fibers acquire twitch properties and are capable of generating action potentials.[47]

The differences in MyHC composition in the whole-muscle extract (Fig. 3) among the ALS EOMs probably reflect heterogeneity in the degree of disease involvement, as well as individual variation.[27]

Grouping of small slow fibers was observed in the primate EOMs by Durston[44] 2 years after nerve section, whereas no fiber grouping was noted by Porter at al.[45] In our specimens, there was little evidence of fiber grouping, but as shown in Figure 4B1, there were sometimes clusters of very hypertrophic fibers containing MyHCIIa, seen over a restricted area, suggesting the possibility of shared reinnervation. Fiber type composition in the human EOMs is more complex than that reported for other species, and classifications based on fiber size and NADH activity are not adequate to classify the human EOM fibers.[27] In the present study, extensive fiber grouping would have been inadequately diagnosed if the fiber size and NADH activity were major criteria for fiber type classification (e.g., Figs. 2B–H).

With the exceptions listed herein regarding MyHCemb and MyHCslow tonic, the fiber type composition of the ALS EOMS was rather normal, which may reflect (1) only mild denervation/reinnervation, (2) successful reinnervation, or (3) capacity of the muscle fibers in the EOMs to retain their MyHC composition despite denervation. All these three alternatives are possible. Extraocular motor neurons and their nuclei appear to be only mildly affected in ALS[21] and differences in calcium-binding protein levels (parvalbumin and calbindin-$D_{28K}$) have been reported in ocular motor neurons and may contribute to their relative sparing in ALS.[48] The EOMs are richly innervated, and theoretically there is a plethora of neighboring axons that could be rapidly attracted to the newly denervated fibers, resulting in successful reinnervation. Finally, there is evidence that the EOM fibers are capable of retaining their MyHC phenotype, even when transplanted to the bed of a limb muscle supplied by a limb nerve.[24]

A major feature of the ALS EOMs was an increased amount of connective tissue, due in part to an apparent loss of fibers and also to fiber atrophy (Table 2). The increase in connective tissue occurred around the individual muscle fibers and generally over larger areas or the whole EOM (Figs. 2E, 2F) whereas in ALS limb samples, the increase in connective tissue was more typically seen in focal areas and in association with impressive fiber atrophy and regeneration (Fig 1A). After denervation, primate EOMs do not show an increase in connective tissue,[44,45] further indicating that there are significant differences between whole-nerve-section experiments and ALS and possibly also differences between species.

Areas of fatty replacement were present in both EOM and limb samples of ALS, although they were far more prominent in the limb muscle samples. The EOMs are very richly innervated, and they are known to have the smallest motor units in the body, with an average of 10 fibers per motoneuron.[29] Therefore, small nerve trunks are usually abundantly present in normal EOMs, as was also the case in the ALS specimens. In the particular case depicted in Figure 2H, there was an abnormal clustering of nerve trunks in the GL in a fashion not seen otherwise. This specimen had a clear paucity of muscle fibers in the GL and the clustering was therefore interpreted as likely to reflect a complete preservation of nerve trunks, at least in the sense of their perineurium being prominent.

Profusion of internal nuclei is suggestive of regeneration and is commonly seen in ALS limb samples. However, there was no increase in the number of internal nuclei in the ALS EOMs. The EOMs normally have an ongoing renewal process whereby nuclei are incorporated into the muscle fibers, and there is a significant population of activated satellite cells in EOMs under normal conditions.[49] In future studies we will evaluate and quantify the satellite cells in ALS EOMs.

The most striking property of EOMs is their unique behavior in disease. We have shown that the EOMs were rather spared, although not completely, in our ALS specimens. The EOMs are classified as a distinct muscle allotype because of their intrinsically different properties that are distinct from those of the jaw and limb muscles.[23,24] Intrinsic EOM-specific differences (e.g., in target-derived neurotrophins), may be responsible for the different susceptibility of the EOMs and their motor neurons to ALS. Recent studies in human ALS and animal models of SOD1-mediated ALS strongly suggest that motor neuron degeneration progresses in a dying-back pattern, from the motor endplate and progressing toward the central nervous system.[50] Furthermore, the EOMs appear to be the key source of brain-derived neurotrophic factor transported retrogradely to the oculomotor neurons during development.[51] Similarly, GDNF (glial cell line-derived neurotrophic factor), another target-derived potent survival factor for motorneurons, is expressed in higher levels in the extraocular muscles than in glial cells.[52] Studies in an ALS mouse model showed a neuroprotective effect of muscle-derived GDNF in contrast to centrally delivered GDNF.[53] Taken together, these data suggest that trophic factors derived from the target muscles may be crucial in the sparing of the oculomotor neurons. The EOMs are therefore a promising model for further study of the pathophysiology of ALS and, reciprocally, further knowledge on the intrinsic properties of the EOMs can be gained from ALS cases and animal models.

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## *References*

1. Haverkamp LJ, Appel V, Appel SH. Natural history of amyotrophic lateral sclerosis in a database population: validation of a scoring system and a model for survival prediction. *Brain.* 1995;118:707–719.
2. Turner MR, Bakker M, Sham P, Shaw CE, Leigh PN, Al-Chalabi A. Prognostic modelling of therapeutic interventions in amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2002;3:15–21.
3. Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron.* 2006;52:39–59.
4. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science.* 1994;264:1772–1775.
5. Wong PC, Pardo CA, Borchelt DR, et al. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron.* 1995;14:1105–1116.
6. Andersen PM, Nilsson P, Ala-Hurula V, et al. Amyotrophic lateral sclerosis associated with homozygosity for an Asp90Ala mutation in CuZn-superoxide dismutase. *Nat Genet.* 1995;10:61–66.
7. Reaume AG, Elliott JL, Hoffman EK, et al. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet.* 1996;13:43–47.
8. Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep.* 2006;6:37–46.
9. Van der Graaff M. Amyotrophic lateral sclerosis. *Orphanet Encyclopedia.* September 2004.
10. Lawyer T Jr, Netsky MG. Amyotrophic lateral sclerosis. *Arch Neurol Psychiatry.* 1953;69:171–192.
11. Mitchell JD, Borasio GD. Amyotrophic lateral sclerosis. *Lancet.* 2007;369:2031–2041.
12. Krasnianski A, Deschauer M, Neudecker S, et al. Mitochondrial changes in skeletal muscle in amyotrophic lateral sclerosis and other neurogenic atrophies. *Brain.* 2005;128:1870–1876.
13. Yoshihara T, Ishii T, Iwata M, Nomoto M. Ultrastructural and histochemical study of the motor end plates of the intrinsic laryngeal muscles in amyotrophic lateral sclerosis. *Ultrastruct Pathol.* 1998;22:121–126.
14. Esteban A, De Andres C, Gimenez-Roldan S. Abnormalities of Bell's phenomenon in amyotrophic lateral sclerosis: a clinical and electrophysiological evaluation. *J Neurol Neurosurg Psychiatry.* 1978;41:690–698.
15. Jacobs L, Bozian D, Heffner RR Jr, Barron SA. An eye movement disorder in amyotrophic lateral sclerosis. *Neurology.* 1981;31:1282–1287.
16. Leveille A, Kiernan J, Goodwin JA, Antel J. Eye movements in amyotrophic lateral sclerosis. *Arch Neurol.* 1982;39:684–686.
17. Averbuch-Heller L, Helmchen C, Horn AK, Leigh RJ, Buttner-Ennerver JA. Slow vertical saccades in motor neuron disease: correlation of structure and function. *Ann Neurol.* 1998;44:641–648.
18. Palmowski A, Jost WH, Prudlo J, et al. Eye movement in amyotrophic lateral sclerosis: a longitudinal study. *Ger J Ophthalmol.* 1995;4:355–362.
19. Gizzi M, DiRocco A, Sivak M, Cohen B. Ocular motor function in motor neuron disease. *Neurology.* 1992;42:1037–1046.
20. Hayashi H, Kato S, Kawada T, Tsubaki T. Amyotrophic lateral sclerosis: oculomotor function in patients in respirators. *Neurology.* 1987;37:1431–1432.
21. Okamoto K, Hirai S, Amari M, et al. Oculomotor nuclear pathology in amyotrophic lateral sclerosis. *Acta Neuropathol.* 1993;85:458–462.
22. Harvey DG, Torack RM, Rosenbaum HE. Amyotrophic lateral sclerosis with ophthalmoplegia: a clinicopathologic study. *Arch Neurol.* 1979;36:615–617.
23. Porter JD, Khanna S, Kaminski HJ, et al. Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc Nat Acad Sci U S A.* 2001;98:12062–12067.
24. Hoh JF, Hughes S. Myogenic and neurogenic regulation of myosin gene expression in cat jaw-closing muscles regenerating in fast and slow limb muscle beds. *J Muscle Res Cell Motil.* 1988;9:59–72.
25. Mayr R. Structure and distribution of fibre types in the external eye muscles of the rat. *Tissue Cell.* 1971;3:433–462.
26. Bottinelli R. Functional heterogeneity of mammalian single muscle fibres: do myosin isoforms tell the whole story? *Pflugers Arch.* 2001;443:6–17.
27. Kjellgren D, Thornell LE, Andersen J, Pedrosa-Domellöf F. Myosin heavy chain isoforms in human extraocular muscles. *Invest Ophthalmol Vis Sci.* 2003;44:1419–1425.
28. Stirn Kranjc B, Smerdu V, Erzen I. Histochemical and immunohistochemical profile of human and rat ocular medial rectus muscles. *Graefes Arch Clin Exp Ophthalmol.* 2009;247:1505–1515.
29. Sadeh M. Extraocular muscle. In: Engel AG, Franzini-Armstrong C, ed. *Myology*. 3rd ed. McGraw-Hill; 2004;119–127.
30. Pedrosa-Domellöf F, Holmgren Y, Lucas CA, Hoh JF, Thornell LE. Human extraocular muscles: unique pattern of myosin heavy chain expression during myotube formation. *Invest Ophthalmol Vis Sci.* 2000;41:1608–1616.
31. Silberstein L, Webster SG, Travis M, Blau HM. Developmental progression of myosin gene expression in cultured muscle cells. *Cell.* 1986;46:1075–1081.
32. Liu JX, Eriksson PO, Thornell LE, Pedrosa-Domellöf F. Myosin heavy chain composition of muscle spindles in human biceps brachii. *J Histochem Cytochem.* 2002;50:171–183.
33. Hughes SM, Cho M, Karsch-Mizrachi I, et al. Three slow myosin heavy chains sequentially expressed in developing mammalian skeletal muscle. *Dev Biol.* 1993;158:183–199.

34. Cho M, Webster SG, Blau HM. Evidence for myoblast-extrinsic regulation of slow myosin heavy chain expression during muscle fiber formation in embryonic development. *J Cell Biol.* 1993;121:795–810.
35. Sawchak JA, Leung B, Shafiq SA. Characterization of a monoclonal antibody to myosin specific for mammalian and human type II muscle fibers. *J Neurol Sci.* 1985;69:247–254.
36. Pedrosa-Domellöf F, Thornell LE. Expression of myosin heavy chain isoforms in developing human muscle spindles. *J Histochem Cytochem.* 1994;42:77–88.
37. Gambke B, Rubinstein NA. A monoclonal antibody to the embryonic myosin heavy chain of rat skeletal muscle. *J Biol Chem.* 1984;259:12092–12100.
38. Dubowitz V SC. *Muscle Biopsy: A Practical Approach*. 3rd ed. London: Saunders 2007.
39. Toniolo L, Maccatrozzo L, Patruno M, Caliaro F, Mascarello F, Reggiani C. Expression of eight distinct MHC isoforms in bovine striated muscles: evidence for MHC-2B presence only in extraocular muscles. *J Exp Biol.* 2005;208:4243–4253.
40. Pette D, Staron RS. Transitions of muscle fiber phenotypic profiles. *Histochem Cell Biol.* 2001;115:359–372.
41. Gutmann E, Zelena J. Morphological changes. In: Gutmann E, ed. *The Denervated Muscle*. Prague: Publishing House of the Czechoslovak Academy of Sciences; 1962;57–75.
42. Hnik P. Rate of denervation muscle atrophy. In: Gutmann E, ed. *The Denervated Muscle*. Prague: Publishing House of the Czechoslovak Academy of Sciences; 1962;341–371.
43. Borisov AB, Carlson BM. Cell death in denervated skeletal muscle is distinct from classical apoptosis. *Anat Rec.* 2000;258:305–318.
44. Durston JH. Histochemistry of primate extraocular muscles and the changes of denervation. *Br J Ophthalmol.* 1974;58:193–216.
45. Porter JD, Burns LA, McMahon EJ. Denervation of primate extraocular muscle: a unique pattern of structural alterations. *Invest Ophthalmol Vis Sci.* 1989;30:1894–1908.
46. Spencer RF, McNeer KW. Botulinum toxin paralysis of adult monkey extraocular muscle. Structural alterations in orbital, singly innervated muscle fibers. *Arch Ophthalmol.* 1987;105:1703–1711.
47. Bondi AY, Chiarandini DJ, Jacoby J. Induction of action potentials by denervation of tonic fibres in rat extraocular muscles. *J Physiol.* 1986;374:165–178.
48. Appel SH, Beers D, Siklos L, Engelhardt JI, Mosier DR. Calcium: the Darth Vader of ALS. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 2001;2(suppl 1):S47–S54.
49. McLoon LK, Wirtschafter J. Activated satellite cells in extraocular muscles of normal adult monkeys and humans. *Invest Ophthalmol Vis Sci.* 2003;44:1927–1932.
50. Fischer LR, Culver DG, Tennant P, et al. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol.* 2004;185:232–240.
51. Steljes TP, Kinoshita Y, Wheeler EF, Oppenheim RW, von Bartheld CS. Neurotrophic factor regulation of developing avian oculomotor neurons: differential effects of BDNF and GDNF. *J Neurobiol.* 1999;41:295–315.
52. Chen J, Butowt R, Rind HB, von Bartheld CS. GDNF increases the survival of developing oculomotor neurons through a target-derived mechanism. *Mol Cell Neurosci.* 2003;24:41–56.
53. Li W, Brakefield D, Pan Y, Hunter D, Myckatyn TM, Parsadanian A. Muscle-derived but not centrally derived transgene GDNF is neuroprotective in G93A-SOD1 mouse model of ALS. *Exp Neurol.* 2007;203:457–471.