Human extraocular muscles (EOMs) have a very complex architecture, muscle fiber composition, and innervation patterns that are thought to reflect their highly specialized function in the execution of the different types of eye movements. The fibers of EOMs are organized into two separate layers: a thin orbital layer facing the orbital wall and a global layer comprising the remaining central part of the muscle. Among the special features of EOMs is the presence of multiply innervated fibers (MIFs), which are not normally found in adult mammalian skeletal muscles, in addition to singly innervated fibers (SIFs). MIFs display multiple small en grappe motor endplates generally closely located in a row, in contrast to the typical, large, single en plaque endings found on the SIFs. En grappe motor endplates exist only in muscle fibers containing myosin heavy chain slow-tonic (MyHCsto) isoform and exhibit tonic mode of contraction. MIFs in the orbital layer generally have several en grappe motor endplates along the fiber length, and a single en plaque motor endplate in the middle portion. Approximately 15% to 20% of fibers are MIFs whereas the remaining are SIFs, innervated by a single large en plaque motor endplate in the middle portion of each muscle fiber. SIFs typically contain MyHC fast Ila (MyHCIIa) and are regarded as fast myofibers or lack both MyHCIIa and MyHC slow (MyHCl) but contain MyHC extraocular (MyHCeom), an isoform almost exclusive to the EOMs, and are therefore classified as MyHCeom fibers. Another striking feature of the EOMs is that their myofibers show unusual content of acetylcholine receptor (AChR) subunits. AChRs are highly concentrated in the postsynaptic region of neuromuscular junctions (NMJs), and are pentameric proteins existing in two isoforms in mammalian NMJs: the fetal isoform δβ and the adult isoform δβ. In limb muscle, the fetal γ subunit is expressed in AChR only during developmental stages or in denervated skeletal muscles. In contrast, the EOMs are the only adult muscles that normally express both the fetal (δβ) and the adult (δβ) subunits of AChR. RT-PCR and immunohistochemical data indicate that en plaque endings of SIFs express the adult ε but not the fetal γ AChR subunit whereas en grappe endings of MIFs fibers express the fetal γ but not the adult ε AChR subunit in the EOMs of rats and mice, respectively. However, more complex results have also been reported in another immunohistochemical study of the rat EOMs, and it remains to be determined whether such direct correlation between innervation pattern, AChR subunits, and fiber types is true for the human EOMs. The present study aimed to systematically investigate the type of motor endplate of the different fiber types and to examine the distribution of adult ε and fetal γ subunits of AChR in human adult EOMs. In addition to the previously described types of motor endplates, we report the wide occurrence of a novel type of multiterminal innervation in MyHCeom fibers and a very complex AChR subunit composition in the human EOMs.

A Novel Type of Multiterminal Motor Endplate in Human Extraocular Muscles

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PURPOSE. To investigate the relation between type of motor endplate, acetylcholine receptor (AChR) subunit composition, and fiber types in human extraocular muscles (EOMs).

METHODS. EOM samples collected from subjects aged 54 to 82 years were serially sectioned and processed for immunohistochemistry, with specific antibodies against different myosin heavy chain (MyHC) isoforms, neurofilament, synaptophysin, and adult epsilon (ε) and fetal gamma (γ) AChR subunits as well as α-bungarotoxin.

RESULTS. A novel type of motor endplate consisting of large, multiterminal en plaque endings was found in human EOMs, in addition to the previously well-described single en plaque and multiple en grappe endplates. Such novel endplates were abundant but exclusively observed in myofibers lacking MyHC slow and fast Ila but containing MyHC extraocular (MyHCeom), isoforms. Multiple en grappe endings were found only in myofibers containing MyHC slow-tonic isoform and contained fetal γ AChR subunit. Adult ε and fetal γ AChR subunits, alone or combined, were found in the multiterminal endplates. Distinct AChR subunits were present in adjacent motor endplates of a given myofiber containing MyHCeom.

CONCLUSIONS. Human EOMs have a more complex innervation pattern than previously described, comprising also a novel type of multiterminal motor endplate present in myofibers containing MyHCeom. The heterogeneity in AChR subunit composition in a given myofiber suggests the possible presence of polynervous innervation in human EOMs.

Keywords: extraocular muscles, motor endplate, multiterminal motor endplate, en grappe, en plaque, polynervous innervation, myosin heavy chain, acetylcholine receptor subunit
**Materials and Methods**

**Human Muscle Samples**

A total of 23 EOM samples (8 superior rectus, 7 medial rectus, 4 lateral rectus, 3 inferior obliques, and 1 unspecified EOM) were collected at autopsy from six adult (34–59 years old, males, 14 specimens) and five elderly (72–82 years old, 3 males and 2 females) subjects. None of the subjects was known to suffer from neuromuscular disease. In addition, a vastus lateralis muscle sample from a 23-year-old male and the tibialis anterior muscle from a fetus collected at 20 weeks of gestation were also used to test the specificities of the AChR antibodies. The muscle samples were collected with the approval of the Regional Ethical Review Board in Umeå, Sweden, in adherence to the recommendations of the Declaration of Helsinki.

The muscle samples were mounted on cardboard with optimal cutting temperature (OCT) Cryomount (Histolab Products AB, Västra Frölunda, Sweden), rapidly frozen in propane chilled with liquid nitrogen, and stored at −80°C until sectioned. Serial sections, 5-μm thickness, comprising the whole muscle cross section or serial longitudinal sections comprising the whole muscle thickness and part of the whole muscle length, were obtained at −23°C using a Reichert Jung cryostat (Leica, Heidelberg, Germany).

**Antibodies and Immunofluorescence**

Motor endplates were detected either by rhodamine-conjugated α-bungarotoxin (α-BTx) labeling (Molecular Probes, Inc., Eugene, OR, USA) or by a mixture of monoclonal antibodies (mAbs) against neurofilament protein (clone NR4; 70kD; Dako, Glostrup, Denmark) and against synaptophysin (SY38; Boehringer Mannheim Biochemica, Indianapolis, IN, USA). The use of a mixture of antibodies against neurofilament protein and synaptophysin allows the simultaneous visualization of the axon and the motor endplates using a single fluorochrome. A rat mAb against the ε AChR subunit15,16 (mAb168; purchased from Socrates J. Tzartos, University of Patras, Rio, Greece) and a mouse mAb against the γ AChR subunit16 (GTX74890; Gentex, Landskorna, Sweden) were used to detect the adult ε AChR and the fetal γ AChR subunits, respectively. In addition, rabbit polyclonal antibody MYH14/7b against MyHCsto isoform17 (gift from Stefano Schiaffino, CNR Institute of Neuroscience, Padova, Italy), mAbs A4,951 and BA-D5 against MyHC1 isoform,18 A4,74 against MyHC1la isoform,19 and N2.261 against MyHC1, MyHC1la, and MyHCem18,19 were used to distinguish different types of muscle fibers (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA). Because each myofiber and its motor endplates can be reliably studied only on a single longitudinal section, given the very small diameter of the myofibers in the EOMs, double or triple immunofluorescent labeling was carried out: AChR subunits (ε or γ) + MyHC isoforms (MyHC1, MyHC1la, or MyHCcom) + α-BTx, AChR subunits (ε or γ) + neurofilament mixed with synaptophysin + MyHC isoform; adult ε AChR subunit + fetal γ AChR subunit + MyHC isoform.

Immunohistochemistry was performed on air-dried tissue sections, as previously described.1 In brief, the tissue sections were air-dried, rehydrated in 0.01 M PBS, and then blocked with 5% donkey serum for 15 minutes. Sections were then incubated with the first primary antibody (AChR subunit) at +4°C overnight. All antibodies were diluted in 0.01 M PBS containing 0.1% bovine serum albumin and used at their optimal dilutions. The next day, after washing in PBS and an additional blocking with 5% donkey serum for 15 minutes, sections were incubated for 30 minutes at 37°C with appropriate secondary antibody. Thereafter, the second primary antibody (the other AChR subunit or neurofilament + synaptophysin) was applied (37°C for 60 minutes), followed by incubation with appropriate secondary antibodies for 30 minutes at 37°C. Subsequently, immunolabeling for the third primary antibody against MyHCs was performed, followed by incubation with the appropriate secondary antibodies for 30 minutes at 37°C. Detailed information on the secondary antibodies used to detect each primary antibody is provided in Supplementary Table S1. Control sections were treated as above, except that the primary antibodies were excluded. No staining was observed in control sections.

**Microscopy and Motor Endplate Quantification**

The sections were examined and photographed with a Spot camera (RT KE slider; Diagnostic Instruments, Inc., Sterling Heights, MI, USA) connected to a Nikon microscope (Eclipse, E800; Tokyo, Japan). The images were processed using the Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA, USA).

The samples were evaluated with respect to motor endplates, fiber types, and AChR subunits. For quantification of labeled motor endplates, double-labeled sections combining α-BTx or neurofilament and synaptophysin (NF+Syn) with one of the antibodies against AChR subunits (α-BTx+γ, α-BTx+ε, NF+Syn+γ, NF+Syn+ε) were evaluated. In addition, triple-labeled sections combining antibodies against fetal γ AChR subunit, adult εAChR subunit, and one of the MyHC isoforms were also evaluated. The total area of each muscle section was examined, and every motor endplate identified with α-BTx or NF+Syn was counted and evaluated as either positive or negative for the antibodies used, carefully taking into account the background staining level and excluding lipofuscin dots present under both green and red filters. The same criteria were used for the antibodies against MyHC isoforms and AChR subunits. In addition, care was taken to properly identify muscle spindles in cross sections and palisade endings as well as myomys junctions in longitudinal sections, to avoid any possible misinterpretation of the results. A total of 768 myofibers, in both cross and longitudinal sections, of all EOMs having motor endplate(s) were typed and evaluated for AChR subunit composition.

When necessary, following microscopic evaluation and photographing, a sequential immunolabeling procedure with an additional MyHC antibody was performed in order to allow typing of all myofibers. For example, a muscle section that had been immunolabeled with antibodies against adult ε AChR subunit (fluorescein isothiocyanate [FITC], green), against fetal γ AChR subunit (Rhodamine Red-X, IgG, red), and against MyHC1la (Alexa Fluor 647) was, after evaluation and photographing, additionally incubated with the antibody against MyHC1 (Alexa Fluor 594, IgG2b, red). Thereby we could examine the section and photograph it again and classify the myofibers into those containing MyHC1 or MyHCII or, in case they were unlabeled by both antibodies, myofibers containing MyHCcom.

**Results**

**Selective Antibody Specificity Against Fetal γ and Adult ε AChR Subunit Epitopes**

The specificity of the two antibodies against fetal γ and adult ε AChR subunits in human tissue was tested on a tibialis anterior muscle from a fetus at 20 weeks of gestation and a sample from adult vastus lateralis muscle (Fig. 1). MAb GTX74890, raised
against AChR γ subunit, immunolabeled all the NMJs detected with z-BTx in fetal limb muscle (Figs. 1A–C) whereas immunoreactivity with this antibody was not found in NMJs of human adult vastus muscle (Figs. 1G–I), indicating that GTX74890 specifically recognizes the fetal γ AChR subunit also in human muscle tissue. In contrast, antibody mAb 168, raised against e AChR subunit, labeled only a few NMJs of limb muscles at 20 weeks gestation very weakly (Figs. 1D–F) but strongly labeled all the NMJs of adult limb muscles (Figs. 1J–L), confirming its specificity to the adult ε AChR subunit in human muscle tissue.

Muscle Fiber Types in Human EOMs

Three groups of muscle fibers exhibiting different staining patterns with the antibodies against MyHC isoforms were identified with immunohistochemistry in the human EOMs, as previously described7,9: (1) muscle fibers strongly labeled with the antibody against MyHCsto and in the vast majority, also strongly labeled with the antibody against MyHCIIa and therefore classified as muscle fibers containing MyHCsto/I, as previously9; (2) muscle fibers strongly or moderately labeled with the antibody against MyHCIIa and hereinafter referred to as muscle fibers containing MyHCIIa; (3) muscle fibers unlabeled with all of the antibodies above but moderately labeled with antibody N2.26, which recognizes MyHCIIa+MyHCIIb+MyHCcom,7,9 therefore referred to as muscle fibers containing MyHCcom.

Three Major Types of Motor Endplates in the Human EOMs

The well-known single large en plaque and small multiple en grappe motor endplates were readily identified in the muscle fibers of the human EOMs. In addition, a novel type of motor endplate was detected, mostly in the myofibers of the global layer. We list all three major types of motor endplates below, together with a schematic illustration (Fig. 2) and relation to AChR subunits and muscle fiber types (Figs. 3–6; Table).

Single En Plaque Motor Endplates. Single large en plaque motor endplates, the typical endplates of SIFs, were readily detected with z-BTx or antibodies against neurofilament and synaptophysin in all the above-mentioned three groups of muscle fibers of the human EOMs (myofibers containing MyHCsto/I, myofibers containing MyHCIIa, and myofibers containing MyHCcom), in both orbital and global layers (Fig. 2A).

The single en plaque motor endplates in muscle fibers containing MyHCIIa were always labeled with the mAb against adult ε AChR subunit but were generally unlabeled with the mAb against fetal γ AChR subunit (Figs. 3A–D). The number of myofibers containing MyHCIIa varied widely between specimens, but generally, NMJs were less frequently found on this fiber type. In muscle fibers containing MyHCIIa and/or MyHCsto/I in the orbital layer, en plaque motor endplates were in most cases labeled with antibodies against fetal γ AChR subunit (Figs. 3I–L) but motor endplates labeled with both fetal γ and adult ε AChR subunits were also encountered (Figs. 3E–H; Table). The AChR composition of myofibers containing MyHCsto/I varied between EOMs from the same subject and between subjects, but we could not find any clear relation between fiber location and AChR subunit composition.

Typical En Grappe Motor Endplates. Typical en grappe motor endplates were readily identified in longitudinal sections of the human EOMs (Figs. 2B, 2C, 4). These multiple small en grappe endplates were exclusively seen in myofibers containing MyHCsto/I, in both the orbital and global layers (Fig. 4). En grappe motor endplates were typically present along a line, only on one side of the longitudinally cut muscle fibers (Figs. 2B, 2C, 4A–C). When it was possible to track nerve terminals, it was clear that these multiple small en grappe endings...
originated from one common axon (Fig. 2C). Thus, we confirmed that these were MIFs.

The multiple en grappe motor endplates were labeled with mAb against fetal αAChR subunit (Figs. 4A, 4C, 4F, 4G) but were not labeled with mAb against adult εAChR subunit (Figs. 4E, 4G).

In the most distal part of the EOMs, palisade endings were found in the global layer in the vicinity of myotendinous junctions and only in muscle fibers containing MyHCsto. The axons supplying the palisade endings ran closely along either side of the myofibers or intertwined with the myofibers. In a subset of MyHCsto fibers receiving palisade endings, at least two or more small motor endplates labeled with the antibody against γAChR subunit were found (not shown).

Novel Multiterminal En Plaque Motor Endplates. In longitudinal sections, a novel type of motor endplates consisting of several large en plaque endplates was observed in the myofibers containing MyHCom isoform in both global and orbital layers, but mostly in the global layer, irrespective of the age of the subject or EOM examined (Figs. 2D, 5). These large multiterminal en plaque motor endplates were readily found along myofiber segments that were up to ~520 μm long (Figs. 2D, 5B, 5I, 6B). Approximately 77 ± 11% of the endplates were rather shallow, in contrast to the deep junctional folds seen in typical single en plaque endplates. The length of each motor endplate was approximately 15 to 40 μm and the distance between adjacent endplates varied between 25 and 240 μm. These multiterminal en plaque motor endplates were clearly distinct from the multiple en grappe endings which were mostly aligned on the same side of the muscle fiber, some of these multiterminal large en plaque motor endplates were present on opposite sides of the longitudinally cut muscle fibers (Figs. 2D, 5B). In addition, the diameter of MyHCom fibers possessing the multiterminal en plaque endings was always larger than that of MyHCsto/I ones possessing the multiple en grappe endings, in agreement with our previous findings that the size of MyHCom fibers was largest whereas that of MyHCsto/I fibers was smallest (590 ± 210 vs. 320 ± 190 μm², respectively).

In the present study, approximately 63 ± 10% of the myofibers containing MyHCom and displaying motor endplates on the sections analyzed had multiterminal en plaque
**FIGURE 3.** AChR subunit composition in single en plaque motor endplate of myofibers containing MyHCIIa (A–D) and MyHCI (E–L) in cross-sectioned (A–H) or longitudinally sectioned (I–L) samples. Adult ε AChR subunit was present (A, C, FITC, green) whereas fetal γ AChR subunit was absent (B, C, Rhodamine Red-X, red) in myofibers containing MyHCIIa (D, Alexa Fluor 647, gray).Copresence of adult ε (E, G, arrow; FITC, green) and fetal γ (F, G, arrow; Rhodamine Red-X, red) AChR subunits was found in motor endplates of myofibers containing MyHCsto/I (H, Alexa Fluor 594, red). Presence of fetal γ AChR subunit (J, arrow; Rhodamine Red-X, red) but absence of adult ε AChR subunit (I, arrow; FITC, green) was also found on motor endplates of MyHCsto/I myofiber (L, Alexa Fluor 647, gray).

**FIGURE 4.** AChR subunit composition in multiple en grappe motor endplates of longitudinally cut myofibers containing MyHCsto. Fetal γ AChR subunit (A, C, F, G, arrows; Alexa Fluor 488, green) was present on all en grappe motor endplates labeled with neurofilament and synaptophysin (NF+Syn; B, C, Rhodamine Red-X, red) in myofibers containing MyHCsto (D, H, Alexa Fluor 594, red). In contrast, the adult ε AChR subunit (E, G, FITC, green) was absent.
**Figure 5.** AChR subunit composition in multiple large en plaque motor endplate of myofibers containing MyHCeom in longitudinally (A–C, K–M) or transversely (D–J) cut EOMs. Note the three combinations of AChR subunit composition of motor endplates. (A–C) Adult ε AChR subunit (A, arrows; FITC, green) was absent but fetal γ AChR (B, arrows; Rhodamine Red-X, red) was present on the multiterminal en plaque endings. (D–J) Copresence of adult ε (D, E, arrowheads; FITC, green; α-BTx, red) and fetal γ AChR subunits (E, G, arrowheads; Alexa Fluor 488, green; α-BTx, red) on motor endings of myofibers lacking MyHC (H, Alexa Fluor 488, green) and MyHCIIa (I, Alexa Fluor 488, green) but labeled with the antibody against MyHCII-Ha+eom (J, Alexa Fluor 488, green), that is, a myofiber containing MyHCeom. (K–M) Two adjacent en plaque motor endplates in a given myofiber showed distinct AChR subunits. The left endplate contained solely adult ε AChR subunit (K, M, FITC, green; marked with single asterisk) whereas the endplate to the right contained solely fetal γ AChR subunit (L, M, Rhodamine Red-X, red; marked with two asterisks).
motor endings, whereas the remaining MyHCcom fibers had single en plaque endings. It should be noted that this percentage was evaluated on longitudinally sectioned specimens where longer and shorter myofiber segments were present, but an analysis of the full length of each myofiber is never possible. In other words, we could not determine whether those MyHCcom myofibers displaying a single endplate in the segment analyzed had additional motor endplates along the rest of their length, and therefore the percentage above may be underestimated. Furthermore, the percentage of MyHCcom myofibers varied within different portions of EOMs and between EOMs from different subjects, but we could not detect any clear-cut differences between different EOMs in terms of incidence and distribution of this novel multiterminal en plaque motor endplate.

Three different patterns regarding AChR subunit composition were noted for the multiterminal large en plaque motor endplates (Fig. 5; Table): (1) fetal $\gamma$ AChR subunit alone (Figs. 5A–C); (2) coexpression of adult $\varepsilon$ and fetal $\gamma$ AChR subunits (Figs. 5D–G); and (3) less frequently, adult $\varepsilon$ AChR subunit alone (Figs. 5K–M). In a number of myofibers containing MyHCcom, the AChR subunit composition varied from one endplate to another, along the length of a single myofiber; that is, one en plaque endplate contained fetal $\gamma$ AChR subunit solely whereas another contained adult $\varepsilon$ AChR subunit only (Figs. 5K–M) or contained both adult $\varepsilon$ and fetal $\gamma$ AChR subunits.

In addition, sporadic MyHCcom myofibers displaying a combination of multiterminal large en plaque and multiple small en grappe motor endplates were observed, displaying at least two small en grappe endplates along the fiber segments examined (Fig. 6). The multiterminal en plaque endplates were labeled with mAb against fetal $\gamma$ AChR subunit (Figs. 6A–C) and in some cases there was coexistence of both adult $\varepsilon$ and fetal $\gamma$ AChR subunits in the same endplate. The small en grappe endplates were generally labeled with mAb against fetal $\gamma$ AChR subunit (Figs. 6B, 6E) and either labeled (Figs. 6A, 6D) or unlabeled with mAb against adult $\varepsilon$ AChR subunit.

Finally, sporadic myofibers containing MyHCsto/I or MyHCcom displayed several small “grape-like” endplates on either side of the muscle fiber (Fig. 7), as previously described in the anterior part of the inferior oblique muscle of children undergoing strabismus surgery and two young adults. These clustered small grape-like endplates were labeled with the mAb against adult $\varepsilon$ AChR subunit (Fig. 7), and we could not determine whether they contained fetal $\gamma$ AChR subunit, as only very sporadic myofibers with this type of motor endings were found.

**TABLE. Relation Between Myofiber Type, AChR Subunit Composition, and Motor Endplate Types in Human EOMs**

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>SIF/MIF</th>
<th>AChR Subunit</th>
<th>Type of Motor Endplate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyHCIIa</td>
<td>SIF</td>
<td>$\varepsilon$, $\gamma$</td>
<td>Large en plaque</td>
</tr>
<tr>
<td>MyHCsto/I</td>
<td>*</td>
<td>$\varepsilon$, $\gamma$</td>
<td>Large en plaque</td>
</tr>
<tr>
<td>MyHCsto/I</td>
<td>MIF</td>
<td>$\varepsilon$, $\gamma$</td>
<td>Multiple small en grappe</td>
</tr>
<tr>
<td>MyHCcom</td>
<td>MIF</td>
<td>$\varepsilon$, $\gamma$</td>
<td>Multiterminal large en plaque</td>
</tr>
</tbody>
</table>

* We were unable to determine whether this type of motor ending was present in the myofibers containing MyHCsto/I and having en grappe endings (MIFs) or whether these could be true SIFs.
neurofilament and synaptophysin (NF mAb against adult a longitudinally cut MyHCeom myofiber showing strong labeling with mAb against adult ε AChR subunit (FITC, green)).

FIGURE 7. Several small grape-like endplates (arrows) identified with neurofilament and synaptophysin (NF+Syn, Rhodamine Red-X, red) on a longitudinally cut MyHCeom myofiber showing strong labeling with mAb against adult ε AChR subunit (FITC, green).

described en plaque and en grappe endings. Furthermore, this is, to the best of our knowledge, the first study clearly correlating fiber types, motor endings, and AChR subunit composition in human EOMs (Table). The major findings can be summarized as follows. (1) A novel type of motor endplate consisting of multiterminal en plaque endings was found along the length of myofibers containing MyHCeom, indicating that this is a new category of multiply or polyneuronally innervated myofibers; (2) adult ε and fetal γ AChR subunits were present simultaneously in a subgroup of en plaque endings of myofibers containing either MyHCeom or MyHCsto/I; (3) distinct AChR subunits were present in neighboring en plaque motor endplates of a given myofiber containing MyHCeom; (4) we confirm that typical en grappe motor endings are present only in myofibers containing MyHCsto/I and contain the fetal γ AChR subunit.

Here we report a novel type of multiterminal motor endplate that differed from traditional multiple en grappe endplates in several aspects. First, this novel type of multiterminal motor endplate consisted of several large en plaque endings, with longer length and greater distance between two adjacent endings than the conventional small multiple en grappe motor endplates. Second, they were exclusively observed in myofibers containing MyHCeom whereas conventional en grappe endplates were found only in myofibers containing MyHCsto/I. Third, unlike en grappe motor endplates, which were always lined up in a row on the same side of the longitudinally cut myofibers, several en plaque motor endplates appeared on both sides of a given myofiber. Finally, care was taken to ensure that these novel en plaque endplates were not incorrectly identified palisade endings or muscle spindles and that no myomysoid junctions were present in these muscle fiber segments. The present findings clearly indicate a new group of myofibers with multiterminal innervation and containing MyHCeom isoform in human EOMs. Although the material of the present study did not allow a clear evaluation of whether these multiterminal en plaque motor endplates are supplied by single or multiple axons, and although there is uncertainty regarding the total percentage of myofibers with such multiterminal endings, it is clear that a higher percentage of myofibers in human EOMs than previously recognized do not have single en plaque motor endings. The myofibers containing MyHCeom have previously been reported to account for approximately 25% of the fibers in the global layer, and in the midbipal of the EOMs they are the absolutely most abundant fiber type. In the present study, we estimated that approximately 63% of these myofibers had multiterminal en plaque motor endplates. In addition, approximately 14% to 16% of the fibers in human EOMs have previously been recognized as MIFs, meaning that, altogether, at least one-third of the myofibers in human EOMs are multiply or polyneuronally innervated.

Kjellevold and Bruenech reported the presence of multiple en plaque motor endplates in serial transverse sections of human EOMs and concluded that these were related to aging. In addition, they did not classify the fiber type possessing these multiple en plaque motor endplates. In the present study investigating a very large number of longitudinally cut myofibers, we found multiterminal en plaque motor endplates in the EOMs from both younger and elderly subjects, indicating that aging is not a confounder for the presence of multiterminal en plaque motor endplates but rather that this is a true major type of endplate in myofibers containing MyHCeom. Oda reported two types of multiple motor endplates, Type B and Type C, in the most anterior portion of the inferior oblique muscles from children undergoing strabismus surgery (4–11 years old) and two young adults (17 and 19 years old). Type C is identical to the well-known multiple small en grappe motor endplates, whereas Type B is formed by endplates consisting of several small particles stained by AChE and regularly spaced on muscle fibers. The latter were called "grape-like" endplates and were present in approximately 5% of the myofibers and only in myofibers of intermediate diameter. Altogether, the morphologic appearance of such multiple grape-like endplates and their myofibers was completely different from the novel multiterminal en plaque endplates in the large MyHCeom fibers of the present study. Furthermore, grape-like motor endplates were sporadically found in the present study, in both MyHCsto/I and MyHCeom fibers. The fact that we did not observe such motor endings as often as reported by Oda may reflect differences between EOMs or may be related to strabismus, given that the study by Oda was performed in the most anterior portion of the inferior oblique muscles of patients undergoing strabismus surgery.

In the present study, single en plaque endplates in myofibers containing MyHCIIa generally contained adult ε AChR subunit whereas multiple en grappe endplates in myofibers containing MyHCsto/I contained only fetal γ AChR subunit, as reported in other species. In contrast, single en plaque endplates seen in myofibers containing MyHCsto/I and multiterminal en plaque endplates in myofibers containing MyHCeom showed a complex AChR subunit composition, including fetal γ and adult ε AChR subunit alone, and the coexpression of both subunits. The current results are partially divergent from previous findings on adult EOMs of rats and mice, where the en plaque endplates always have adult ε AChR subunit only. Furthermore, the coexpression of adult ε and fetal γ AChR subunits in small en grappe endplates in rat EOMs was never observed in human EOMs in the current study, suggesting that differences between species or antibodies used may exist.

The distinct AChR subunit composition is likely related to the fiber type, its physiological properties, and motor axon supply. AChRs containing fetal γ subunit differ from those containing adult ε subunit in their ion conductance and ion channel open speed. The opening time of ion channel is reduced from 11 to 6 ms during the switch from fetal γ to adult.
\[ \varepsilon \text{ AChR subunit during limb muscle development.}^{22} \text{ Thus, the present findings that single en plaque endplates of myofibers containing MyHCIIa exclusively contained adult } \varepsilon \text{ AChR subunits whereas multiple en gruppe endplates of myofibers containing MyHCSto/I exclusively expressed fetal } \gamma \text{ AChR subunits, suggests that the differences in contraction speed between these two fiber types. Abnormalities in NMJ size, number, and AChR subunit composition have been previously reported in the EOMs of subjects with infantile nystagmus syndrome.}^{23} \text{ It would be important to study the muscles of such patients in longitudinally cut sections and taking into consideration the new knowledge of an additional type of motor endings and the complex AChR subunit composition of the human EOMs.} \\

Unfortunately, with the freshly frozen muscle samples available, we did not succeed in determining whether the multiterminal en plaque endings were supplied by a single or by several axons, that is, whether these myofibers were multiply or polynervously innervated. In order to assess whether the multiterminal en plaque motor endings are polynervously or multiply innervated, further studies using, for example, silver teasing and/or acetylcholinesterase processed. However, the finding of neighboring en plaque endings with distinct AChR subunit composition on a given myofiber favors the possibility of polynervous innervation. The presence of polynervous innervation has been recognized electrophysiologically in the EOMs of mammals, including cat,24–28 monkey,26,29 and rat.30 Electrophysiological data elegantly show that the sum of twitch and tetanic tensions in response to individual nerve branch stimulation is greater than that when the whole EOM nerve is simultaneously stimulated, indicating the presence of polynervous innervation.27,28 In addition, by using silver teasing and/or acetylcholinesterase staining method, Dietert3 and Oda30 demonstrate multiterminal endplates that are innervated by several different motor axons, indicating the presence of polynervous innervation also in human EOMs. The multiterminal and possible polynervous innervation of EOM myofibers could play a role in protecting the EOMs and maintaining normal eye motility in neuromuscular disease involving loss of motor neurons, for example, amyotrophic lateral sclerosis (ALS). We have previously shown that EOMs are remarkably well preserved in comparison to the severely affected limb muscles from the same ALS patients, although some morphologic alterations were observed.31–34 However, we very recently reported significant loss of myofibers containing MyHCSto/I in both layers of the medial rectus in terminal ALS patients, suggesting that the EOMs as a whole are relatively spared but that the MyHCSto/I fibers, which are multiply innervated from endings arising from the same motor axon, are vulnerable.35 In summary, human EOMs have a more complex innervation pattern with respect to motor endplate number and AChR composition than previously recognized. Further studies are needed to determine the extent of multiple and polynervous innervation and its relation to fiber types, motor endplate morphology, and AChR subunit composition.

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