Palisade Endings of Extraocular Muscles Develop Postnatally Following Different Time Courses

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Purpose. To analyze in a frontal-eyed mammal (cat) the postnatal development of palisade endings in extraocular muscles (EOMs) and to compare the spatiotemporal and quantitative patterns of palisade endings among individual rectus muscles.

Methods. Cats of different ages ranging from birth to adult stage were studied. EOM whole-mount preparations were fluorescently labeled using six combinations of triple staining and analyzed in the confocal laser scanning microscope.

Results. Palisade endings developed postnatally and passed in each rectus muscle through the same, three developmental steps but in a heterochronic sequence and to a different final density per muscle. Specifically, palisade ending development was first completed in the medial rectus and later in the inferior, lateral, and superior rectus. The highest density of palisade endings was observed in the medial rectus and the lowest in the lateral rectus whereas values for the inferior and superior rectus were in between. Palisade endings expressed high levels of growth associated protein 43 during development and were supplied by axons that established motor terminals.

Conclusions. Cats open their eyes 7 to 10 days after birth and later develop a complex three-dimensional visuomotor climbing and jumping behavior depending on accurate binocular vision and fine tuning of the ocular movements. Our findings indicate that palisade ending development correlates with important landmarks in visuomotor behavior and provide support for our previous notion that palisade endings play an important role for convergence eye movements in frontal-eyed species.

Keywords: oculomotor, GAP43, multiply-innervated muscle fibers, singly-innervated muscle fibers, convergence, frontal-eyed animals, extraocular muscles, eye movements

The eyes are one of the most complex organs and allow perceiving objects in terms of shape, color, and detail. Each eye is moved by three pairs of extraocular muscles (EOMs) and knowledge in which direction the eyes are pointing is essential for proper vision. Eye position signals have been found in various brain regions including the primary somatosensory cortex and it has been supposed that they arise from sensory organs (proprioceptors) in EOMs.[1,2] Surprisingly, classical proprioceptors (muscle spindles and Golgi tendon organs) are exceptional in mammalian EOMs and have only been found in even-toed ungulates where they are present in very high numbers.[3] In eye muscles of primates, muscle spindles are present whereas Golgi tendon organs are missing.[4–6] In EOMs of other species like cat, dog, rabbit, guinea pig, rat, and mouse, muscle spindles and Golgi tendon organs are both absent.[3] The reason for these interspecies variations is still unclear and there is no evolutionary and/or functional explanation for their pattern of appearance.

However, mammals possess a unique structure in their EOMs, the so-called "palisade ending" or alternatively the "innervated myotendinous cylinder."[7–9] Palisade endings are regularly present in the EOMs of frontal-eyed species (human, monkey, cat, dog, and ferret) but infrequent in lateral-eyed species (i.e., they are present in ungulates [pig, cow, horse, and sheep] and rabbit), but absent in most rodents.[10–12] Palisade endings are located at the myotendinous junction and formed by nerve fibers that come in from the muscle and extend into the tendon. There, axons make a u-shaped turn, branch, and establish terminal varicosities at the tendon level and around single muscle fiber tips. The muscle fibers associated with palisade endings belong to the inner layer of the EOMs (global layer) and they possess several en grappe terminals along their length and are consequently classified as multiply-innervated muscle fibers (MIFs).[7,9] MIFs exhibit a nontwitch form of contraction and in frontal-eyed species, the cell bodies of MIF motoneurons lie separated in the EOM motor nuclei, although the specific pattern of arrangement is different across species.[13,14]

Their location at the muscle-tendon junction has placed palisade endings for many years as candidates for providing eye position signals.[7,9,11,15] Their sensory role has been put into question when molecular analyses and neuronal tracing studies have demonstrated that palisade endings are cholinergic and originate from the EOM motor nuclei, most likely from MIF motoneurons.[16–20]

Little is known how palisade endings are established during pre- and/or postnatal development and additionally there is discrepancy in two studies on human subjects. Specifically, whereas Bruenech and Ruskell[21] have reported that palisade endings are absent in human infants, Lukas et al.[22] have found them in a 2-year-old child. Here, we chose another frontal-eyed mammal (cat) and performed a comprehensive analysis of the developmental steps of establishing palisade endings from neonate to adult and compared the spatiotemporal and quantitative patterns of palisade endings among individual rectus muscles. We show that palisade endings pass through the same developmental steps but in a heterochronic sequence and to a different final density per muscle. Our findings indicate that the time course of palisade ending development correlates with important landmarks in the development of visuomotor behavior and thus, provide novel developmental support for our previous hypothesis that palisade endings are particularly important for convergence eye movements in frontal-eyed species.

Material and Methods

Animals

Cats of either sex were obtained from our breeding colony at Universidad de Sevilla and also supplied by the animal house of the Universidad de Córdoba (Spain). In the present study, cats of different postnatal (P) ages were analyzed including the day of birth (P0); 2 days after birth (P2); eight days (P8); 22 days (P22); 45 days (P45); 95 days (P95); and adult age of more than 1 year. From each postnatal stage three animals were analyzed with the exception of P95 where two animals were studied. Procedures for experiments followed the guidelines of the National Institute of Health (http:/oacu.od.nih.gov, in the public domain), specific recommendations for maintenance of higher mammals during neuroscience experiments (NIH publication #94–3207,1994), and were in accordance with Spanish legislation for the use and care of laboratory animals (RD 53/2013, BOE 34/11370–421, 2013), and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation

Cats were deeply anesthetized with a terminal dose of sodium pentobarbital (100 mg/kg, intraperitoneally [IP]) and intracardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Then, eyeballs including the EOMs were dissected and postfixed for 2 hours in the same fixative solution. Thereafter, tissue was cryoprotected in 40% sucrose, frozen, and kept at −80°C before further processing. For analysis, EOM whole-mount preparations were used which in contrast to sectioning allow studying the overall morphology of palisade endings. In P0 and P2 kittens, the muscles were small and the complete EOMs, including the distal tendon, were used as whole-mounts. In the rest of the animals, EOMs were large and therefore cut transversally into two pieces: one piece containing the muscle belly and the second containing the distal part of the muscle with the attached tendon. The distal EOM myotendinous pieces were used as whole-mount preparations. EOM whole-mounts were labeled using triple-fluorescent labeling.

Antibodies and Toxins. For labeling of EOM whole-mount preparations, the following antibodies and toxins were used. Antibodies: chicken anti-neurofilament (Cat# AB5539, PRID:AB_177520; 1:2000; Millipore, Billerica, MA, USA); goat anti-choline acetyltransferase (Cat# AB144P, PRID:AB_2079751; 1:100; Millipore); mouse anti-synaptophysin (Cat# MAB329, PRID:AB_95786; 1:300; Millipore); and rabbit anti-growth associated protein 43 (Cat# AB5312, PRID:109488; 1:500; Millipore). Toxins: α-bungarotoxin (Cat# B13422; 1:500; Thermo Fisher Scientific, Waltham, MA, USA) and phalloidin (Cat# 65906; 1:150; Sigma-Aldrich Corp., St. Louis, MO, USA).

Immunofluorescence. Six different combinations of triple fluorescent labeling were used and included labeling with**:** 1) antibodies against neurofilament, synaptophysin, and phalloidin staining; 2**)** antibodies against neurofilament, growth associated protein 43 (GAP43) along with phalloidin; 3) antibodies against GAP43, synaptophysin along with phalloidin; 4) antibody against GAP43, along with α-bungarotoxin and phalloidin stainings; 5) antibody against choline acetyl transferase (ChAT), along with α-bungarotoxin and phalloidin stainings; and 6) antibodies against ChAT, synaptophysin, and phalloidin staining. Staining combination 1 was used to label axons by anti-neurofilament[23] including its nerve terminals by anti-synaptophysin.[24] Staining combinations 2 through 4 were used to label growing axons by GAP43 expression.[25] Axons were counterstained by anti-neurofilament (staining 2); nerve terminals were demonstrated by synaptophysin (staining 3); and nicotinic acetylcholine receptors by α-bungarotoxin (staining 4).[26] Staining combinations 5 and 6 were used to label cholinergic axons by anti-ChAT[27] in addition to acetylcholine receptors (staining 5) and nerve terminals by synaptophysin (staining 6). In all staining combinations, phalloidin was used as a counterstaining for muscle fibers.[28]

Before labeling, frozen muscles were thawed and connective tissue was carefully removed from the surface of the muscles. To facilitate penetration of the antibodies in whole mounts, tissue was frozen once again in −80°C cold isopentane and immediately thawed and kept in PBS containing 1% Triton X-100 (PBS-T) overnight at room temperature. The tissue was blocked for 2 hours in 10% normal goat serum (staining combinations 1–4) or alternatively in 10% normal rabbit serum (staining combinations 5 and 6) in PBS-T. Then the tissue was incubated for 48 hours with the primary antibodies, washed with PBS-T, and incubated for 24 hours with one of the secondary antibodies. Following another washing step, tissue was incubated overnight with the other secondary antibody and phalloidin (staining combinations 1–3 and 6), or with α-bungarotoxin and phalloidin (staining combinations 4 and 5). Finally, the tissue was rinsed again and mounted in vol/vol 60% glycerine + 40% PBS. Primary antibodies were applied at room temperature; secondary antibodies phalloidin, and α-bungarotoxin were applied at 37°C. For negative control experiments, primary antibodies were omitted and secondary antibodies were used alone. In all cases, the omission of the primary antibodies resulted in a complete lack of immunostaining.

Fluorescently labeled whole-mounts were analyzed with a confocal laser scanning microscope (CLSM; LSM 700; Carl Zeiss Meditec, Oberkochen, Germany) at low magnification (×10; Figs. 4A, 4C, 9A–D) and ×20 (Figs. 2, 4B) as well as high magnification (×40; Figs. 6A–D", 7, 8) and ×63 (Figs. 3, 5)]. A series of virtual CLSM sections of 0.9-µm thickness were cut through the structures of interest. Each section was photodocumented with a 1024 × 1024 pixel resolution and 3D projections were rendered on a computer using LSM software (ZEN; Carl Zeiss Meditec). Images were generated in three different fluorescence channels: excitation wavelength of 488, 568, and 633 nm.

Quantification of GAP43 Immunoreactivity. The intensity of GAP43 immunoreactivity was determined in kittens of all postnatal stages analyzed in this study until adult age. For intensity measurements, identical settings including objec-

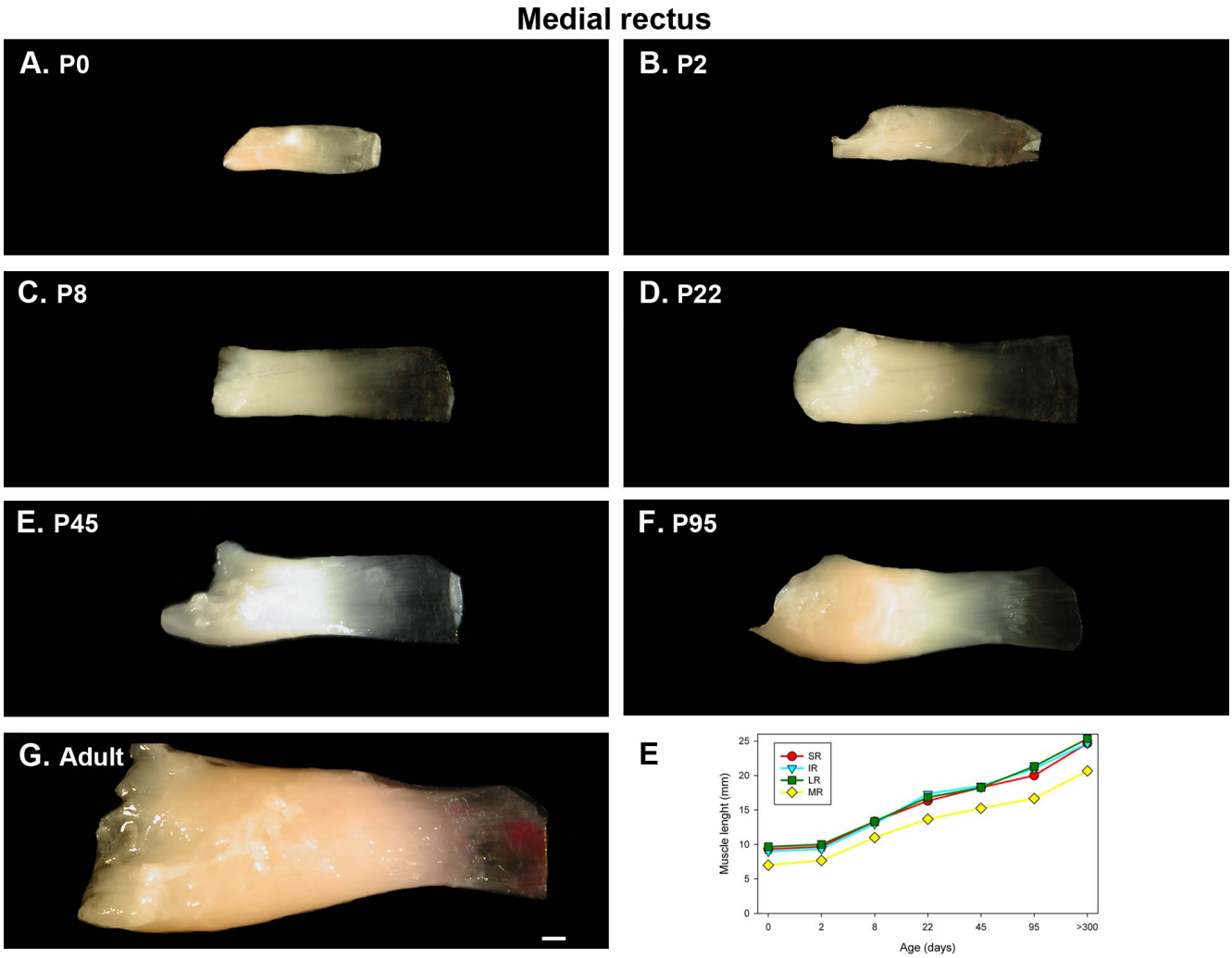

FIGURE 1. Showing the increase in muscle length of the rectus muscles during postnatal development. (**A–G**) Showing a medial rectus including the distal tendon and the increase of size from P0 until adult stage. The proximal tendon of the muscle is short and normally not dissected out from the annulus of Zinn. *Scale bar*: 1 mm. (**E**) Graph showing the increase in muscle length for all rectus muscles during development. For consistency, length measurement included the distal tendon and the muscle but not the proximal tendon. Measurements show that the medial rectus was shorter than the other three EOMs at all ages (2-way ANOVA, Holm-Sidak method for post hoc comparisons, $P < 0.001$).

tive, laser intensity, and photomultiplier were applied on the CLSM. Images were analyzed by using the program ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MA, USA). Since the axons forming palisade endings normally coursed in parallel, we performed random perpendicular transects and the optical density of GAP43 immunostaining was measured at the points of transection. We collected a total of 50 measurements per *z*-stack in three stacks per muscle. A longitudinal study of GAP43 optical density during the different developmental stages until the adult stage was performed for medial rectus muscle. A cross-sectional study of GAP43 optical density was performed at P45 for all four recti muscles.

Quantification and Statistics

The number of palisade endings was counted in the two vertical (superior and inferior) and two horizontal (medial and lateral) EOMs. Counts were done in three animals per age (with the exception of the P45 animals where two animals were available) and for both orbits. Data were expressed as mean and standard error of the mean (SEM). For comparison between groups, we used the 2-way ANOVA followed by the Holm-Sidak method at an overall significance level of $P < 0.05$ for the analysis of the number of palisades in the different muscles, and to analyze muscle length at different ages. The analysis of GAP43 optical density was performed with the Kruskal-Wallis 1-way ANOVA on ranks, followed by post hoc analysis by means of Tukey's method at an overall significance level of $P < 0.05$. Statistics were carried out using a commercial program (SigmaPlot; Systat Software, Inc., San Jose, CA, USA).

RESULTS

Figure 1 includes images showing the increase in muscle size of the medial rectus muscle during postnatal development up to adult age, and a line diagram showing the increase in muscle length for all rectus muscles. There was a continuous increase in length up to the adult state. Medial rectus was systematically shorter than the other three muscles at all ages tested (2-way ANOVA, Holm-Sidak method for post hoc comparisons; $F = 3.266$, degrees of freedom [*df*] = 18, $P < 0.001$).

Palisade Endings Develop Postnatally Following a Different Time Course

To analyze the developmental signatures of palisade endings, EOM whole-mount preparations were labeled with antibodies against neurofilament and synaptophysin along with phalloidin staining. Anti-neurofilament is a general marker for axons[23] and anti-synaptophysin for vesicles in nerve terminals.[24] Phalloidin binds to actin filaments and labels muscle fibers.[28]

Around Birth Precursors of Palisade Endings Are Only Present in the Inferior and Medial Rectus. At P0 and P2, typical, mature palisade endings were absent in all rectus muscles. Nevertheless, there were differences between muscles in the degree of axon growth toward the tendon. Specifically, all axons in the superior and lateral rectus stopped at variable distances away from the muscle-tendon junction and no axons were observed penetrating the tendon (Figs. 2A, A', 2B, B'). In both muscles, axons stopped more far away from the tendon at P2 than at P0 and a possible explanation is the differential growth between muscle and axons resulting in a shift of axons away from the tendon. In contrast to the superior and lateral rectus, some axons in the inferior rectus and many axons in the medial rectus extended straightly from the muscle into the tendon (Figs. 2A", A"', 2B", B"'). High resolution images showed that axonal extensions exhibited few, if any, branches in the tendon of the inferior rectus but, to the contrary, many branches in the medial rectus tendon (Figs. 3A, A', 3B, B'). In both muscles, axonal branches established vesicle-filled terminal varicosities as demonstrated by synaptophysin-immunoreactivity, most of them at the level of the tendon and only few close to the muscle fiber tip (Figs. 3A, A', 3B, B'). Such terminal varicosities were features consistent with palisade endings of adult animals. However, in contrast to adult animals, where axons turned back in the tendon to form palisade endings, axonal expansions in perinatal kittens did not exhibit such a feature. Therefore, nerve structures observed in the tendon of newborn kittens (in medial and inferior rectus) were considered as palisade endings at an early stage of development and such structures were further on termed precursors of palisade endings. Due to extensive axonal branching at the tendon level and higher number of terminal varicosities, palisade ending precursors were more complex in the medial than in the inferior rectus.

Figure 4 shows the innervation of the whole medial rectus at birth (P0) including singly innervated muscle fibers, multiply-innervated muscle fibers, and palisade ending precursors. In P0 kittens, en plaque motor endings of singly innervated muscle fiber covered a broad region extending from the nerve entry site to the distal third of the muscle. This is very different from other mammalian EOMs where the endplates form a band across the middle third of the muscle.[13]

At P8 Immature Palisade Endings Are Only Present in the Medial Rectus. At P8 all axons in the superior rectus stopped at the level of the muscle without crossing the muscle-tendon border (Fig. 2C). In the lateral rectus, we observed at P8 few palisade ending precursors exhibiting very few axonal branches and comparable in their morphology with those observed earlier in the inferior rectus (Fig. 2C'). In the inferior rectus, palisade endings were still at precursor stage 8 days after birth (Figs. 2C", 3C). Exclusively in the medial rectus, palisade ending maturation was more advanced at P8. Specifically, in addition to axons forming precursors of palisade endings, we observed axons that turned back at the tendon level and by approaching single muscle fiber tips they divided into few axonal branches. Axonal branches exhibited synaptophysin-positive terminal varicosities that were frequent at the tendon level and sparse around the muscle fiber tips (Figs. 2C"', 3C'). Recurrent axons establishing terminal varicosities were features consistent with palisade endings in adult animals. However, P8 palisade endings in the medial rectus had fewer axonal branches and only few terminals were located around the muscle fiber tip, which was different to palisade endings in adult animals. Therefore, they were designated immature palisade endings. Taking together these data, 1 week after birth there were marked differences in the degree of maturation of palisade endings. Thus, palisade ending development occurred later in the superior rectus. Less developed, palisade endings were observed in the inferior and lateral rectus, but much further developed palisade endings in the medial rectus muscle.

At P22, Immature Palisade Endings Are Present in the Inferior Rectus. At P22 we observed palisade ending precursors for the first time in the superior rectus. This represents a 3-week delay with respect to the fastest developing muscle, the medial rectus. In the lateral rectus, however, there was no further change with respect to the prior stage (Figs. 2D, D'). In the inferior rectus, an important step of maturation was observed at P22. Thus, by 3 weeks after birth, recurrent axons forming immature palisade endings were found in the inferior rectus, and as expected, the complexity of immature palisade endings was increased in the medial rectus (Figs. 2D", D"', 3D, D'). Moreover, P22 palisade endings of the medial rectus showed most of their terminal varicosities concentrated now around the muscle fiber tip and fewer at the tendon level (Fig. 3D').

At P45 Immature Palisade Endings Are Present in the Superior/Lateral Rectus. At P45, simple palisade endings with few axonal branches were observed for the first time in the superior and lateral rectus (Figs. 2E, E'). However, palisade endings were more complex in the inferior rectus and medial rectus, with the highest complexity in the medial rectus (Figs. 2E", E"', 3E, E'). The morphology of medial rectus palisade endings at P45 was similar to that of adult muscles (see the series of Fig. 3A'–G').

At P95 Mature Palisade Endings Are Present in all Rectus Muscles. At P95, palisade endings in all rectus muscles exhibited a complex morphology and were qualitatively indistinguishable from palisade endings in adult animals (Figs. 2F–F"', 2G–G"', 3F, F', 3G, G'). Exclusively in the superior rectus, we found few palisade endings which were at an immature stage.

Developing Palisade Endings Express Growth Associated Protein 43

In addition to the developmental morphology of palisade endings, we analyzed the spatiotemporal expression pattern of a molecular marker for axonal development. Therefore, we visualized growth associated protein 43 (GAP43) which is strongly expressed in axons during development and regeneration.[25] For GAP43 detection, EOM whole-mounts were immunolabeled with anti-GAP43 and counterstained with anti-neurofilament or alternatively, with anti-GAP43 along with anti-synaptophysin. Muscle fibers were labeled with phalloidin. GAP43 was quantified by measuring the optical density of the GAP43 signal for each postnatal stage in different animals.

First, we tested the expression of GAP43 in palisade endings of the medial rectus from P0 until adult stage. Precursors of palisade endings around birth (P0/P2) as well as immature palisade endings at P8 exhibited high and almost the same GAP43 staining intensity (Figs. 5A–A", 5B–B", 5C–C", 6E). GAP43 expression decreased significantly (Kruskal-Wallis 1-way ANOVA on ranks, $H = 649.175$, $df = 6$, $P < 0.001$, followed

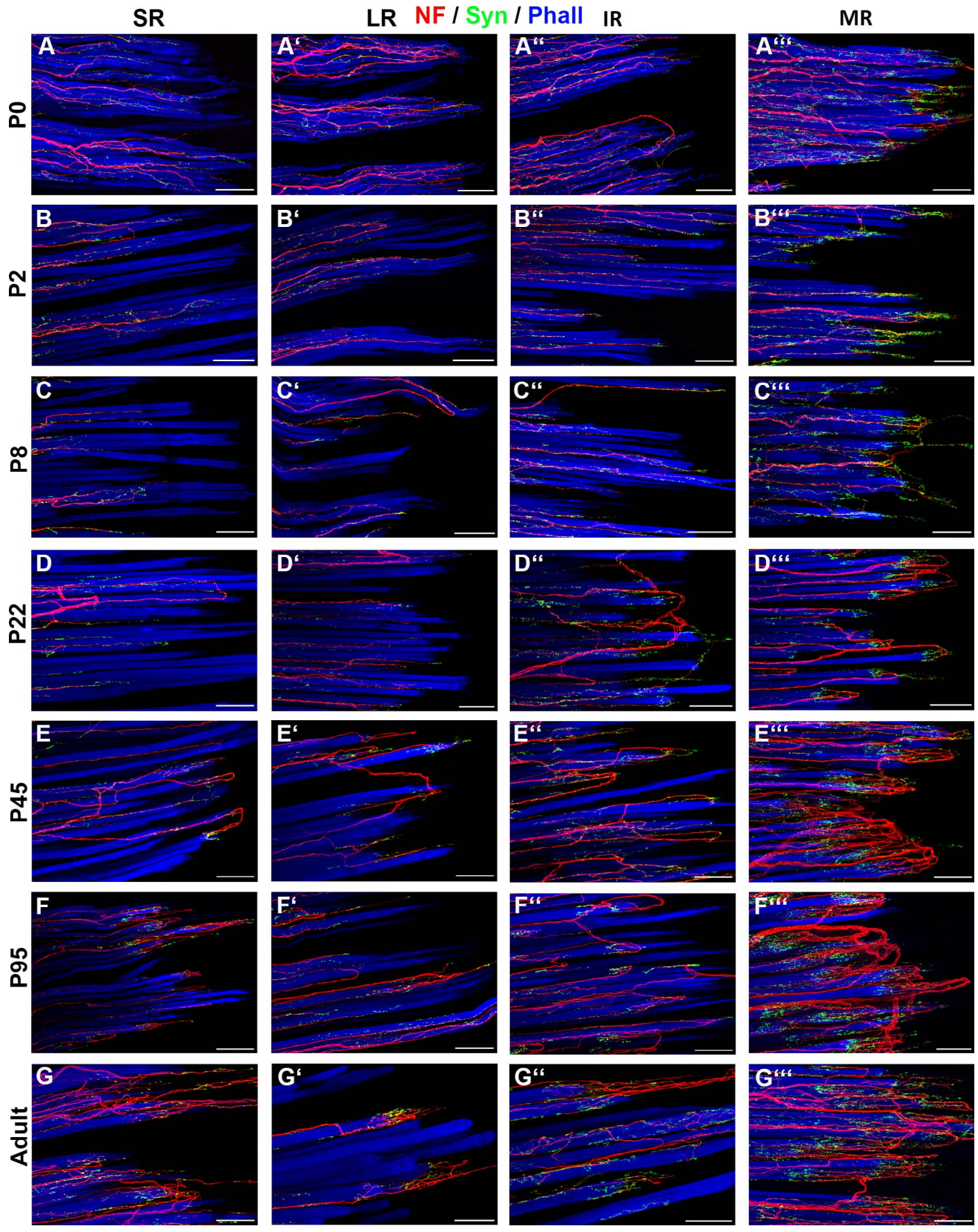

FIGURE 2. Images of CLSM *z*-stacks showing the heterochronic time courses of palisade ending development in the rectus muscles from P0 until adult stage. IR, inferior rectus; LR, lateral rectus; MR, medial rectus; SR, superior rectus. Images were done with low magnification and show a part of the muscle-tendon junction. Nerve fibers were labeled with anti-neurofilament (NF); nerve terminals with anti-synaptophysin (Syn); and muscle fibers with phalloidin (Phall). In this and other figures, the distal tendon extends from the muscle fibers toward the right hand side of each picture and is not labeled. At P0 and P2 all axons stop at variable distances away from the tendon in the SR (**A**, **B**) and LR (***A'***, ***B'***). Different to that, few axons in the IR (**A''**, **B''**) and many axons in the MR (**A'''**, **B'''**) extend straightly into the tendon where they establish synaptophysin-positive nerve

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terminals. Such nerve structures represent precursors of palisade endings. Palisade ending precursors are more frequent in the medial rectus compared with the inferior rectus. At P8, all axons stop in the SR (**C**) before reaching the tendon whereas in the LR (**C'**), single axons penetrate the tendon, thereby forming palisade ending precursors. In the IR (**C''**), there is no change to the prior stage and all palisade endings are still at the stage of precursors. Exclusively in the medial rectus (**C'''**), recurrent axons supply less elaborated palisade endings which are designated immature palisade endings. At P22, palisade ending precursors are found in the SR for the first time (**D**) and palisades are still at the stage of precursors in the LR (**D'**). At P22, formation of palisade endings starts in the inferior rectus (**D''**) and palisade endings increase their complexity in the medial rectus (**D'''**). At P45, palisade endings are present in all rectus muscles. They are simple and immature in the SR (**E**) and LR (**E'**), development is more advanced in the IR (**E''**) and most advanced in the MR (**E'''**). At P95 (**F**, **F'**, **F''**, **F'''**), palisade endings in all rectus muscles appear qualitatively almost equal to palisade endings in adult animals (**G**, **G'**, **G''**, **G'''**). *Scale bars*: 100 μm.

by Tukey's test, $P < 0.05$) in later postnatal stages to intermediate values in P22 and P45 palisade endings (Figs. 5D–D", 5E–E", and 6*E*) and was significantly lower (same test as above) and almost absent in palisades of P95 and adult stages, respectively (Figs. 5F–F", 5G–G", and 6E).

Since the development of palisade endings was heterochronic, we wanted to know whether the GAP43 expression varied in different muscles of the same postnatal stage. For our analyses we chose P45 animals where palisade endings were simple in the superior and lateral rectus, more elaborated in the inferior rectus and most elaborated in the medial rectus. GAP43 expression was higher in palisade endings of the superior and lateral rectus, compared with the inferior and medial rectus (Fig. 6A–D", 6F). In turn, medial rectus showed the lowest level of GAP43, significantly smaller than the other three muscles (Fig. 6F; Kruskal-Wallis 1-way ANOVA on ranks, $H = 111.003$, $df = 3$, $P < 0.001$, followed by Tukey's test for post hoc comparisons, $P < 0.05$). Therefore, GAP43 expression was an inverse indicator of the degree of maturation and complexity of the palisade endings.

When combining anti-GAP43/anti-neurofilament staining, there was not a complete overlap of the signals (Fig. 5A–E"). Specifically, GAP43-positive varicosities mostly in the periphery of palisade endings lacked a neurofilament signal in P0 to P45 animals. To test whether GAP43 was confined to axons, we combined anti-GAP43 with another neuronal marker (synaptophysin) that labels synaptic nerve terminals. In a medial and inferior rectus of a P22 animal, almost all varicosities of the palisade endings exhibited GAP43/synaptophysin immunoreactivity. Only in very few GAP43-positive varicosities at the periphery of palisade endings a synaptophysin signal was not detected (Fig. 7).

Palisade Endings Are Formed by Expansions of MIF Motoneurons

To determine the innervation characteristics of axons supplying palisade endings, we traced axons in EOM whole-mounts. Although this task was sometimes limited by topologic constraints (i.e., axons suddenly got out of focus or intermingled with other axons), we were able to trace axons long enough to examine whether they established motor terminals. Analyses were done in all rectus muscles and Figure 8 provides an image series of medial rectus palisade endings. We selected three combinations of triple labeling including anti-GAP43/α-bungarotoxin/phalloidin, anti-ChAT/α-bungarotoxin/phalloidin, and anti-ChAT/anti-synaptophysin/phalloidin. Anti-GAP43 was used to label developing axons[25] and anti-ChAT to demonstrate cholinergic axons.[27] Anti-synaptophysin was used to label nerve terminals[24] and α-bungarotoxin to label acetylcholine receptors of motor nerve terminals.[26] Muscle fibers were stained with phalloidin.[28]

In P2 animals, GAP43-positive axons established multiple motor terminals as shown by α-bungarotoxin staining alongside the muscle fiber before extending straightly into the tendon to form precursors of palisade endings (Fig. 8A). In later postnatal stages (P8, P22, and P95) and the adult stage, we showed that axons supplying palisade endings were cholinergic and established multiple synaptophysin/α-bungarotoxin-positive terminals alongside the muscle fibers (Fig. 8B–F). Terminal varicosities in palisade endings expressed synaptophysin as well (Fig. 8C–D) but lacked α-bungarotoxin (Fig. 8B, E, F). Multiple neuromuscular contacts are the morphologic signature of MIF motoneurons and based on the present findings, we conclude that the MIF motoneurons initially extend into the tendon and later turn back to form palisade endings.

At P2, many α-bungarotoxin binding sites appeared larger than in the later postnatal stages but further analyses is needed to evaluate this observation.

Quantification of Palisade Endings

We counted palisade endings in the rectus muscles of different postnatal ages to demonstrate the dynamics of development. Quantification could only be done for immature and mature palisade endings but not for precursors of palisade endings for the following reasons. Immature and mature palisade endings established a cuff of terminal varicosities at the end of single muscle fibers and by counting these cuffs assigned to individual muscle fibers, we could easily determine the number of palisade endings for each muscle. Palisade ending precursors did not exhibit such cuffs and especially in the medial rectus terminal varicosities spread broadly in the tendon and could not be assigned to individual muscle fibers (see Figure 3B' where a palisade ending precursor is associated with more than one muscle fibers). Therefore, the exact number of palisade endings precursors was difficult to determine and postnatal stages with palisade endings at the level of precursors were excluded.

Quantification of immature and mature palisade endings was done in three animals per stage in rectus muscles of both orbits with the exception of the P95 stage where two animals were available. In adult animals, the lowest number of palisade ending was counted in the lateral rectus and the highest number in the medial rectus. Values for the inferior and superior rectus were in between. The adult number of palisade endings was established at different time points for the rectus muscles. Specifically, adult values of palisade endings were established first (at P45) in the medial and inferior rectus, and later (at P95) in the lateral rectus. The most delayed muscle was the superior rectus since its number of palisade endings increased progressively during development until reaching its maximum at the adult stage (Table and Figs. 9A–E; 2-way ANOVA, $F = 52.997$, df muscle × age = 12, $P < 0.001$, followed by Holm-Sidak comparisons, $P < 0.05$).

A schematic diagram summarizing the results of the present study is shown in Figure 10.

Discussion

Palisade endings are nerve specializations regularly present in frontal but not in lateral-eyed species.[10] Little is known about

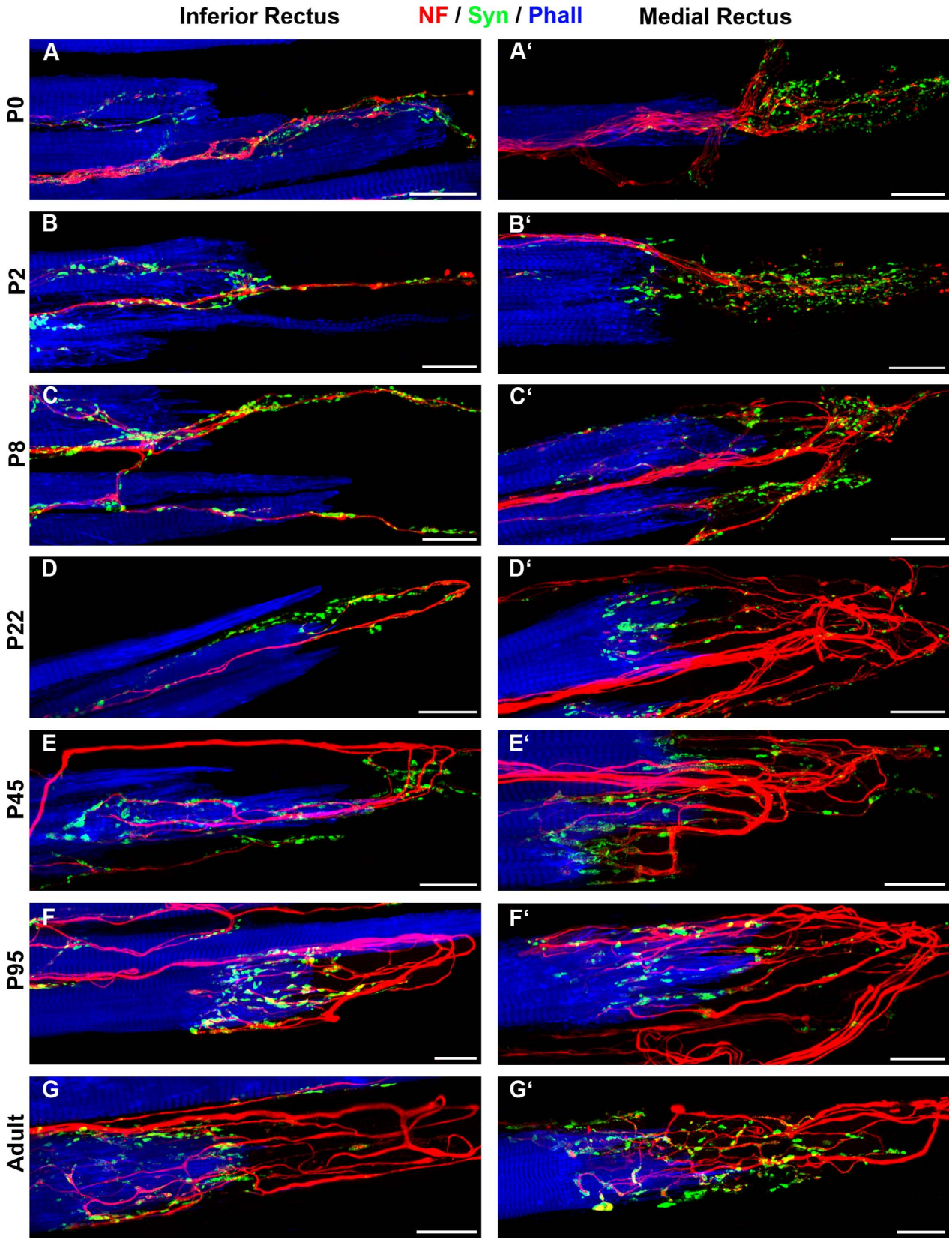

FIGURE 3. Images of CLSM *z*-stack at high magnification showing the heterochronic development of palisade endings in the inferior rectus (IR) and medial rectus (MR). Nerve fibers were labeled with anti-NF, nerve terminals with anti-Syn, and muscle fibers with Phall. At P0 (**A**, **A'**) and P2 (**B**, **B'**), axonal expansions forming precursors of palisade endings are present both in the inferior and medial rectus muscles. Axonal expansions exhibit few if any branches in the inferior rectus muscle, but many in the medial rectus and establish synaptophysin-positive terminal varicosities, most of them at the tendon level, and only few close to the muscle fiber tip. At P8 (**C**, **C'**), palisade endings precursors are still in the inferior rectus (**C**) whereas immature palisade endings formed by recurrent axons are observed in the medial rectus (**C'**). Immature palisade endings are less elaborated and

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establish synaptophysin-positive terminal varicosities, most of them at the tendon level. At P22 (**D**, **D'**), immature palisade endings with a simple morphology are observed in the inferior rectus (**D**) for the first time, whereas their complexity is increased in the medial rectus (**D'**) and terminal varicosities are now more concentrated around the muscle fiber tip and only few are at the tendon level. At P45 (**E**, **E'**), maturation of palisade endings is still delayed in the inferior rectus (**E**) and more advanced and similar to adult stage in the medial rectus (**E'**). At P95 (**F**, **F'**), palisade endings in the inferior (**F**) and medial rectus (**F'**) appear almost indistinguishable from palisade endings in adult animals (**G**, **G'**). *Scale bars*: 20 μm.

their development and the only two studies in humans reveal discrepancies. Specifically, in a limited number (seven) of rectus muscles obtained from five human infants between the ages of 3 days and 47 months, no palisade endings have been found and only scattered nerve terminals at the tendon level have been seen in the 47-month-old child.[21] On the other hand, Lukas et al.[22] has described typical palisade endings in EOMs of a 2-year-old child.

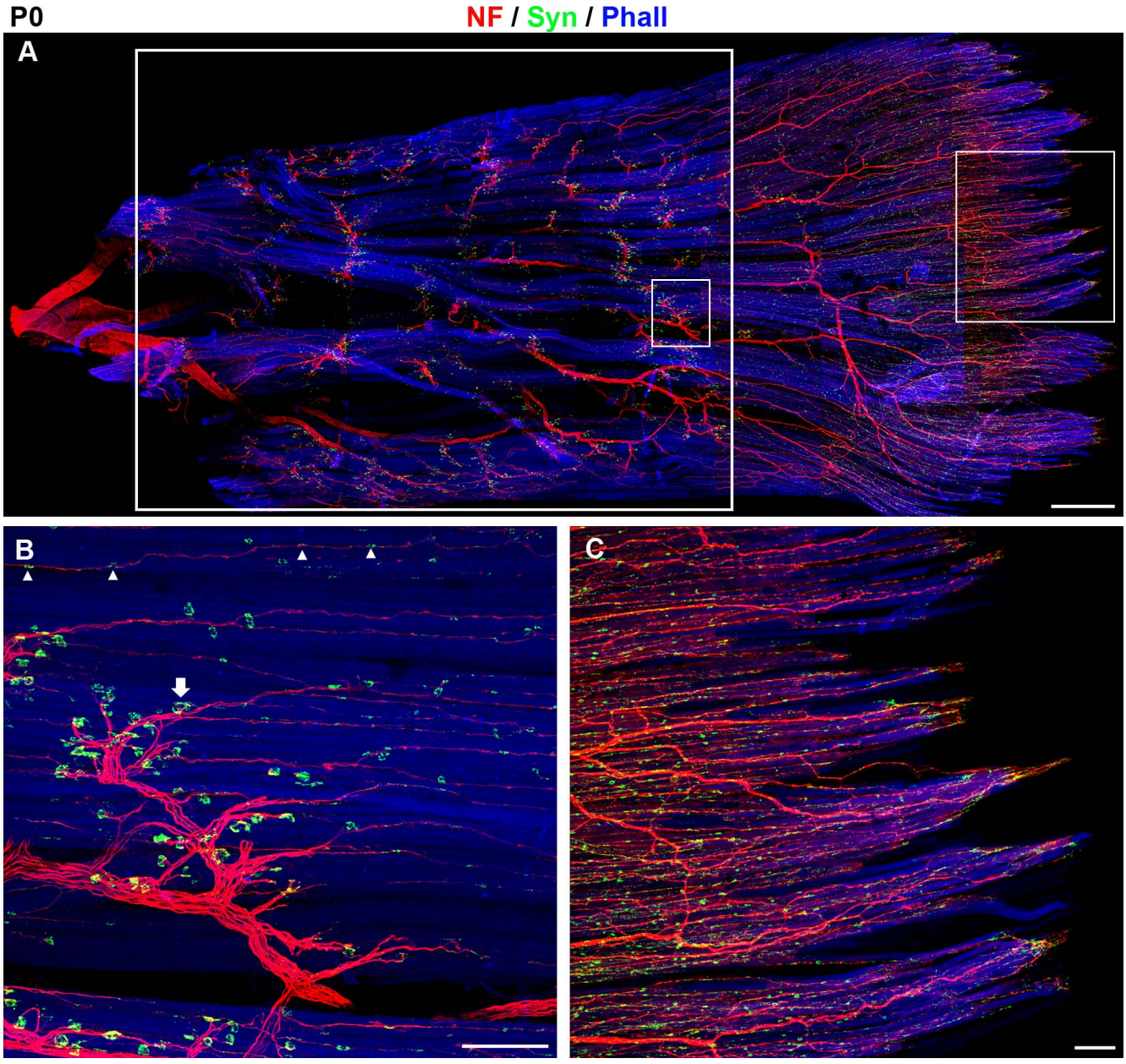

Figure 4. Photomontages of CLSM *z*-stacks showing a P0 medial rectus and its pattern of innervation. Nerve fibers were labeled with anti-NF, nerve terminals with anti-Syn, and muscle fibers with Phall. (**A**) Shows the nerve entering the muscle at its proximal end (*left side*) and dividing into several branches. Most axons establish single en plaque motor endplates on muscle fibers (singly innervated muscle fibers). The endplates form a broad band reaching from the nerve entry site to the distal third of the muscle (large inset). Other axons with a smaller diameter establish numerous small boutons along the length of muscle fibers (multiply-innervated muscle fibers). At the distal muscle-tendon junction (right side) palisade endings are present which are at the stage of precursors in P0 animals. (**B**, **C**) Insets of (**A**) at higher magnification. (**B**) Small, *left inset* of (**A**) showing en plaque motor endplates (*arrow*) on singly innervated muscle fiber and en grappe motor endings (*arrowheads*) on multiply-innervated muscle fibers (**C**) Small, *right inset* of (**A**) showing axons extending into the tendon and forming palisade ending precursors. *Scale bars*: 500 μm (**A**), 100 μm (**B**, **C**).

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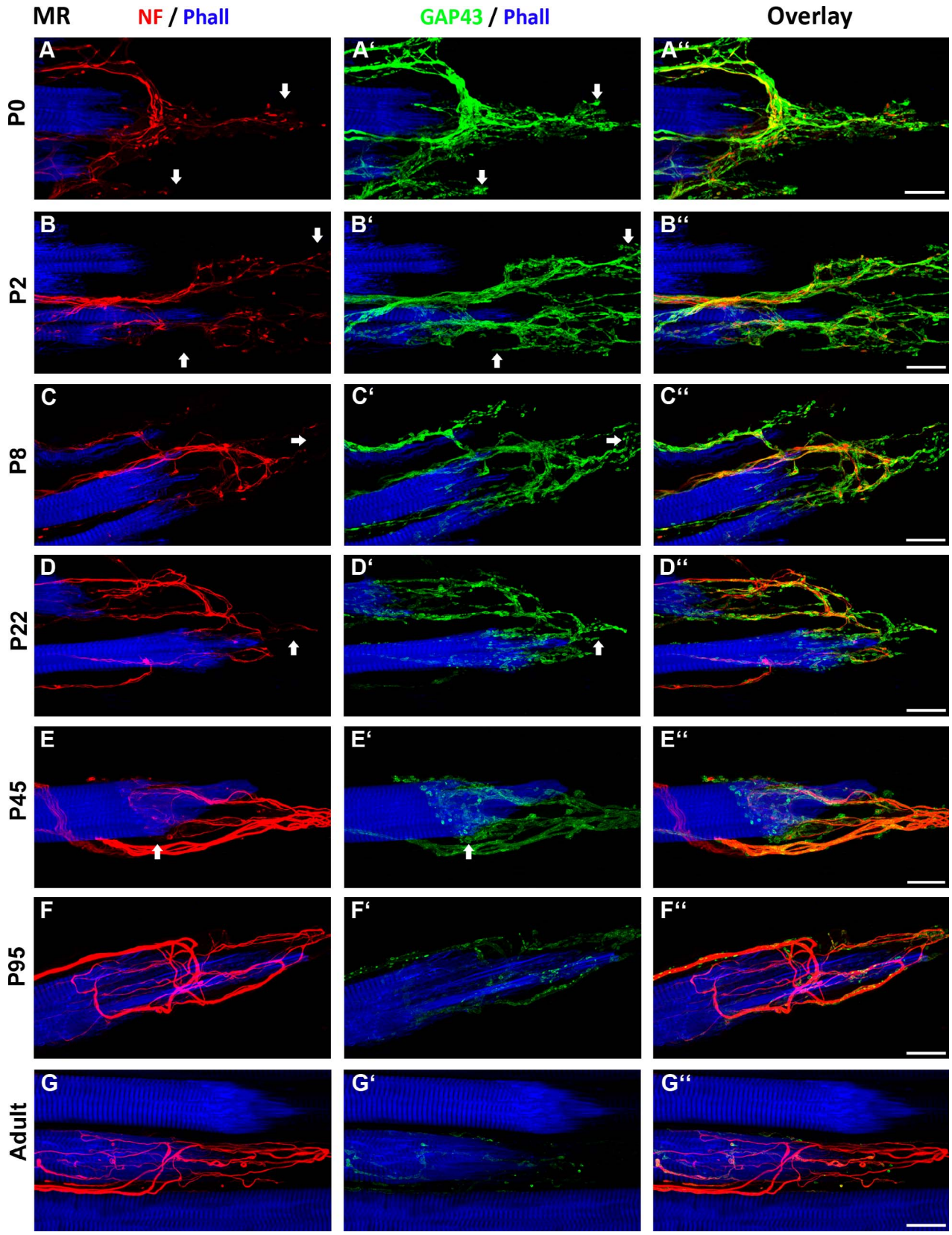

FIGURE 5. Images of CLSM *z*-stacks showing the expression of GAP43 in palisade endings of MR muscles starting from P0 until adult age. Axons were double-labeled with anti-NF/anti-GAP43, and muscle fibers with Phall. (**A**–**G**) Showing palisade endings in neurofilament staining. (**A'**–**G'**) Palisade endings after GAP43 staining. (**A''**–**G''**) The overlay of neurofilament and GAP43 staining is shown. GAP43 expression is strong in palisade ending precursors of P0 (**A'**) and P2 animals (**B'**), as well as in immature palisade endings of P8 animals (**C'**). It is reduced in palisade endings of P22 (**D'**) and P45 (**E'**) animals and almost absent in palisade endings of P95 (**F'**) and adult animals (**G'**). *Arrows* in (**A**–**E'**) show varicosities in palisade endings of P0 to P45 animals which lack neurofilament signals but are GAP43-positive. *Scale bars*: 20 μm.

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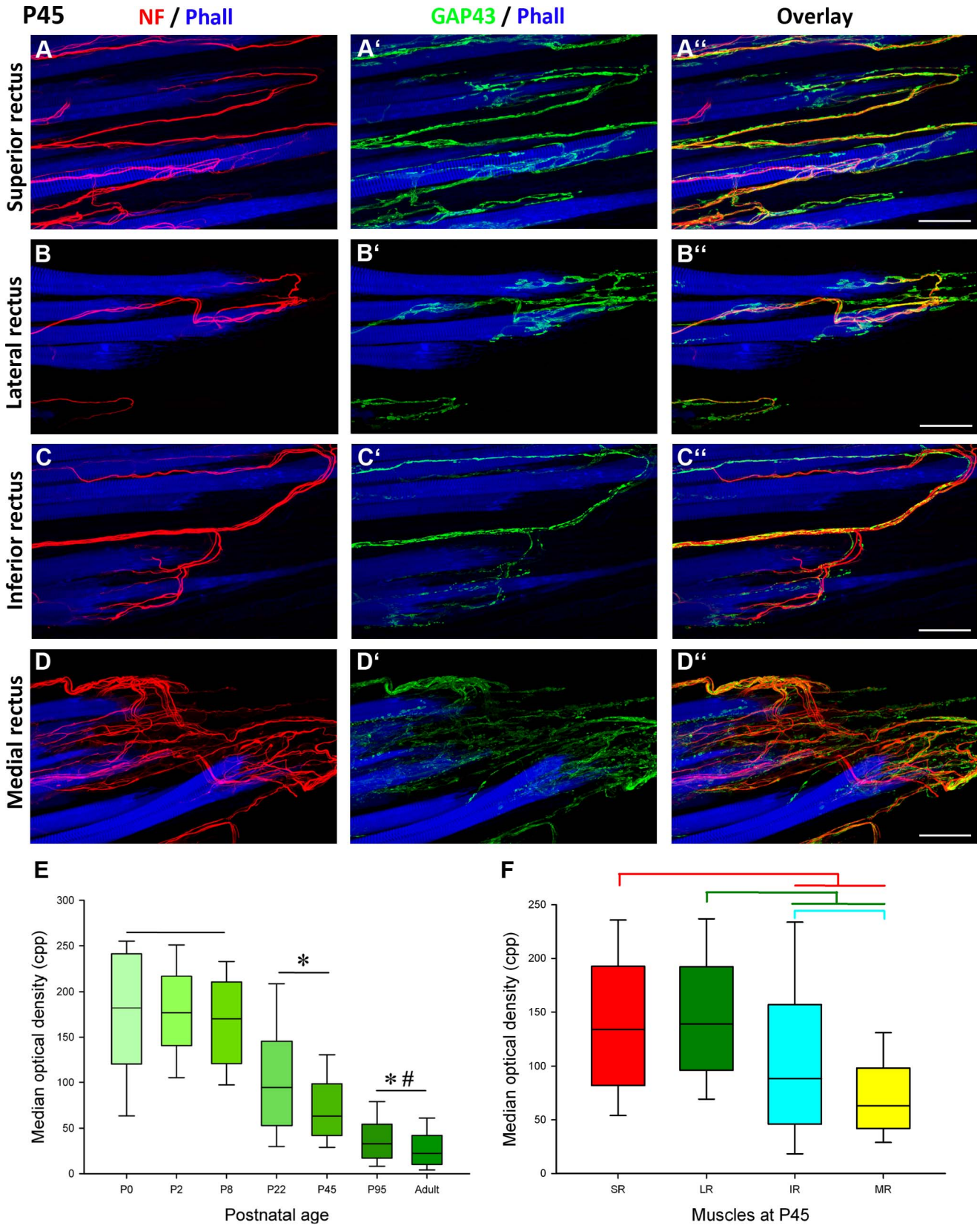

Figure 6. Images of CLSM *z*-stacks of the four rectus muscles of a P45 animal showing the expression of GAP43 in palisade endings. (**A–D''**) Axons were double-labeled with anti-NF/anti-GAP43, and muscle fibers with Phall. (**A–D**) Showing palisade endings in NF staining. (**A'–D'**) Palisade endings after GAP43 staining. (**A''–D''**) The overlay of NF and GAP43 is shown. At P45 palisade endings in the SR (**A**) and LR (**B**) are less elaborated than palisade endings in the IR (**C**) and MR (**D**). (**A'-D'**) Showing the relative GAP43 staining intensity in different muscles of the same age. Less elaborated palisade ending in the superior and lateral rectus (**A'**, **B'**) exhibit a stronger GAP43 immunoreactivity than further elaborated palisade endings in the inferior and medial rectus (**C'**, **D'**). *Scale bars*: 50 μm. (**E**, **F**) Measurements of the optical density of GAP43 immunostaining

(expressed in counts per pixel) in MR muscles at different postnatal stages until adult (**E**) and in the four rectus muscles of P45 animals (**F**). (**E**) In three palisade endings per group arising from three different animals, except two animals in P95, comparisons were carried out by means of Kruskal-Wallis ANOVA on ranks, followed by Tukey's post hoc test at a level of significance of $P < 0.05$. The *box* represents the 25, 50 and 75 percentiles, the *lower* and *upper error bars* represent the 10 and 90 percentiles. The *horizontal lines* above groups indicate similarity in the data between the underlying groups. Thus, the early postnatal groups P0, P2, and P8 were similar, the intermediate postnatal P22 and P45 were similar and the late postnatal and adult were similar. *Asterisks* indicate significant differences with each of the early postnatal groups; hashtag indicates significant differences with each of the mid-postnatal group. (**F**) Same analysis as in (**E**), but for the four recti muscles studied at P45. *Horizontal lines* establish the differences of the underlying muscles with the muscle of the representative color of the line. For instance, IR and MR were significantly different from SR.

The present work is the first comprehensive study analyzing the development of palisade endings in a frontal-eyed mammal (cat). We show that palisade endings develop postnatally and pass through the same steps of development, but in a striking heterochronic sequence, thus reaching a significantly different final density per muscle. Additionally, we show that palisade endings express high levels of growth-associated proteins (GAP43) during development and are supplied by axons that establish multiple motor contacts alongside the muscle fibers.

Palisade Endings Undergo the Same Developmental Steps in All Rectus Muscles but in a Heterochronic Sequence

Palisade endings developed entirely postnatally in three phases. In the first phase, precursors of palisade ending were formed; in the second phase, immature palisade endings developed; and in the third phase, palisade endings attained adult characteristics. Although stereotypical in all rectus muscles, these developmental steps were heterochronic between muscles.

Around birth, palisade ending precursors (axonal expansions extending straightly into the tendon) were solely observed in the inferior and medial rectus. They were detected in the lateral rectus first at P8 and in the superior rectus as late as P22. Palisade ending precursors appeared complex in the medial rectus because axonal expansions branched extensively at the tendon level and were richly endowed with terminal varicosities. In the other rectus muscles, palisade ending precursors were much simpler because axonal branching was almost absent in the tendon. We never found complex precursors in the other rectus muscles, although we analyzed several stages in the early postnatal period and three animals per stage. We concluded that complex palisade endings precursors are only a feature of the medial rectus. The scattered nerve terminals at the tendon level of a 47-month-old child may represent precursors of palisade endings[21] indicating that palisade ending development is delayed in humans.

In the second phase of development, supplying axons made a sharp U-shaped turn in the tendon and immature palisade endings appeared. Whether this axonal turning back is

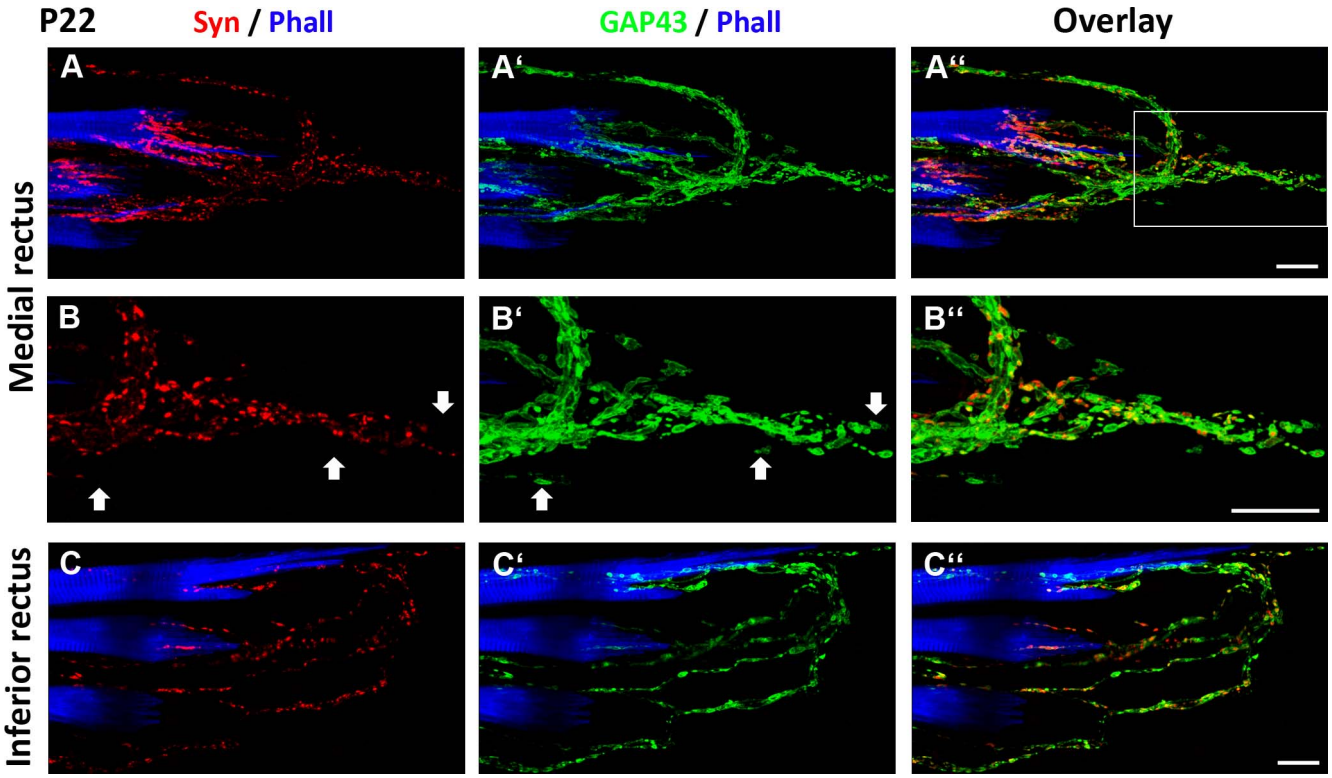

Figure 7. Images of CLSM *z*-stacks of a medial and inferior rectus of a P22 animal showing that GAP43 colocalizes with terminal varicosities in palisade endings. Nerve terminals were labeled with anti-Syn, axons with anti-GAP43, and muscle fibers with Phall. (**A–C**) Palisade endings after synaptophysin staining. (**A'-C'**) Palisade endings after GAP43 staining. (**A''-C''**) The overlay of synaptophysin and GAP43 staining. (**B–B''**) Inset of (**A''**) at higher magnification. Terminal varicosities in palisade endings express Syn (**A–C**) and GAP43 (**A–C'**). Few GAP43-positive varicosities in palisade endings (*arrows* in [**B**, **B'**]) lack Syn signals. *Scale bars*: 20 µm.

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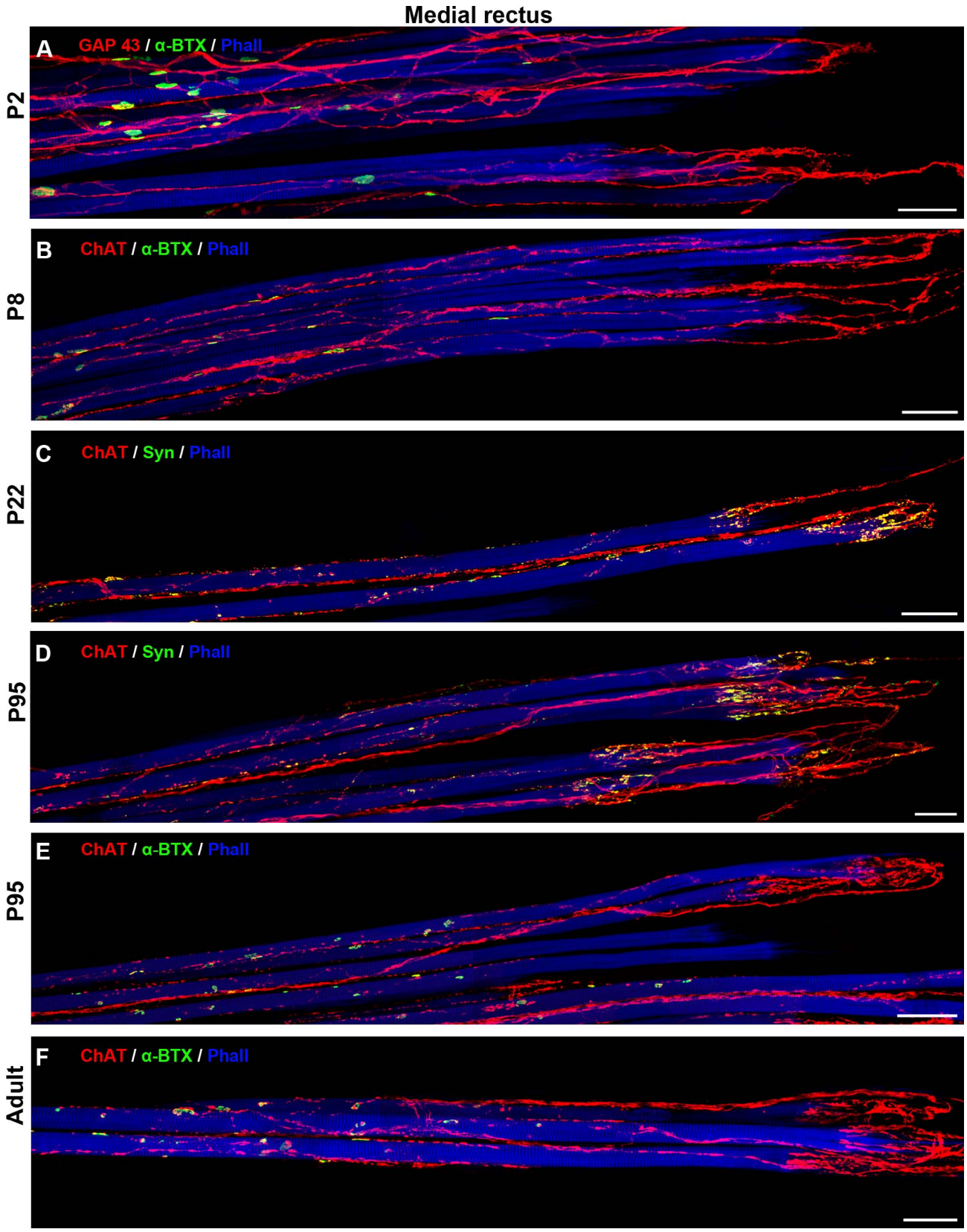

Figure 8. Photomontages of CLSM *z*-stacks showing the innervation characteristics of axons supplying palisade endings in different stages of development for a medial rectus muscle. (**A**) Axons were labeled with anti-GAP43 and motor terminals with α-bungarotoxin (α-BTX). (**B**, **E**, **F**) Axons were labeled with anti-ChAT and motor terminals with α-bungarotoxin. (**C**, **D**) Axons were labeled with anti-ChAT and nerve terminals with anti-Syn. In all staining combinations, muscle fibers were labeled with Phall. (**A**) GAP43-positive axons form palisade ending precursors and establish α-bungarotoxin-positive nerve terminals alongside the muscle fiber. (**B**, **E**, **F**) Show that ChAT-positive axons supplying palisade endings at P8 (**B**), P95 (**E**), and adult stage (**F**) establish α-bungarotoxin–positive nerve terminals alongside the muscle fibers. (**C**, **D**) ChAT-positive axons supplying palisade endings at P22 (**C**) and P95 (**D**) form Syn-positive nerve terminals alongside the muscle fiber as well as Syn-positive terminal varicosities in the palisade endings. *Scale bars*: 50 μm.

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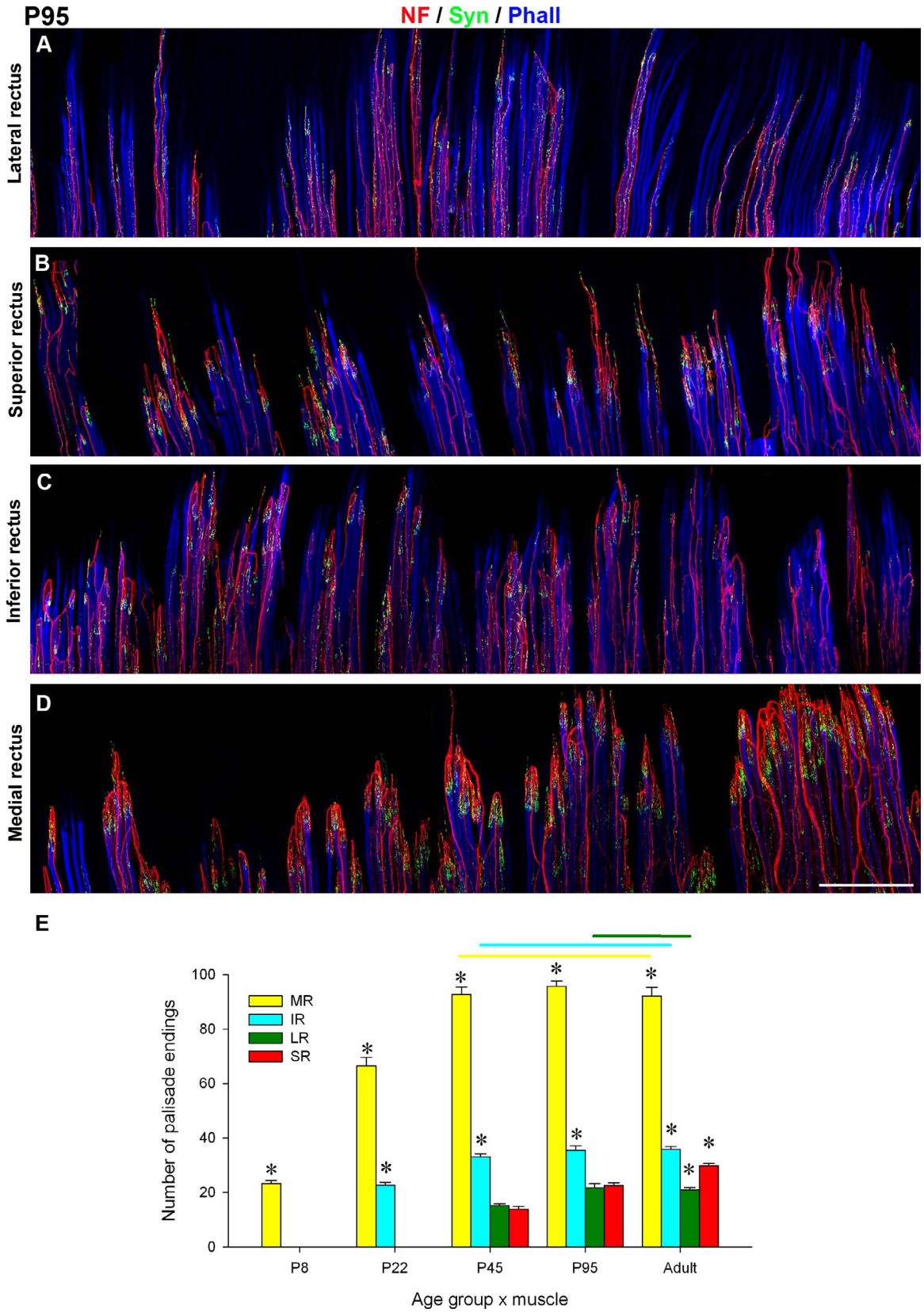

FIGURE 9. Showing the relative abundancy of palisade endings in rectus muscles and their increase in number over time. (**A**–**D**) Photomontage of CLSM *z*-stacks showing the muscle-tendon junction of the four rectus muscles. Images are from a P95 animal and were done at the same magnification to give a realistic impression of the relative abundancy of palisade endings. The whole muscle-tendon junction is shown in the MR (**D**) whereas due to the larger size, the muscle-tendon junction is not completely shown in the other rectus muscles (**A**–**C**). Nerve fibers were labeled with anti-NF, nerve terminals with anti-Syn, and muscle fibers with Phall. The tendon, not labeled, continues the muscle fibers to the *top*. (**A**) The lowest number of palisade endings is in the LR and (**D**) the highest in the MR. (**B**, **C**) The values for the SR and IR are in between. *Scale bars*: 500

μm. (**E**) *Bar chart* showing the increase in number of palisade endings during development. *Horizontal lines* indicate absence of differences in those underlined groups of the same color. *Asterisks* indicate significant differences with the other three muscles within the same age group (2-way ANOVA, interaction muscle × age, $P < 0.001$, followed by post hoc Holm-Sidak comparisons, $P < 0.05$).

genetically or environmentally driven is unclear, but this step appears crucial in palisade ending development to attain adult-like character. Immature palisade endings were present at P8 in the medial rectus, at P22 in the inferior, and at P45 in the lateral and superior rectus.

In the third maturation phase, palisade endings were refined by increasing axonal branching and shifting their terminal varicosities toward the muscle fiber tip. They reached adult quality at P45 in the medial rectus and at P95 in the other rectus muscles. Inferior rectus palisade endings were likely completed between P45 and P95 since their developmental status was constantly more advanced than that of lateral/superior rectus palisades. The developmental steps as well as the heterochronic pattern of palisade ending development in cat was not observed in human,[21,22] but it would require more detailed data from humans to figure out whether there is a true species difference in developmental pattern.

Palisade Endings of the Medial Rectus Are More Frequent and Reach Earlier Final Density

During development, the number of palisade endings increased progressively in the rectus muscles, but there were differences with respect to density per muscle as well as the time point at which final density was reached. Concordant with recent quantitative analyses,[10] the highest number of palisade endings was counted in the medial rectus and the lowest in the lateral rectus, whereas values for the inferior and superior rectus lay in between. Additionally, the final density of palisade endings was reached at P45 in the medial and inferior rectus, at P95 in the lateral rectus and not as early as P95 in the superior rectus. The preponderance and early accomplishment of adult-like quantity as well as quality indicate that palisade endings are particular important for the medial rectus muscle.

Although length measurements showed that rectus muscles were still growing at P95, the adult quantity of palisade endings was accomplished at this time point in three rectus muscles and almost adult values in the fourth muscle (superior rectus). These findings indicate that the maturation of palisade endings is independent from that of the eye muscles.

The GAP43 expression correlates with developmental steps of palisade endings

Because GAP43 is enriched in axons during development or regeneration,[25,29] we analyzed the spatiotemporal expression of this growth protein in developing palisade endings. GAP43 expression was high at the beginning of palisade ending development but decreased with time and was nearly absent in mature palisade endings. Moreover, GAP43 downregulation occurred in a heterochronic pattern since in animals of the same age (P45), the GAP43 signal was stronger in those muscles where palisade ending maturation was delayed (superior and lateral rectus) and weaker in muscles (inferior and medial rectus) where development was advanced. Thus, the spatiotemporal expression of GAP43 closely correlates with the developmental steps of palisade endings and suggests a possible role for this growth protein in palisade ending maturation.

Although there were GAP43-positive varicosities in palisade endings which lacked neurofilament signals, an additional staining combination using the synaptic vesicle marker synaptophysin and GAP43 showed that almost all varicosities were double-positive, indicating that GAP43 was confined to neurons. Only in exceptional cases, a synaptophysin signal was lacking in GAP43-positive structures and in this respect, it is worth mentioning that GAP43 is also found in Schwann cells as shown previously.[30,31] A possible explanation for the absence of a neurofilament signal in GAP43-positive structures could be that the neurofilament in the most terminal parts of axons was at a concentration too low for antibody detection.

Table. Number of Palisade Endings (mean ± SEM) in Rectus Muscles at Different Postnatal Ages

	P8	P22	P45	P95	Adult
MR	23.3 ± 1.5	66.5 ± 1.5	92.6 ± 1.5	95.7 ± 1.7	92.2 ± 1.5
IR	–	22.7 ± 1.5	33.2 ± 1.5	35.5 ± 1.8	35.8 ± 1.5
SR	–	–	13.8 ± 1.5	22.5 ± 1.8	29.8 ± 1.5
LR	–	–	15.2 ± 1.5	21.7 ± 1.8	21.0 ± 1.5

The number of sampled muscles was always six, except at P95 where four muscles were inspected.

Developmental Support for a MIF Motoneuron-Palisade Ending Construction

There is consensus that palisade endings originate from the EOM motor nuclei, but it is discussed whether they arise from motor-like or sensory-like neurons in the EOM motor nuclei.[18–20] Combining anterograde tracer labeling and molecular analyses, we demonstrated that axons supplying palisade endings established several motor contacts alongside the muscle fibers in a manner equal to that of en grappe motor terminals on MIFs.[20] These findings strongly suggest that palisade endings arise from MIF motoneurons in the EOM motor nuclei although receptors for cholinergic transmission have not been found in palisade endings so far with the exception of rabbit and rat.[10,20]

Following tracer injection into the distal myotendinous junction (the region of the palisade endings), two populations of neurons have been retrogradely labeled at the periphery of the oculomotor nucleus, i.e., multipolar motor-like neurons and spindle shaped sensory-like neurons.[32] It has been suggested that the latter group are the source of palisade endings.[32] Present findings do not support this hypothesis. Specifically, we showed that axons exhibiting multiple motor neuromuscular contacts supplied precursors of palisade endings as well as immature and mature palisade endings. Due to anatomic constraints, it was not possible to confirm this innervation pattern in every case. However, the present findings exclusively revealed that MIF motoneurons grow out into the tendon and form palisade endings. Thus, our results provide developmental support for an MIF motoneuron-palisade ending ensemble.

The Development of Palisade Endings Correlates With the Development of Visuomotor Behavior

Cats open their eyes 7 to 10 days after birth[33] and develop a complex three-dimensional visuomotor behavior in the first 3 months of life.[34] Visuomotor behavior begins to appear in kittens at 15 days of age and while simple tasks like tracking,

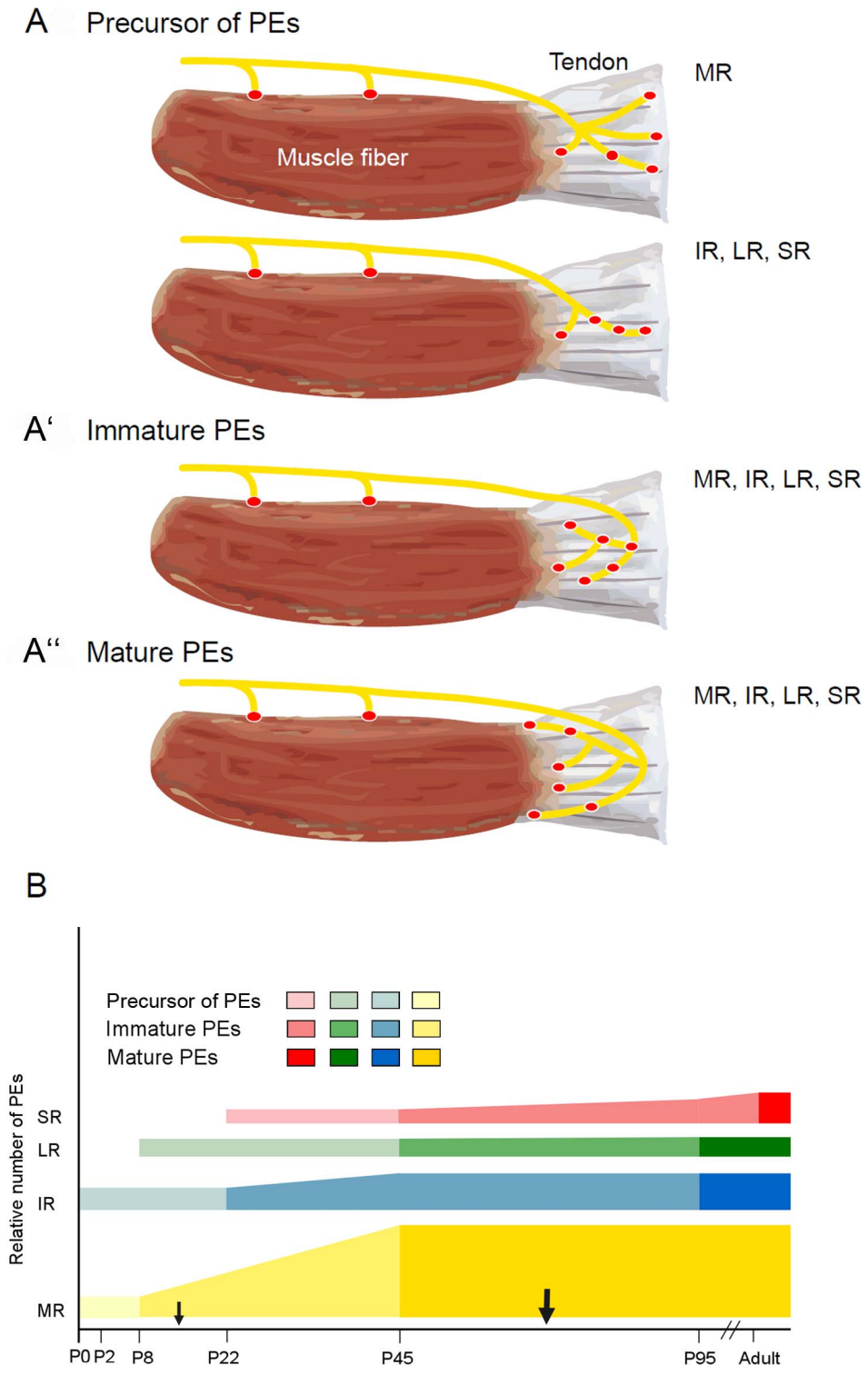

Figure 10. Schematic drawings summarizing the developmental characteristics of palisade endings. (**A–A"**) Showing the different stages of palisade ending development. (**A**) Showing palisade ending precursors in the MR and the other rectus muscles (IR, LR, SR). In all rectus muscles, palisade ending precursors arise from axons extending straightly into the tendon where they extensively sprout in the MR, but not in the other rectus muscles. The majority of nerve terminals (*red*) are at the tendon level and only few close to the muscle fiber tip. (**A'**) Showing an immature palisade ending formed by a recurrent axon but with poorly elaborated axonal branching. Most terminals are at the tendon level. (**A"**) Showing a mature palisade ending with increased axonal branching and most terminals around the muscle fiber tip and fewer at the tendon level. (**B**) Scheme showing the timeline of palisade ending development. The thickness of the *bars* corresponds to the number of palisade endings and shows the increase in number of palisade endings in the course of time. Color variations indicate developmental stages showing that the development of palisade endings is most accelerated in the medial rectus followed by the inferior, lateral, and superior rectus. At P15 (*small arrow*) visuomotor behavior starts to develop, and adult-like but immature palisade endings are present in the MR, and 10 weeks after birth (*large arrow*), when visuomotor behavior is almost completely developed, palisades are mature in the MR and at late immature stage in the other rectus muscles (see discussion).

obstacle avoidance as well as optokinetic nystagmus become positive 3 weeks after birth, more complex tasks like visual guided reaching and jumping become positive between the 5th and 10th week after birth [34,35]. Binocularity is the foundation for proper visuomotor coordination and physiologic experiments have shown that binocular neurons in the visual cortex of cat develop in the first 3 months after birth.[36] Our results showed that the crucial period for development of binocularity[36] is accompanied by palisade endings maturation and the pattern of palisade ending development might correlate with important hallmarks in the development of visuomotor behavior. Specifically, when visuomotor behavior starts to develop at P15,[34] palisade endings at an immature stage were already present in the medial rectus. Moreover, when visuomotor behavior becomes more complex between the 5th and 10th postnatal week and comprises visual guided reaching and jumping behavior[34]—that in turn requires binocularity and fine alignment of the eyes—palisade endings were adult-like in the medial rectus and at late immature stages in the other rectus muscles.

Cats are frontal-eyed species, have retinal specializations (area centralis), and use convergence eye movements to align both eyes on target. Convergence is generated by contraction of both medial rectus muscles and accompanied by eye accommodation. The preponderance, early final density as well as accelerated development of palisade endings in the medial rectus strengthens our previous hypothesis that palisade endings play an important role in convergence.[10] Additional support comes from findings that the cell bodies supplying palisade endings of the medial and inferior rectus lie in a distinct group of neurons (C-group) close to the Edinger-Westphal nucleus. Dendrites of the C-group neurons extend into the Edinger-Westphal nucleus increasing their synaptic density there, indicating that they might share synaptic input with preganglionic neurons related to pupil constriction and lens accommodation during convergence.[13,37]

Although palisade endings are well characterized with respect to origin, innervation pattern, and molecular properties, it remains elusive whether they are motor or sensory structures. However, present developmental findings along with our previous results suggest that palisade endings are particularly important for convergence eye movements in frontal-eyed species.

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References

1. Wang X, Zhang M, Cohen IS, Goldberg ME. The proprioceptive representation of eye position in monkey primary somatosensory cortex. *Nat Neurosci*. 2007;10:640–646.
2. Donaldson IM, Long AC. Interactions between extraocular proprioceptive and visual signals in the superior colliculus of the cat. *J Physiol*. 1980;298:85–110.
3. Maier A, DeSantis M, Eldred E. The occurrence of muscle spindles in extraocular muscles of various vertebrates. *J Morphol*. 1974;143:397–408.
4. Lukas JR, Aigner M, Blumer R, Heinzl H, Mayr R. Number and distribution of neuromuscular spindles in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 1994;35:4317–4327.
5. Blumer R, Lukas JR, Aigner M, Bittner R, Baumgartner I, Mayr R. Fine structural analysis of extraocular muscle spindles of a two-year-old human infant. *Invest Ophthalmol Vis Sci*. 1999; 40:55–64.
6. Greene T, Jampel R. Muscle spindles in the extraocular muscles of the macaque. *J Comp Neurol*. 1966;126:547–549.
7. Alvarado Mallart RM, Pinçon Raymond M. The palisade endings of cat extraocular muscles: a light and electron microscope study. *Tissue Cell*. 1979;11:567–584.
8. Dogiel A. Die Endigungen der sensiblen Nerven in den Augenmuskeln und deren Sehnen beim Menschen und den Saeugetieren [in German]. *Arch Mikroskop Anal*. 1906;68: 501–526.
9. Ruskell GL. The fine structure of innervated myotendinous cylinders in extraocular muscles of rhesus monkeys. *J Neurocytol*. 1978;7:693–708.
10. Blumer R, Maurer-Gesek B, Gesslbauer B, et al. Palisade endings are a constant feature in the extraocular muscles of frontal-eyed, but not lateral-eyed, animals. *Invest Ophthalmol Vis Sci*. 2016;57:320–331.
11. Eberhorn AC, Horn AK, Eberhorn N, Fischer P, Boergen KP, Büttner Ennever JA. Palisade endings in extraocular eye muscles revealed by SNAP-25 immunoreactivity. *J Anat*. 2005; 206:307–315.
12. Rungaldier S, Pomikal C, Streicher J, Blumer R. Palisade endings are present in canine extraocular muscles and have a cholinergic phenotype. *Neurosci Lett*. 2009;465:199–203.
13. Büttner Ennever JA, Horn AK, Scherberger H, D'Ascanio P. Motoneurons of twitch and nontwitch extraocular muscle fibers in the abducens, trochlear, and oculomotor nuclei of monkeys. *J Comp Neurol*. 2001;438:318–335.
14. Bohlen MO, Warren S, Mustari MJ, May PJ. Examination of feline extraocular motoneuron pools as a function of muscle fiber innervation type and muscle layer. *J Comp Neurol*. 2017; 525:919–935.
15. Billig I, Buisseret Delmas C, Buisseret P. Identification of nerve endings in cat extraocular muscles. *Anat Rec*. 1997;248:566–575.
16. Blumer R, Konakci KZ, Pomikal C, Wieczorek G, Lukas JR, Streicher J. Palisade endings: cholinergic sensory organs or effector organs? *Invest Ophthalmol Vis Sci*. 2009;50:1176–1186.
17. Konakci KZ, Streicher J, Hoetzenecker W, Blumer MJ, Lukas JR, Blumer R. Molecular characteristics suggest an effector function of palisade endings in extraocular muscles. *Invest Ophthalmol Vis Sci*. 2005;46:155–165.
18. Lienbacher K, Mustari M, Ying HS, Büttner-Ennever JA, Horn AK. Do palisade endings in extraocular muscles arise from neurons in the motor nuclei? *Invest Ophthalmol Vis Sci*. 2011;52:2510–2519.
19. Zimmermann L, May PJ, Pastor AM, Streicher J, Blumer R. Evidence that the extraocular motor nuclei innervate monkey palisade endings. *Neurosci Lett*. 2011;489:89–93.
20. Zimmermann L, Morado-Diaz CJ, Davis-Lopez de Carrizosa MA, et al. Axons giving rise to the palisade endings of feline extraocular muscles display motor features. *J Neurosci*. 2013; 33:2784–2793.
21. Bruenech R, Ruskell GL. Myotendinous nerve endings in human infant and adult extraocular muscles. *Anat Rec*. 2000; 260:132–140.
22. Lukas JR, Blumer R, Denk M, Baumgartner I, Neuhuber W, Mayr R. Innervated myotendinous cylinders in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2000;41: 2422–2431.

23. Miller N, Feng Z, Edens BM, et al. Non-aggregating tau phosphorylation by cyclin-dependent kinase 5 contributes to motor neuron degeneration in spinal muscular atrophy. *J Neurosci*. 2015;35:6038–6050.
24. De Camilli P, Vitadello M, Canevini MP, Zanoni R, Jahn R, Gorio A. The synaptic vesicle proteins synapsin I and synaptophysin (protein P38) are concentrated both in efferent and afferent nerve endings of the skeletal muscle. *J Neurosci*. 1988;8:1625–1631.
25. Chidlow G, Ebneter A, Wood JP, Casson RJ. The optic nerve head is the site of axonal transport disruption, axonal cytoskeleton damage and putative axonal regeneration failure in a rat model of glaucoma. *Acta Neuropathol*. 2011;121:737–751.
26. Anderson MJ, Cohen MW. Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J Physiol*. 1974; 237:385–400.
27. Gilmor ML, Erickson JD, Varoqui H, et al. Preservation of nucleus basalis neurons containing choline acetyltransferase and the vesicular acetylcholine transporter in the elderly with mild cognitive impairment and early Alzheimer's disease. *J Comp Neurol*. 1999;411:693–704.
28. Faulstich H, Zobeley S, Rinnerthaler G, Small JV. Fluorescent phallotoxins as probes for filamentous actin. *J Muscle Res Cell Motil*. 1988;9:370–383.
29. Gonzalez-Hernandez T, Rustioni A. Nitric oxide synthase and growth-associated protein are coexpressed in primary sensory neurons after peripheral injury. *J Comp Neurol*. 1999;404: 64–74.
30. Woolf CJ, Reynolds ML, Chong MS, Emson P, Irwin N, Benowitz LI. Denervation of the motor endplate results in the rapid expression by terminal Schwann cells of the growth-associated protein GAP-43. *J Neurosci*. 1992;12:3999–4010.
31. Curtis R, Stewart HJ, Hall SM, Wilkin GP, Mirsky R, Jessen KR. GAP-43 is expressed by nonmyelin-forming Schwann cells of the peripheral nervous system. *J Cell Biol*. 1992;116:1455–1464.
32. Lienbacher K, Mustari M, Hess B, Büttner-Ennever J, Horn AK. Is there any sense in the Palisade endings of eye muscles? *Ann N Y Acad Sci*. 2011;1233:1–7.
33. Villablanca JR, Olmstead CE. Neurological development of kittens. *Dev Psychobiol*. 1979;12:101–127.
34. van Hof-van Duin J. Development of visuomotor behavior in normal and dark-reared cats. *Brain Res*. 1976;104:233–241.
35. Norton TT. Receptive-field properties of superior colliculus cells and development of visual behavior in kittens. *J Neurophysiol*. 1974;37:674–690.
36. Hubel DH, Wiesel TN. The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J Physiol*. 1970;206:419–436.
37. Erichsen JT, Wright NF, May PJ. Morphology and ultrastructure of medial rectus subgroup motoneurons in the macaque monkey. *J Comp Neurol*. 2014;522:626–641.

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