Palisade Endings: Cholinergic Sensory Organs or Effector Organs?

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PURPOSE. This study aims to complement the authors’ prior findings on palisade endings in extraocular muscles (EOMs) of monkeys, and to clarify whether palisade endings are cholinergic motor or cholinergic sensory.

METHODS. Macaque monkeys (Macaca fascicularis, n = 10) of both sexes were analyzed using three-dimensional (3D) reconstructions, confocal laser scanning microscopy (CLSM), and conventional/immuno transmission electron microscopy (TEM). For CLSM, we used three combinations of triple fluorescent labeling. EOM wholemounts were labeled with cholinergic markers, including choline acetyltransferase (ChAT), choline transporter (ChT), vesicular acetylcholine transporter (VACHt), and a classical postsynaptic marker for motor terminals, namely α-bungarotoxin. Muscle fibers were counterstained with phalloidin. 3D reconstructions were done of triple-labeled palisade endings. For immuno TEM, tissue was labeled with antibody against ChAT.

RESULTS. Concordant with prior findings, the authors demonstrated that palisade endings at the muscle fiber tips arose from nerve fibers that are ChAT-positive. In 25% of the cases, axons forming palisade endings established multiple neuromuscular contacts outside the palisade complex. Such additional neuromuscular contacts were motor terminals, as demonstrated by α-bungarotoxin binding. All palisade endings established nerve terminals on the tendon. In 40% of the palisade endings, nerve terminals were observed on the muscle fiber as well. Neurotendinous contacts and neuromuscular contacts in palisade endings were ChT/ChAT/VACHt-immunoreactive. Neuromuscular contacts exhibited structural features of motor terminals and were also α-bungarotoxin positive.

CONCLUSIONS. The present study ascertained that palisade endings are cholinergic motor organs. Therefore, it was concluded that palisade endings are not candidates to provide eye-position signals. (Invest Ophthalmol Vis Sci. 2009;50:1176–1186) DOI:10.1167/iovs.08-2748

Knowledge of the position of objects in space is of practical importance for activities in everyday life. Simple tasks such as reaching for an object, as well as more complex tasks such as driving a car, need spatial information. To accurately locate objects in space, the central nervous system needs visual information from the retina as well as additional information from the eye’s position in the orbit. It is believed that such nonvisual information comes from sensory organs (proprioceptors) in extraocular muscles (EOMs). In fact, experimental findings in mammals and psychophysical studies in man provide important support that proprioceptive input from EOMs plays a role in the development of a normal binocular vision, depth perception, and orienting behavior.1–7

Interestingly, classical proprioceptors (muscle spindles and Golgi tendon organs) as known from other skeletal muscles, are absent in the EOMs of most mammals.8 Thus, it is not clear where the source of EOM proprioception lies. One possible origin of this information is the palisade ending (myotendinous cylinder), a nerve-end organ that is unique to EOMs.

Palisade endings, which consist of a dense ramification of preterminal axons and their vesicle-loaded nerve terminals around the tip of a muscle fiber, are encapsulated organs located at the distal and proximal myotendinous junction. Dogiel9 was one of the first scientists to describe palisade endings in the EOMs of several mammals. So far, palisade endings have been found in the EOMs of almost all species investigated, including monkeys,10,11 cats,12–15 rabbits,16,17 sheep,18 rats,19 and humans.20–22 In most species (cat,12,15 sheep,18 monkey,10 and human20), palisade nerve terminals establish contacts on the tendon and on the muscle fiber. In fact, in cats,12,15 sheep,18 monkeys,10 and humans,20 neurotendinous contacts are more numerous than neuromuscular contacts. Palisade endings in rabbits12 and rats19 appear to be an exception, because exclusively neuromuscular contacts have been observed. Palisade endings arise from nerve fibers that, coming from the muscle, extend into the tendon, and then turn back 180° to terminate around the tip of a muscle fiber. Palisade endings are exclusively found in the global (inner) layer of the EOMs and they are associated with non-twitch, multiply innervated muscle fibers that have several motor contacts along their length.12,21,25 The multiply innervated muscle fibers have a unique innervation from small motoneurons located outside the borders of the main EOM nuclei.24

Although direct physiologic evidence is still lacking, the literature to date suggests that palisade endings are sensory organs providing important information about the eye position.2–5,6,12,13,15,19,25,26 In a single, earlier study, Sas and Schab27 suggested a motor role for palisade endings; more recently, Lukas et al.20 proposed a sensory/motor function.

Surprisingly, we recently showed in cats and macaque monkeys that palisade endings are supplied by cholinergic axons, and that the palisade complexes are cholinergic as well.11,14,15 In some cases, we determined that nerve fibers supplying palisade endings establish neuromuscular contacts outside the palisade complex.11,14,15 In monkeys, such additional neuromuscular contacts were observed in 30% of the cases stud-
ied. Our recent findings have reopened the debate about the functional significance of palisade endings and have advanced the question: Are palisade endings cholinergic sensory structures or effector organs involving collagen fibrils? The presence of nerve terminals in apposition to collagen fibrils seems to argue for sensory as opposed to motor function. However, in this case, the additional neuromuscular contacts established by the nerve fiber that supplies palisade endings would have to be interpreted as sensory as well.

In this study, we have continued our analysis on palisade endings in monkeys to complement our prior findings on this EOM-specific organ in a primate species. Specifically, we labeled palisade endings with all commercially available cholinergic markers, including antibodies against choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VAChT), and choline transporter (ChT). In the nervous system, ChT is used for the uptake of choline and ChAT synthesizes acetylcholine, which is packed into vesicles by VACHT. To distinguish between sensory and motor terminals, we used α-bungarotoxin, a form of snake venom that binds to postsynaptic nicotinic acetylcholine receptors and is widely used to detect motor terminals in vertebrate skeletal muscles including EOMs. For our analysis, we used various techniques including three-dimensional (3D) reconstructions, confocal laser scanning microscopy (CLSM), immuno light microscopy (LM), and conventional/immuno transmission electron microscopy (TEM). Here, we provide novel data indicating that palisade endings are effector (motor) organs.

**MATERIALS AND METHODS**

All animals used in this study were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Captive-bred cynomolgus monkeys (Macaca fascicularis, n = 10), five females and five males, their age varying between 4 and 6 years and their weight between 2.2 and 4.1 kg, were used for this study. The eyeballs including both rectus and oblique muscles. The whole EOM myotendons were labeled with: 1) phalloidin (counterstaining the muscle fibers), an antibody against ChAT and α-bungarotoxin; 2) phalloidin and antibodies against ChAT and ChT; and 3) phalloidin and antibodies against neurofilament and VACHT. The sources and working dilutions of phalloidin, α-bungarotoxin, primary antibodies, and secondary antibodies are listed in Table 1.

**Labeling with Phalloidin, Anti-ChAT and α-Bungarotoxin.** Fixed tissue was rinsed in PBS, frozen in liquid nitrogen, and subsequently thawed in PBS containing 1% Triton X. After blocking the nonspecific binding sites in 10% donkey serum for 1 hour, wholemounts were incubated in the primary antibody goat anti-ChAT at 20°C and in darkness for 48 hours. Wholemounts were extensively rinsed in PBS and then incubated in the secondary antibody (conjugated donkey anti-goat, Alexa Fluor 488; Invitrogen, Carlsbad, CA) and diluted in PBS containing 1% Triton X for 4 hours in darkness for another 4 hours. After rinsing again, specimens were incubated overnight in a mixture containing rhodamine conjugated α-bungarotoxin and conjugated phalloidin (Alexa Fluor 633; Invitrogen). Finally, wholemounts were rinsed once more and mounted (Citifluor; Agar Scientific Ltd., Stansted, Essex, UK).

**Labeling with Phalloidin, Anti-ChAT, and Anti-ChT.** Wholemounts were processed using the method described above. After freezing and thawing, wholemounts were blocked in 10% donkey serum and incubated in a mixture of the primary antibodies goat anti-ChAT and mouse anti-ChT. Then, wholemounts were incubated in the secondary antibodies (Alexa Fluor 488 conjugated donkey anti-goat and rhodamine conjugated donkey anti-mouse) in each antibody for 4 hours at 20°C. After phalloidin incubation, wholemounts were mounted (Citifluor; Agar Scientific Ltd.). Between the incubation steps, specimens were extensively rinsed.

**Labeling with Phalloidin, Anti-Neurofilament and Anti-VACHT.** Frozen and thawed wholemounts were blocked in 10% donkey serum and incubated in the primary antibodies rabbit anti-neurofilament and goat anti-VACHT (Santa Cruz Biotechnology, Santa Cruz, CA). Then, specimens were labeled with the secondary antibodies rhodamine conjugated donkey anti-rabbit, conjugated donkey anti-goat (Alexa Fluor 488; Invitrogen), and phalloidin. Finally, wholemounts were mounted (Citifluor; Agar Scientific Ltd.).

**Wholemount Immunostaining and Confocal Laser Scanning Microscopy**

The EOMs of five animals were analyzed by CLSM. Distal EOM myotendons (60) were divided into three groups, each group containing 20 EOMs including both rectus and oblique muscles. The whole EOM myotendons were labeled with: 1) phalloidin (counterstaining the muscle fibers), an antibody against ChAT and α-bungarotoxin; 2) phalloidin and antibodies against ChAT and ChT; and 3) phalloidin and antibodies against neurofilament and VACHT. The presence of nerve terminals in apposition to collagen fibrils seems to argue for sensory as opposed to motor function. However, in this case, the additional neuromuscular contacts established by the nerve fiber that supplies palisade endings would have to be interpreted as sensory as well.

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8.0.1: Adobe, San Jose, CA). An individual threshold for one of the structures of interest was set once by the operator in a single sample image and was then automatically adjusted to all other images by a macro. The segmentation procedure resulted in multiple series of binary (black and white) image files, each containing one of the structures of interest (muscle fiber, nerve fiber, palisade ending). 3D models of the required structures were generated by recombining the respective sets of binary image files into a single 3D object using information clustering software (Velocity 4.2; Vivisimo, Pittsburgh, PA) running on a Mac computer (Power Mac G5; Apple, Cupertino, CA).

The sectional outlines of the respective structures were placed in a z-distance corresponding to the sectional thickness. Polygonal surfaces were then generated using a marching cube algorithm. The final models were visualized either alone or as combinations of solid or transparent objects.

**Conventional Electron Microscopy**

After immersion fixation was completed, EOMs containing the tendon were cut longitudinally into small strips. Specimen were postfixed in 1% osmium tetroxide, dehydrated in graded solutions of alcohol and embedded in resin (Epon; Hexion Specialty Chemicals, Houston, TX). Semithin cross-sections were cut through the tissue blocks and examined in the light microscope. When palisade endings were identified, ultrathin sections were cut at appropriate intervals. Sections were mounted on dioxane formvar-coated (Formvar; SPI-Chem, West Chester, PA) copper grids and stained in a 2% uranyl acetate solution, followed by 0.4% lead citrate solution. Sections were analyzed with a transmission electron microscope (Zeiss EM 10; Zeiss).

**Immono Light/Immono Electron Microscopy**

For immunolabeling we used the pre-embedding method. After immersion fixation, EOMs including the tendons were rinsed in PBS containing 0.1% Tween 20. Then, specimens were frozen and thawed. To inhibit the endogenous peroxidase, wholemounts were incubated in 0.05 M Tris buffered saline pH 7.4 (TBS) containing 0.05% phenylhydrazine. After blocking in 10% rabbit serum, tissue was incubated in the primary antibody goat anti-ChAT (1:100) for 48 hours at 20°C followed by the secondary antibody horseradish peroxidase conjugated rabbit anti-goat (1:200; Chemicon International Inc., Temecula, CA). After antibody labeling was completed, wholemounts were immersion fixed in 2% PFA and 0.2% glutaraldehyde for superior preservation of ultrastructure. For enzymatic detection of horseradish peroxidase, wholemounts were incubated in 0.05 M tris buffered saline pH 7.4 (TBS) containing 0.05% phenylhydrazine, which inhibited the endogenous peroxidase. Conventional Electron Microscopy was used to analyze the morphology of palisade endings and the overall morphology of palisade endings became apparent. We observed nerve fibers that came from the muscle belly and extended for variable distances into the tendon. The diameter of these nerve fibers varied from 2 to 4 μm. Within the tendon the nerve fibers made a 180° loop and returned to the muscle. At the muscle-tendon junction, the returning axons divided into preterminal branches which surrounded single muscle fiber tips. Preterminal axons established nerve terminals in the tendon and around the muscle fiber tips. Such a neural specialization at the junction of a muscle fiber with the tendon represents the principle of a palisade ending. Palisade endings were usually supplied by a single axon with few exceptions in which two axons contributed to supply palisade endings (Figs. 1, 2, 3, and 4).

In immunolabeled EOM wholemounts, the nerve fibers supplying palisade endings were traced backward. In about one-fourth of the cases, we observed that the nerve fibers forming palisade endings established numerous delicate neuromuscular contacts outside the palisade/muscle fiber complex. Such additional neuromuscular contacts were described either on the same muscle fiber or on the neighboring muscle fiber (Figs. 2 and 3). In other cases, the axons supplying palisade endings came from far away and when we traced the axons, we observed that they intermingled with others. Within such an axonal bundle it was not possible to trace individual axons further. However, in such cases we did not observe other nerve fibers which establish nerve terminals on the muscle fibers associated with palisade endings.

**Control Experiments**

In negative controls, the primary antibodies were omitted and the secondary antibodies were used exclusively. In all cases, the omission of the primary antibodies resulted in a complete lack of immunostaining. To demonstrate the specificity of the cholinergic markers, positive controls were performed. Cryostat sections of monkey EOM muscle bellies were labeled with α-bungarotoxin and then either with antibodies against ChT, ChAT, or VACHT. Muscle fibers were counterstained with phalloidin. Examinations by CLSM demonstrated that α-bungarotoxin positive motor endplates were also positive for ChT, ChAT, and VACHT.

**RESULTS**

**Number of Palisade Endings**

We observed palisade endings in the distal myotendons of all types of EOMs. The number of palisade endings was counted in three medial recti and in three superior oblique. In the medial recti we counted between 50 and 73 palisade endings, and in the superior oblique, between 35 and 45. Generally, the number of palisade endings was higher in the rectus EOMs than in the oblique EOMs.

**Morphology of Palisade Endings**

**3D Reconstruction and Confocal Laser Scanning Microscopy.** In 3D reconstructions and immunolabeled wholemounts, the overall morphology of palisade endings became apparent. We observed nerve fibers that came from the muscle belly and extended for variable distances into the tendon. The diameter of these nerve fibers varied from 2 to 4 μm. Within the tendon the nerve fibers made a 180° loop and returned to the muscle. At the muscle-tendon junction, the returning axons divided into preterminal branches which surrounded single muscle fiber tips. Preterminal axons established nerve terminals in the tendon and around the muscle fiber tips. Such a neural specialization at the junction of a muscle fiber with the tendon represents the principle of a palisade ending. Palisade endings were usually supplied by a single axon with few exceptions in which two axons contributed to supply palisade endings (Figs. 1, 2, 3, and 4).

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**Conventional Electron Microscopy.** We analyzed the fine structure of 50 palisade endings in different EOMs. Muscle fibers associated with palisade endings had a capsule sleeve that continued forward into the tendon. The capsule consisted of two to five layers of fibrocytes. Nerve fibers forming palisade endings penetrated the capsule at the level of the tendon. In the tendinous compartment, the axon lost its perineural envelope and myelin sheath. The nerve fiber divided into preterminal axons, which were directed toward the muscle fiber tip. Preterminal axons were completely encircled by Schwann cells, which were covered with a basal lamina. Along their further course, preterminal axons exhibited repeated expansions that obviously represented nerve terminals on the neighboring collagen fibrils. These so-called neurotendinous contacts were only partly covered with Schwann cells, whereas in the Schwann cell free areas, only a basal lamina was interposed.
Wholemounts were labeled with phalloidin, anti-ChAT, and anti-VAChT; and phalloidin, anti-ChAT, and anti-ChT.

In wholemounts labeled with phalloidin, anti-ChAT, and α-bungarotoxin, we observed ChAT-immunoreactive nerve fibers forming palisade endings. In some cases, we detected that ChAT-positive nerve fibers forming palisade endings also established multiple nerve contacts on the same muscle fiber or neighboring muscle fiber. These additional neuromuscular contacts were ChAT/α-bungarotoxin-positive (Figs. 2A, 2B).

The palisade endings, including preterminal axons and palisade nerve terminals, were ChAT-positive. In about two-thirds of the palisade endings, we observed exclusively neurotendinous contacts which were ChAT-positive but α-bungarotoxin-negative (Fig. 2A). In about one-third of the palisade endings, we found additional neuromuscular contacts exhibited ChAT/α-bungarotoxin-reactivity (Figs. 2B, 2C). The ratio of palisade endings exhibiting neuromuscular contact matches our findings from TEM, in which 40% of the palisade endings had neuromuscular contacts.

In wholemounts labeled with phalloidin, anti-neurofilament, and anti-VAChT, we found neurofilament positive nerve fibers supplying palisade endings. We also observed additional neuromuscular contacts exhibiting VACHT-immunoreactivity either on the same or neighboring muscle fiber (Fig. 3C). Within the palisade ending, the nerve terminals were VACHT-immunoreactive as well (Figs. 3A–C).

In wholemounts labeled with phalloidin, anti-ChAT, and anti-ChT, we detected ChAT-positive nerve fibers forming palisade endings. Palisade nerve terminals exhibited ChAT/CHT-immunoreactivity (Fig. 4). Unfortunately, we did not find additional neuromuscular contacts due to the fact that nerve fibers forming palisade endings intermingled with others and could not be traced any further.

**Immuno Light Microscopy and Immuno Electron Microscopy.** We analyzed 30 palisade endings of different EOMs. By LM, ChAT-labeled axons were identified by the brown deposits of the DAB reaction product. By TEM, ChAT-immunoreactivity was identified by the electron-dense deposits of the DAB reaction product.

ChAT-positive axons formed palisade endings at the muscle fiber tips. In the tendinous compartment of the palisade endings, the ChAT-immunoreactive axons divided into preterminal axons sheathed by Schwann cells. Preterminal axons established ChAT-positive neurotendinous contacts that were partly invested with Schwann cells; at the point of contact, only a basal lamina was interposed between the axolemma and the collagen fibrils (Figs. 7, 8). As demonstrated by TEM, the internal structure of the neurotendinous contacts was partly masked by the dark DAB-reaction product. Whereas mitochondria were visible, clear vesicles were completely covered. ChAT-positive neurotendinous contacts were observed in each palisade ending. In 11 of 30 palisade endings, we observed ChAT-positive neuromuscular contacts as well. Such contacts were found on the finger-like muscle fiber processes attaching the muscle fiber to the tendon (Figs. 7, 8). Neuromuscular contacts had a basal lamina in the synaptic cleft (Fig. 8). Importantly, we did not find ChAT-negative nerve terminals in the palisade endings.

**Motor Terminals**

We analyzed the structural and molecular characteristics of motor terminals on singly innervated muscle fibers (SIFs) and multiply-innervated muscle fibers (MIFs) and compared them with palisade nerve terminals. Observations by TEM showed that en plaque motor endplates on SIFs and en grappe motor terminals on MIFs contained mitochondria and clear vesicles.
and had a basal lamina in the synaptic cleft. Whereas en plaque motor endplates exhibited deep folding of the subsynaptic membrane, en grappe terminals showed only shallow subsynaptic folding. The size of en plaque motor endplates varied between 30 and 50 μm and that of en grappe terminals between 5 and 8 μm. En plaque motor endplates and en grappe motor terminals bound α-bungarotoxin and immunohistochemically, they exhibited ChAT/VACHT/ChT reactivity (Figs. 9, 10). The morphologic and molecular characteristics of motor terminals on SLFs and MIFs and palisade nerve terminals are summarized in Table 2.

**DISCUSSION**

We have recently demonstrated in cats14,15 and monkeys11 that palisade endings are cholinergic. These novel findings have reopened the debate about the functional significance of palisade nerve terminals. (C) Two neurofilament-labeled nerve fibers running alongside a muscle fiber and establishing VACHT/neurofilament-positive neuromuscular contacts on a neighboring muscle fiber outside the palisade complex. Both nerve fibers supply a palisade ending on the same muscle fiber and also extend further into the tendon. Palisade nerve terminals are VACHT/neurofilament-positive. Scale bars, 100 μm.
Palisade endings and have advanced the question: Are palisade endings cholinergic sensory organs or effector organs involving collagen fibrils? We have extended our prior finding on palisade endings in monkeys. Herein, we show that nerve fibers supplying palisade endings establish ChAT/ChT/VAChT-immunoreactive neuromuscular contacts outside the palisade complex; that all palisade endings exhibit ChAT-immunoreactivity; and that neuromuscular contacts, when present in palisade endings, exhibit features of motor terminals and are α-bungarotoxin-positive as well. The implications of these findings with respect to the function of palisade endings are discussed below.

In line with our prior CLSM findings, we observed that ChAT-immunoreactive nerve fibers supply palisade endings in monkey and also establish multiple neuromuscular contacts outside the palisade complex. Such additional neuromuscular contacts were observed either on the same muscle fiber of the palisade/muscle fiber complex or—in a new finding—on a neighboring muscle fiber. Additional neuromuscular contacts were found in about 25% of the palisade endings, which is slightly less (30% of the palisade endings) than in our prior study on monkeys. Concordant with our prior study, we showed that additional neuromuscular contacts are ChAT-immunoreactive. Here, we further demonstrated that these contacts are VAChT-immunoreactive and, more importantly, α-bungarotoxin-positive as well. α-Bungarotoxin binding proves that additional neuromuscular contacts are motor; this finding has a direct consequence with respect to palisade endings. In fact, palisade endings arising from axons that supply motor neuromuscular contacts in other locations would have to be interpreted as motor as well.

By TEM, we demonstrated that each palisade ending in monkey establishes nerve terminals targeting collagen fibrils. In 40% of the palisade endings, we observed nerve terminals targeting the muscle fibers as well. Such neurotendinous and neuromuscular contacts contain mitochondria and clear vesicles. Neuromuscular contacts in palisade endings of monkeys usually have a basal lamina in the synaptic cleft, which is a defining feature of motor terminals. We observed that the basal lamina is discontinuous in only a few neuromuscular contacts. Such an interruption of the basal lamina was also detected in motor terminals on muscle fibers of rat EOMs. By immunohistochemistry, we confirmed our previous findings that neurotendinous and neuromuscular contacts in palisade endings of this primate species are ChAT/VACHT immunoreactive; here we demonstrated that they are ChT-immunoreactive as well. Neuromuscular contacts, when present in palisade endings, also exhibit α-bungarotoxin staining. The co-localization of ChT, ChAT, and VACHT demonstrates that neurotendinous and neuromuscular contacts in palisade endings contain all components for the synthesis of acetylcholine. α-Bungarotoxin-binding shows that neuromuscular contacts in palisade endings have nicotinic acetylcholine receptors. Applying morphologic and molecular criteria, the present study proves that neuromuscular contacts in palisade endings are definitely motor. In neurotendinous contacts of palisade endings, α-bungarotoxin staining, which is typical for motor terminals, is absent. On the other hand, we provided evidence that the palisade endings themselves are motor structures and in this case,
palisade neurotendinous contacts would have to be interpreted as motor as well.

Taken together, the present study confirms our prior findings that palisade endings are cholinergic and provides novel data clearly indicating that palisade endings are cholinergic motor and not cholinergic sensory.

A major argument to classify palisade endings as putative effectors is based on the finding that nerve fibers supplying palisade endings also supply motor neuromuscular contacts outside the palisade complex. Such additional neuromuscular contacts were observed in 25% of the cases, and in fact, it is a critical question whether all palisade endings have these contacts. To detect additional neuromuscular contacts, the nerve fibers supplying palisade endings had to be traced over a long distance, and often the axons intermingled with others and could not be traced any further. In such cases we did not

Figure 6. Micrographs from conventional transmission electron microscopy. (A) Micrograph at low magnification showing a cross-section through a palisade ending at the level of the muscle fiber/tendon attachment. The muscle fiber (MF) exhibits processes that are separated by COL. Palisade nerve terminals (T) contact the muscle fiber surface. (B, C) Micrographs at high magnifications showing cross-sections through T contacting the MF. A basal lamina (arrow) fills the synaptic cleft. The neuromuscular contacts contain mitochondria and clear vesicles. (B, inset) Detail of a neuromuscular contact. (D) Micrograph at high magnification showing a cross-section through a palisade nerve terminal contacting the muscle fiber with the synaptic cleft only partly filled with a basal lamina (arrow). Other areas of the synaptic cleft lack a basal lamina investment (arrowheads). Scale bar: (A) 10 μm; (B, C, D) 1 μm.

Figure 7. Micrographs from immuno light microscopy. Nerve fibers and nerve terminals are labeled with anti-ChAT. After the DAB-reaction, ChAT-positive neurons appear brown in color. (A) Semithin cross-section through the tendinous compartment of a palisade ending showing ChAT-positive preterminal axons (arrowbeads) and a ChAT-positive palisade nerve terminal (arrow). (B) Semithin longitudinal section through a palisade ending. Palisade nerve terminals (arrowbeads) contacting the collagen fibrils are visible. Other palisade nerve terminals (arrow) contact the muscle fiber processes that attach the MF to the tendon. On a neighboring muscle fiber a ChAT-positive motor terminal (asterisk) is visible. Scale bars, 10 μm.
such a construction could not be confirmed represent another population of palisade endings. If this were indeed the case, we would have to distinguish between two categories of palisade endings.

With respect to innervation, EOMs in mammals have two kinds of muscle fibers, SIFs and MIFs. SIFs have a single motor endplate (en plaque ending), whereas MIFs receive multiple neuromuscular contacts (en grappe endings) throughout their length.32-35 Palisade endings are exclusively found on the tip of a particular muscle fiber that is the MIF of the global EOM layer.12,21,25 There can be no doubt that the additional neuromuscular contacts ascertained in palisade endings of the present study are en grappe endings. We therefore conclude that palisade endings arise from axons that also supply MIFs. Interestingly, palisade endings were either found on the tip of the same muscle fiber innervated by the MIF motoneurons or, alternatively, on a neighboring muscle fiber, indicating a variability of the MIF motoneuron/palisade ending unit.

To date, physiological investigations on palisade endings are completely missing; their morphology, however, is well described. Ultrastructural investigations which focused on palisade nerve terminals have presented anatomic evidence to classify palisade endings as sensory organs. Specifically, in palisade endings of cats,12,15 sheep,18 and humans,29 and in Ruskell’s10 earlier study on palisade endings in rhesus monkeys, neurotendinous contacts were constantly observed and, despite the presence of clear vesicles, nerve terminals in apoposition to collagen fibrils seem to point to a sensory function. With the exception of humans, neuromuscular contacts in palisade endings of these species lack a basal lamina in the synaptic cleft, a feature common with sensory nerve terminals on intrafusal muscle fibers of muscle spindles.34-37 Surprisingly, there are differences between Ruskell’s10 findings and ours with respect to neuromuscular contacts in palisade endings of monkeys. In Ruskell’s study,10 neuromuscular contacts appear morphologically sensory-like, whereas in the present study neuromuscular contacts are morphologically motor-like, which is confirmed by α-bungarotoxin binding. By TEM, we analyzed 50 palisade endings of different EOMs in detail and it is extremely unlikely that we missed morphologically sensory-like neuromuscular contacts. At the moment, the discrepancies between Ruskell’s10 and our study regarding neuromuscular contacts in palisade endings of the same species are not explainable.

The most compelling argument that palisade endings are sensory structures has come from Billig et al.15 who injected neuronal tracer into the trigeminal ganglion, which is presumed to exclusively contain cell bodies ofafferent nerve fibers. In cats, Billig et al.15 found three kinds of labeled nerve endings, one resembling palisade endings. Recently, Wang et al.26 provided indirect evidence that palisade endings are sensory. Using rhesus monkeys, Wang and colleagues26 recorded eye position signals from the contralateral side in the primary somatosensory cortex. Since muscle spindles and Golgi tendon organs are rare or absent in monkey EOMs and palisade endings are numerous, the authors concluded that the signals arise from the palisades.5,26,58

Despite the impressive support in the literature that palisade endings are sensory organs, the present report challenges this view for three reasons. First, nerve fibers supplying palisade endings also supply motor neuromuscular contacts. Second, palisade endings contain acetylcholine, the neurotransmitter of motor terminals. Third, neuromuscular contacts in palisade endings are endowed with nicotinic acetylcholine receptors, which are otherwise present in motor terminals. Indication that palisade endings are putative effectors was
provided by α-bungarotoxin binding in palisade endings of humans and cats. Specifically, in palisade endings of humans, neuromuscular contacts are α-bungarotoxin-positive, and in palisade endings of cats, the sparse neuromuscular contacts are α-bungarotoxin positive as well. Further evidence that palisade endings are putative effectors comes from a nerve degeneration experiment. Sas and Schab made lesions of the oculomotor nuclei and showed that in addition to the expected loss of motor terminals on the EOMs, palisade endings were degenerated. The authors concluded that palisade endings are supplied by axons which originate from the EOM motor nuclei.

**Functional Considerations**

In the present study, we ascertained that palisade endings are supplied by axons that also supply MIFs. The palisade endings are associated with the same muscle fibers innervated by MIF motoneurons or, alternatively, with neighboring muscle fibers. MIF motoneurons establish en grappe endings and on activation, such motoneurons induce local contractions of the muscle fiber at the site of the nerve terminals. But what can be the function of palisade endings arising from MIF motoneurons?

On activation, MIF/palisade motoneurons would excite en grappe endings as well as palisade endings, including palisade neurotendinous contacts and, when present, palisade neuromuscular contacts at the muscle fiber tip. After neurotransmitter release, en grappe endings would elicit focal contractions of the muscle fiber body, whereas palisade neuromuscular contacts would elicit contraction of the most terminal part of the muscle fiber, either of the same or neighboring muscle fiber. Neurotendinous contacts in palisade endings are surrounded by collagen fibrils and it is unclear what an effect neurotransmitter release could have on the tendon. With respect to their position, palisade neurotendinous contacts lie in serial to the muscle fibers, which is analogous to nerve terminals in Golgi tendon organs. Thus, neurotendinous contacts in palisade endings are ideally located to register muscle fiber contraction and could, theoretically, function as sensory endings. However, in this case the nerve fibers supplying palisade endings would have to conduct information in two directions: from the central nervous system to the palisade neuromuscular contacts (efferent) and, in the opposite direction, from palisade neurotendinous contacts to the central nervous system (afferent). In fact, such an idea is highly speculative and at present it is too early to determine the functional sense of palisade endings. Nevertheless, based on their frequency, morphology, and molecular characteristics, there can be no doubt that palisade endings are functional.

It is unlikely that palisade endings receive a double innervation arising from a motor and a sensory nerve fiber. First, the
The present study shows that, with few exceptions, palisade endings are supplied by a single axon. Second, by double-labeling of palisade endings with a general marker for nerve fibers (antibody against neurofilament) and a marker for cholinergic nerve fibers (antibody against ChAT) all axons of the palisade complexes exhibit ChAT-immunoreactivity. Third, by immunoelectron microscopy, we observed that all palisade nerve terminals are ChAT-positive. Fourth, by labeling palisade endings with substance P, which is present in some sensory axons, no staining was detected at all in palisade endings of humans, monkeys, and rats. Afferent signals from mammalian EOMs reach several regions within the central nervous system, including the superior colliculus, the lateral geniculate body, the pulvinar of the thalamus, the tegmentum, the vestibular nucleus, the nucleus prepositus hypoglossi, the cerebellum, Brodman area 17 and 18, the Clare Bishop area, the frontal cortex, and the somatosensory cortex. Muscle spindles and Golgi tendon organs are absent in the EOMs of most mammalian species, whereas palisade endings, so far, have been found in each species investigated. Therefore, it has been suggested that palisade endings could be the source of proprioceptive input to the central nervous system. The findings of the present study call this view into question. Palisade endings are present in human EOMs and due to their localization at the myotendinous junction, a finding of particular interest for strabismus surgeons. Observations of patients after strabismus surgery indicate alteration in spatial perception, and it is supposed that the damage of the palisade endings during the surgical procedure could be the reason. The results of the present study could indicate that surgical procedures to treat strabismus would have no side effects with respect to eye position signals.

TABLE 2. Morphological and Molecular Characteristics of Palisade Nerve Terminals and Motor Nerve Terminals on Singly-innervated Muscle Fibers (SIFs) and Multiply-innervated Muscle Fibers (MIFs)

<table>
<thead>
<tr>
<th></th>
<th>Palisade Nerve Terminals</th>
<th>Motor Terminals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neurotendinous Contacts</td>
<td>Neuromuscular</td>
</tr>
<tr>
<td>Clear vesicles</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basal lamina in the synaptic cleft</td>
<td>−*</td>
<td>+</td>
</tr>
<tr>
<td>ChAT/ChAT/VChAT reactivity</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Bungarotoxin-reactivity</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Motor Terminals on SIFs

Motor Terminals on MIFs

* Neurotendinous contacts are covered by a basal lamina, but are not separated by a synaptic cleft from a neighboring structure.
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References


