Palisade Endings Have an Exocytotic Machinery But Lack Acetylcholine Receptors and Distinct Acetylcholinesterase Activity

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RB and AMP designed research. RB performed whole-mount immunostaining and transmission electron microscopy. All authors analyzed data. RB wrote manuscript. All authors made critical revision of the manuscript.

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Purpose. The purpose of this work was to test whether palisade endings express structural and molecular features of exocytotic machinery, and are associated with acetylcholine receptors, and enzymes for neurotransmitter breakdown.

Methods. Extraocular rectus muscles from six cats were studied. Whole-mount preparations of extraocular muscles (EOMs) were immunolabeled with markers for exocytotic proteins, including synaptosomal-associated protein of 25 kDa (SNAP25), syntaxin, synaptobrevin, synaptotagmin, and complexin. Acetylcholine receptors (AChRs) were visualized with α-bungarotoxin and with an antibody against AChRs, and acetylcholinesterase (AChE) was tagged with anti-AChE. Molecular features of multicolor labeled palisade endings were analyzed in the confocal scanning microscope, and their ultrastructural features were revealed in the transmission electron microscope.

Results. All palisade endings expressed the exocytotic proteins SNAP25, syntaxin, synaptobrevin, synaptotagmin, and complexin. At the ultrastructural level, vesicles docked at the plasma membrane of terminal varicosities of palisade endings. No AChRs were associated with palisade endings as demonstrated by the absence of α-bungarotoxin and anti-AChR binding. AChE, the degradative enzyme for acetylcholine exhibited low, if any, activity in palisade endings. Axonal tracking showed that axons with multiple en grappe motor terminals were in continuity with palisade endings.

Conclusions. This study demonstrates that palisade endings exhibit structural and molecular characteristics of exocytotic machinery, suggesting neurotransmitter release. However, AChRs were not associated with palisade endings, so there is no binding site for acetylcholine, and, due to low/absent AChE activity, insufficient neurotransmitter removal. Thus, the present findings indicate that palisade endings belong to an effector system that is very different from that found in other skeletal muscles.

Keywords: eye muscles, proprioception, palisade endings, SNARE proteins

Afferent (proprioceptive) signals coming from extraocular muscles (EOMs) have been identified in different regions of the brain,[1,2] including the somatosensory cortex[3] and sensorimotor cortex,[4] so it is assumed that EOM proprioception is of value for visually guided behavior. Surprisingly, the classical proprioceptor pair (muscle spindles and Golgi tendon organs) is absent in the EOMs of most mammals and only found in even-toed ungulates (pig, cow, and sheep) where they occur in high numbers.[5] Muscle spindles with simplified morphology are present in human EOMs but not Golgi tendon organs. In other mammalian species (cat, dog, rat, guinea pig, and rabbit) these classical proprioceptors are lacking.[5–7] The reason for these interspecies variations remains unclear and to date there is no evolutionary and/or functional explanation for their pattern of occurrence.

A peculiar axonal termination named palisade endings has been found in the EOMs of all mammalian groups analyzed so far, with the exception of rodents.[8–11] Based on this wide distribution, it has long been suggested that palisade endings might substitute for classical proprioceptors.[12,13]

Palisade endings are exuberant axon terminations at the muscle-tendon junction of EOMs. Axons supplying palisade endings come from the muscle and enter the tendon where they then turn back to establish terminal varicosities around individual muscle fiber tips. The muscle fibers associated with palisade endings possess several en grappe motor terminals along their course and are consequently classified as multiply innervated muscle fibers (MIFs) as opposed to singly innervated muscle fibers, which have a large single endplate (en plaque motor terminal) in the middle third of

the muscle.[10,11] Muscle fibers displaying multiple endplates have been recently described in EOMs of humans, even though it is not known whether they exist in other species.[14] For many years, there has been consensus that palisade endings are sensory structures because of their similarities to sensory Golgi tendon organs. However, their sensory role was put into question when more recent studies in cats and monkeys showed that palisade endings are cholinergic and originate from the oculomotor nuclei, most likely from MIF motoneurons.[15–17] Other studies suggest that palisade endings might arise from a separate population of neurons in the oculomotor nuclei with morphology reminiscent of sensory pseudounipolar neurons.[18,19] So far, no receptors for cholinergic transmission (i.e. acetylcholine receptors [AChR]), have been found on muscle fibers opposed to palisade endings in investigated species with the exception of rabbits and rats,[20] whose palisade endings, however, have a simplified morphology. Taken together, motor features seem to dominate palisade endings but their functional role is still not clear.

A hallmark of palisade endings are clear vesicles inside their terminal varicosities suggesting the storage of neurotransmitter, potentially acetylcholine, because palisade endings express acetylcholine transferase (ChAT), vesicular acetyl choline transporter, and choline transporter.[21] It is, however, unknown whether palisade endings have the necessary exocytotic machinery required for neurotransmitter release in response to neuronal activity. Key proteins of exocytosis include synaptobrevin on the vesicle membrane, and synaptosomal-associated protein 25 kDa (SNAP-25) and syntaxin that form the SNARE complex on the presynaptic membrane.[22] Synaptotagmin, a Ca^{2+} sensing protein on the vesicle membrane, triggers vesicle fusion,[23] and finally complexin, a small cytoplasmic protein plays a role in vesicle release.[24] The present study set out to examine whether these core proteins of the exocytotic machinery are expressed in palisade endings. Additionally, we examined whether palisade endings are associated with AChRs by using α-bungarotoxin and an antibody against AChRs, and whether acetylcholine esterase (AChE), the degradative enzyme for acetylcholine is expressed in association with palisade endings. Here, we show that palisade endings have a molecular machinery for neurotransmitter (acetylcholine) release, but lack AChR and exhibit only low or no AChE activity.

Materials and Methods

Animals

In the present study six adult cats were analyzed. Handling procedures for experiments followed the guidelines of the National Institutes of Health (NIH; http:/oacu.od.nih.gov), 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 (R.D. 53/2013, BOE 34/11370-421, 2013).

Tissue Preparation

Cats were deeply anesthetized with a terminal dose of sodium pentobarbital (50 mg/kg, IP) and intracardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Then, their eyeballs, including the EOMs, were dissected free. From every animal the four rectus muscles of both sides were collected. Muscles were cut transversally into two pieces: one piece containing the muscle belly and the other the distal part of the muscle with the attached tendon. The distal EOM myotendons contained predominantly multiply innervated muscle fibers, because it is supposed that only multiply innervated muscle fibers are attached to the distal tendon, whereas singly innervated muscle fibers are shorter and do not reach the distal tendon.[25,26] Muscle bellies were cryoprotected in graded concentrations of sucrose (10%, 25%, and 40%) in sodium phosphate-buffered saline (PBS). Afterward, preparations were embedded using cryomatrix (Thermo Fisher Scientific, Waltham, MA, USA), frozen in ice-cooled 2-methylbutane and stored at −80°C. The distal EOM myotendinous junctions of the rectus muscles were stored at 4°C in PBS containing 0.05% sodium azide to avoid bacteriological affection and underwent whole-mount preparation. EOM muscle bellies and distal myotendinous junctions of five cats were used for multicolor immunofluorescence. The distal myotendinous junction of the rectus muscles (n = 8) from one cat, were processed for transmission electron microscopy.

Antibodies and Toxins

Different neuronal markers and toxins for labeling nerve fibers, synaptic proteins, and muscle fibers were used in the present study. The primary antibodies/toxins including working dilutions, RRID numbers, and suppliers are listed in the Table.

Immunofluorescence

Control Experiments Using Double-Immunofluorescence. The validity of the markers for SNAP25, synaptobrevin, synaptotagmin, syntaxin, complexin, and AChE was tested on the motor endplates of EOMs muscle bellies. Following cryosectioning of the EOM muscle bellies at 10 µm thickness, motor endplates were visualized with fluorescently tagged α-bungarotoxin, a snake neurotoxin that binds to AChR. An α-bungarotoxin incubation was combined with antibodies against SNAP25, synaptobrevin, syntaxin, synaptotagmin, complexin, or AChE. For immunostaining, sections were blocked for 1 hour with 10% normal goat serum followed by incubation with one of the primary antibodies for 48 hours at 4°C. After washing in PBS containing 0.1% Triton X-100, sections were incubated with Alexa fluor 488 conjugated secondary antibodies along with rhodamine-conjugated α-bungarotoxin for 2 hours at 37°C. Sections were rinsed again and coverslipped with fluorescence mounting medium.

Triple-Immunofluorescence. A series of triple-labeling experiments were performed in EOM whole-mount preparations (n = 40) of the distal muscle-tendon junctions: (1) to visualize the overall innervation of palisade endings, we used anti-neurofilament protein (a general marker for nerve fibers) and anti-synaptophysin (a marker for vesicles in nerve terminals). In this and other staining combinations (2, 3, 5, 6), phalloidin (a marker for actin filaments) was used to counterstain muscle fibers. (2) To distinguish between non-cholinergic and cholinergic nerve fibers/nerve terminals, we performed incubation with anti-neurofilament protein or anti-synaptophysin in combination with

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Table. List of Markers Used in the Present Study Including Working Dilution, RRID Number, and Suppliers

Markers	Working Dilution	RRID Number	Supplier
Presynaptic markers			
Rabbit anti-syntaxin	1:500	AB_887900	Synaptic Systems
Rabbit anti-complexin	1:500	AB_2619793	Synaptic Systems
Rabbit anti-synaptophysin	1:300	AB_887905	Synaptic Systems
Mouse anti-synaptophysin	1:300	AB_95786	Merck/Millipore
Mouse anti-synaptobrevin	1:100	AB_2272637	Merck/Millipore
Mouse anti-synaptotagmin	1:250	AB_10013783	Zebrafish International Resource Center
Mouse anti-SNAP25	1:500	AB_510034	Covance
Marker for neurotransmitter depletion			
Mouse anti-acetylcholinesterase	1:200	AB_11213984	Merck/Millipore
Postsynaptic markers			
Rat anti-nicotinic acetylcholine receptor	1:500	AB_260473	Sigma-Aldrich
α-bungarotoxin	1:500		Thermo Fisher
Markers for axons			
Chicken anti-neurofilament	1:2000	AB_177520	Merck/Millipore
Goat anti-choline acetyltransferase	1:100	AB_2079751	Merck/Millipore
Marker for muscle			
Phalloidin	1:200		Sigma-Aldrich

anti-ChAT (a marker for cholinergic axons). (3) For detection of molecules involved in exocytosis, we used antibodies against SNAP25, synaptobrevin, synaptotagmin, syntaxin, or complexin in combination with anti-ChAT or anti-neurofilament protein, respectively. (4) To verify whether synaptobrevin/synaptotagmin colocalize with the synaptic vesicle marker synaptophysin, anti-synaptobrevin/anti-synaptophysin and anti-synaptotagmin/anti-synaptophysin along with anti-neurofilament protein immunolabeling was performed. In these staining combinations, the anatomic structure of the tissue was visualized using bright field images. (5) AChR were visualized by α-bungarotoxin or alternatively by antibody against AChR and staining combinations anti-ChAT/α-bungarotoxin and anti-ChAT/anti-AChR were performed. (6) To visualize AChE, the degradative enzyme for acetylcholine, anti-AChE along with anti-neurofilament protein immunolabeling was performed. Two to three rectus muscles were treated with the staining combinations 1, 2, and 4. Staining combinations 3, 5, and 6 were performed in all 4 rectus muscles.

Prior to immunolabeling, EOM whole-mounts were shock frozen in ice cold 2-methylbutane at −80°C and immediately thawed. Afterward, tissue was incubated overnight in PBS containing 1% Triton X-100 (PBS-T) at room temperature. The tissue was blocked for 2 hours with 10% normal goat serum (staining combinations 1, 3 [staining with anti-neurofilament protein along with anti-syntaxin anti-complexin, anti-synaptophysin, anti-synaptobrevin, and synaptotagmin], 4, and 6) or alternatively with 10% normal rabbit serum (staining combinations 2, 3 [staining anti-ChAT along with SNAP25, synaptobrevin, and synaptotagmin,] and 5) in PBS-T. Then the tissue was incubated for 48 hours with the primary antibodies. Following extensive washing in PBS-T, tissue was incubated for 6 hours with secondary antibodies and phalloidin, or with secondary antibodies along with α-bungarotoxin and phalloidin (staining combination 5). Finally, the tissue was rinsed again and mounted in *v/v* 60% glycerin + 40% PBS. Primary antibodies were applied at 4°C, secondary antibodies, phalloidin, and α-bungarotoxin at 37°C. Alexa Fluor 488, 568, and 647-conjugated secondary antibodies were used at a concentration of 1:500 and Alexa Fluor 647-conjugated phalloidin at a concentration of 1:150.

For negative controls, 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.

Data Analyses of Immunofluorescence

Fluorescently labeled muscle belly sections and EOM whole-mounts were analyzed with a confocal laser scanning microscope (CLSM; Olympus FV3000, Olympus Europa SE & Co. KG, Hamburg, Germany). A series of virtual CLSM sections of 1 µm thickness were cut through the structures of interest. Each section was photo-documented with a 1024 × 1024 pixel resolution and 3D projections were rendered using Image J software (National Institutes of Health [NIH], Bethesda, MA, USA). Double-colored images (Fig. 1) were generated using lasers with excitation wavelength 488 and 568 nm and triple-colored images (Figs. 2A, 2B and 3–8) were generated using an addition laser with an excitation wavelength 633 nm. In some cases, bright field images were recorded in the CLSM to correlate immunolabeling structures to morphological structures (Figs. 2B and 5E-E", F-F").

Transmission Electron Microscopy

Following perfusion fixation, the rectus muscles of one cat were dissected and immersion fixed in modified Karnovsky solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS at pH 7.4 for 24 hours at 4°C. After rinsing in PBS, tissue was postfixed in 1% osmium tetroxide in PBS for 12 hours at 4°C, dehydrated in graded dilutions of ethanol, and embedded in epon. Semithin sections of epon-embedded tissue were cut with an Ultracut UCT (Leica, Wetzlar, Germany), and stained with toluidine blue and viewed under the light microscope. When structures of interest were detected at light microscopic level, ultrathin sections were cut, mounted on dioxane-formvar coated copper grids, immersed in an aqueous solution containing 2% uranyl acetate followed by a solution

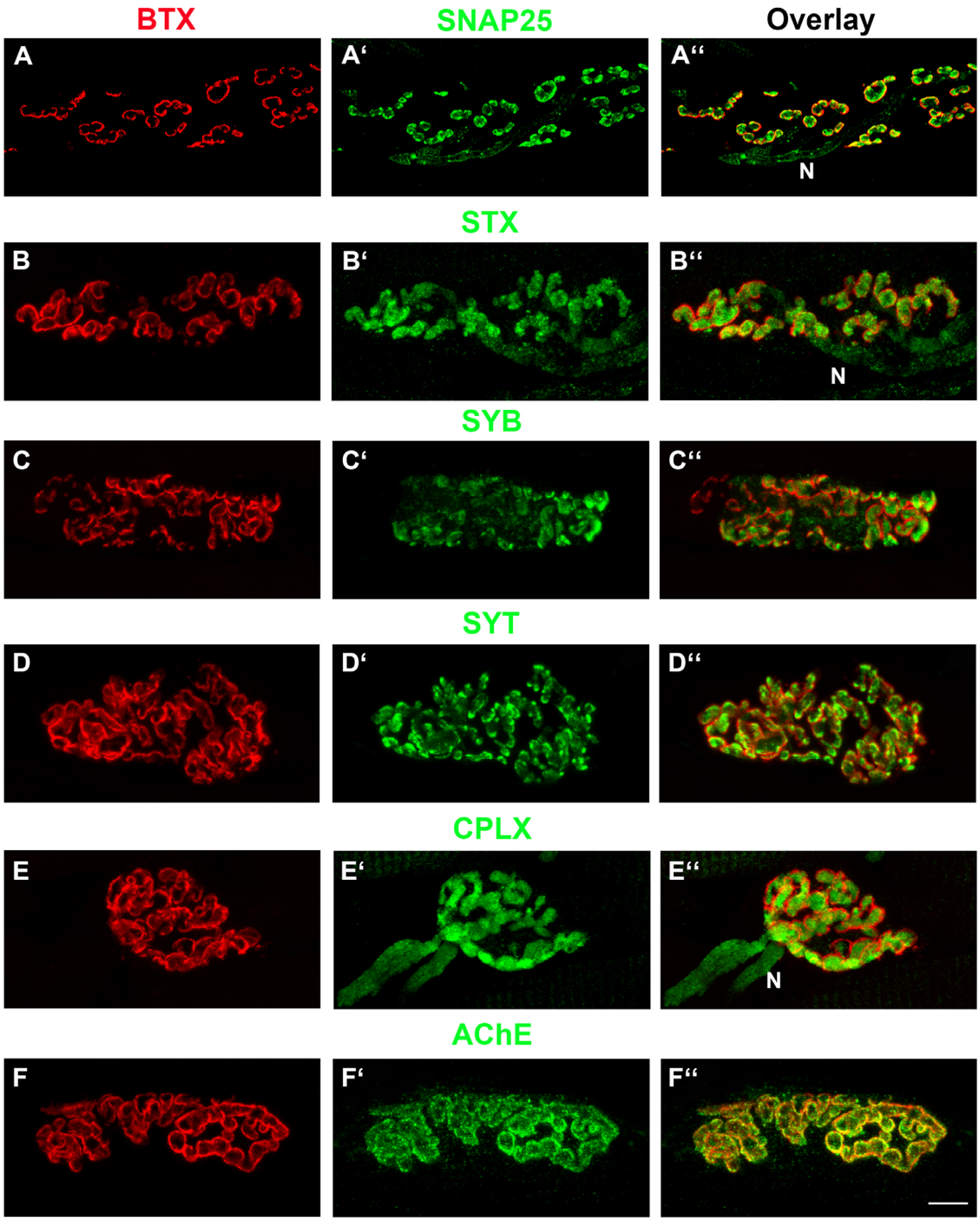

Figure 1. CLSM images of immunofluorescently labeled en plaque motor terminals which served as reference structure for the validity of the antibodies used in the present study. (**A–F**) Showing en plaque motor terminals labeled with α-bungarotoxin (BTX) and **A'-F'**, with antibodies against SNAP25 (**A'**), syntaxin (STX, **B'**), synaptobrevin (SYB, **C'**), synaptotagmin (SYT, **D'**), complexin (CPLX, **E'**), and acetylcholinesterase (AChE, **F'**). (**A"–F"**) Overlays of α-bungarotoxin staining and immunolabeling showing that α-bungarotoxin-positive motor terminals expressed SNAP25 (**A"**), syntaxin (**B"**), synaptobrevin (**C"**), synaptotagmin (**D"**), complexin (**E"**), and AChE (**F"**) as well. Areas with superimposition of the *red* and *green color* appear in *yellow color*. There is not complete overlap between α-bungarotoxin and antibody signals (**A"–E"**). This is because SNAP25, syntaxin, synaptobrevin, synaptotagmin, and complexin are located at the presynaptic site whereas α-bungarotoxin binds to acetylcholine receptors at the postsynaptic site. In panel **F"**, there is more co-localization between α-bungarotoxin and AChE because AChE is concentrated in the synaptic cleft, very close to postsynaptic AChRs. In addition to en plaque motor terminals, SNAP25 (**A', A"**), syntaxin (**B', B"**), and complexin (**E', E"**) are also enriched in axons (N) supplying motor terminals. Scale bar: 10 µm.

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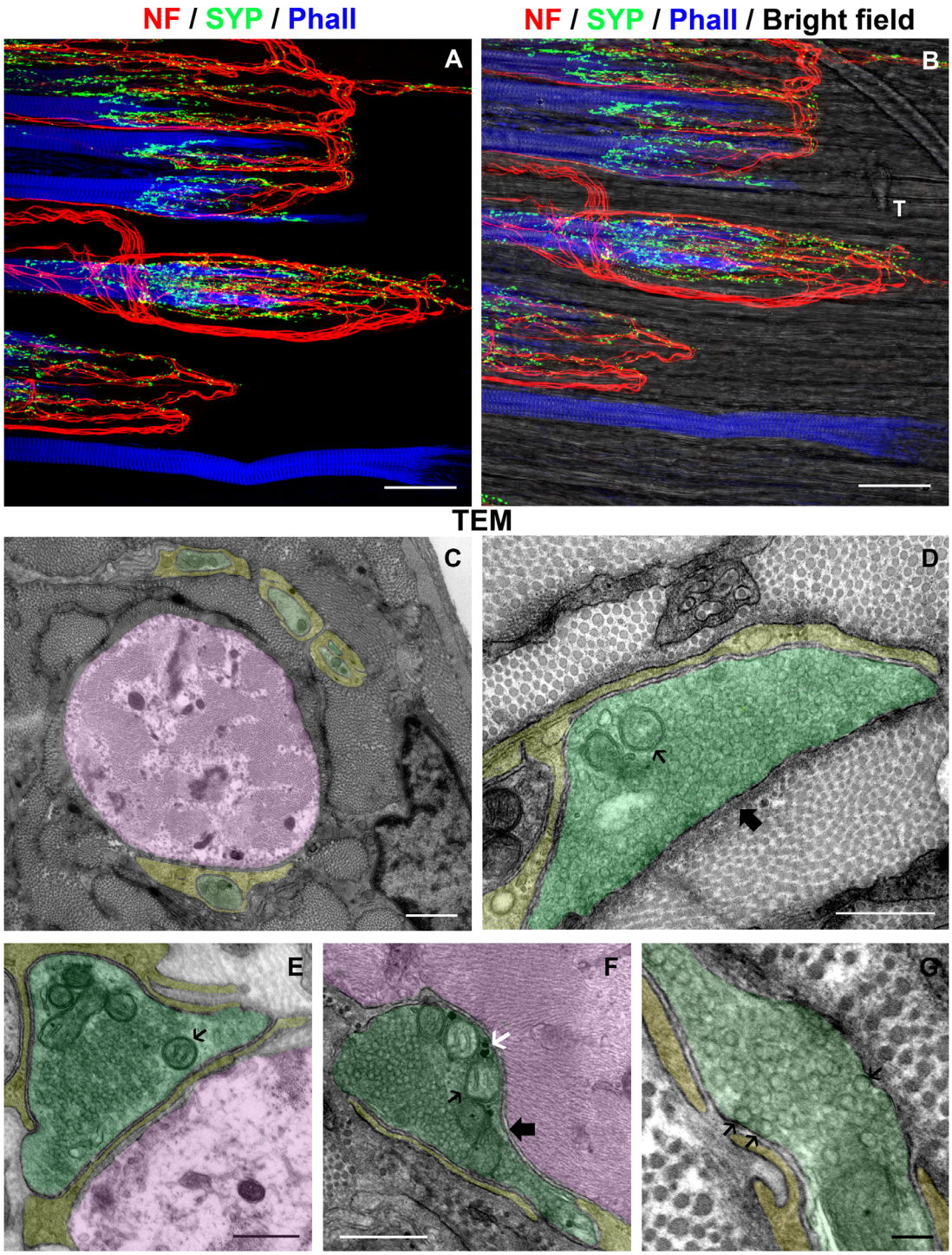

Figure 2. Morphological features of palisade endings at CLSM and TEM. (**A, B**) The 3D projection of immunofluorescently labeled palisade endings viewed in the CLSM. Immunolabeling with anti-neurofilament protein (NF, *red*) and anti-synaptophysin (SYP, *green*). Muscle fibers are counterstained with phalloidin (Phall, *blue*). The tendon is not labeled and continues to the right from the muscle fibers (**A**). The tendon (T) is visualized in the image that includes a bright field overlay (**B**). Neurofilament protein-positive nerve fibers coming from the muscle penetrate the tendon where they turn back to form palisade endings on individual muscle fiber tips. Palisade endings establish terminal varicosities which are synaptophysin immunoreactive. Terminal varicosities appear in *yellow color* and where the neurofilament protein signal is weak, they appear in *green color*. Terminal varicosities are serially arranged along axons and are found at the level of the tendon and muscle fiber tips. Scale bars: 50 µm. (**C–G**) TEM images of palisade endings. Muscle fibers are highlighted in *magenta*, Schwann cells in *yellow*, and nerve terminals in *green*. (**C**) A cross section at the level of the muscle fiber tip show a muscle fiber tip surrounded by preterminal axons, which are completely enveloped by Schwann cells. The collagen fibrils of the tendon appear as *gray dots*. (**D–G**) Terminal varicosities in palisade endings have a few mitochondria and are otherwise full of clear vesicles. (**D**) The terminal varicosity at the tendon level is associated with the collagen fibrils and only partly invested by a Schwann cell. In the Schwann cell free area, a basal lamina (*thick arrow*) covers the axolemma. Mitochondria (*small arrow*). (**E**) A terminal varicosity close to the muscle fiber is separated from it by Schwann cell

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processes. Mitochondria (*small arrow*). (**F**) A terminal varicosity contacting the muscle fiber. The synaptic cleft (*thick black arrow*) is free from a basal lamina. In addition to clear vesicles, a few dark granules (*white arrow*) are visible. Mitochondria (*small black arrow*). (**G**) Examples of vesicles (*thin black arrows*), which are docked at the axolemma of a terminal varicosity. Scale bars: 1 µm **C**, 500 nm **D–F**, and 100 nm **G**.

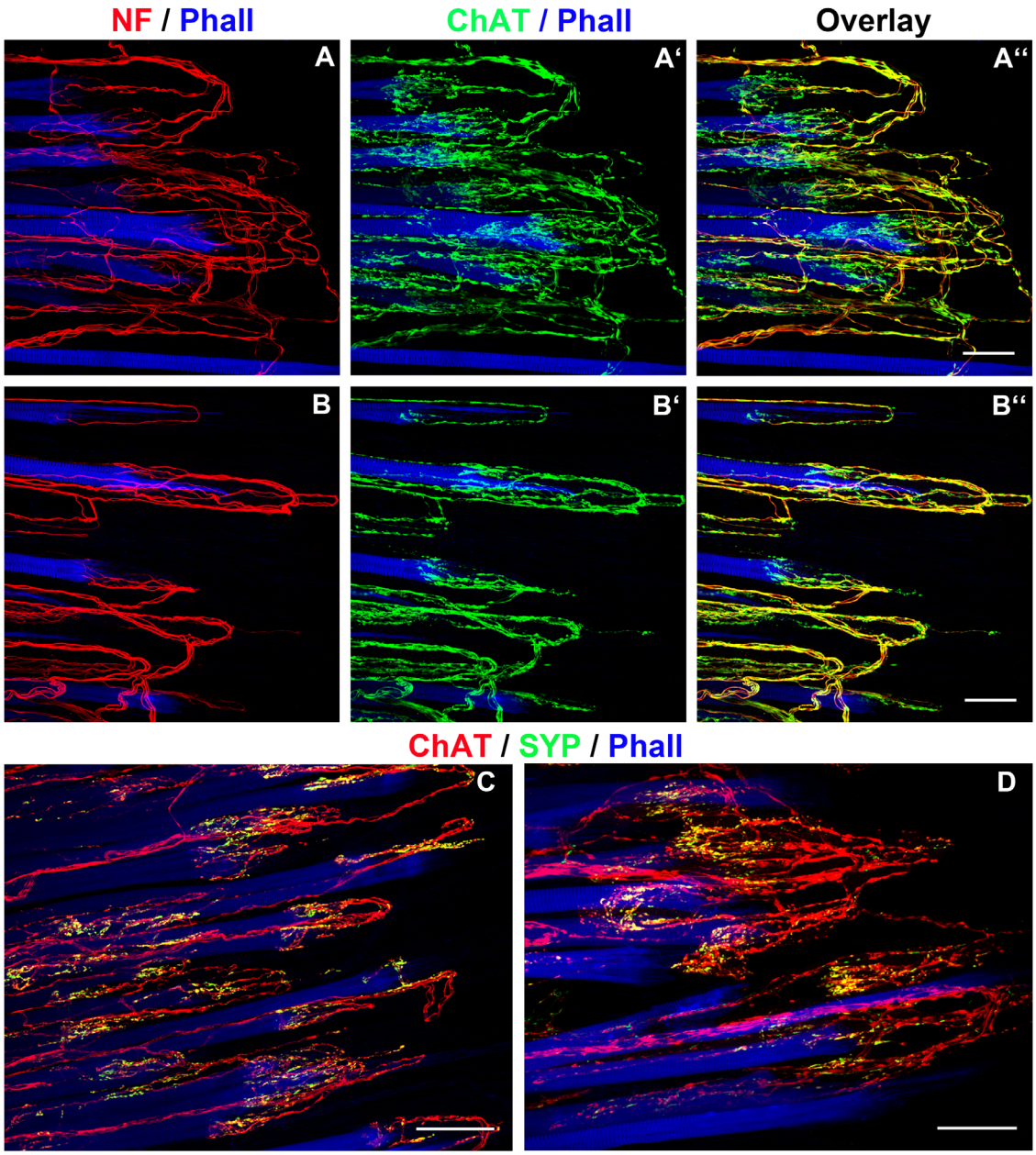

Figure 3. The 3D projection of CLSM images showing that palisade endings are cholinergic. (**A–A”, B- B”**) Palisade endings are immunolabeled with anti-neurofilament protein (NF, *red*) and anti-ChAT (*green*). The tendon is not visible, but extends to the right of the phalloidin (Phall, *blue*) labeled muscle fibers. Showing palisade endings from medial rectus (**A–A”**) and inferior rectus muscle (**B–B”**) in neurofilament protein staining (**A, B**) and ChAT staining (**A’, B’**). Labeling with anti-ChAT shows that the staining intensity varies and axonal sections with low and high ChAT signals alternate (**A’, B’**). (**A”, B”**) Overlays of neurofilament protein and ChAT immunoreactivity showing that all neurofilament protein-positive palisade endings are ChAT-positive. However, due to the variations of the ChAT signal intensity, axonal sections with low ChAT intensity appear in *reddish-yellow color*, whereas axonal parts with high ChAT intensity appear in *yellow color*. Terminal varicosities of palisade endings around muscle fiber tips are only faintly stained with anti-neurofilament protein but show intense ChAT signals. They appear in *green-yellow color*. Scale bars: 50 µm. (**C, D**) Palisade endings are immunolabeled with anti-ChAT (*r*ed) and anti-synaptophysin (SYP, *green*), and are shown in an inferior rectus muscle at lower magnification **C** and a medial rectus muscle at high magnification **D**. ChAT-positive nerve fibers forming palisade endings establish terminal varicosities, which are ChAT/synaptophysin immunoreactive. Co-localization of ChAT and synaptophysin results in *yellow color*. Muscle fibers are labeled with palloidin. Scale bars: 100 µm **C** and 50 µm **D**.

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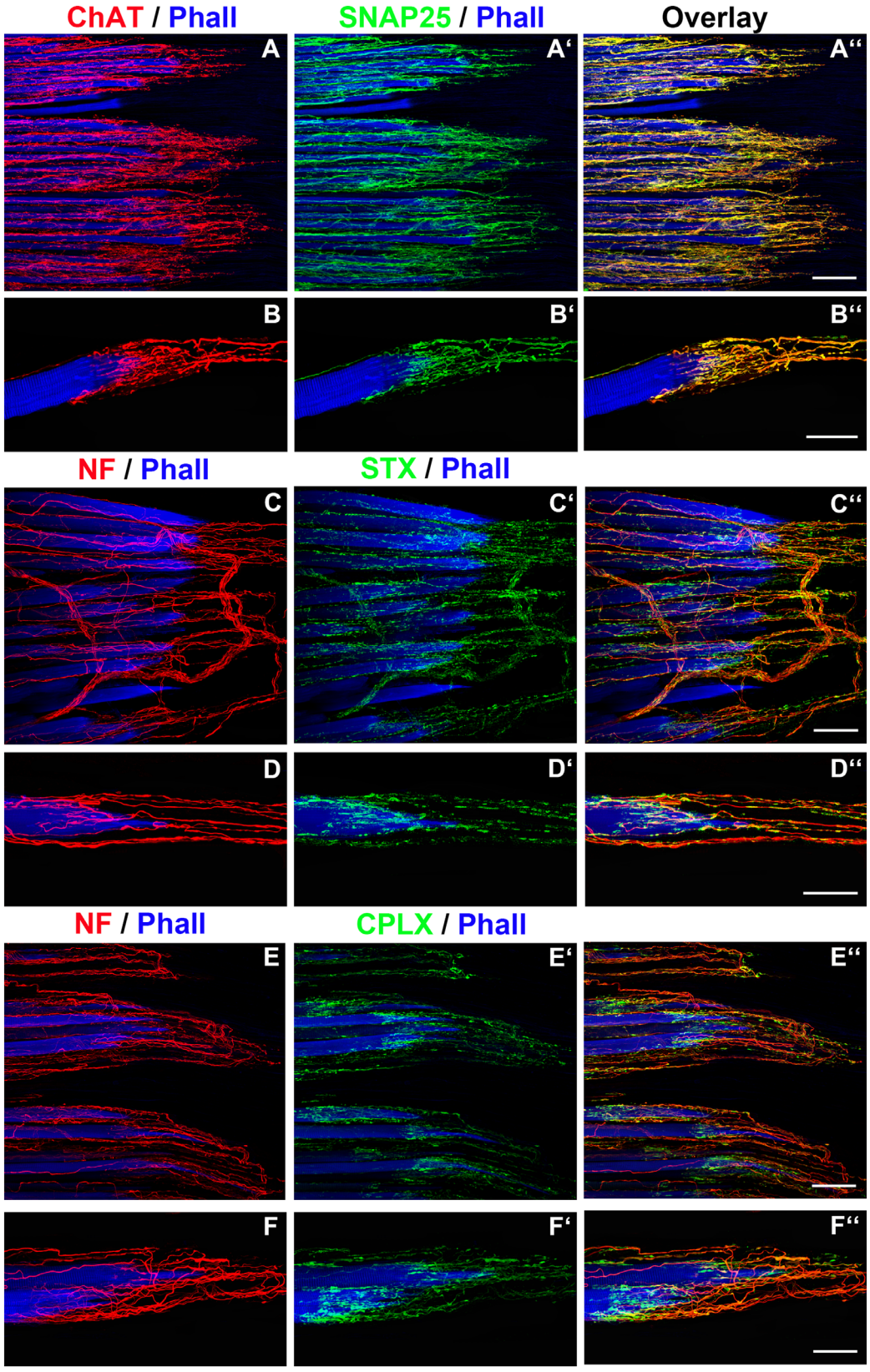

Figure 4. The 3D projections of CLSM images at lower (**A-A"**, **C-C"**, **E-E"**) and higher magnification (**B–B", D–D", F–F"**) showing that palisade endings express SNAP25, syntaxin, and complexin. (**A–A", B–B"**) Immunolabeling with anti-ChAT (*red*) and anti-SNAP25 (*green*). The tendon which is not visible, continues to the right of the phalloidin (Phall, *blue*) labeled muscle fibers. Palisade endings shown in ChAT (**A, B**) and SNAP25 (**A', B'**) staining. SNAP25 immunoreactivity is also present throughout axons forming palisade endings. (**A", B"**) Overlay shows co-labeling of ChAT/SNAP25. However, due to variation of the ChAT and SNAP25 staining intensity, some axonal branches are *yellow* and other *reddish-yellow*. Scale bars: 100 µm **A"** and 50 µm **B"**. (**C–C", D-D"**) Immunolabeling with anti-neurofilament protein (NF, *red*) and anti-

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syntaxin (STX, *green*) and phalloidin (*blue*). Palisade endings are shown following NF (**C, D**) and syntaxin (**C', D'**) staining. However, lower lever syntaxin immunoreactivity is also present in the axons that form palisade endings, which is more clearly seen in the high magnification overly (**D'**). (**C", D"**) Overlay shows co-labeling of NF/syntaxin. However, terminal varicosities of palisade exhibit higher levels of syntaxin immunoreactivity and appear in *yellow* whereas axonal branches with lower syntaxin immunoreactivity are *reddish-yellow*. Scale bars: 100 µm **C"** and 50 µm **D"**. (**E-E", F-F"**) Immunolabeling with anti-neurofilament protein (NF, *red*), and anti-complexin (CPLX, *green*), and phalloidin (*blue*). Palisade endings are labeled following NF (**E, F**) and complexin (**E', F'**) labeling. However, fainter complexin immunoreactivity is also present in axons of palisade endings. (**E", F"**) Overlay demonstrates co-labeling of NF/complexin. Terminal varicosities of palisade endings have higher levels of complexin and are *yellow*, whereas axonal branches with lower levels of complexin are *reddish-yellow*. Scale bars: 100 µm **E"** and 50 µm **F"**.

of 0.4% lead citrate, and examined under a transmission electron microscope Phillips 10 (Phillips, Amsterdam, The Netherlands).

Quantification of AChE

The regions of interest were detected with a wand automeasure tool based on contrast thresholding with the background (Image J, NIH, USA). The mean optical density of the pixels enclosed was automatically computed. Comparison between palisade endings and en grappe terminals were carried out using the Mann-Whitney Rank sum test at a significant level of 0.05 using Sigma Plot version 11 (Systat Software, Erkrath, Germany).

Results

Positive Controls

The specificity of markers for exocytotic proteins (SNAP-25, syntaxin, synaptobrevin, synaptotagmin, and complexin) and AChE was validated in motor endplates (en plaque motor endings) of cat EOMs. Motor endplates were visualized by α-bungarotoxin, which selectively binds to AChRs on the muscular membrane (postsynaptic membrane). An α-bungarotoxin incubation was combined with antibodies against SNAP-25, syntaxin, synaptobrevin, synaptotagmin, complexin, or AChE. All motor endplates in cat EOMs also expressed SNAP25, syntaxin, synaptobrevin, synaptotagmin, complexin, and AChE (see Figs. 1A-F"). There was not complete overlap of α-bungarotoxin and antibody signals due to different binding sites. Specifically, α-bungarotoxin-labeled AChR are located at the postsynaptic site, whereas proteins involved in exocytosis (SNAP25, syntaxin, synaptobrevin, synaptotagmin, and complexin) are found at the presynaptic site. AChE is concentrated in the gap between the pre- and postsynaptic membranes (synaptic cleft). In line with the literature[27–29] SNAP-25, syntaxin, and complexin immunoreactivity were not confined to motor terminals, but were also present in axons supplying motor terminals (see Figs. 1A'-A", B'-B", E'-E"). Taken together, our positive controls proved the specificity of the antibodies and were in accordance with findings on motor endplates in other skeletal muscles.[30,31] Following adaption of the staining protocols for triple-immunofluorescence labeling, we continued to analyze palisade endings in muscle-tendon whole-mount preparations. In contrast to sectioning, whole-mount preparations preserve the morphological integrity of the tissue and the true three-dimensional architecture of structures can be visualized in the CLSM by superimposing a series of optical sections along the z-axis. This approach allows a high standard of analysis.

Morphological Features of Palisade Endings

The overall morphology of palisade endings was visualized in the CLSM. For this purpose, nerve fibers were immunolabeled with anti-neurofilament protein and nerve terminals with anti-synaptophysin. In this and other staining combinations (Figs. 2A, 2B; 3–5A-D; 6–8) muscle fibers were counterstained with phalloidin. Cellular and subcellular features of palisade endings were imaged in the transmission electron microscope (TEM).

In accordance with literature, we observed palisade endings at the distal myotendinous junction of each rectus muscle and as previously shown, many more palisades were present in the medial recuts.[10,17,21] Palisade endings were formed by nerve fibers that came from the muscle and extended into the tendon. There, nerve fibers turned back to approach single muscle fiber tips and, by further branching they established a network of bulb-like terminal varicosities as demonstrated by synaptophysin immunoreactivity (see Figs. 2A, 2B). Terminal varicosities were serially arranged along axons and were distributed at both the level of the tendon and the muscle fiber tips, where they extended for a variable distance (50–150 µm) along the muscle fibers surface. TEM images showed that palisade endings were supplied by thin, myelinated axons that approached single muscle fibers. Following ramification, myelinated axons lost their myelin sheath to form preterminal axons that were completely enveloped by Schwann cells processes (Fig. 2C). Preterminal axons established terminal varicosities which were only partly enveloped by Schwann cells. The Schwann cell-free area was of variable dimension and the axolemma of the terminal varicosities was only covered with a basal lamina. At the tendon level, terminal varicosities were associated with the collagen fibrils of the tendon (Fig. 2D). At the level of the muscle fiber tips, terminal varicosities surrounded the muscle fiber (Fig. 2E). TEM images clearly visualized the relation of terminal varicosities to the muscle fiber. Specifically, many terminal varicosities were located very close to the muscle fiber surface and were only separated from the muscle fiber by collagen fibrils or Schwann cell processes (see Fig. 2E). However, we also found terminal varicosities that contacted the muscle fiber surface. Such neuromuscular contacts were only found in very few palisade endings of the rectus muscles and when neuromuscular contacts were present, their number was low. Neuromuscular contacts of palisade endings lacked a basal lamina in the synaptic cleft (Fig. 2F). All the terminal varicosities in palisade endings contained a few mitochondria, but they were otherwise full of clear vesicles (Figs. 2D–G). These vesicles were often very close to the plasma membrane (see Fig. 2D), and sometimes vesicles docked at the plasma membrane of the terminal varicosities (see Fig. 2G). In terminal varicosities contacting the muscle fiber, we found a few dark granules, which were not limited by any membrane

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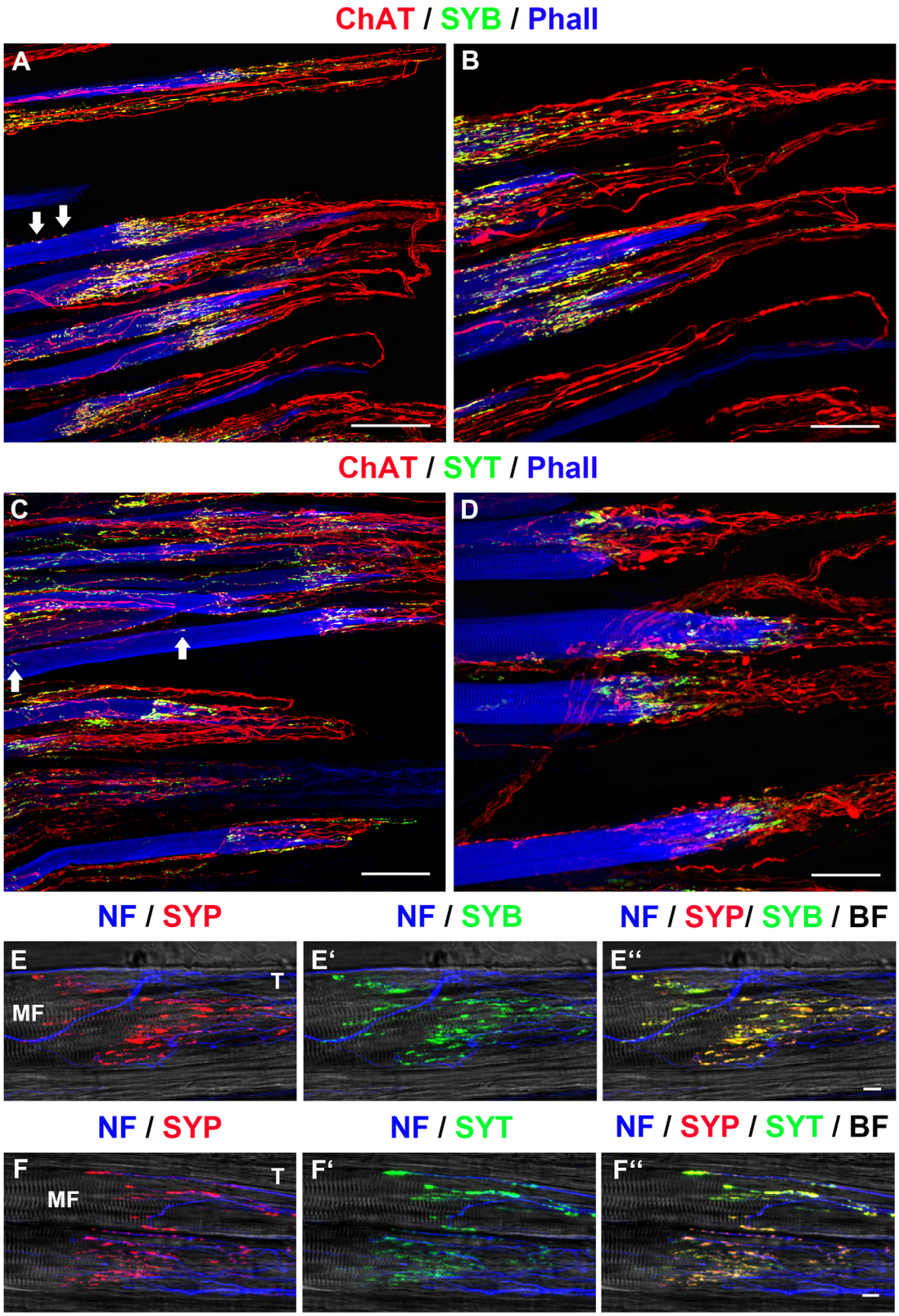

Figure 5. The 3D projections of CLSM images show that palisade endings express synaptobrevin and synaptotagmin. Images are presented at lower (**A, C**) and higher magnification (**B, D, E – E”, F – F”**). **A, B** Immunolabeling with anti-ChAT (*red*), anti-synaptobrevin (SYB, *green*), and (**C, D**) with anti-ChAT (*red*) and anti-synaptotagmin (SYT, *green*). Muscle fibers are visualized with phalloidin (*blue*). Palisade endings positive for ChAT express synaptobrevin **A, B** and synaptotagmin **C, D** in restricted areas of the palisade endings. Co-localization of ChAT and synaptobrevin or synaptotagmin results in *yellow color*. Outside the palisade endings, synaptobrevin and synaptotagmin-positive puncta (*arrows*) are present along muscle fibers consistent with en grappe motor terminals. Scale bars: 100 µm **A**, **C** and 50 µm **B**, **D**. (**E-E”, F-F”**) Immunolabeling with anti-NF (*blue*) and anti-synaptophysin (SYP, *red*), in combination with anti-synaptobrevin (SYB,

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green) or anti-synaptotagmin (SYT, *green*), respectively. **E, F** Palisade endings display synaptophysin-positive terminal varicosities. (**E', F'**) An identical staining pattern is visible following synaptobrevin and synaptotagmin immunolabeling. The overlays demonstrate co-localization of synaptophysin/synaptobrevin **E"** and synaptophysin/synaptotagmin **F"**, which results in *yellow*, *yellow-reddish*, and *green-yellow color*. **E", F"** Fluorescent and bright field (BF) images are merged. Bright field helps to visualize the anatomic structure of muscle fibers (MF) and the tendon (T). Scale bars: 10 µm.

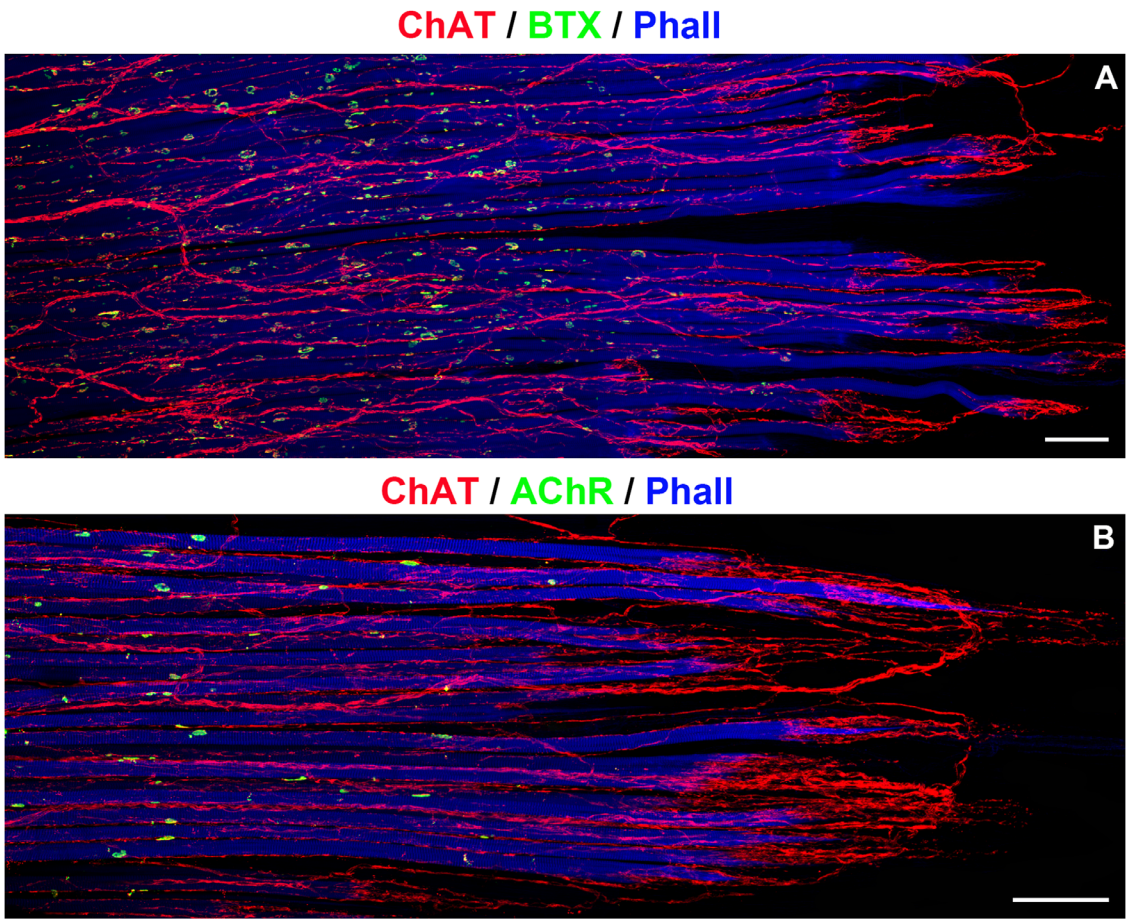

Figure 6. Stitched CLSM images show the characteristics of palisades and en grappe terminations. (**A, B**) Immunolabeling with anti-ChAT (*red*) in combination with labeling for **A** α-bungarotoxin (BTX, *green*) or **B** anti-AChR (*green*). Muscle fibers are counterstained with phalloidin (Phall, *blue*). No α-bungarotoxin **A** or AChR signals **B** are visible in palisade endings (at *right*). Exclusively, en grappe motor terminals on multiply innervated muscle fibers exhibit α-bungarotoxin **A** or anti-AChRs signals **B**. En grappe motor terminals appear in *yellow-green color*. Scale bars: 100 µm.

(see Fig. 2G). We assume that they represent glycogen granules, which are also found in other nerve terminals.[32]

Palisade Endings are Cholinergic Structures

To demonstrate the cholinergic nature of palisade endings, we performed labeling with anti-ChAT in combination with anti-neurofilament protein and anti-synaptophysin, respectively. ChAT is the synthesizing enzyme of the neurotransmitter acetylcholine, so anti-ChAT selectively visualizes cholinergic axons.

Consistent with previous studies,[21,33,34] nerve fibers forming palisade endings exhibited ChAT immunoreactivity. We found palisade endings which were supplied by single axons and others by up to four axons (Figs. 3A-A", B-B"). When several axons contributed to palisade ending formation, all the neurofilament protein-positive axons nevertheless exhibited ChAT immunoreactivity. ChAT-negative axons were never found in palisade endings (see Figs. 3A-A", B-B"). Concordantly with two previous studies,[21,35] all terminal varicosities of palisade endings exhibited ChAT/synaptophysin immunoreactivity (Figs. 3C, 3D). Taken together, immunolabeling with a specific marker for cholinergic axons in combination with general markers for axons/terminal varicosities confirmed the exclusive cholinergic phenotype of palisade endings, ruling out the presence of noncholinergic nervous elements.

Expression of Exocytotic Proteins in Palisade Endings

Key proteins of neurotransmitter exocytosis are SNAP25, syntaxin, synaptobrevin, synaptotagmin, and complexin. We

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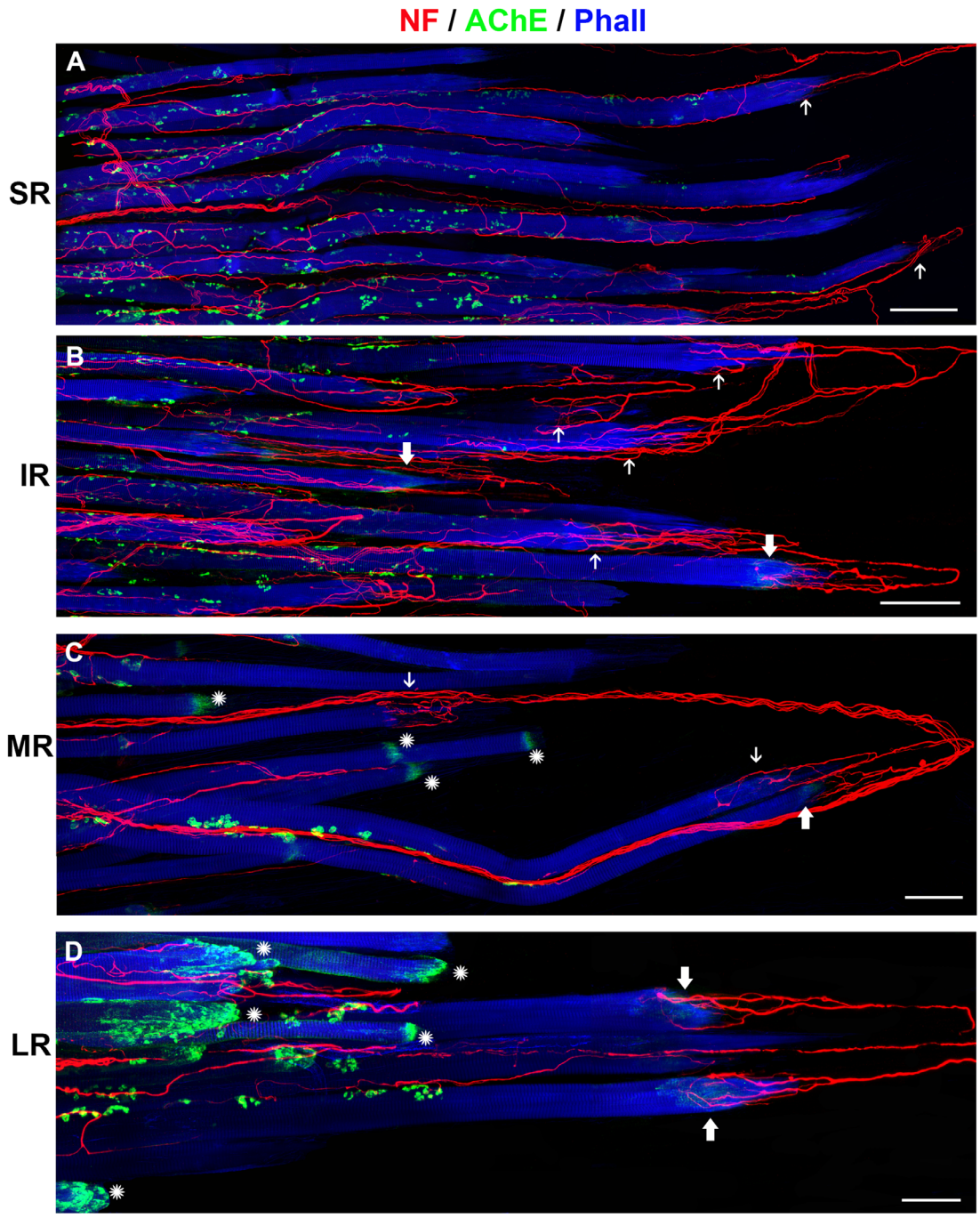

Figure 7. Stitched CLSM images showing that the AChE expression is low or absent in palisade endings. (**A, B, C, D**) Immunolabeling with anti-neurofilament protein (NF, *red*), anti-AChE (*green*), and phalloidin (Phall, *blue*) is shown for the four rectus muscles (superior rectus [SR]; inferior rectus [IR]; medial rectus [MR]; and lateral rectus [LR]). Images are at lower magnification (20×, **A, B**) and higher magnification (40×, **C, D**). The region containing the palisade endings in some rectus muscles fibers exhibit low AChE activity **B, C, D,** *thick arrows*, which appears as diffuse *yellow-blue spots* on the muscle fiber tip associated with the palisade ending. However, in most palisade endings, AChE activity is absent **A, B, C,** *thin arrows*. En grappe motor terminals on the same fibers as the palisade endings express high AChE activity. **C, D** Some muscle fibers display high AChE activity at the muscle fiber tips (*asterisks*). Scale bars: 100 µm **A, B** and 50 µm **C, D**.

tested whether these proteins are expressed in palisade endings.

All palisade endings in the four rectus muscles expressed SNAP25, syntaxin, synaptobrevin, synaptotagmin, and complexin. However, the pattern of immunoreactivity was different among the different exocytotic proteins. Specifically, SNAP25, syntaxin, and complexin were enriched throughout palisade endings as well as in axons giving rise to palisade endings, although at lower concentration (Figs. 4A–F"). This was more clearly seen when viewing palisade endings at high magnification (Figs. 4B'-B", 4D'-D", and 4F'-F"). Synaptobrevin and synaptotagmin were exclusively concentrated in restricted regions of the palisade endings (Figs. 5A–D), thereby copying

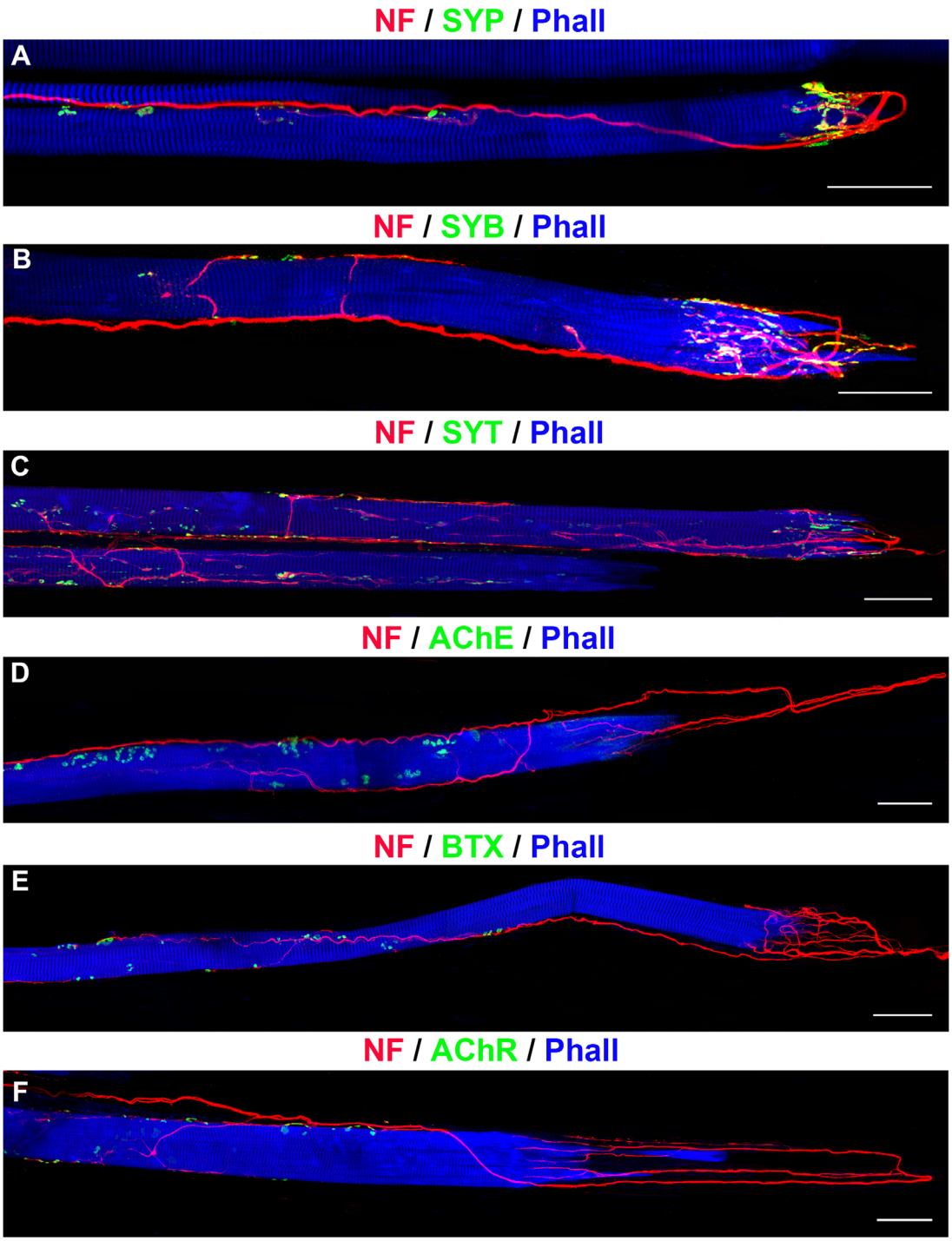

Figure 8. Stitched CLSM images showing that palisade endings are in continuity with axons displaying en grappe motor terminals. (**A–F**) Examples show different staining combinations where axons are visualized by anti-neurofilament protein (NF, *red*) and muscle fibers by phalloidin (Phall, *blue*). Nerve terminals are visualized by anti-synaptophysin **A,** SYP, *green*, anti-synaptobrevin **B,** SYB, *green*, anti-synaptotagmin **C,** SYT, *green*, anti-AChE **D,** *green*, α-bungarotoxin **E,** BTX, *green*, and anti-AChR **F**, AChR, *green*. Nerve fibers run alongside muscle fibers and establish multiple en grappe motor terminals, which are positive for synaptophysin **A**, synaptobrevin **B**, synaptotagmin **C**, AChE **D**, α-bungarotoxin **E**, and anti-AChR **F**. Axons with multiple motor terminals extend into the tendon and return to form palisade endings on single muscle fiber tips. Terminal varicosities of palisade endings are positive for synaptophysin **A**, synaptobrevin **B**, and synaptotagmin **C**, but exhibit only week AChE activity **D**, and no α-bungarotoxin **E**, and anti-AChR signals. Scale bars: 50 µm.

the synaptophysin terminal distribution. To test whether synaptobrevin/synaptotagmin colocalized with synaptophysin, we performed two additional staining experiments and combined either anti-synaptobrevin/anti-synaptophysin or anti-synaptotagmin/anti-synaptophysin immunolabeling, along with anti-neurofilament protein staining. Individual high magnification micrographs revealed corresponding distributions of synaptobrevin, synaptotagmin, and

synaptophysin, and the overlay images demonstrated precise overlap of these proteins (Figs. 5E–F").

Synaptobrevin/synaptotagmin-positive signals were also observed outside the palisade endings (see Figs. 5A, 5C). These signals were present in ChAT-positive axons accompanying muscle fibers, which is consistent with en grappe motor endings on multiply innervated muscle fibers.

Absence of α-Bungarotoxin and Anti-AChR Labeling in Palisade Endings

To screen palisade endings for AChRs, we used two markers: first, α-bungarotoxin (a snake neurotoxin) and second, antibody against AChR. Adjacent confocal images were stitched together to view palisade endings as well as neighboring synapses on multiply innervated muscle fibers.

In line with previous studies,[17,35] no α-bungarotoxin signal was detected in palisade endings. Instead, α-bungarotoxin binding was observed outside the palisade endings, specifically involving en grappe motor terminals located alongside multiply innervated muscle fibers (Figs. 6A, 8E). Following immunolabeling with antibody against AChR, antibody signals were absent in palisade endings. They were only associated with en grappe motor terminals found along multiply innervated muscle fibers (Figs. 6B, 8F). Therefore, α-bungarotoxin and AChR labeling was qualitatively confirmed in the same tissue that indicated AChRs were absent in palisade endings. It is important to note that in all the rectus muscles, palisade endings lacked AChRs.

Acetylcholinesterase Activity is Low or Absent in Palisade Endings

To test the AChE activity in palisade endings, we performed immunolabeling with anti-AChE. For analysis, confocal images were stitched together to allow simultaneous viewing of AChE activity in palisade endings and neighboring synapses on multiply innervated muscle fibers. Data are shown for the four rectus muscles.

Our analyses showed that the majority of palisade endings lacked AChE immunoreactivity (Figs. 7A–C, thin arrows) and the remaining palisade endings exhibited only low levels of AChE activity (Figs. 7B–D, thick arrows). In contrast, en grappe motor endings, which were observed outside the palisade endings, exhibited intense AChE activity (see Figs. 7A–D, 8D). In fact, AChE intensity was almost equal in en grappe and en plaque motor endings (compare Figs. 1F–F", 7A–D, 8D). We quantified the optical density of AChE immunostaining in those palisade endings showing AChE signals, as well as in en grappe terminals as an index of the intensity of the immunolabeling. We found that the intensity of AChE immunostaining in palisade endings was 8.2% of the intensity of en grappe motor terminals. This difference was significant (Mann-Whitney Rank Sum Test; $P = 0.001$). Thus, our findings clearly demonstrate that cholinergic palisade endings and cholinergic motor terminals (en plaque and en grappe motor endings) differ with respect to AChE activity. Note that images of palisade endings and motor terminals were acquired with identical CLSM settings excluding the possibility that AChE intensity differences were the result of CLSM measuring parameters.

Additionally, we observed individual muscle fibers that exhibited intense, cap-like AChE expression at the muscle fiber tip, and lower AChE activity along the muscle fiber between neuromuscular junctions (see Figs. 7C, 7D, asterisks). Such muscle fibers were not associated with palisade endings. The muscle fibers exhibiting cap-like AChE labeling were shorter, in that they did not extend far into the tendon compared with muscle fibers associated with palisade endings. In the lateral and medial rectus muscles (see Figs. 7C, 7D) they came closer to the muscle tendon junction compared to the other rectus muscles indicating muscle-specific fiber length variations.

Palisade Endings are Elongations of Axons Forming Multiple En Grappe Terminals

By tracking axons, we tried to determine the innervation characteristics of nerve fibers supplying palisade endings. Nerve fibers were labeled with anti-neurofilament protein and nerve terminals with presynaptic markers (anti-synaptophysin, anti-synaptobrevin, and anti-synaptotagmin) as well as extrasynaptic anti-AChE or postsynaptic α-bungarotoxin and anti-AChR. Adjacent confocal images at high magnification (40 times) were stitching together to view palisade endings and neighboring en grappe synapses.

We observed that nerve fibers running alongside muscle fibers were in continuity with palisade endings. These nerve fibers established multiple en grappe motor terminals positive for the presynaptic markers synaptophysin, synaptobrevin, and synaptotagmin as well as extrasynaptic AChE, and postsynaptic α-bungarotoxin, or anti-AChR (see Figs. 8A–F). Palisade endings emerging from axons with en grappe motor terminals expressed synaptophysin, synaptobrevin, and synaptotagmin in their terminal varicosities, but they exhibited only low amounts of AChE, and no α-bungarotoxin or anti-AChR signals in association with the palisade terminals (see Figs. 8A–F). Altogether, our findings demonstrated that palisade endings were peripheral extensions of axons that established multiple en grappe motor terminals (MIF motoneurons). However, the terminal varicosities of palisade endings and en grappe motor terminals of the same axon differed in their molecular repertoire. These differences pertain to AChE and AChRs, but not to proteins of the presynaptic site, including synaptophysin, synaptobrevin, and synaptotagmin.

Discussion

We identified key proteins of neurotransmitter secretion in palisade endings, including SNAP-25, syntaxin, synaptobrevin, synaptotagmin, and complexin. AChRs for cholinergic transmission were not associated with palisade endings as demonstrated by the general absence of α-bungarotoxin and anti-AChR signals. AChE, the degradative enzyme for acetylcholine, was at low concentration or absent in palisade endings. Figure 9 summarizes the results of the present study.

Palisade Endings are Formed by Recurrent Nerves and are Cholinergic Structures

In agreement with previous studies,[10,11,21] palisade endings were formed by myelinated nerve fibers that extended into the tendon and turned back to establish synaptophysin-positive terminal varicosities at the level of the tendon and the muscle fibers tips. Rarely, terminal varicosities contacted the muscle fiber surface and such neuromuscular

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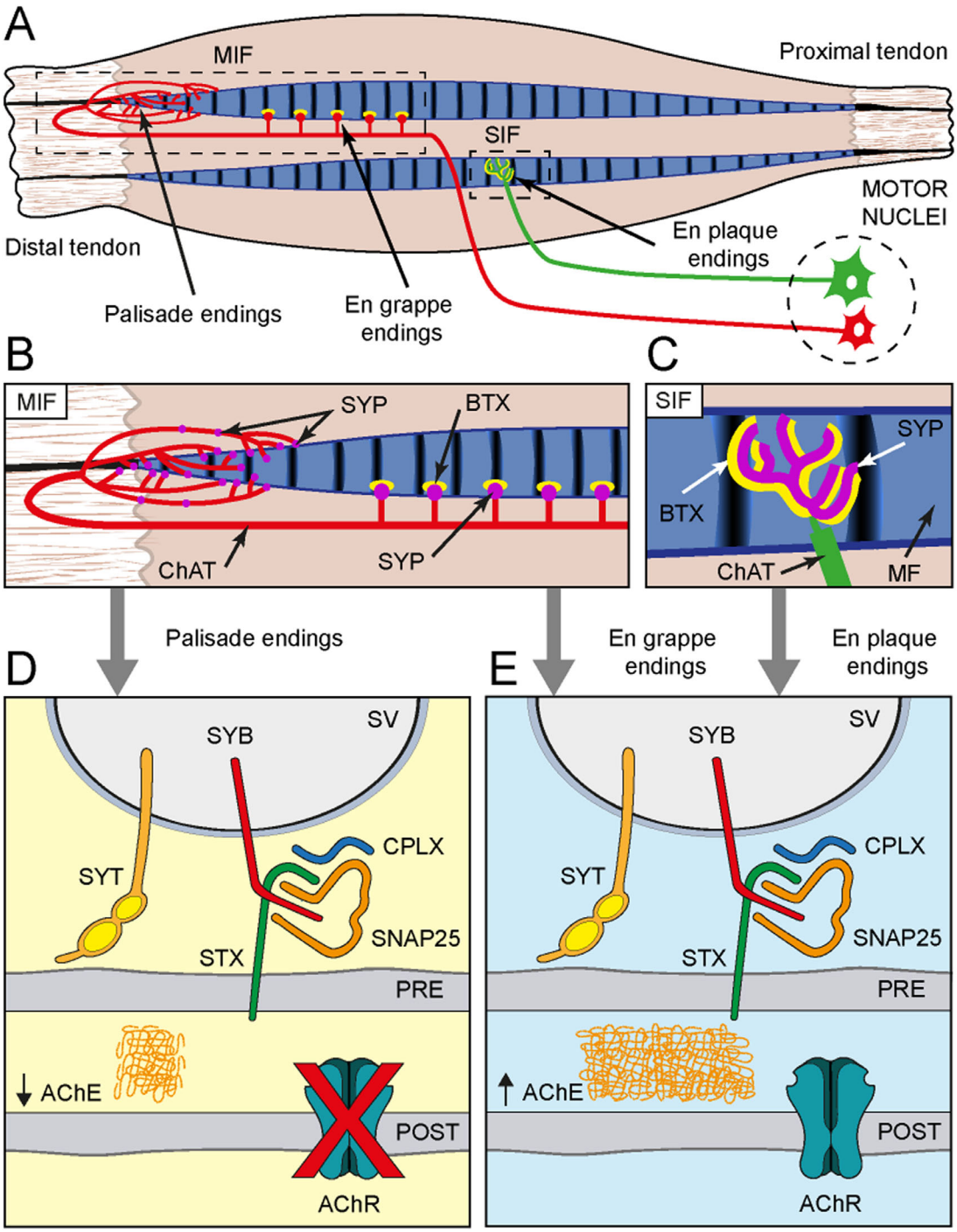

Figure 9. Summary diagram of the present study. (**A**) Axons forming palisade endings establish multiple en grappe terminals alongside multiply innervated muscle fibers (MIFs). Singly innervated muscle fibers (SIFs) have a single en plaque motor terminal in the middle third of the muscle fiber. MIF and SIF motoneurons are located in the EOM motor nuclei. (**B**) Axons forming palisade endings express ChAT. (**C**) En grappe motor terminals and terminal varicosities of palisade endings express synaptophysin (SYP), but only en grappe motor terminals bind BTX. (**C**) En plaque motor terminal express synaptophysin and bind BTX. (**D, E**) Molecular organization at terminal varicosities of palisade endings **D** and en grappe and en plaque motor terminals **E**. **D** Terminal varicosities of palisade endings contain synaptic vesicles (SVs) and express exocytotic proteins including synaptobrevin (SYB), syntaxin (STX), SNAP25, synaptotagmin (SYT), and complexin (CPLX), but lack AChRs and have only low level AChE activity. (**E**) En grappe and en plaque motor terminals express the same repertoire of exocytotic proteins but have AChRs and AChE.

contacts were without a basal lamina in the synaptic cleft. This arrangement is different from other neuromuscular contacts (en plaque and en grappe motor terminals), which usually have a basal lamina in the synaptic cleft.[15,36] Consistent with other studies, all terminal varicosities of palisade endings were full of clear vesicles and displayed a few

mitochondria.[10,11,21] By immunolabeling, we demonstrated that all nerve fibers forming palisade endings expressed ChAT and all terminal varicosities of palisade endings expressed ChAT as well. These findings confirmed previous studies[17,21,34] that showed that palisade endings are exclusively cholinergic.

Structural and Molecular Evidence for an Exocytotic Machinery

Palisade endings express ChAT and vesicular acetylcholine transporter.[21] This suggests synthesis and storage of the neurotransmitter acetylcholine in clear vesicles of terminal varicosities. We tested whether palisade endings express the molecular machinery for neurotransmitter (acetylcholine) release. Neurotransmitter exocytosis is a highly regulated process that is driven by specialized proteins. Specifically, synaptobrevin at the vesicular membrane together with syntaxin and SNAP-25 at the presynaptic membrane form a protein complex (SNARE complex), which docks synaptic vesicles to the presynaptic membrane.[22,37] Synaptotagmin, a Ca^{2+} binding protein at the vesicle membrane, and complexin, a cytoplasmic neuronal protein, interact with the SNARE complex to trigger synaptic vesicles fusion.[23,24,28,38]

Docked vesicles were observed in terminal varicosities of palisade endings indicating the initial step of neurotransmitter exocytosis at the ultrastructural level.[39] At the molecular level, we identified key proteins of the exocytotic machinery in palisade endings including SNAP25, synaptobrevin, syntaxin, synaptotagmin, and complexin. As previously shown in monkeys,[34] SNAP25 was enriched throughout the palisade endings and was also present along axons supplying palisade endings albeit at lower concentration. The same distribution pattern existed for syntaxin, and complexin, whereas synaptobrevin and synaptotagmin were located in restricted areas of palisade endings. Distribution of SNAP25, syntaxin, and complexin along axons, including terminal specializations, has also been observed in neurons of the brain[29] and in motoneurons supplying the skeletal muscles,[28] including EOMs (control experiments of the present study). Because SNAP25, syntaxin, and complexin were expressed along the entire axons and were not restricted to specialized regions (terminal varicosities) of palisade endings, it is possible that the role of these proteins is not limited to neurotransmitter exocytosis.

Synaptobrevin and synaptotagmin were focally distributed in palisade endings and colocalized with synaptophysin. Synaptophysin is a vesicle membrane protein[40] and is enriched in terminal varicosities of palisade endings.[21,41] The full overlap of these three molecules suggests that they, at the subcellular level, are associated with vesicles in terminal varicosities of palisade endings. In fact, synaptobrevin and synaptotagmin are essential for neurotransmitter release in central and peripheral (neuromuscular) synapses,[37,42] and it is therefore most likely that these proteins have secretory function in palisade endings as well.

In conclusion, palisade endings exhibit structural and molecular characteristics of exocytotic machinery. Although other proteins are implicated in vesicle exocytosis as well,[43] the expression of key proteins in palisade endings and their subcellular location is consistent with other secretory axons. This demonstrates that a pathway for neurotransmitter (acetylcholine) release is available in palisade endings.

Receptors for Cholinergic Transmission are Missing

Because we found evidence for the presence of the molecular apparatus for neurotransmitter (acetylcholine) release, we looked for AChRs on muscle fiber tips opposed to palisade endings. To visualize AChRs, previous studies[8,44] have used α-bungarotoxin, a snake neurotoxin. Despite the reliability of α-bungarotoxin,[45] we additionally applied a monoclonal antibody (mouse anti-AChR) because there are examples in the literature where antibodies targeting AChRs are more sensitive.[46,47] Specifically, by using the same antibody against AChRs (M217, RRID:AB_260473) that we used, they were visualized on the slow muscle fibers of zebra fish, when they could not been seen with α-bungarotoxin.[46] Moreover, in rat cortex, the number of neurons expressing AChRs is higher when labeling is performed with anti-AChR compared to α-bungarotoxin.[47] In conclusion, our strategy to visualize AChRs with different markers substantially advanced the reliability of the findings.

Labeling of EOM whole-mounts with α-bungarotoxin and antibody against AChR provided a consistent staining pattern. Specifically, no α-bungarotoxin/antibody signals were associated with palisade endings, but were instead exclusively observed in en grappe motor terminals along multiply innervated muscle fibers. This agrees with previous studies[8,15] that have consistently shown that α-bungarotoxin is absent in palisade endings of most species. Exceptions are palisade endings of rabbits and rats, which usually bind α-bungarotoxin.[8,20] It is, however, important to note that palisade endings of these species are different from canonical palisades found in higher mammals (i.e. their palisades are simpler and exclusively have terminals varicosities contacting the muscle fiber tips).[8,20] A few palisade endings of primates (monkey and man) bind α-bungarotoxin, albeit at very low levels.[8,44]

Because labeling with α-bungarotoxin and a potentially more sensitive antibody was qualitatively identical, we conclude that AChRs are either absent from muscle fiber tips associated with palisade endings or are alternatively, at a concentration too low for detection at the CLSM level. In this respect, it is important to mention that the density of AChRs at central synapses is higher at the CLSM level compared to the TEM level. This may reflect a higher sensitivity for confocal microscopy compared with immunoelectron microscopy, where the molecular quality of the tissue might be altered during extensive processing.[48]

Indications that Neurotransmitter Breakdown is Almost Absent in Palisade Endings

AChE terminates neurotransmission of acetylcholine. We tested the AChE expression in palisade endings. We demonstrated that the majority of palisade endings lacked AChE and other palisade endings exhibited low AChE activity. Intense AChE activity was associated with en grappe motor terminals, and was present at the tips of shorter muscle fibers, although they lacked palisade innervation.

Only a single older study from 1975 of the cat has analyzed the AChE activity in EOMs.[25] Using a histochemical, as opposed to an immunofluorescence approach, AChE activity was observed both in en grappe and en plaque motor endings as well as at the muscle-tendon junction.[25] Consistent with the present findings, the prior study[25] has demonstrated that longer muscle fibers that penetrate

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deeper into the tendon exhibit less conspicuous AChE activity at the muscle fiber tips than shorter muscle fibers that stop some distance away from the muscle tendon junction.[25] In the present study, muscle fibers with low AChE activity at the muscle fiber tips were associated with palisade endings but this has not been examined in the previous study. Mayr et al.[25] have hypothesized that shorter muscle fibers with high AChE activity at their tips are singly innervated muscle fibers, but this could not be confirmed in the present study, because our whole-mount preparations did not include the muscle belly where en plaque motor endplates are located.

Assuming that palisade endings with a molecular exocytosis machinery release neurotransmitter, the shortage or lack of AChE suggests that neurotransmitter degradation is insufficient or absent in palisade endings resulting in higher levels and prolonged acetylcholine activity. Due to the relative great distance from the palisade zone, it is not clear whether acetylcholine, likely secreted from palisade endings, might affect shorter muscle fibers that have high levels of AChE activity at the muscle fiber tips.

Anatomical Verification of Continuity Between MIF Inputs and Palisade Endings

Following tracer injection into the EOM motor nuclei, palisade endings have been visualized suggesting that these structures are supplied by MIF motoneurons.[16,17] In the present study, axon tracking in EOM whole-mounts preparations revealed that axons with en grappe motor terminals were continuous with palisade endings. Thus, our findings confirmed that MIF motoneurons and palisade endings belong to the same anatomic entities, although specialized regions of MIF motoneuron axons (en grappe motor terminals and terminals varicosities of palisade endings) displayed distinct molecular profiles. Specifically, en grappe motor terminals and terminals varicosities expressed synaptophysin, synaptobrevin, and synaptotagmin at the presynaptic site, but unlike en grappe motor terminals, terminals varicosities of palisade endings exhibited low or no AChE activity and were not associated with AChRs. These molecular differences suggest that en grappe motor terminals and terminal varicosities of palisade endings likely serve different functions, although both are specialized regions of the same neuron.

That palisade endings originate from sensory-like neurons has been proposed in the two previous studies.[18,19] Specifically, following distal EOM tracer injection, involving the palisade zone and en grappe motor terminals, two morphologically distinct populations of neurons have been identified in the EOM motor nuclei of monkeys: multipolar neurons and another group, resembling spindle-shaped neurons. It has been assumed that the latter group, which might be similar to mesencephalic sensory neurons, is the source of palisade endings.[18,19] If this is true, sensory nerve fibers with molecular characteristics of motoneurons and MIF motoneuron innervations pattern, would supply palisade endings. As a consequence, this would mean that MIF motoneurons are both sensory and motor, which, however, does not seem to be easily compatible.

Even analyses of EOM whole-mount preparations confirmed a MIF motoneuron/palisade ending continuity in each staining combination, it was not possible to do this for every palisade ending. Due to anatomic constraints, axonal tracking was limited to examples where axons did not go out of focus or intermingled with other axons. Thus, we cannot verify that every palisade ending is formed by an MIF motoneuron axon.

Functional Considerations

Structural and molecular findings suggest that palisade endings have an exocytosis machinery, but lack key synaptic and postsynaptic features. Thus, following excitation, the terminal varicosities of palisade endings likely release acetylcholine, but the effect of acetylcholine remains unclear because the muscle fiber tips opposed to palisade endings lack AChRs. Additionally, the absence of AChE in association with palisade endings indicates that fast acetylcholine removal is insufficient resulting in the possibility of prolonged neurotransmitter activity. Because palisade endings are extensions of MIF motoneurons axons, excitation of MIF motoneurons would produce neurotransmitter release at multiple en grappe motor terminals as well as palisade endings. En grappe motor terminals endowed with AChRs would provoke contraction of the muscle fiber body, whereas palisade endings would, due to the absence of AChRs exert no effect on the terminal portion of the muscle fiber. This suggests that palisade endings belong to an effector system, which is very different from that found in other skeletal muscles. It is therefore possible that acetylcholine set free from palisade endings binds to hitherto unknown receptors or serves a receptor-independent, modulatory function.

There is supporting evidence that palisade endings are important for convergence eye movements, which are generated by simultaneous contraction of both medial rectus muscles and which are accompanied by pupil constriction and lens accommodation. Specifically, in frontal-eyed animals, many more palisade endings are present in the medial rectus muscle compared with the other rectus muscles and a developmental study in a frontal-eyed species (cat) has demonstrated that palisade endings with adult-like characteristics appear much earlier in the medial rectus muscle.[8,35] Further, in two primate species (man and monkey) many palisade endings located in the medial rectus muscle and a lower number of palisade endings of the inferior rectus muscle express calretinin, whereas palisade endings of the other two rectus muscles lack calretinin.[19,34] Calretinin is a calcium-blinding protein and, although its functional role is unclear, the authors have hypothesized that calretinin-positive palisade endings represent a specialized, probable more excitable, type of palisade ending needed for convergent eye movement.[19,34] Recently, it has been demonstrated that MIF motoneurons participate in the whole repertoire of eye movements.[49] Due to the fact that palisade endings are extensions of MIF motoneurons axons, this suggests that palisade endings despite their unknown function, are relevant for all types of eye movement.[49]

In summary, our structural and molecular findings show that palisade endings have intact exocytotic machinery. It is therefore most likely that palisade endings release neurotransmitter (acetylcholine) following excitation. However, palisade endings lack currently known receptors for cholinergic transmission and the degradative enzyme of acetylcholine is almost absent. This suggests that palisade endings belong to an effector system, which is different from that usually observed in skeletal muscle.

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References

1. Porter JD, Spencer RF. Localization of morphology of cat extraocular muscle afferent neurones identified by retrograde transport of horseradish peroxidase. *J Comp Neurol*. 1982;204(1):56–64.
2. Porter JD. Brainstem terminations of extraocular muscle primary afferent neurons in the monkey. *J Comp Neurol*. 1986;247(2):133–143.
3. Wang X, Zhang M, Cohen IS, Goldberg ME. The proprioceptive representation of eye position in monkey primary somatosensory cortex. [see comment]. *Nat Neurosci*. 2007;10(5):640–646.
4. Balslev D, Albert NB, Miall C. Eye muscle proprioception is represented bilaterally in the sensorimotor cortex. *Hum Brain Mapp*. 2011;32(4):624–631.
5. Maier A, DeSantis M, Eldred E. The occurrence of muscle spindles in extraocular muscles of various vertebrates. *J Morphol*. 1974;143(4):397–408.
6. Ruskell GL. The fine structure of human extraocular muscle spindles and their potential proprioceptive capacity. *J Anat*. 1989;167:199–214.
7. 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(13):4317–4327.
8. 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(2):320–331.
9. Eberhorn AC, Horn AK, Eberhorn N, Fischer P, Boergen KP, Buttner Ennever JA. Palisade endings in extraocular eye muscles revealed by SNAP-25 immunoreactivity. *J Anat*. 2005;206(3):307–315.
10. Alvarado Mallart RM, Pincon Raymond M. The palisade endings of cat extraocular muscles: a light and electron microscope study. *Tissue Cell*. 1979;11(3):567–584.
11. Ruskell GL. The fine structure of innervated myotendinous cylinders in extraocular muscles of rhesus monkeys. *J Neurocytol*. 1978;7(6):693–708.
12. Billig I, Buisseret Delmas C, Buisseret P. Identification of nerve endings in cat extraocular muscles. *Anat Rec*. 1997;248(4):566–575.
13. Buttner Ennever JA, Eberhorn A, Horn AK. Motor and sensory innervation of extraocular eye muscles. *Ann N Y Acad Sci*. 2003;1004:40–49.
14. Liu JX, Domellof FP. A novel type of multiterminal motor endplate in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2018;59(1):539–548.
15. 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(1):155–165.
16. Lienbacher K, Mustari M, Ying HS, Buttner-Ennever JA, Horn AK. Do palisade endings in extraocular muscles arise from neurons in the motor nuclei? *Invest Ophthalmol Vis Sci*. 2011;52(5):2510–2519.
17. 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(7):2784–2793.
18. Lienbacher K, Mustari M, Hess B, Buttner-Ennever J, Horn AK. Is there any sense in the palisade endings of eye muscles? *Ann N Y Acad Sci*. 2011;1233:1–7.
19. Lienbacher K, Sanger K, Strassburger S, et al. Extraocular muscles involved in convergence are innervated by an additional set of palisade endings that may differ in their excitability: a human study. *Prog Brain Res*. 2019;248:127–137.
20. Blumer R, Wasicky R, Hotzenecker W, Lukas JR. Presence and structure of innervated myotendinous cylinders in rabbit extraocular muscle. *Exp Eye Res*. 2001;73(6):787–796.
21. Konakci KZ, Streicher J, Hoetzenecker W, et al. Palisade endings in extraocular muscles of the monkey are immunoreactive for choline acetyltransferase and vesicular acetylcholine transporter. *Invest Ophthalmol Vis Sci*. 2005;46(12):4548–4554.
22. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. 1998;395(6700):347–353.
23. Fernandez-Chacon R, Konigstorfer A, Gerber SH, et al. Synaptotagmin I functions as a calcium regulator of release probability. *Nature*. 2001;410(6824):41–49.
24. Mohrmann R, Dhara M, Bruns D. Complexins: small but capable. *Cell Mol Life Sci*. 2015;72(22):4221–4235.
25. Mayr R, Gottschall J, Gruber H, Neuhuber W. Internal structure of cat extraocular muscle. *Anat Embryol (Berl)*. 1975;148(1):25–34.
26. Spencer RF, Porter JD. Structural organization of the extraocular muscles. *Rev Oculomot Res*. 1988;2:33–79.
27. Galli T, Garcia EP, Mundigl O, Chilcote TJ, De Camilli P. v- and t-SNAREs in neuronal exocytosis: a need for additional components to define sites of release. *Neuropharmacology*. 1995;34(11):1351–1360.
28. Lin MY, Rohan JG, Cai H, Reim K, Ko CP, Chow RH. Complexin facilitates exocytosis and synchronizes vesicle release in two secretory model systems. *J Physiol*. 2013;591(10):2463–2473.
29. Garcia EP, McPherson PS, Chilcote TJ, Takei K, De Camilli P. rbSec1A and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but are not in a stable complex with syntaxin. *J Cell Biol*. 1995;129(1):105–120.
30. Bernard V, Girard E, Hrabovska A, et al. Distinct localization of collagen Q and PRiMA forms of acetylcholinesterase at the neuromuscular junction. *Mol Cell Neurosci*. 2011;46(1):272–281.
31. Rozas JL, Gomez-Sanchez L, Tomas-Zapico C, Lucas JJ, Fernandez-Chacon R. Increased neurotransmitter release at the neuromuscular junction in a mouse model of polyglutamine disease. *J Neurosci*. 2011;31(3):1106–1113.
32. Thornell LE, Sjostrom M, Karlsson U, Cedergren E. Variable opacity of glycogen in routine electron micrographs. *J Histochem Cytochem*. 1977;25(9):1069–1073.
33. 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(3):1176–1186.
34. Lienbacher K, Ono S, Fleuriet J, Mustari M, Horn AKE. A subset of palisade endings only in the medial and inferior rectus muscle in monkey contain calretinin. *Invest Ophthalmol Vis Sci*. 2018;59(7):2944–2954.

Investigative Ophthalmology & Visual Science

35. Blumer R, Streicher J, Davis-Lopez de Carrizosa MA, de la Cruz RR, Pastor AM. Palisade endings of extraocular muscles develop postnatally following different time courses. *Invest Ophthalmol Vis Sci*. 2017;58(12):5105–5121.
36. Salpeter MM, McHenry FA, Feng HH. Myoneural junctions in the extraocular muscles of the mouse. *Anat Rec*. 1974;179(2):201–224.
37. Liu Y, Sugiura Y, Lin W. The role of synaptobrevin1/VAMP1 in Ca^{2+}-triggered neurotransmitter release at the mouse neuromuscular junction. *J Physiol*. 2011;589(Pt 7):1603–1618.
38. Sudhof TC, Rizo J. Synaptotagmins: C2-domain proteins that regulate membrane traffic. *Neuron*. 1996;17(3):379–388.
39. Verhage M, Sorensen JB. Vesicle docking in regulated exocytosis. *Traffic*. 2008;9(9):1414–1424.
40. Navone F, Jahn R, Di Gioia G, Stukenbrok H, Greengard P, De Camilli P. Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J Cell Biol*. 1986;103(6 Pt 1):2511–2527.
41. 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(5):1625–1631.
42. Pang ZP, Melicoff E, Padgett D, et al. Synaptotagmin-2 is essential for survival and contributes to Ca^{2+} triggering of neurotransmitter release in central and neuromuscular synapses. *J Neurosci*. 2006;26(52):13493–13504.
43. Jahn R, Fasshauer D. Molecular machines governing exocytosis of synaptic vesicles. *Nature*. 2012;490(7419):201–207.
44. 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(9):2422–2431.
45. Anderson MJ, Cohen MW. Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J Physiol*. 1974;237(2):385–400.
46. Park JY, Mott M, Williams T, et al. A single mutation in the acetylcholine receptor delta-subunit causes distinct effects in two types of neuromuscular synapses. *J Neurosci*. 2014;34(31):10211–10218.
47. Murakami K, Ishikawa Y, Sato F. Localization of alpha7 nicotinic acetylcholine receptor immunoreactivity on GABAergic interneurons in layers I-III of the rat retrosplenial granular cortex. *Neuroscience*. 2013;252:443–459.
48. Jones IW, Wonnacott S. Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J Neurosci*. 2004;24(50):11244–11252.
49. Hernandez RG, Calvo PM, Blumer R, de la Cruz RR, Pastor AM. Functional diversity of motoneurons in the oculomotor system. *Proc Natl Acad Sci USA*. 2019;116(9):3837–3846.

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