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A Subset of Palisade Endings Only in the Medial and Inferior Rectus Muscle in Monkey Contain Calretinin

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PURPOSE. To further chemically characterize palisade endings in extraocular muscles in rhesus monkeys.

METHODS. Extraocular muscles of three rhesus monkeys were studied for expression of the calcium-binding protein calretinin (CR) in palisade endings and multiple endings. The complete innervation was visualized with antibodies against the synaptosomal-associated protein of 25 kDa and combined with immunofluorescence for CR. Six rhesus monkeys received tracer injections of cholera toxin subunit B or wheat germ agglutinin into either the belly or distal myotendinous junction of the medial or inferior rectus muscle to allow retrograde tracing in the C-group of the oculomotor nucleus. Double-immunofluorescence methods were used to study the CR content in retrogradely labeled neurons in the C-group.

RESULTS. A subgroup of palisade and multiple endings was found to express CR, only in the medial and inferior rectus muscle. In contrast, the en plaque endings lacked CR. Accordingly, within the tracer-labeled neurons of the C-group, a subgroup expressed CR.

CONCLUSIONS. The study indicates that two different neuron populations targeting nontwitch muscle fibers are present within the C-group for inferior rectus and medial rectus, respectively, one expressing CR, one lacking CR. It is possible that the CR-negative neurons represent the basic population for all extraocular muscles, whereas the CR-positive neurons giving rise to CR-positive palisade endings represent a specialized, perhaps more excitable type of nerve ending in the medial and inferior rectus muscles, being more active in vergence. The malfunction of this CR-positive population of neurons that target nontwitch muscle fibers could play a significant role in strabismus.

Keywords: eye muscles, oculomotor nucleus, en grappe endings, vergence

The presence of proprioception in extraocular muscles is one of the most discussed issues in oculomotor control.^{1–3} Although the roles of muscle spindles and Golgi tendon organs in proprioception of limb muscles are well understood, their roles in oculomotor function are not clear. There are some differences between limb and extraocular muscles in different aspects that deserve further study.⁴

During saccades the extraocular muscles show contraction speed that is among the fastest of any muscle in the body.^{5,6} In addition, extraocular muscles function in a sustained fashion during eccentric fixations.^{7,8} Both of these extremes are necessary to support the highly precise movements of the eye during behavior. This extraordinary feature can exist only because of the interaction of small motor units within the oculomotor neurons and an elaborate sensory control system.⁹ In addition, the different muscle fiber types in the extraocular muscles support this highly efficient and precise system. The singly innervated twitch fibers (SIF) resemble the classical skeletal muscle fibers and are innervated by single en plaque endings. Their activation results in a fast contraction (twitch). The multiply innervated nontwitch fibers (MIF) are targeted by

en grappe endings along the whole length of the muscle fiber and respond with slow tonic local contractions.^{3,10,11}

The proprioceptive system, whose presence has been long debated,¹² is considered by some researchers to be very important for exact eye position feedback.¹³ Unfortunately, this role is much more complex than in the trunk and limb skeletal muscles. The amount and even the presence of proprioceptors vary not only between different species,^{1,3,14,15} but even between the eye muscles themselves.^{16–18} In addition, the extraocular muscles contain a unique type of nerve ending, the palisade endings, that could fulfill such a proprioceptive function. A sensory role is supported by the location of palisade endings at the myotendinous junction with mainly neurotendinous contacts¹⁹ and their resemblance to immature Golgi tendon organs.²⁰ A motor or sensory effector function has been suggested by their cholinergic signature including α -bungarotoxin binding at the postsynaptic site of neuromuscular terminals.^{3,19,21,22}

Previous studies revealed that central tract-tracer injections in the vicinity of the oculomotor and abducens nucleus labeled palisade endings anterogradely in the respective extraocular muscle.^{23,24} Since small tracer injections into the myotendinous



TABLE 1. Relative Populations of Calretinin-Expressing Neurons in the C-Group

Case	Tracer	Injection Site	C-Group	CR+ in C-Group
1	CTB	MR belly	+	15%
2	CTB	MR distal	+	>40%
3	CTB	IR distal, small	+	0
4	WGA	LR distal, IO, IR distal	+	7%
5	CTB	IR distal, MR distal	+	9%, 23%
6	WGA	SR belly and distal, MR distal	+	33%

IO, inferior oblique muscle.

junctions of extraocular muscles, which contain the palisade endings, retrogradely label neurons exclusively in the periphery of the oculomotor nuclei, these peripheral neurons are now considered the location of the palisade ending cell bodies.^{23,25} In contrast, the motoneurons of the SIFs are located within the motor nuclei of extraocular muscles.²⁵ To date, the peripherally located neurons have been considered MIF motoneurons.²⁵ With the recent identification of palisade ending cell bodies within the peripheral cell groups, two possible morphologic features were assumed: Firstly, the peripheral neurons represent a homogenous population that gives rise to multiple nerve endings and palisade endings innervating the nontwitch muscle fibers in the extraocular muscle.⁴ Secondly, the peripheral neurons consist of two different groups: a sensory population, which represents the cell bodies of palisade endings, and a motoneuron population, which innervates the MIFs via en grappe endings. Thereby the palisade endings would register the tension of the muscle within the myotendinous junction and activate the MIF motoneurons in a γ motoneuron-like fashion to keep the system sensitive during muscle contraction.⁴

A sensory and motor innervation of single muscle fibers is known from intrafusal fibers of muscle spindles. In their central part they are contacted by proprioceptive sensory type Ia afferent nerve fibers, whereas the polar ends are innervated by motor endings of γ motoneurons.²⁶ The observation that the sensory axons in the muscle spindles contain the calcium-binding protein calretinin (CR),²⁷ but are also associated with acetylcholine receptors at the sensory neuromuscular contact sites,²⁸ initiated a similar comparative investigation of multiple and palisade endings in the extraocular muscles in the present study. A different histochemical signature of the two types of nerve endings would also indicate differences in the histochemical profile of the neurons in the peripheral groups and therefore better support one of the two hypotheses.

METHODS

All surgical interventions and perfusion conformed to the state and university regulations on laboratory animal care, including the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by animal care officers and the Institutional Animal Care and Use Committees at the University of Washington. For surgery under aseptic conditions, the animals were anesthetized with isoflurane (1.25%–2.5%). Blood pressure, heart rate, blood oxygenation, body temperature, and CO₂ in expired air were monitored (SurgiVet monitor; Smiths Medical, Dublin, OH, USA) and maintained within physiological limits. Under sterile conditions the extraocular muscles were exposed after retraction of the eyelids and a small conjunctival incision. Small tracer volumes

(5–10 μ L) were injected with a thin cannula attached to a Hamilton syringe. Four macaque monkeys received a tracer injection of either 5 to 10 μ L cholera toxin subunit B (CTB, 1% in aqua bidistilled; List Biological Laboratories, Campbell, CA, USA) or 5 to 10 μ L wheat germ agglutinin (WGA, 2.5% in aqua bidistilled; Sigma-Aldrich Corp., St. Louis, MO, USA) into the extraocular muscle. Specifically, the injections were placed into either the distal tip (myotendinous region) or the belly of the inferior rectus (IR) or the medial rectus (MR) muscle. In addition, one case with a WGA injection into the distal part of the lateral rectus (LR) with contamination of the IR and one case with a WGA injection into the distal part of the superior rectus (SR) with contamination of the MR distal part were examined (Table 1).

After a survival time of 3 days, the animals were sedated with ketamine, euthanized with an overdose of pentobarbital (>90 mg/kg), and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) as described previously.^{23,25} The brainstem and all extraocular muscles were removed from the skull and transferred through increasing concentrations of sucrose in 0.1 M PB for frozen sectioning. The brainstem sections were cut at 40 μ m in the transverse plane and processed free floating. The extraocular muscles were cut flat from the orbital to the global layer at 20 μ m thickness using a cryostat (Thermo Scientific Microm HM 560; Fisher Scientific, Germany) and thaw-mounted to glass slides (Superfrost Plus; Thermo Scientific, Germany).

Immunoperoxidase Staining for Calretinin in the Extraocular Muscle

For the immunocytochemical detection of CR-positive nerve endings, the extraocular muscle tissue was processed on slides. Sections were pretreated with 10% methanol and 3% H₂O₂ to suppress endogenous peroxidase activity and were then preincubated in 0.1 M PB at pH 7.4 containing 0.3% Triton X-100 with 5% normal goat serum for 1 hour. Then, the tissue was processed with rabbit anti-CR (1:1000; Swant, Marly, Switzerland) for 24 hours at room temperature. The sections were washed in 0.1 M PB three times and treated with biotinylated goat anti-rabbit (1:200; Vector Labs, Burlingame, CA, USA) for 1 hour at room temperature. After three washes in 0.1 M PB, sections were incubated in ExtrAvidin peroxidase (1:1000; Sigma-Aldrich Corp.) for 1 hour at room temperature. Following two rinses in 0.1 M PB and one rinse in 0.05 M Tris buffer solution (TBS, pH 7.6), the antigenic sites were visualized with 0.025% diaminobenzidine (DAB), 0.2% ammonium nickel sulfate (Riedel-De Haën; Germany), and 0.01% H₂O₂ in 0.05 M TBS (pH 7.6) for 5 to 10 minutes, which yielded a black reaction product. The sections were mounted, air dried, dehydrated, and coverslipped in DPX mountant (Merck, Darmstadt, Germany).

Combined Immunofluorescence for the Tracers and Calretinin in the Brain

Midbrain sections containing the oculomotor nucleus were simultaneously stained for retrogradely transported tracer (CTB or WGA) and CR. The free-floating tissue was washed with 0.05 M TBS (pH 7.6) and preincubated in 0.1 M TBS at pH 7.4 containing 0.3% Triton X-100 with 5% normal donkey serum for 1 hour at room temperature. Then the tissue was processed either with goat anti-WGA (1:250; Vector Laboratories, Burlingame, CA, USA) or with goat anti-CTB (1:5000; List Biological Laboratories) and rabbit anti-CR (1:1000; Swant) in 0.1 M TBS (pH 7.4) containing 0.3% Triton X-100 with 5% normal donkey serum for 24 hours at room temperature. After

TABLE 2. Comparison of the Relative Amount of Calretinin-Positive Palisade Endings in Different Extraocular Muscles in Monkey

	Case 7 MR	Case 7 IR	Case 7 LR	Case 8 MR	Case 2 IR	Case 2 LR
PE number	36	8	17	60	5	16
CR+ PE	32	3	0	50	1	0
Percentage of CR+ PE	88.9	37.5	0	86.7	20	0

three washes with 0.1 M TBS (pH 7.4) the sections were treated with a cocktail consisting of Cy³-donkey anti-rabbit (1:200; Dianova, Hamburg, Germany) and Alexa Fluor 488 donkey anti-goat (1:200; Molecular Probes, Eugene, OR, USA) for 1 hour. After three washes with 0.1 M TBS (pH 7.4) and a short washing in distilled water, the sections were air dried and coverslipped in Fluoromount medium (Sigma-Aldrich Corp.).

Combined Fluorescence Immunostaining for Calretinin and Other Markers in the Extraocular Muscles

The extraocular muscle sections of all cases were immunostained for the calcium-binding protein CR (rabbit anti-CR; 1:1000; Swant), combined with the immunocytochemical detection of one of the following markers: (1) synaptosomal-associated protein of 25 kDa (SNAP-25; mouse SMI 81, 1:2000; Biogen, San Diego, CA, USA) to visualize the complete innervation; (2) the cholinergic marker choline acetyltransferase (ChAT, goat anti-ChAT, 1:80; Merck, Darmstadt, Germany); (3) the tracer CTB (goat anti-cholera toxin B subunit, 1:5000; List Biological Laboratories) or WGA (goat anti-WGA, 1:250; Axxora); (4) in one section series the combined detection of

SNAP-25 and ChAT was applied. After blocking nonspecific staining with 0.3% Triton X-100 with 5% normal donkey serum for 1 hour, the tissue was processed with a cocktail containing anti-CR in combination with the antibody against one of the markers listed above for 48 hours at 4°C.

After several buffer washes the sections were reacted with a mixture of fluorochrome-tagged secondary antibodies of Cy³ combined with Alexa Fluor 488 (for [1] Cy³ anti-rabbit with Alexa Fluor 488 anti-mouse or Cy³ anti-mouse with Alexa Fluor 488 anti-rabbit; for [2, 3] Cy³ anti-goat with Alexa Fluor 488 anti-rabbit; for [4] Cy³ anti-goat with Alexa Fluor 488 anti-mouse) and incubated for 2 hours at room temperature. After washing, the sections were coverslipped with GEL/MOUNT permanent aqueous mounting medium (Merck, Darmstadt, Germany) and stored in the dark at 4°C.

Analysis

The fluorescence-labeled sections were examined with a epifluorescence microscope (DMRB; Leica, Bensheim, Germany) equipped with appropriate filters for red fluorescent Cy³ (N2.1, excitation filter BP 515–560 nm, dichromatic mirror 580 nm, suppression filter LP 590 nm) and Alexa Fluor 488 (I3, excitation filter BP 450–490 nm, dichromatic mirror 510 nm, suppression filter LP 515 nm).

The slides with immunoperoxidase-based staining were examined with the same microscope under brightfield illumination. Microphotographs were taken with a digital camera (Pixera Pro 600 ES; Klughammer, Markt Indersdorf, Germany), captured on a computer (Pixera Viewfinder software, Klughammer), and processed with image analysis software (Photoshop 11.0; Adobe Systems, Mountain View, CA, USA). The images were arranged and labeled using drawing

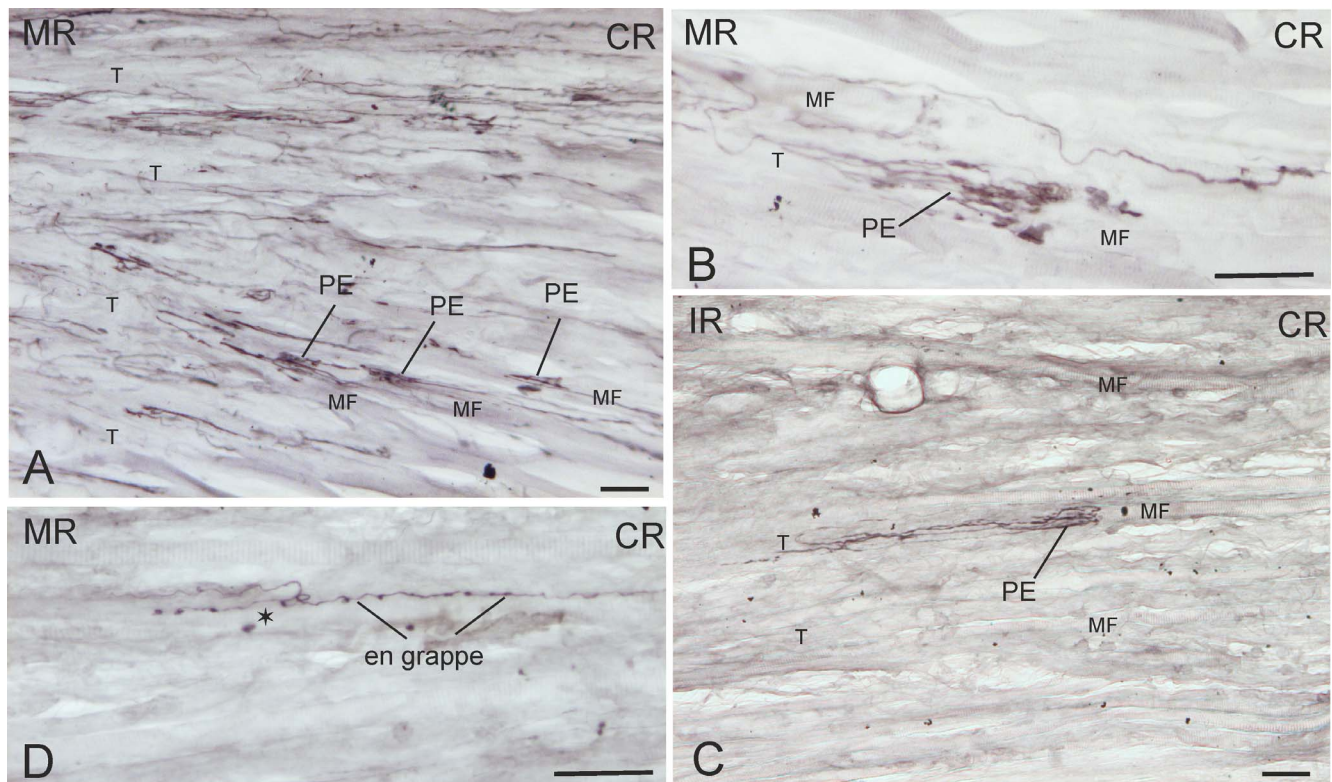


FIGURE 1. Immunoperoxidase staining for calretinin (CR). In contrast to numerous CR-positive palisade endings in the medial rectus muscle (MR) (A, B), the inferior rectus muscle (IR) contains only few CR-positive palisade endings (C). In both muscles an additional few CR-positive en grappe endings were found (D). T, tendon; MF, muscle fiber. Scale bar: 50 μm.

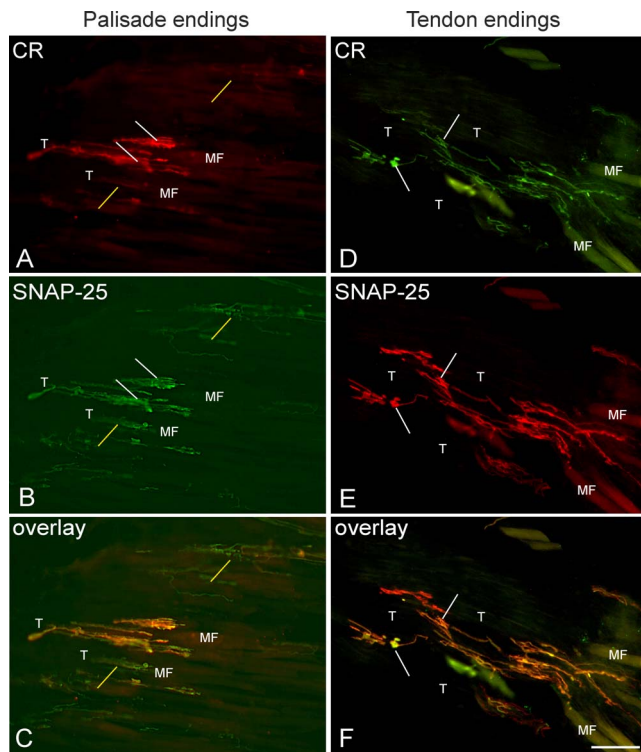


FIGURE 2. Immunofluorescence. Numerous palisade endings (A–C) in the distal part of the medial rectus muscle stained for calretinin (CR) (A, red, white lines) and SNAP-25 (B, green, white lines) show a coexpression of both markers (C, yellow). In addition, CR-negative palisade endings are present (A–C, yellow lines). Tendon endings were found in the distal tendon of the medial rectus muscle identified by SNAP-25 (E, red, white lines) that stained for CR (D, green, white lines) as clearly seen in the overlay (F, yellow, white lines). T, tendon; MF, muscle fiber. Scale bar: 50 μ m.

software (CorelDraw 11.0; Corel, Ottawa, ON, Canada). The sharpness, contrast, and brightness were adjusted to reflect the appearance of the labeling seen through the microscope.

CR-positive and CR-negative palisade endings in the respective extraocular muscles were counted on every third section (20 μ m/section) of the series containing the myotendinous junction area. In addition, every third section was carefully inspected for other CR-positive nerve endings.

RESULTS

Calretinin-Positive Nerve Endings in the Extraocular Muscles

Immunostaining for SNAP-25 revealed the complete innervation of all extraocular muscles, which included large en plaque endings in a broad band at the middle third, and smaller focal endings and en grappe endings across the whole muscle length. In the myotendinous junction of all extraocular muscles numerous palisade endings were found, occasionally associated with tendinous ending complexes as described previously.²³ Furthermore, thin nerve fibers meandering across and traveling between the muscle fibers in the connective tissue were observed in addition to small varicose nerve endings associated with blood vessels.

The systematic analysis of the nerve endings in all extraocular muscles immunostained for the simultaneous expression of CR revealed that only the MR and IR contained CR-positive palisade endings (Fig. 1; Table 2). Therefore the

following description of palisade endings refers only to these two muscles.

Close inspection of every third slide of the myotendinous junctions of MR and IR revealed that both muscles contained a considerable fraction of CR-immunoreactive palisade endings, which were more numerous in the MR (Fig. 1; Table 2), in addition to the total number of palisade endings (Figs. 2A–C; Table 2). More than 86% of identified SNAP-25-positive palisade endings in the MR expressed CR, and more than 20% in the IR (Figs. 2A–C; Table 2). Furthermore, the quantitative analysis of the palisade endings in every third section of the extraocular muscles demonstrated that the MR contained far more palisade endings at the myotendinous junction (36/60) compared to the other eye muscles (IR 5/8, LR 16/17) (Table 2).

The CR-positive palisade endings were scattered over the full extent of the myotendinous junction and showed the same distribution as the CR-negative ones. Furthermore, CR-negative and CR-positive palisade endings did not differ in their morphology or size. As found for CR-negative palisade endings, some CR-positive palisade endings were associated with CR-positive tendon endings (Figs. 2D, 2E, 2F). Similarly, en grappe endings in the distal part of the MR and IR were identified that expressed CR immunoreactivity (Figs. 1D, 3A–C) in addition to those that lacked CR (Figs. 3D–F). We did not find any en plaque ending expressing CR within the broad innervation band in the midbelly of all extraocular muscles (Figs. 3G–I).

Other CR-positive nerve fibers and endings were identified in the distal third of the muscles, which included spiral endings, and compact and complex endings as described by Billig et al.²⁹ in 1997 in cat extraocular muscles. Accordingly, thick nerve endings covering the whole width of a muscle fiber, which arose from coiled nerve fibers, were considered as compact endings (Fig. 4A). Complex endings were characterized by a thin axon approaching the muscle fiber orthogonally to split up into several collateral branches into opposite directions along the muscle fiber to terminate in numerous small boutons (Fig. 4D). Thin nerve fibers with numerous boutons, which encircled single muscle fibers, were considered as spiral endings (Fig. 4B). In addition, very thin nerve fibers meandering across the muscle fibers were found, few of them expressing CR immunoreactivity (Fig. 4C). These thin CR-positive fibers were also found in the other extraocular muscles and may represent autonomic nerve fibers. However none of the other extraocular muscles contained any CR-positive en grappe endings.

We performed simultaneous immunostaining for the cholinergic marker ChAT and CR to study whether CR-positive palisade endings represent a specific class of nerve endings not described before. The systematic analysis of the MR revealed that all CR-positive palisade endings showed cholinergic features, as found for CR-negative ones (Figs. 5A–C). The same observation of an additional ChAT immunoreactivity was made for the CR-positive en grappe endings in MR and IR, as well as for CR-positive focal endings (Figs. 5D–I).

Calretinin-Positive Neurons in the C-Group of the Oculomotor Nuclei

Based on the finding that distal tracer injections into the myotendinous junction of MR or IR resulted in exclusive back-labeling of neurons in the C-group^{23,25} filling the cell bodies of palisade endings and en grappe endings, a systematic study of the neurons in the C-group for the expression of CR was performed in the present study. Combined immunostaining for the retrograde tracer (WGA or CTB) revealed a consistent population of retrogradely labeled neurons in the C-group that were CR positive (Fig. 6). Only one case (case 3) with a very

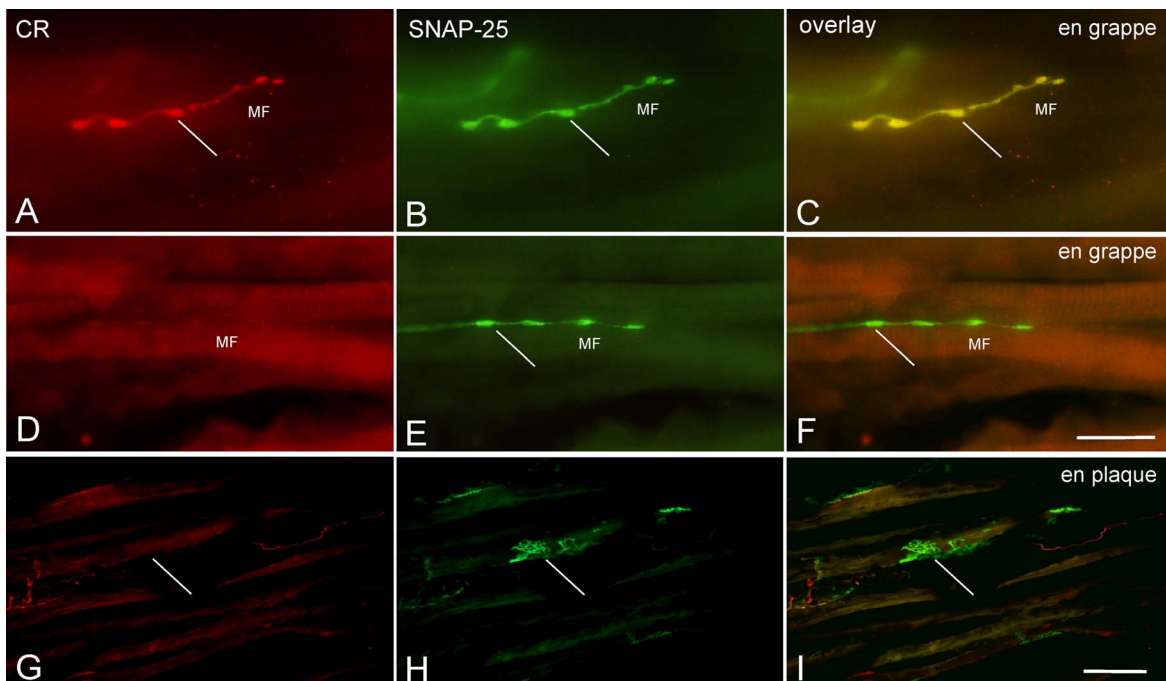


FIGURE 3. Immunofluorescence. High-power photograph of a calretinin (CR)-positive axon with en grappe endings along a muscle fiber (A, red) in the medial rectus muscle, visualized by SNAP-25 (B, green; C, yellow). Other multiple endings along muscle fibers (MF) did not express CR (D–F, white line). The photograph of the belly of the medial rectus muscle demonstrates the lack of CR in en plaque endings (G, red, white line) identified by SNAP-25 (H, green; I, green, white line). Scale bar: 30 μm.

small tracer injection site resulted in only few retrogradely labeled neurons in the C-group, none of them expressing CR immunoreactivity.

As found previously, no CR expression was found in SIF motoneurons within the oculomotor nucleus.³⁰ The systematic

analysis of six cases with tracer injections revealed a consistent population (7% to over 40%) of CR-expressing neurons within the C-group (Table 1). This was most obvious in cases with tracer injections into the myotendinous junction of the MR muscle, which exclusively labeled the peripheral neurons in

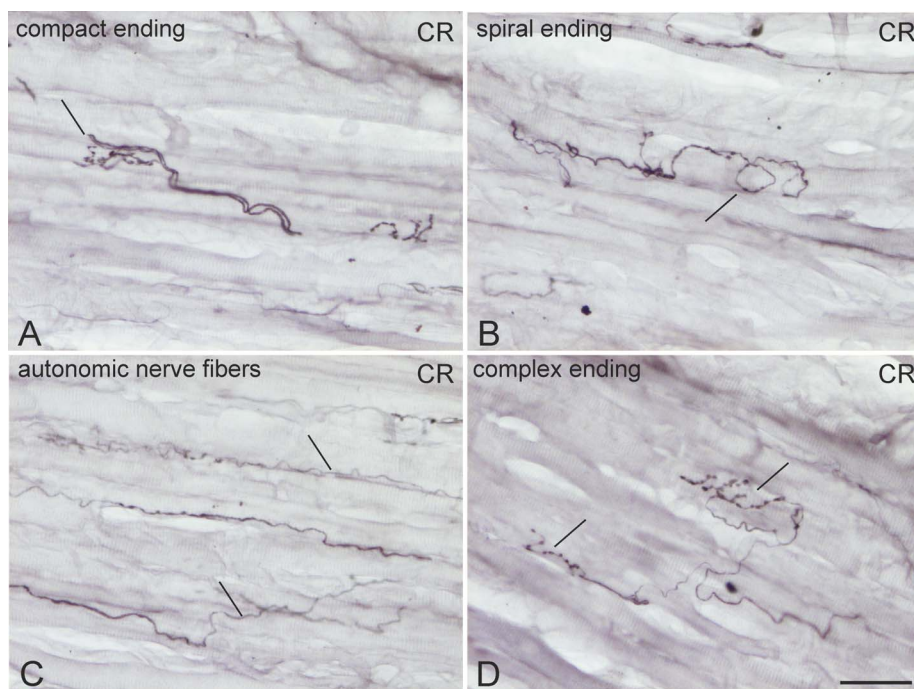


FIGURE 4. Examples of additional calretinin (CR)-positive nerve fibers in the medial rectus and inferior rectus muscle including a compact ending (A), a spiral ending (B), and thin fibers meandering across muscle fibers as putative autonomic nerve endings (C, lines) and complex endings (D). Scale bar: 50 μm.

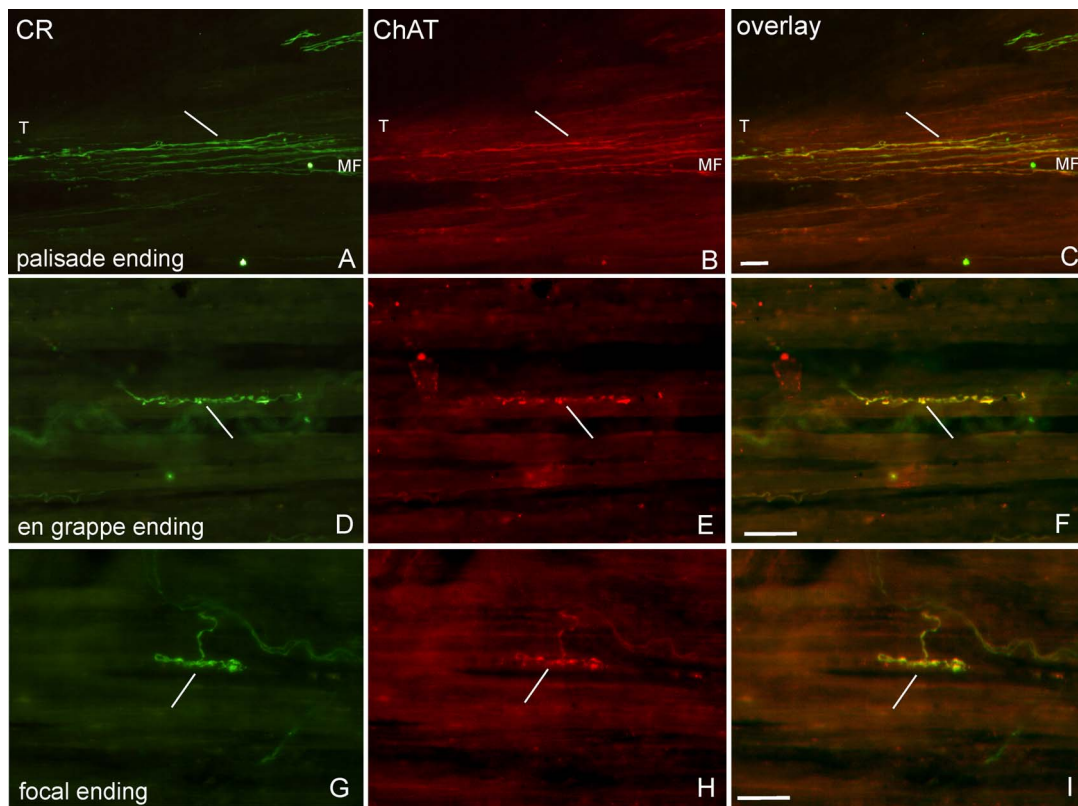


FIGURE 5. Immunofluorescence. Calretinin (CR)-positive palisade endings (A, green), en grappe endings (D, green), and small focal endings (G) in the medial rectus muscle all expressed the cholinergic marker choline acetyltransferase (ChAT) (B, E, H, red, line), which is more clearly seen in the overlay (C, E, I, yellow, line). Scale bar: 50 μ m.

the C-group extending further rostral to oculomotor nucleus, where 23% to over 40% CR-positive neurons of all tracer-labeled neurons were counted (Table 1). A muscle belly injection into the MR led to tracer uptake in multiple en grappe endings distributed along the whole length of the extraocular muscle, but spared the distal muscle part with palisade endings, showing a considerable number of CR-positive neurons of 15%, as well (Table 1). In two cases for a different project, tracer injections were aimed at other extraocular muscles, but contaminated the myotendinous junction of MR or IR and led to retrograde labeling in the C-group as well. Therefore these cases were incorporated in this study (see Table 1: Case 4, injection into the LR contaminated the IR; case 5, IR injection contaminated the MR; case 6, injection of the SR contaminated the MR). In case 5 tracer uptake occurred from both, IR and MR, which led to retrograde tracing of both populations in the C-group. The assignment of the labeled neurons to the respective extraocular muscle was based on the findings in a previous study.³¹ The systematic analysis and plotting of CR-positive and CR-negative tracer-labeled neurons in the C-group in all cases revealed that the CR-positive and CR-negative neurons were intermingled throughout the C-group in the MR population as shown for one example (Fig. 7).

DISCUSSION

Our studies further characterized palisade endings histochemically to infer potential functional properties. We found a subpopulation of palisade endings only in the MR and IR that expressed the calcium-binding protein CR. More than 86% of the palisade endings in the MR and more than 20% in the IR were CR positive. In addition, both muscles contained CR-

positive multiple en grappe endings. In line with this result, tracer injections into the myotendinous junctions of MR or IR resulted in back-labeling of a subpopulation of CR-positive neurons in the C-group of the oculomotor nucleus.

Additional CR-positive nerve endings included spiral, compact, and complex endings as described by Billig et al. in 1997.²⁹ Since compact and complex endings were anterogradely labeled after tracer injections into the trigeminal ganglion, they have been considered as sensory.²⁹ Therefore it is quite possible that the small population of CR-positive neurons within the trigeminal ganglion that were back-labeled from tracer injection into the MR muscle in monkey represent their parent cell bodies.³² Based on their anterograde labeling following tracer injections into the oculomotor nucleus or nerve, the spiral nerve endings have been considered as motor endings.^{29,32} Whether these endings form their own functional group of terminals or are a modification of en grappe endings, whose cell bodies are located in the C-group as well, is not known.

The CR Expression in Palisade Endings Does Not Support a Motor or Sensory Function

With immunostaining for ChAT, vesicular acetylcholine transporter, choline transporter, or calcitonin gene-related peptide palisade endings have been shown to express the same histochemical signature as multiple en grappe and single en plaque motor endings.³³⁻³⁵ The cholinergic characteristics of palisade endings are one property that favors a motor function,³⁶ whereas calcitonin gene-related peptide has been identified in motor^{37,38} and sensory neurons.³⁹ With the additional CR immunoreactivity in a

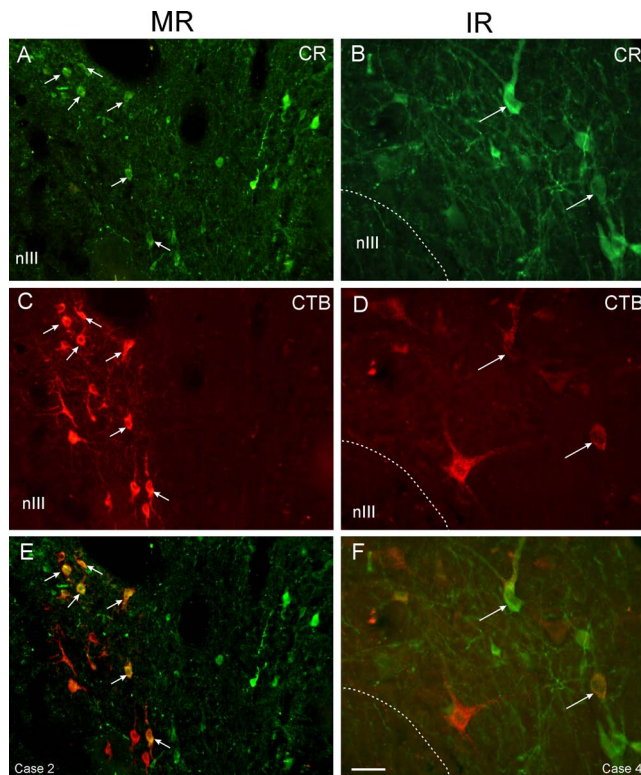


FIGURE 6. Immunofluorescence of tracer-labeled neurons in the C-group after cholera toxin subunit B (CTB) injection into the myotendinous junction of the left medial rectus muscle (C, red) and left IR (D, red). Combined immunofluorescence for calretinin (CR) (A for MR, green; B for IR, green) revealed a considerable portion of double-labeled neurons in the C-group (E for MR, F for IR, arrows, yellow). nIII, oculomotor nucleus. Scale bar: 100 μ m.

subset of palisade endings and multiple endings, but lacking in en plaque endings, we here report for the first time on a marker that is differentially expressed in cholinergic endings targeting muscle fibers in the extraocular muscle. Until now, CR has not been identified in motoneurons of extraocular muscle,^{30,40,41} but is present in sensory afferents that are considered to be rapidly adapting mechanoreceptors including annulospiral nerve endings of muscle spindles in skeletal muscles, palisades of lanceolate nerve endings in hair follicles and vibrissae of rat.^{42,43} More recently, CR was also found in proprioceptive terminals surrounding the posterior and reticular ciliary muscle tips and their elastic tendons thought to measure stretch of the tendon.⁴⁴ In general, CR does not seem to be a good marker to distinguish between sensory or motor neurons or nerve endings, since it is expressed in neurons of sensory ganglia⁴⁵ and also in motoneurons of the spinal cord.⁴¹ Although the present findings showing CR expression in a subset of palisade endings do not allow us to assign a sensory versus motor function to palisade endings, a specialized role of palisade endings in MR and IR is supported.

Anatomic Arrangement of Palisade Endings and Multiple Endings

Since in the present study CR was expressed in a subset of both palisade endings and multiple endings in MR and IR, this marker did not help to resolve the question whether palisade endings and multiple endings derive from a

common cell body or from different cell bodies as discussed previously.³² The partial CR expression in nerve endings was corroborated by the identification of a rather robust CR-positive population of tracer-labeled C-group neurons of the MR and a smaller one of the IR, which reflect the frequency of CR-positive palisade endings/multiple endings in the respective extraocular muscles. We know from previous studies that tracer injections into the myotendinous junction of extraocular muscles led to retrogradely labeled neurons exclusively in the periphery of oculomotor nucleus including the C-group.^{25,31} Based on the findings that central tracer injections into the vicinity of extraocular muscle motoneuclei anterogradely labeled palisade endings,^{24,32} the peripheral cell groups are now considered to contain the MIF motoneurons and palisade ending cell bodies.²³ Given such an arrangement and the frequency of CR-positive palisade endings/multiple endings and cell bodies in the C-group as a consideration, it is reasonable to assume that palisade endings and en grappe endings are supplied by a single neuron as indicated from whole-mount extraocular muscle preparations.³⁵ Further support comes from a recent developmental study in cats, where the innervation pattern in extraocular muscle at different time points after birth was studied.⁴⁶ The data suggest that the axons with multiple nerve endings grow out toward the tendon and then turn back to form palisade endings.

In summary, two populations of these cell bodies may exist, CR-positive and CR-negative ones. However, it still cannot be ruled out that (apart from the CR expression) two categories of neurons exist in the C-group: cell bodies of palisade endings, which in addition provide multiple endings along the distal and proximal nontwitch muscle fibers, and cell bodies giving rise to exclusively multiple endings along the complete extent of nontwitch muscle fibers (Fig. 8).

Specialized Role of Palisade Endings in MR and IR in Convergence

Taken together, our present findings identified two histochemically different forms of nerve endings (palisade endings and multiple endings) that are associated with nontwitch muscle fibers and are thought to control eye stabilization and alignment (for review see Refs. 3 and 18). The additional CR-positive population of both nerve ending types in the MR and IR may represent a specialization in these muscles for a specific task, such as convergence. This is in accordance with recent investigations. A comparative quantitative study on the numbers of palisade endings in the rectus muscles in different animals including lateral-eyed and frontal-eyed species revealed two major points: Only in frontal-eyed species like cats, dogs, monkeys, and humans are well-developed palisade endings a constant feature, whereas in lateral-eyed species the presence, frequency, and morphology varied.¹⁹ In all species with palisade endings, their frequency was highest in the MR and the lowest number was found in the LR.¹⁹ The developmental study in cats revealed that the adult-like morphology and density of palisade endings was reached first in MR followed by IR, and only 50 days later in the LR.⁴⁶ Thereby the time course of palisade ending development accompanies the development of binocularity, which requires vergence, in the first 3 months after birth in cats,⁴⁷ or in the first 6 weeks in monkeys.⁴⁸ The selective presence of a CR-positive subpopulation of palisade endings in the MR, and to a lesser extent in the IR, which participates in vergence mainly in the lower visual field, supports the view of a special palisade ending function that is most strongly required in vergence.

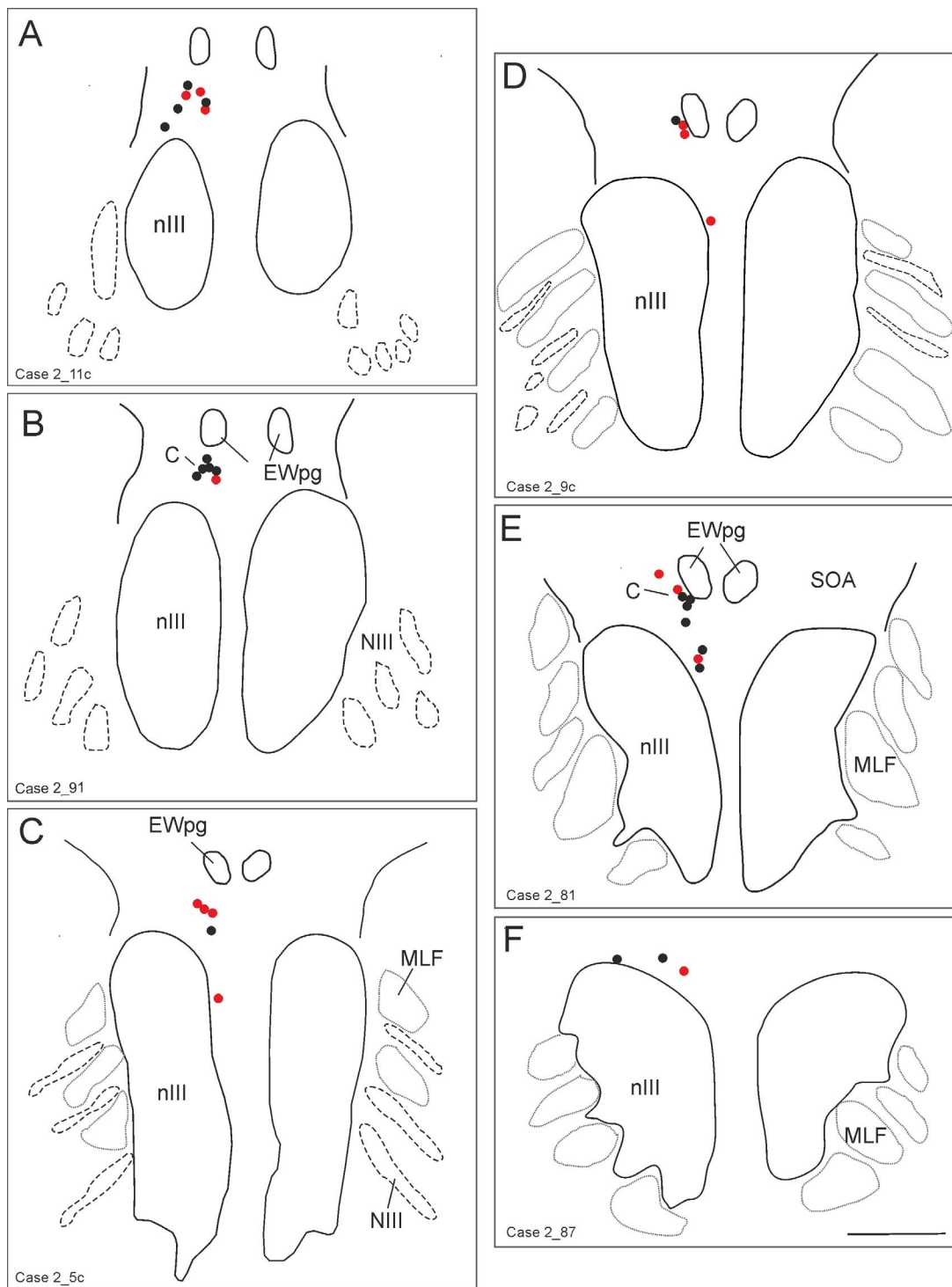


FIGURE 7. Plot of the C-group neurons from rostral to caudal (A-F). *Black dots* indicate the retrogradely labeled neurons after a cholera toxin B injection into the distal part of the medial rectus muscle. *Red dots* indicate tracer-labeled neurons that express calretinin. Note that calretinin-positive neurons are distributed throughout the whole extent of the C-group. EWpg, Edinger-Westphal nucleus, preganglionic subdivision; SOA, supraoculomotor area; nIII, oculomotor nucleus; MLF, medial longitudinal fasciculus. *Scale bar:* 500 μ m.

Functional Significance of CR-Positive Palisade Endings in MR and IR

Like parvalbumin and calbindin, CR is a calcium-binding protein present in many neurons of the peripheral and central nervous system, and may be involved in the maintenance of the homeostasis of intracellular calcium ions.⁴⁹ Neither the

functional significance of CR in certain cell groups nor the mechanisms through which CR or the other calcium-binding proteins produce their diverse effects are clear. Among current hypotheses, there are consistent indications that CR has a role in the modulation of neuronal excitability.⁵⁰ This has been most clearly shown in the cerebellum of CR knockout mice (reviewed in Ref. 51). The cerebellar granule cells in these

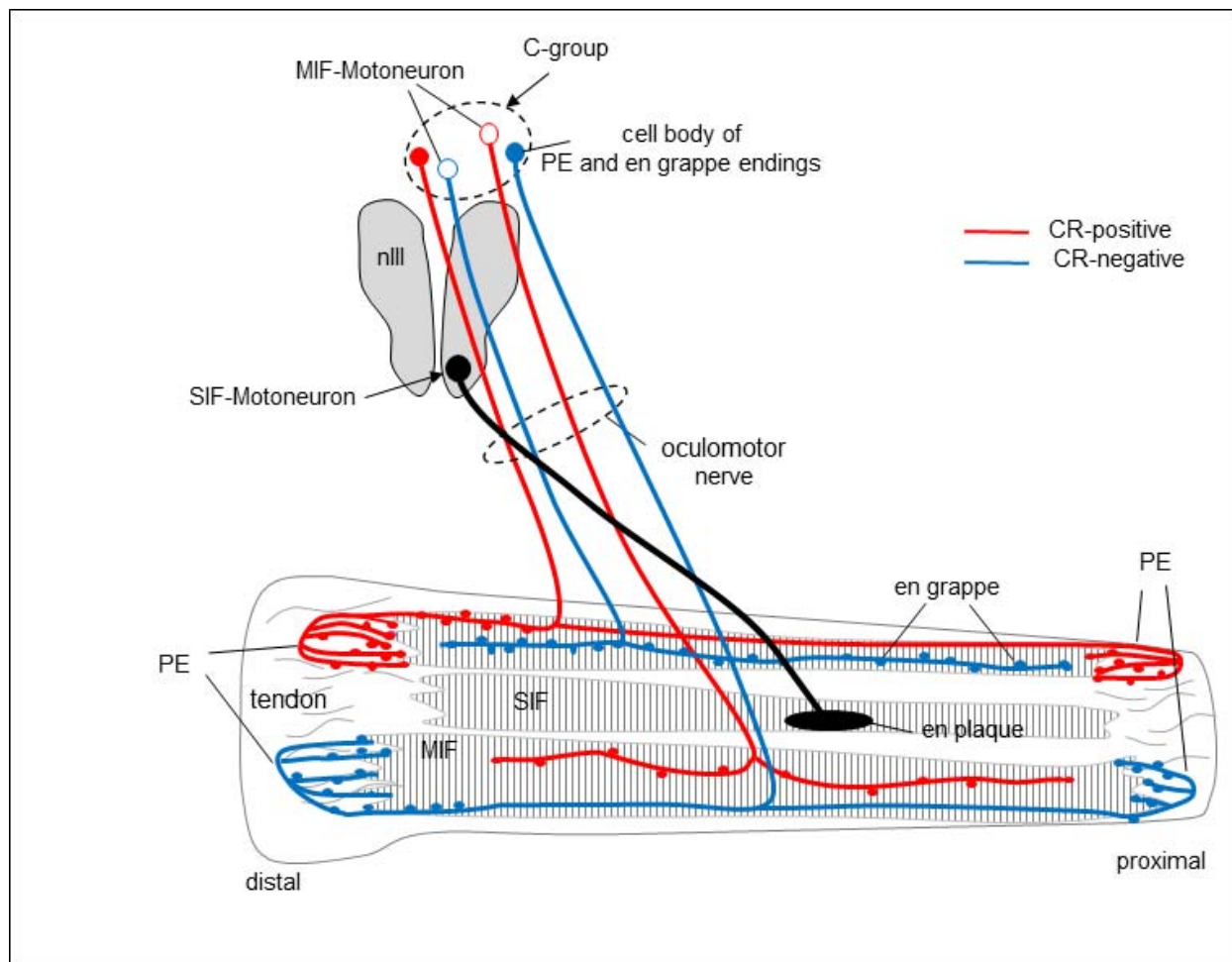


FIGURE 8. Summary diagram describing the possible innervation pattern of the global layer in the medial and inferior rectus muscle based on the present findings: Motoneurons within the oculomotor nucleus (nIII) correspond to motoneurons of skeletal muscles and target the twitch muscle fibers by a single en plaque endplate (SIF) (black). Two histochemically different sets of palisade endings (PE) and multiple en grappe endings were found in the medial and inferior rectus muscle. This includes one group of calretinin (CR)-positive (red) and CR-negative (blue) palisade and multiple endings, respectively. Two populations of neurons are present in the C-group targeting the nontwitch muscle fibers, a CR-positive and a CR-negative group. The present study could not determine whether PEs and multiple endings form an anatomic entity or originate from different neuronal groups within the C-group, or whether both forms are present in parallel. Therefore, in theory, four populations of neurons in the C-group can be anticipated: CR-positive neurons giving rise to palisade endings and multiple en grappe endings (*filled neuron soma*), CR-positive neurons providing only multiple endings (*open neuron soma*), and the respective populations for CR-negative neurons including their nerve endings. The CR-positive innervation unique to the medial and inferior rectus muscle may represent an additional specialized more excitable set of neurons targeting nontwitch muscle fibers required for eye alignment in convergence.

mice showed an increased excitability in the absence of CR leading to altered firing properties.⁵² A recent study of the expression of calcium-binding proteins in motoneurons in the spinal cord of the locomotor system in zebrafish revealed that CR was highly expressed in fast motoneurons, but not in slow motoneurons.⁵³ This is in contrast to our findings in the oculomotor system, where CR is present only in a subpopulation of neurons activating multiply innervated tonic muscle fibers with slow contractions,⁵⁴ whereas the motoneurons activating singly innervated twitch muscle fibers lack CR, but contain parvalbumin.^{55,56} On the other hand, in the rostral mesencephalon a subgroup of saccadic premotor burst neurons (only those involved in upgaze) contain CR.⁵⁷ These neurons have high firing rates and thereby are histochemically characterized by their additional content of parvalbumin and the presence of perineuronal nets.^{58,59} Therefore the CR expression of a given neuron does not seem to depend on the firing rate, but it is possible that the CR-positive palisade

ending subgroup is a more specialized group of neurons with an increased sensitivity by modulation of the excitability. This is most obvious in the MR muscle, which contains not only the largest palisade ending population, but also the highest number of CR-positive palisade endings. The fact that all extraocular muscles contain palisade endings suggests that they play an important role for eye movements in all directions, such as eye alignment and stabilization during fixation of a visual target or during smooth pursuit. The CR-positive palisade and multiple endings of the MR and IR muscles, which may represent a more excitable and thereby more sensitive system, could provide the fast precise eye alignment during vergence when changing the viewing distance. In infants, vergence movements are adult-like at the age of 2 months.⁶⁰ To date it is not known at what age CR-positive palisade endings are present in MR and IR muscles, and this has to be resolved in future developmental studies.

CONCLUSIONS

Taken together, our findings provide information that palisade endings (and multiple endings) are not a uniform, homogeneous group of nerve endings across all extraocular muscles, but differ in their histochemical properties in those extraocular muscles that are involved in vergence. Thereby it is possible that the CR-negative palisade endings and multiple endings represent the basic population present in all extraocular muscles. Whether the palisade endings with associated multiple endings may serve as proprioceptive or effector organs is still not clear. In contrast, the CR-positive palisade endings and multiple endings may form a specialized, perhaps more excitable type of nerve endings in MR and IR most active in vergence. The malfunction of this CR-positive population of neurons targeting nontwitch muscles fibers may play a role in strabismus, which is characterized by a tonic misalignment of the eyes.⁴⁸

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