The source of innervation and function of palisade endings in the extraocular muscles of Macaca mulatta

Dissertation

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If you can dream - and not make dreams your master

If you can think - and not make thoughts your aim

If you can meet with Triumph and Disaster

And treat those two impostors just the same

R. Kipling

Veröffentlichte Publikationen:

- **Lienbacher K.**, Mustari M., Hess B., Büttner-Ennever J., Horn A.K. (2011) Is there any sense in the palisade endings of eye muscles? Ann N Y Acad SCI 1233:1-7.*
- **Lienbacher, K.**, Mustari, M., Ying, H.S., Büttner-Ennever, J.A., Horn, A.K.E. (2011) Do palisade endings in extraocular muscles arise from neurons in the motor nuclei? Invest Ophthalmol Vis Sci 52:2510-2519.*
- Ying, H.S., **Fackelmann, K.**, Messoudi, A., Tang, X.F., Büttner-Ennever, J.A., Horn, A.K. (2008) Neuronal signalling expression profiles of motoneurons supplying multiply or singly innervated extraocular muscle fibres in monkey. Prog Brain Res171:13-16.
- **Fackelmann, K.**, Nouriani, A., Horn, A.K., Büttner-Ennever, J.A. (2008) Histochemical characterisation of trigeminal neurons that innervate monkey extraocular muscles. Prog Brain Res 171:17-20.*
- **Lienbacher, K.** and Horn, Anja K. E., Biol Cybern, Palisade endings and proprioception in extraocular muscles: a comparison with skeletal muscles (2012) (in press), DOI: 10.1007/s00422-012-0519-1.*

Teile dieser Doktorarbeit wurden in den mit * gekennzeichneten Publikationen veröffentlicht.

Veröffentlichte Kongressbeiträge

- **Lienbacher K.**, Kimura H. and Horn A.K.E. (2011) Analysis of sensory afferents of extraocular muscles in the trigeminal ganglion and their sensory endings in eye muscle of the rat. International Brain Research Organization, Annual Meeting.
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- **Lienbacher, K.**, Mustari, M., Hess, B., Peisker, N., Horn, A.K. (2009) Proprioception in the extraocular eye muscles of different species. Neuroforum, Februar Vol. XV(1), Neurowissenschaftliche Gesellschaft.
- Schulze, Ch., Rothermel, M., **Lienbacher, K.,** Curie, T., Klupp, B.G., Mettenleiter, T.C., Distler, C., Hatt, H., Hoffmann, K.-P., Horn, A.K.E. (2009) Transsynaptic retrograde labelling in the oculomotor system in rodent using tetanus toxin fragment C and Pseudorabies virus: opportunities and limitations. Neuroforum, Februar Vol. XV (1), Neurowissenschaftliche Gesellschaft.

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Abbreviations

CCN	central caudal nucleus
cGMP	cyclic guanosine monophosphate
ChAT	choline acetyltransferase
CR	calretinin
CT	choleratoxin (subunit B)
EWpg	Edinger-Westphal nucleus
GTO	Golgi tendon organ
i.p.	intraperitoneal
i.v.	intravenous
IO	inferior oblique muscle
IR	inferior rectus muscle
LP	levator palpebrae muscle
LR	lateral rectus muscle
MHC	myosin heavy chain
MHCs	myosin heavy chain slow
MIF	multiply innervated fiber
MLF	medial longitudinal fasciculus
MR	medial rectus muscle
N III	oculomotor nerve
n III	oculomotor nucleus
N IV	trochlear nerve
n IV	trochlear nucleus
N VI	abducens nerve
n VI	abducens nucleus

NDS	normal donkey serum
NO	nitric oxide
NOS	nitric oxide synthase (neural)
OKR	optokinetic reflex
PV	parvalbumin
SIF	singly innervated fiber
SNAP-25	synaptosomal-associated protein 25
SO	superior oblique muscle
SR	superior rectus muscle
SYN	synaptophysin
TBS	tris buffered saline
TG	trigeminal ganglion
TH	tyrosine hydroxylase
V1	ophthalmic branch of the trigeminal ganglion
V2	maxillary branch of the trigeminal ganglion
V3	mandibular branch of the trigeminal ganglion
VMes	mesencephalic trigeminal nucleus
VOR	vestibulo-ocular reflex
WGA	wheat germ agglutinin
WGA-HRP	wheat germ agglutinin – horseradish peroxidase

1 Abstract

Vertebrate extraocular muscles show a highly complex anatomy, which differs in many respects from skeletal muscles. Furthermore, there is a considerable variation among different species with regard to the presence of proprioceptive organs. Whereas muscle spindles and Golgi tendon organs are well developed in sheep and pig, neither are found in cat, and only poorly developed muscle spindles are present in human and monkey. In all vertebrates studied so far cuffs of nerve terminals around multiply innervated muscle fibers of the global layer, termed palisade endings (PE) are present at the myotendinous junction.

Palisade endings (PE) are specialized nerve endings unique to extraocular muscles. There is still an ongoing debate on PE function. A proprioceptive function is supported by their ultrastructural morphology, and the location of the majority of their terminals within the muscle tendon. A motor function of PEs is suggested by the expression of different cholinergic markers, and the binding of α-bungarotoxin to at least a small proportion of PE nerve endings. So far the location of the somata giving rise to the PEs is unknown.

After eye muscle injections with different tracers, and the investigation of retrogradely labeled cells after three days of survival, there are two possible options for the location of the cell bodies of PEs: The trigeminal ganglion (TG), where almost all of the sensory afferents of eye muscles come from, or the motor neurons of the abducens (nVI), trochlear (nIV) and oculomotor nucleus (nIII) in the brainstem, where the motor neurons of the extraocular muscles are situated.

In the first part of this thesis the histochemical and morphometric properties of TG cells projecting to the extraocular muscles were studied and related to different nerve fibers and terminals in these muscles, to obtain more information about their function. Retrogradely labeled TG neurons and eye muscle terminals were processed for the presence of substance P (SP), nitric oxide synthase (NOS), calretinin (CR) and cholinacetyltransferase (ChAT). Injections of the tracer were placed into the medial rectus (MR) or the lateral recuts muscle (LR), either in the belly or in the distal, myotendinous part of the muscle. PEs were only labeled by CR or ChAT and none of the other markers that were used. Furthermore, CR positive retrogradely labeled tracer cells in the TG were

rarely found (under 1 % of all tracer cells). The results indicate that the possible function of TG afferents could be mainly vasodilatation or nociception whereas the probability of proprioception via PEs is very low. These results point to the fact, that the source of the PE cell body is probably localized in the brainstem. In a second attempt to solve this problem, rhesus monkey received tract-tracer injections (WGA or CT) into the oculomotor nucleus, which contains the motor neurons of the medial, inferior and superior recti and inferior oblique muscles, as well as the trochlear nucleus, which contains the motor neurons of the superior oblique. All extraocular muscles were processed for the combined immunocytochemical detection of the tracer and non-phosphorylated neurofilament for the visualization of the complete muscle innervation. In all muscles (except lateral rectus) numerous anterogradely tracer labeled PEs were found, as well as tracer-filled tendon organs. In addition the en plaque and en grappe motor endings, were also strongly tracer positive. Double immunolabeling revealed that all types of nerve endings including tendon organs were anterogradely labeled, except the thin tyrosine hydroxylase positive autonomic nerve fibers of the sympathetic system. No anterograde labeling was found within the trigeminal ganglia.

These results suggest that the somata of palisade endings are located within the brainstem, in or around the oculomotor nuclei, and confirm several previous studies. In how far the multiple nerve endings of non-twitch muscle fibers and the PEs form an anatomical entity with one parent soma in the periphery of the oculomotor nuclei was studied by morphological and histochemical analysis of the peripheral neurons around the motor nuclei. This data revealed two populations: one group of cholinergic multipolar neurons represent the motor neurons supplying the multiple motor innervation, one group of round calretinin positive cholinergic neurons giving rise to PEs. If the palisade endings do have a sensory function, then their cell body location amongst the non-twitch motor neurons would be an ideal location to control the tension in the non-twitch extraocular muscle fibers, and the data brought together in this thesis points strongly in this direction.

To fully establish this hypothesis more physiological experimental data is required.

2 Introduction

An understanding of the control of eye movements leads to fundamental insights into how the brain works. Irregularities in the motility of eye muscles are often the result of abnormal processes in the brainstem. However in almost every area of the brain, in monkey and human, some activity related to eye movements, can be found. Furthermore, the complexity of these movements is reflected not only in the organization of central activity, but also in the construction and innervation of the individual extraocular eye muscle (EOM) itself.

2.1 Extraocular muscles

2.1.1 Arrangement and function

The eye is moved by six extraocular muscles (EOMs), four recti muscles (the superior-, inferior-, medial- and lateral rectus) and two oblique muscles (superior- and inferior oblique). A seventh EOM, the levator palpebrae superioris (LP), does not affect the movement of the eye, but elevates the upper eyelid and appears only in mammals (Spencer and Porter, 2006). The presence of these four recti and two oblique muscles is consistent across vertebrates, only the pattern of innervation and the site of insertion differs (Isomura, 1981). In the bony orbit the eyeball is surrounded by the EOMs, together with connective tissue and orbital fat. All muscles, with the exception of the inferior oblique (IO) and LP muscle, have their origin in the annulus of Zinn, a tendinous ring which surrounds the optic foramen and a portion of the superior orbital fissure (Sevel, 1986). The LP has its origin at the sphenoid bone, above the optic foramen, whereas the IO arises from the maxillary bone in the medial wall of the orbit (Fig. 1).

The medial rectus (MR) lies medial to the globe and inserts posterior to the corneoscleral junction; the lateral rectus (LR) lies on the lateral aspect of the globe and inserts on the sclera via a long and broad tendon. Besides these two horizontal muscles the two vertical muscles, the superior rectus (SR) and inferior rectus (IR), insert dorsally and ventrally on the globe, anterior to the equator. The superior oblique muscle (SO) inserts at the posterotemporal

surface of the globe, passes medially, through the trochlea, a chondral ring at the upper edge of the medial orbit, and courses proximally to the tendinous annulus. Lastly, the IO originates from the maxillary bone behind the lacrimal fossa, passes ventral to the tendon of the IR and inserts on the lateral eyeball, between the IR and LR (Miller, 1998) (Fig. 1).

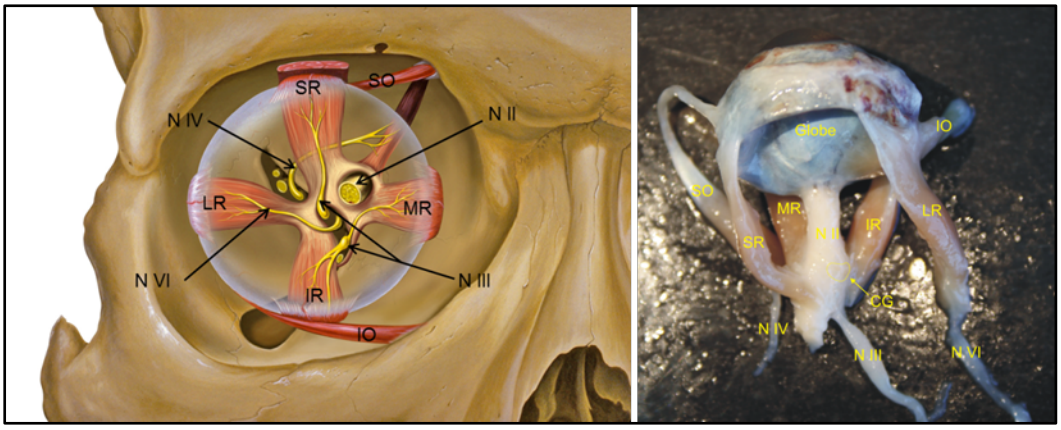

Fig. 1 Alignment of the eye muscles: The left drawing shows the alignment of eye muscles within the orbit. All muscles, with the exception of the inferior oblique (IO) and LP muscle, have their origin at the annulus of Zinn. The LP has its origin at the sphenoid bone, above the optic foramen, whereas the IO arises from the maxillary bone in the medial wall of the orbit. All eye muscles except the LP insert on the globe. The right picture shows a dissected monkey right eye with the extraocular muscles and corresponding nerves. NII – optic nerve; NIII – oculomotor nerve; NIV – trochlear nerve; NVI – abducens nerve. *(Illustration left: modified from Patrick Lynch, Yale University School of Medicine).*

According to their location and insertion to the globe, the EOMs have different functions in eye movements (Tab. 1). The horizontal muscles of the MR and LR insert on the opposite sides of the globe, as do the vertical muscles of SR and IR, therefore these muscle pairs are functional antagonists. The MR is the principal adductor and the LR the principal abductor of the eye. These two are the only muscles with no secondary function. In contrast, the SR and IR have, in addition to their function for elevation and depression a secondary function. Depending on the starting position, the SR participates in intorsion and adduction as tertiary function, while the IR contributes also to extorsion and adduction. The main action for the SO is intorsion, the secondary and tertiary actions are depression and abduction, respectively. The IO is mainly involved in the extorsion, but also in elevation and abduction (Horn and Leigh, 2011).

Muscle	Primary Function	Secondary Function	Tertiary Function
Medial rectus	adduction	none	none
Lateral rectus	abduction	none	none
Superior rectus	elevation	intorsion	adduction
Inferior rectus	depression	extorsion	adduction
Superior oblique	intorsion	depression	abduction
Inferior oblique	extorsion	elevation	abduction

Tab. 1 The eye muscles and their different functions in eye movements

2.1.2 Gross and fine anatomy

Compared to skeletal muscles, the EOMs show unique functional features in morphology and physiology, which is reflected in their composition. They are among the fastest and most fatigue resistant muscles in mammals, but also possess slow, non-twitch muscle fibers, a phylogenetically older muscle type which has been well studied in avians and amphibians (Morgan and Proske, 1984, Spencer and Porter, 1988, Ruff et al., 1989).

Since 1938 it has been known, that the EOMs are compartmentalized into an outer orbital and an inner global layer (Kato, 1938). The inner global layer faces the optic nerve and the globe and inserts via a tendon on the sclera of the eye ball, whereas the orbital layer faces the periorbital and orbital bone and inserts onto the fibroelastic connective tissue, present around the equator of the globe, called Tenon's capsule (Demer et al., 2000). In some species a third layer has been described: in sheep there is a C-shaped portion of the muscle lying distally, and external to the orbital layer: it is called the peripheral patch layer (Harker, 1972). In humans a marginal layer has been identified using histochemical markers (Wasicky et al., 2000).

All EOMs consist of two basic muscle fiber categories (Spencer and Porter, 2006). One of them corresponds to the twitch fibers (type IIA) of mammalian skeletal muscles, the singly innervated fibers (SIFs), which respond with an 'all-or-nothing' potential to electrical stimulation. The second type are multiply innervated fibers (MIFs), which are atypical for mammalian skeletal muscles, and besides in EOMs are described in the laryngeal muscles and in the tensor tympani muscle of the middle ear (Fernand and Hess, 1969, Mascarello et al., 1982, Périé et al., 1997, Han et al., 1999, Schiaffino and Reggiani, 2011). They respond with local potentials to electrical stimulation resulting in a slow tonic contraction (Siebeck and Kruger, 1955).

Since the first distinction between the MIFs and SIFs, the classification of mammalian EOM fiber types has been complemented by studying the histochemical properties (Ringel et al., 1978), morphology with light and electron microscopy (Cheng and Breinin, 1966) and electrophysiology (Hess and Pilar, 1963, Bach-y-Rita and Ito, 1966). Nowadays, the broadly accepted scheme of classification includes six different fiber types, characterized on the basis of their location in the orbital or global layer, on their mitochondrial content and hence their fatigue properties and also on their classification into a singly or multiply innervation type (Spencer and Porter, 1988). The orbital layer of muscle consists of two fiber types, one MIF type and one SIF type, the global layer of one MIF type and three SIF types. This arrangement is a common pattern seen across different species (Spencer and Porter, 2006).

Up to 80 % of the orbital muscle fibers represent SIFs, which have small myofibrils (myofibril volume 60 % is smaller than in skeletal muscles with 70-85 %) (Hoppeler and Fluck, 2002) and a relatively high percentage of mitochondria (20 % volume of the orbital SIFs) and accordingly also a high oxidative enzyme content. In addition their capillary network is higher developed than in the global layer. These features implicate that they are fast twitch and highly fatigue resistant muscles, but they have also the competence for anaerobic metabolism (Spencer and Porter, 1988). Furthermore, the orbital layer shows different expressions of the myosin gene, unique to the mammalian EOMs and the laryngeal muscle, and an embryonic myosin heavy chain isoform, usually associated with developing skeletal muscles (Wieczorek et al., 1985, Jacoby et al., 1990, Brueckner et al., 1996). The myosin isoforms are specialized to

provide specific contractile force/velocity profiles (Spencer and Porter, 2006) and they are distributed heterogeneously along the length of a muscle fiber (Rubinstein and Hoh, 2000, Lucas and Hoh, 2003).

The MIFs make up 20 % of the orbital layer fibers. Their myofibrils are larger than those of the orbital SIFs, and along one fiber, where the multiple nerve terminals are distributed, they show characteristics of twitch, as well as none twitch muscle fibers (Pachter, 1984). These MIFs express, apart from the slow twitch isoform, an embryonic myosin and a unique neonatal myosin heavy chain isoform (Wieczorek et al., 1985, Rubinstein and Hoh, 2000, Briggs and Schachat, 2002).

In the global layer there are three different, types of SIFs: red, intermediate and white, all of them with characteristics of fast twitch fibers. The global red SIFs represent one third of the global layer. They are suggested to be significant fatigue resistant, and furthermore express a IIA myosin isoform (Rubinstein and Hoh, 2000). The intermediate SIFs incorporate one fourth of the fast twitch global fibers. With their intermediate contraction speed and intermediate fatigue resistance they are classified between the red and white SIFs. The latter comprise one third of all fibers of the global layer with characteristics of fast twitch, low fatigue resistance fibers (Spencer and Porter, 1988).

The remaining 10 % of the global fibers are MIFs, with an ultrastructural profile similar to slow, tonic muscle fibers of amphibians. After electrical stimulation they exhibit slow graded, local contractions with non-propagated responses (Chiarandini and Stefani, 1979).

2.2 Eye movements

Keeping images stable on the retina is a prerequisite for accurate visual perception. Movement of an image across the retina as during head movements would lead to blurred vision without eye movements. There are at least six different types of eye movements: The vestibulo-ocular reflex (VOR), the optokinetic response (OKR), smooth pursuit eye movements, vergence, saccades and visual fixation (Horn and Leigh, 2011). The VOR is a compensatory eye movement in response to head movements in the opposite

direction. By moving the eyes at the same speed as the head it prevents slippage of retinal images. The activation is mediated by canal and otolith receptors in the labyrinth. While the VOR operates best at brief and high frequency changes in head position, it is poor at low frequency changes (Büttner and Büttner-Ennever, 2006). The optokinetic responses (OKR), in contrast are slow, conjugated eye movements responding to large moving visual fields. Extended stimulation in one direction leads to optokinetic responses consisting of alternate slow phases and contralateral quick phases. VOR and OKN are sufficient eye movements to stabilize vision for species with no fovea centralis, the retinal region with the highest density of cones. Therefore, these two types of eye movements are present in all vertebrates investigated so far (Baker, 1998, Fritzsch, 1998). Species with a fovea must focus stationary visual objects there to obtain clear vision, a task accomplished by saccadic eye movements. They move both eyes rapidly in a conjugate manner to a desired target without head movements. In primates, saccades last between 15 and 100 milliseconds and reach speeds of up to 800°/s. They are one of the fastest and most accurate movements of vertebrates and can be modified voluntarily, with the exception of their velocity, which depends on the angular distance of a target (Büttner and Büttner-Ennever, 2006). But saccades can also occur spontaneously e.g. in an alert state, and even in complete darkness. In afoveate animals they usually occur together with head movements (Leigh and Zee, 2006). Smooth pursuit eye movements are needed to track a moving target, shifting over a stationary background smoothly. These eye movements are limited, and are often associated with saccadic movements, because the image on the fovea slides away and has to be captured again there (Leigh and Zee, 2006). It is a voluntary movement that requires attention and can reach velocities up to 100°/s, but it is considered as a slow eye movement (Lisberger et al., 1981, Simons and Büttner, 1985). Normally the eyes track moving objects together with the head, but the VOR automatically brings the eyes in an opposite direction of the head movement, and must therefore be suppressed under the conditions of smooth pursuit. It is suggested that a smooth pursuit signal cancels the VOR (Leigh, 1999). In contrast, gaze holding or visual fixation, holds a fixed image steadily on the fovea and permits a stable eye position between the eye movements (Büttner and Büttner-Ennever, 2006).

To keep a target in focus on both foveae, even when the distance is less than 30 meters, frontal eyed animals perform disconjugated eye movements, e.g. vergence eye movements. The vergence system allows precise alignment of the visual axes for bifoveal fixation and stereopsis. Generally vergence movements are slow (latency 150-200 ms), but can be much faster when they are associated with saccades (Leigh and Zee, 2006).

All these types of eye movements use the same muscles and moreover the same motor neurons. David Robinson pointed out in the 1970's that different eye movements are achieved through different, and relatively separate, premotor neural circuits driving the motor neurons. However, nowadays research shows that extraocular motor neurons are not a homogenous population that participates equally in all types of eye movements (Büttner-Ennever et al., 2001).

2.3 Motor innervation of extraocular eye muscles

2.3.1 Organization of motor neurons

The motor neurons of the extraocular muscles lie in three distinct nuclei within the brainstem (Fig. 2.). The oculomotor nucleus (nIII) and the trochlear nucleus (nIV) are located in the mesencephalon, the abducens nucleus (nVI) more caudal at the pontomedullary junction.

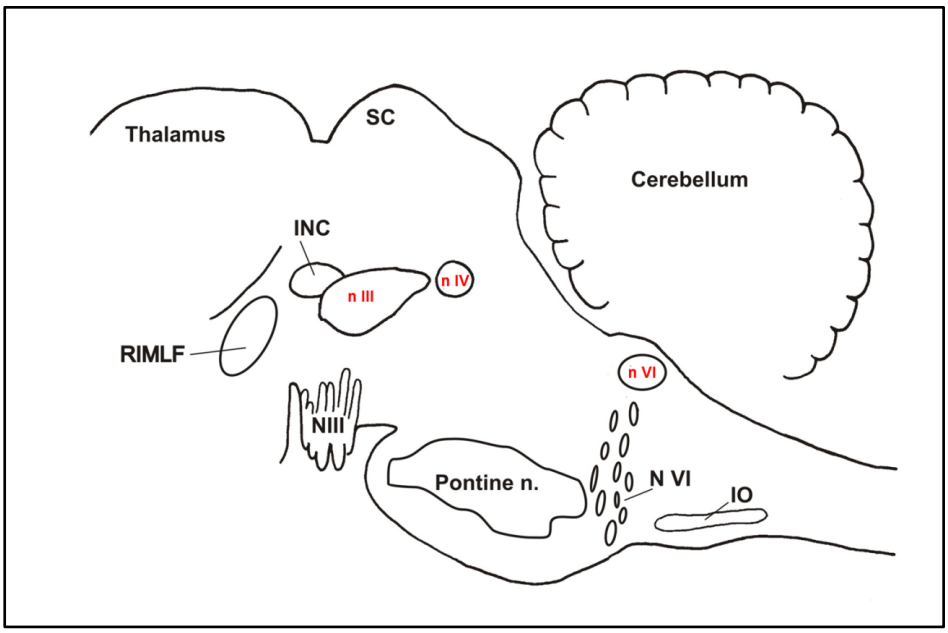

Fig. 2 A sagittal section of the monkey brainstem: this figure demonstrates the location of the oculomotor (nIII), trochlear (nIV) in the mesencephalon and abducens nucleus (nVI) more caudal at the pontomedullary junction. RIMLF - mesencephalic rostral interstitial nucleus of the medial longitudinal fasciculus; INC – interstitial nucleus of Cajal; NIII – oculomotor nerve; SC – superior colliculus; IO – inferior olive.

The nVI lies in the tegmentum of the pontomedullary junction near the midline and underneath the genu of the facial nerve, and contains the motor neurons of the ipsilateral LR muscle. Some species, e.g. cat, contain the motor neurons of additional muscles, the retractor bulbi muscles, accessory eye muscles controlling the nictitating membrane, and therefore participating in the retraction reflex of the eye (Spencer et al., 1980). Besides the motor neurons the abducens nucleus contains internuclear neurons, which project to the MR motor neurons in the contralateral nIII via the medial longitudinal fasciculus (MLF) and provide the neuroanatomical basis for conjugate, horizontal eye movements (Glicksman, 1980, Büttner-Ennever and Akert, 1981, Evinger et al., 1987, Straka and Dieringer, 1991).

The trochlear nucleus (nIV) is located in the mesencephalic tegmentum and adjoins the oculomotor nucleus caudally. Almost all of its motor neurons innervate the contralateral SO muscle, only a few of them project to the ipsilateral SO muscle (Evinger et al., 1987).

The oculomotor nucleus (nIII) contains motor neurons of the ipsilateral MR, IR, IO and the contralateral SR muscles. Systematic investigations of the location of individual motor neuron groups within nIII began with clinical, neuroanatomical and electrophysiological methods in the late nineteenth century (Edinger, 1885, Bernheimer, 1897, Brouwer, 1918). However the presently accepted topographical map of nIII was investigated not until 1953 by retrograde degeneration techniques in non-human primates (Warwick, 1953). After introduction of retrograde tract-tracing methods, for example with horseradish peroxidase (HRP) more detailed information about the organization of oculomotor nuclei was achieved in different species including primates (Fig. 3) (Gacek, 1977, Akagi, 1978, Glicksman, 1980, Büttner-Ennever, 2006). These subgroups have a topographic arrangement, illustrated for monkey and rat in Fig. 3.

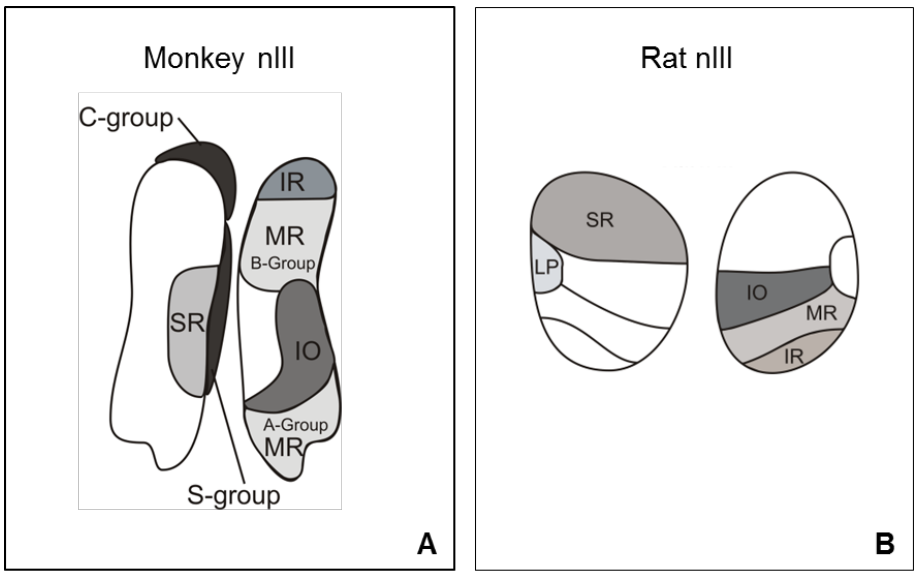

Fig. 3 A topographical organization of motor neurons in the nIII of monkey (A) and rat (B); whereas the motor neurons of almost all eye muscles, which are innervated by nIII, lay on the ipsilateral side, the SR motor neurons lay contralateral. Motor neurons of singly-innervated twitch muscle fibers (SIFs) lie within the boundaries of nIII, whereas motor neurons of multiply innervated fibers non-twitch fibers (MIFs) are located in the periphery of nIII in the C- and S-group most obvious in non-human primates (A).

In all investigated species the subgroups for motor neurons of eye moving muscles within nIII follow an IR, MR, IO and SR sequence from rostral to caudal. In frontal eyed species, like monkey, the motor neurons of the LP form a separate subgroup caudal to the nIII, in the central caudal nucleus (CCN),

whereas in lateral eyed animals, like rat, the LP population lies laterally on the contralateral side (Evinger, 1988).

Another difference in the organization of the nIII between primates and non primates is the threefold representation of the MR in the primate nIII. The A-group, at the ventral portion of the nIII, extends into the MLF and contains the largest number of MR motor neurons. The B-group appears as a circle-round group at the dorsolateral within nIII and dorsomedially, at the peripheral border of the nucleus lies the C-group (Büttner-Ennever and Akert, 1981, Porter et al., 1983) (Fig.3).

2.3.2 Singly- and multiply innervated fiber motor neurons

Although the multiple representation of the MR in the primate nIII is not clear yet, tract-tracer experiments in monkey suggest the C-group of the nIII comprises multiply innervated muscle fiber motor neurons, while the A- and B-group motor neurons give rise to the singly innervated muscle fibers (Büttner-Ennever et al., 2001) (Fig.4 A). Tracer injections into the belly or the distal myotendinous junction of EOMs in monkey were made by Büttner-Ennever et al. (Büttner-Ennever et al., 2001). Only the distal injections, enclosing exclusively the endplates of the MIFs and some special nerve endings called palisade endings revealed a second set of motor neurons in all of the three oculomotor nuclei, smaller in diameter with a completely different organization from that of the “classical” large motor neurons (Büttner-Ennever et al., 2001). The second set of motor neurons was shown to have a different morphology and different histochemical properties (Eberhorn et al., 2005a). The “traditional” SIF motor neurons were exclusively found within the boundaries of the oculomotor nuclei, whereas the motor neurons of the MIFs were located in the periphery of the nuclei: the C-group, located dorsomedially at the peripheral border of the nIII, containing the MIF motor neurons of MR and IR (Fig. 4 B); the S-group, at the midline between the nIII, containing the MIF motor neurons of IO and SR. The peripheral MIF groups of nIV form a cap over the dorsal surface of the nucleus, whereas the MIF group of the VI lay around the medial borders of this nucleus and between the rootlets of the NVI, or around the facial genu (Büttner-Ennever et al., 2001, Wasicky et al., 2004).

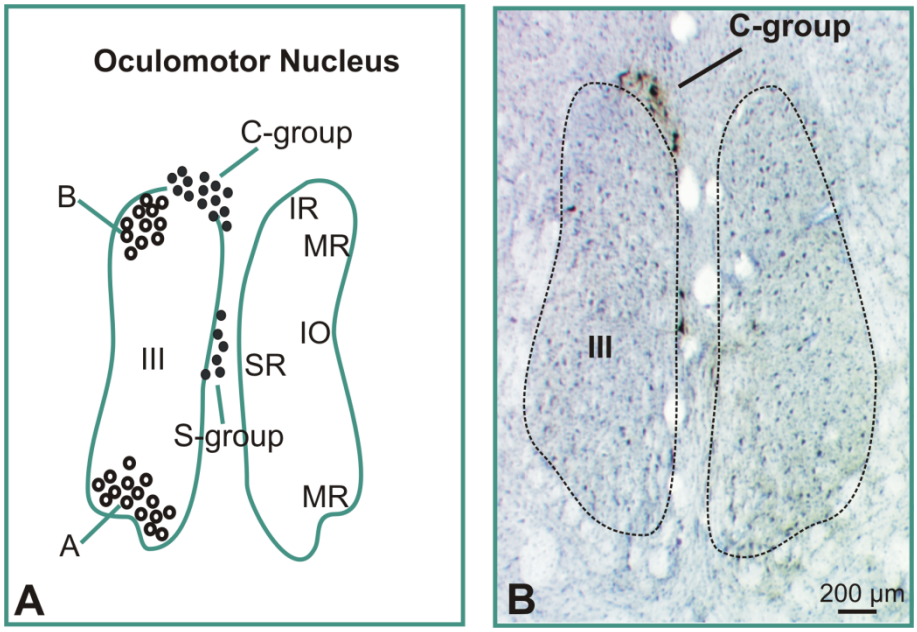

Fig. 4 Location of the singly- and multiply innervated fiber neurons in the nIII (A) in the monkey (and other primates) the MR motor neurons are separated in an A-group, at the ventral portion of the nIII, which contains the largest number of MR motor neurons, dorsolateral the B-group and dorsomedially, at the peripheral border of the nucleus the C-group. The C-group contains the MIF motor neurons of the MR and the IR (B), the S-group contains the MIF motor neurons of the SR and IO. (B) Transverse section of monkey nIII demonstrating retrogradely labeled neurons after a very distal tracer injection into MR. Note that only the peripheral cells of the C-group are tracer positive. (Figure from Lienbacher et al. 2011a)

2.3.3 Neuromuscular junctions

The EOMs consist mainly of twitch fibers that show an 'all-or-nothing' contraction, activated by an endplate that lies approximately in the middle of each fiber, called en plaque ending (Namba et al., 1968, Spencer and Porter, 2006). These nerve terminals are embedded in the deep depressions of the sarcolemma, usually encircling the muscle fiber. The neuromuscular junctions of the singly innervated, twitch fibers in the global layer are morphologically identical to that of the orbital SIFs. Only the en plaque terminals in the global layer of the intermediate SIFs show clusters of large nerve endings, located in synaptic depressions (Spencer and Porter, 1988). At the proximal third, in the region where the nerve enters the muscle, the en plaque endings form a dense innervation band across the global layer.

The non-twitch fibers of the EOMs are multiply innervated along their whole length, and generate only a local contraction that is not propagated throughout

the muscle fiber by the so-called en grappe endings (Spencer and Porter, 1988, Porter et al., 1995). In the orbital layer, the motor endings at the mid-belly resemble those of the orbital SIFs, but proximal and distal to the center the terminals are small and look like a string of pearls (Fig. 5).

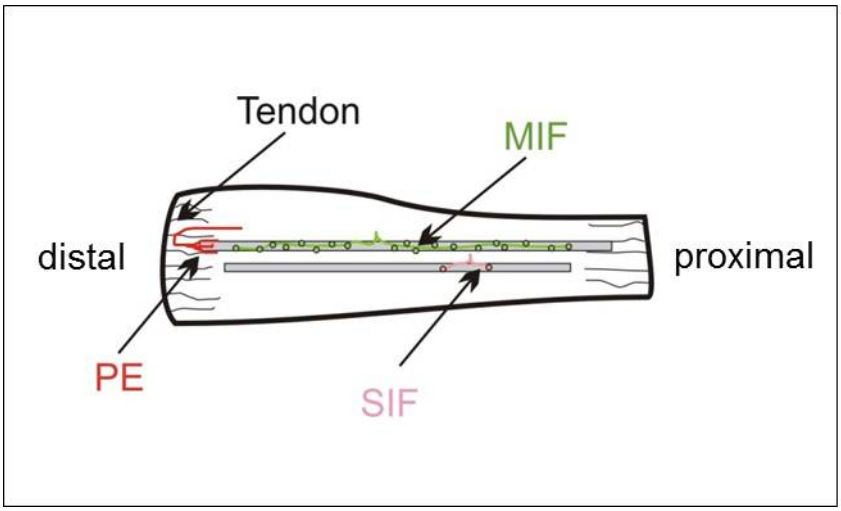

Fig. 5 Eye muscle with its different fiber types and endings; the singly innervated muscle fibers (SIFs) are innervated by only one terminal per fiber with an "all or nothing" action potential signal, whereas the multiply innervated fibers (MIFs) have more than one nerve terminal distributed all over the fiber length, with only local potentials. The MIFs extend proximal and distal into the tendon ,where the palisade endings (PEs) are located at the distal tip of theses fibers at the myotendinous junction.

2.4 Sensory innervation of extraocular eye muscles

2.4.1 Sensory ganglion cells

Based on the general organization of sensory innervation of head structures, it is expected that the cell bodies of sensory neurons innervating the eye muscles lie in the trigeminal ganglion (TG) (Brodal, 1981, Usunoff et al., 1997). Accordingly, tracer injections into the eye muscles result in retrogradely labeled neurons not only in the motor nuclei, but also labeled cell bodies in the ophthalmic division of the TG providing sensory innervation of the eye muscle (Daunicht et al., 1985, Porter and Donaldson, 1991). Some reports suggest the mesencephalic trigeminal nucleus (VMes) as a further source of sensory afferents to the extraocular muscles (Alvarado-Mallart et al., 1975b, Buisseret-Delmas and Buisseret, 1990, Wang and May, 2008).

The trigeminal ganglion (also called semilunar or gasserian ganglion) is situated on the anterior, superior surface of the petrous bone in the middle fossa of the skull, and is surrounded by arachnoid and dura. It contains the cells of origin of all trigeminal sensory axons with different cell sizes (from 20 µm to 80 µm in diameter), and different neurochemical properties (Lazarov, 2002). The axons arising from each cell body bifurcate to form an anterior branch, which proceeds to the periphery in one of the three trigeminal nerve divisions, and a posterior branch, which enters the brainstem at the pontine level and travels within the brainstem to terminate within the principal and spinal trigeminal nucleus (Brodal, 1981, Parent, 1996).

The TG has three peripheral branches, which provide the somatosensory innervation of the face including the orbita and eye: the ophthalmic branch (V1) supplies the forehead, upper eye lid, cornea, conjunctiva, frontal sinuses and the dorsum of the nose; the maxillary branch (V2) innervates the upper lip, the lateral portion of the nose, part of the oral cavity, the maxillary sinus, upper jaw and the roof of the mouth as well as the upper dental arch, the third, mandibular branch (V3) innervates the lower lip and chin, the cheek, the lower teeth, the lower jaw, the floor of the mouth and part of the tongue (Brodal, 1981, Usunoff et al., 1997, Voogd et al., 1998). Furthermore, motor axons travel through the mandibular nerve of the sensory TG to the masticatory muscles. The TG shows a clear topographical organization, where the mandibular aspect lies posterolateral, the ophthalmic division anteromedian and the maxillary fraction located in between (Kerr et al., 1964, Marfurt, 1981).

2.4.2 Proprioceptors in the eye muscles

One type of sensory receptors in the EOMs are mechanoreceptors, which are classified as proprioceptors. They lie deep in the muscle tissue and respond to contractions, stretch, tension and generally to changes in muscle position. Sherrington was the first to define proprioception: “a reflex system for the maintenance of body position and coordination of movement” (Sherrington, 1906). Until now it is suggested that there are two putative proprioceptive structures in the EOMs: the muscle spindles and the Golgi tendon organs.

Muscle spindles are found in the majority of somatic muscles of all tetrapod vertebrates, but the first description of muscle spindles in the EOMs was made by Cilimbaris (Cilimbaris, 1910). A typical spindle consists of some small intrafusal muscle fibers that receive a motor as well as a sensory innervation; it lies in parallel with the extrafusal muscle fibers. It is enclosed within a fluid filled capsule of perineurium, with a fusiform appearance. The equatorial region occupies in large part the intracapsular space, in which the muscle fibers are thinner and enclosed by a fibrous capsule. The intrafusal fibers appear as nuclear chain fibers and nuclear bag fibers. Nuclear chain fibers, making most of the intrafusal fibers, are shorter and thinner than the nuclear bag fibers and their central nuclei are arranged in a continuous row, in contrast to bag fibers which have clustered nuclei (Barker, 1974). Both types are enwrapped by annulospiral primary sensory endings, the chain fibers furthermore secondary sensory flower-spray and annulospiral endings on the distal sides of the primary endings. The motor innervation is the same for both fiber types: efferent axons of small caliber terminating on the contractile polar regions. They arise from gamma motor neurons which are considered to regulate the sensitivity of the spindles during contraction of the corresponding extrafusal muscle fibers (Barker, 1974). (The motor neurons innervating the extrafusal fibers are, in contrast, alpha neurons). The sensory afferent endings of the intrafusal fibers respond to stretch.

Most of the extraocular muscle spindles seem to deviate from the classical muscle spindle found in the skeletal muscles (Cooper and Daniel, 1949, Ruskell, 1999, Donaldson, 2000). Furthermore there are large variations in the distribution of these receptors between different species (Harker, 1972, Barker, 1974), and some species possess no spindles at all (Ruskell, 1999) (Tab. 2).

Golgi Tendon Organs (GTOs) are fusiform receptors at the musculotendinous junction, with thin, encapsulated bundles of small tendon fascicles and a number of muscle fibers, attached on one pole. An afferent nerve fiber innervating the GTO enters the capsule and branches via numerous terminals between the bundles of collagen. These proprioceptors lie in series with the extrafusal fibers and serve as a force transducer that responds more to tension than to stretch (Hunt, 1974). They respond to active tension, generated by contracting muscle fibers, with a low threshold (Jami, 1992).

Although GTOs are important proprioceptors in mammalian striated somatic muscles, second to muscle spindles, they show large variations in morphology and distribution in eye muscles of different species, and they are completely absent in a lot of species (Ruskell, 1999) (Tab. 2).

Even in species with very few, or no muscle spindles or GTOs, representations of eye position or stretch reflexes were found (Dancause et al., 2007, Wang et al., 2007, Balslev and Miall, 2008).

There is a third possible proprioceptive terminal in the eye muscles, found in all species investigated so far: the palisade endings (Tab. 2).

	Muscle Spindles	Golgi Tendon Organs	Palisade Endings
Human	+/-	+	+
Monkey	+/-	+	+
Sheep	+	+	+
Pig	+	+	+
Cat	-	-	+
Rat	-	-	+

Tab. 2 Classical and possible eye muscle proprioceptors like muscle spindles, Golgi tendon organs and palisade endings and their appearance in different species.

2.4.3 Palisade endings

Palisade endings (PE) were described and named by Dogiel in 1906 (Dogiel, 1906) (Fig.6). PEs with a surrounding fibroblast like cell capsule were also called myotendinous cylinders (Ruskell, 1978). The PEs arise from nerve fibers that enter the tendon from the central muscle nerve and turn back 180° to re-enter the muscle zone distally, to form a cuff of nerve endings around the tip of MIFs of the global layer (Chiarandini and Stefani, 1979, Ruskell, 1999). Until now, PEs were found in human and monkey (Tozer and Sherrington, 1910, Richmond et al., 1984, Ruskell, 1999, Donaldson, 2000, Lukas et al., 2000), cat (Alvarado-Mallart and Pincon Raymond, 1979), dog (Rungaldier et al., 2009), sheep (Blumer et al., 1998), rabbit (Blumer et al., 2001b) and rat (Eberhorn et al., 2005b). In monkey up to 300 PEs were found in one single MR muscle

(Ruskell, 1978). In human and cat PEs were also found in the proximal myotendinous part of EOMs (Cooper and Daniel, 1949, Alvarado-Mallart and Pincon Raymond, 1979) and may be a general feature in other species, but this has not yet been proven. Like GTOs they lie in series, at one end connected to a multiple innervated, non-twitch fiber, at the other related to the tendon of the muscle.

Since the first descriptions of PEs (Huber, 1900, Dogiel, 1906), there has been an ongoing debate about their function. Sas and Scháb were the first to suggest, by degeneration experiments with cranial nerves or degenerations around the motor nuclei, that the PEs are motor structures (Sas and Schab, 1952). Recent research has tried to confirm the fact that PEs are motor endings. More specifically, the PEs in humans show a basal membrane around their terminals, which is taken as a typical feature for motor endings (Lukas et al., 2000); secondly, they use acetylcholine as a transmitter, as motor neurons do (Konakci et al., 2005a, Konakci et al., 2005b, Blumer et al., 2006, Blumer et al., 2009). On the other hand a sensory function was proposed by other groups: for example the degeneration experiments of Tozer and Sherrington revealed an afferent and efferent function of the oculomotor nerves (Tozer and Sherrington, 1910). Detailed studies of the ultrastructural morphology support the function of a sensory terminal (Ruskell, 1978, Alvarado-Mallart and Pincon Raymond, 1979, Blumer et al., 1998). Furthermore, tracer injections targeting the trigeminal nerve of the cat were reported to lead to anterograde labeling of PEs in the extraocular muscles (Billig et al., 1997).

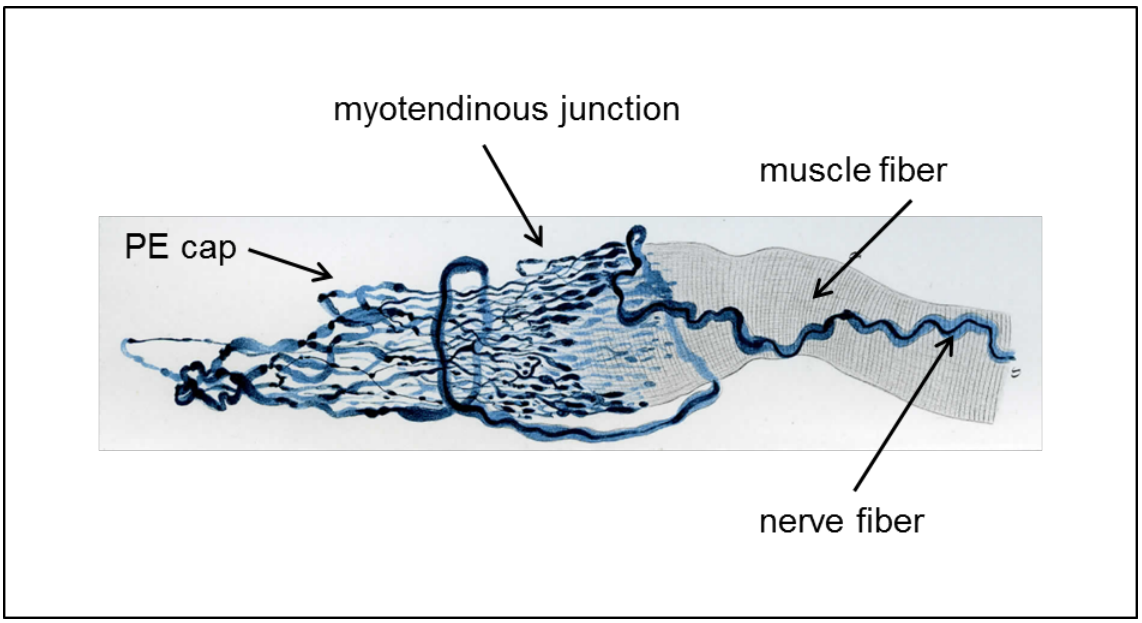

Fig. 6 Drawing of a palisade ending taken from the study by Dogiel in 1906. PEs arise from nerve fibers that enter the tendon from the central muscle nerve and turn back 180° to re-enter the muscle zone distally and form a cap of nerve endings around the tip of MIFs of the global layer.

The debate on the palisade ending function was compounded by the lack of knowledge about the location of their cell bodies. For sensory function they would be expected to lie in the trigeminal ganglion or mesencephalic trigeminal nucleus, for motor function in the motor nuclei of extraocular muscles.

2.5 Aims of the project

1. The TG accommodates most of the cell bodies for sensory terminals of the face. Furthermore, tracer injections in the EOMs showed retrogradely labeled cells in the ophthalmic subdivision of the TG (Porter and Donaldson, 1991). Until now there is no information about their function and what kind of terminals these TG cells subtend in the eye muscles. In order to characterize retrogradely labeled TG cells and their corresponding endings in the EOM, we analyzed these neurons with immunohistochemistry and morphometrical methods.

2. Taking into account that the oculomotor nuclei within the brainstem contain the motor neurons innervating SIF and MIF muscle fibers, tracer injections were placed into the oculomotor nucleus or nerve to find out if PEs can be anterogradely labeled.

3. A close investigation of the histochemical properties of nerve endings was performed to find potential proteins unique to palisade endings. In a next step the putative PE cell bodies were studied for the presence of these proteins.

In this thesis I have been able to provide evidence that the PE cell bodies are not located in the TG, but are intermingled with the peripheral neurons around the oculomotor nuclei. The evidence here supports the hypothesis that they may be sensory cells which could provide a proprioceptive signal and modulate the activity of the adjacent oculomotor neurons.

3 Methods

The tracer injections in macaque monkeys were conducted in collaboration with Prof. Mike Mustari from the Washington National Primate Research Center of the University of Washington, Seattle and Prof. Dr. Bernhard Hess from the Universitätsspital Zürich, Neurologische Klinik. All experimental procedures conformed to the state and university regulations on laboratory animal care, including the Principles of Laboratory Animal Care (NIH Publication 85-23, Revised 1985), and were approved by their animal care officers and Institutional Animal Care and Use Committees.

3.1 Methods for the characterization of retrogradely labeled trigeminal ganglion cells and their terminals in the extraocular muscles

Six Macaque monkeys were anesthetized with Ketalar (500 mg Ketamin in 9 ml, i.p.) following Isoflurane. Under sterile conditions, the extraocular eye muscles were exposed by retracting the eyelids, making a conjunctival incision, and partially collapsing the eye ball. Large or small volumes of the neuronal tracers cholera toxin subunit B (CT, 5–10 μl, 1 % from List Campbell, CA, USA) or wheat germ agglutinin (WGA, 5–10 μl, 2.5 % Sigma, St. Louis, MO, USA) were injected through a Hamilton syringe into the belly or the distal tip of the eye muscle, respectively. In one monkey only the conjunctiva was injected.

After a survival time of 3 days the animals were killed with an overdose of Nembutal (1-2 ml = minimum 50 mg Pentobarbital, i.p.) and transcardially perfused with 0.9 % saline (35 °C) followed by 4 % paraformaldehyde in a 0.1 M TBS (Tris buffered saline) (pH 7.4), after an injection of 1 ml Heparin. Eye muscles and TGs were removed and transferred through 10 %, 20 % and 30 % sucrose in 0.1 M TBS (pH 7.4), until they were cut into 20 µm longitudinal or cross sections on a cryostat (Thermo Scientific Microm HM 560, Fisher Scientific, Germany). The brains were immersed in 10 % sucrose in 0.1 M TBS (pH7.4) and transferred to 30 % sucrose for 4 days. The brain was cut into 40 µm frontal sections also on a cryostat.

3.1.1 Combined immunostaining for the tracers and markers in the trigeminal ganglion

Frozen sections of the brain, the TG, and the eye muscles were processed for the immunocytochemical detection of the tracers cholera toxin subunit B and wheat germ agglutinin (goat anti-choleragenoid, 1:5000; List Biological Laboratories, Campbell, CA, USA; goat-anti-WGA, 1:250; Axxora, Germany; alternatively rabbit anti-WGA, 1:500, EY-Lab, CA, USA, or rabbit anti-CT, 1:5000, Sigma, MO, USA) (see also Tab. 3 for primary and secondary antibodies) and one of the following markers: calretinin (CR, rabbit anti-CR; 1:1000; Swant, Marly, Swiss), Parvalbumin (PV, mouse anti-PV, 1:2500, Swant Marly, Swiss) , substance P (SP, rabbit anti-SP; 1:100; Zymed, Invitrogen, Germany), neuronal nitric oxide synthase (NOS, rabbit anti-NOS; 1:2000; Chemicon Millipore, MA, USA) or cholinacetyltransferase (ChAT, goat anti-ChAT; 1:80; Chemicon Millipore, MA, USA), incubated for 48 h at 4°C. The sections were then reacted with Cy^3-anti-rabbit or Cy^3-anti-mouse (1:200, Dianova, Jackson Immuno Research, Baltimore, USA) and Alexa 488-anti-goat (1:200; Molecular Probes, Oregon, USA) for 2 h. Then the sections were mounted and coverslipped in Fluoromount medium (SIGMA-ALDRICH, MO, USA).

In selected sections ChAT (1:80) and CR (1:2500) were visualized with an immunoperoxidase procedure with subsequent incubation in biotinylated secondary antibodies and extravidin-peroxidase and a final reaction in 0.025 % diaminobenzidine (DAB) and 0.015 % H_2O_2 in 0.1M TBS (ph 7.6) for 10 minutes (Tab. 3).

3.1.2 Cholinacetyltransferase blocking test

To test the reliability of the ChAT immunostaining in the TG, selected TG sections were used for a preabsorption test with the antigen ChAT (ChAT antigen, Biomol). Different concentrations of the ChAT antigen with 8, 4, 2 and 1 µg/ml T antibody were used, at a dilution of 1:80. As a control an additional slide without the ChAT antigen was stained for comparison. All TG sections were immunostained with DAB as described above.

3.1.3 Combined immunostaining for the nerve endings innervating the extraocular muscles and associated histochemical markers

To identify the complete innervation of an extraocular muscle and investigate its histochemical properties, flat eye muscle sections of monkey were stained on-slide for simultaneous detection of SP, NOS, CR, PV or ChAT, combined with either mouse anti-synaptosome-associated protein SNAP-25 (SMI 81; 1:2000; Sternberger Monoclonals Inc., Maryland, USA) or mouse anti-synaptophysin, SYN, 1:20; DAKO, Glostrup, Denmark) using double immunofluorescence. In addition the same staining procedures were performed on flat eye muscle sections of sheep, which are known to contain numerous well developed muscle spindles (Ruskell, 1999). After blocking with 2 % normal donkey serum (NDS, Sigma, St. Louis, MO, USA) in 0.1 M TBS, containing 0.3 % Triton X-100 for 1 hour, the sections were processed with a mixture of antibodies: either rabbit-anti-CR, PV, SP, NOS or goat-anti ChAT, together with either mouse anti-SYN or mouse anti-SNAP-25 for 48 hours at room temperature. After several buffer washes a mixture of fluorochrome-tagged secondary antibodies of Cy^3 - tagged donkey anti-rabbit (1:200, Dianova, Jackson Immuno Research, Baltimore, USA, for CR, NOS or SP), or donkey anti-goat (1:200, Dianova, Jackson Immuno Research, Baltimore, USA, for ChAT) combined with Alexa 488- tagged donkey anti-mouse (1:200, Molecular Probes, Oregon, USA) for synaptophysin or SNAP-25 were incubated for 2 hours at room temperature. Immunofluorescence for tyrosine hydroxylase (TH) was used to identify noradrenergic sympathetic nerve fibers in the eye muscles. Sections were incubated in rabbit anti-TH (1:100; Chemicon Millipore, MA, USA) for 24 hours at room temperature. After washing sections were treated with donkey anti-rabbit tagged with the fluorescent dye Alexa 488 (1:200; Molecular Probes, Oregon, USA) for 2 hours.

All fluorochrome stained sections were coverslipped with GEL/MOUNT permanent aqueous mounting medium (Biomeda, California, USA) and stored in the dark at 4°C.

3.2 Identification of cell bodies of palisade endings

In order to investigate whether the cell bodies of palisade endings are localized in the brainstem, two rhesus monkeys (Macaca mulatta) received tracer injections into the oculomotor nucleus in the midbrain tegmentum: in one monkey tetramethylrhodamine dextran (TMR-DA) into the left (case 1) and cholera toxin subunit B (CT) into the right oculomotor nucleus (nIII) (case 2), and TMR-DA into the oculomotor nerve (NIII) of a second animal (case 3). The animals were between two and three years old. For surgery under aseptic conditions the animals (born in captivity at Yerkes National Primate Research Center, Atlanta, GA) were anesthetized using isoflurane (1.25-2.5 %). Blood pressure, heart rate, blood oxygenation, body temperature and CO_2 in expired air were monitored with a Surgivet Instrument (Waukesha, WI) and maintained in physiological limits. Using stereotaxic methods a titanium head stabilization post and titanium recording chamber (Crist Instruments, Hagerstown, MD) were implanted (anterior = 2 mm; lateral = 1 mm; tilted 20º away from midline) and aimed such that an electrode track located in the center of the chamber intersected a point near the oculomotor nucleus. Postsurgical analgesia (Buprenorphine. 0.01 mg/kg, every 6 hours) and anti-inflammatory (Banamine 1.0 mg/kg, every 6 hours) treatments were administered for several days, as indicated.

In all animals the injection sites were identified in the lab of Prof. Mike Mustari with single-unit recording using tungsten microelectrodes. Motor neurons were recognized by their characteristic burst tonic responses in association with appropriately directed saccades. For injection the recording electrode was replaced either by a custom made micropipette equipped with a beveled glass tip (20-50 µm diameter) and attached by polyethylene tubing to a picoliter pump (WPI 830) (case 1 and 2) or a thin Hamilton syringe (case 3). Short duration (50 ms) pressure pulses delivered over several minutes ejected small tracer volumes at each site. The pipette was left in place for 5-10 minutes following injection and then gradually removed. In case 1, 0.2 µL non-toxic CT (C-167; Sigma/List Biological Laboratories, California, USA; 1 % in aqua bidest) and in case 2 0.5 µL TMR-DA (Molecular Probes; D-3308; Oregon, USA; 3000 MW; 15

% in acetate buffer, pH 3) was injected. Case 3 received 1 µl TMR-DA directly through the Hamilton syringe, which was also left in place for 10 min.

After three days survival time the animals were sedated with ketamine, sacrificed with an overdose of sodium pentobarbital (>90 mg/kg, i.v.) and then transcardially perfused with 0.9 % saline followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PBS; pH 7.4) (Lienbacher et al., 2011b).

The brain, the orbital content and both trigeminal ganglia were removed. The extraocular muscles were carefully dissected keeping the myotendinous junction complete. All tissues were equilibrated in 10 %, 20 % and 30 % sucrose in 0.1 M TBS for freeze cutting. The brainstems were cut transversely in 40 µm sections with a cryostat (MICROM HM 560). Eye muscles and trigeminal ganglia were cut longitudinally in 20 µm sections and directly thaw-mounted onto glass slides (Superfrost Plus; M&B Stricker).

In addition, in a third monkey (case 4), 2 tracers were injected into the myotendinous junction (the location of palisade- and en-grappe endings), of the medial rectus muscle (MR) (CT) and the inferior rectus muscle (IR) (horseradish peroxidase-conjugated wheat germ agglutinin, WGA-HRP, Sigma, St. Louis, MO, USA) in order to analyze the course of the axons of passage through nIII that could be labeled by the injections of cases 1-3, and hence contribute to the labeled terminals in the eye muscles. A detailed description of the eye muscle injections and tracer detection is given above (Tab. 6, Fig. 27).

3.2.1 Tracer detection in the brain and trigeminal ganglion

Brainstem sections were immunocytochemically treated free floating with antibodies against CT or TMR-DA (Molecular Probes, Oregon, USA; 1:6000). The antigenic sites were visualized with a reaction in 0.025 % diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) and 0.015 % H_2O_2 in 0.1M TBS (ph 7.6) for 10 minutes.

An on-slide immunofluorescence detection of the tracer was performed for the trigeminal ganglion. After blocking with 2 % NDS in 0.1 M TBS containing 0.3 % Triton X-100 for 1 hour the slides were processed with goat anti-CT or rabbit anti-TMR-DA for 48 hours at room temperature. After washing, the CT or TMR-

DA-containing sections were treated with the secondary antibody (Cy^3 donkey anti-goat or Cy^3 donkey anti-rabbit, respectively; 1:200) for 2 hours at room temperature.

3.2.2 Combined immunofluorescence labeling in the extraocular muscles

To identify the complete innervation and verify the presence of tracer in nerve fibers and terminals the eye muscle sections were stained on-slide for simultaneous detection of CT or TMR-DA combined with either mouse anti-synaptosome-associated protein SNAP-25 or mouse anti-synaptophysin (SYN) using double immunofluorescence. After blocking with 2 % NDS in 0.1 M TBS, containing 0.3 % Triton X-100 for 1 hour the sections were processed with a mixture of goat anti-CT or rabbit anti-TMR-DA and either mouse anti-SYN or mouse anti-SNAP-25 for 48 hours at room temperature. After several buffer washes a mixture of fluorochrome-tagged secondary antibodies of either Cy^3-tagged donkey anti-goat or donkey anti-rabbit combined with Alexa 488 tagged donkey anti-mouse for synaptophysin or SNAP-25 were incubated for 2 hours at room temperature. Combined immunofluorescence for TH and CT was used to specify sympathetic nerve fibers in the eye muscles. Following the immunostaining for CT as described above sections were incubated in rabbit anti-TH for 24 hours at room temperature. After washing sections were treated with donkey anti-rabbit tagged with the fluorescent dye Alexa 488 for 2 hours.

All fluorochrome stained sections were coverslipped with GEL/MOUNT permanent aqueous mounting medium (Biomeda, California, USA) and stored in the dark at 4°C.

3.2.3 Combined immunoperoxidase labeling in extraocular muscles

In selected sections the detection of CT was combined with immunostaining for the slow isoform of myosin heavy chain (mouse anti-myosin heavy chain (slow (MHCs), 1:100; Novocastra Laboratories Ltd; United Kingdom) to identify non-twitch muscle fibers (Billeter et al., 1980). After immunostaining for CT (see above) the antigenic sites were visualized with 0.025 % DAB, 0.2 % ammonium

nickel sulphate (Riedl-De Haën; Hannover, Germany) and 0.015 % H_2O_2 in 0.1 M TBS, pH 7.4, for 10 minutes to yield a black staining. After blocking residual peroxidase activity with 1 % H_2O_2 in 0.1 M TBS, pH 7.4, for 30 minutes, sections were blocked with 5 % normal horse serum (Vector Laboratories, Burlingame, CA, USA), in 0.1 M TBS, pH 7.4, containing 0.3 % Triton X-100 for 1 hour and subsequently processed with mouse anti-MHCs for 48 hours at 4°C. After several buffer washes the sections were treated with biotinylated horse anti-mouse (1:200; Vector Laboratories; Burlingame, CA, USA) for 1 hour at room temperature. The antigenic sites were visualized with 0.025 % DAB and 0.015 % H_2O_2 in 0.1 M TBS, pH 7.4, for 10 minutes to yield a brown staining of non-twitch muscles fibers.

3.3 Methods for the investigation and differentiation of peripheral oculomotor nuclei cells

Macaque monkeys were injected into the belly or the distal tip of the medial rectus muscle with choleratoxin subunit B (CT) or wheat-germ-agglutinin (WGA). After a survival time of 3 days, the animals were killed and transcardially perfused as described above. The brainstem and the orbital contents were removed and cut transversely at 20 µm (eye muscles) and 40 µm (brainstem).

For the immunocytochemical detection of CT or WGA, free floating brain sections were pretreated with 1 % H_2O_2 in 0.1 M TBS buffer pH 7.4 for 30 minutes to suppress endogenous peroxidase activity, following thoroughly washing. For the detection of CT and WGA the sections were blocked with 5 % rabbit serum (Vector Laboratories, Burlingame, CA, USA) in 0.1 M TBS pH 7.4 containing 0.3 % Triton X-100 for 1 hour and subsequently processed with rabbit anti-CT or rabbit anti-WGA (see Tab. 3) for 48 h at 4°C. After several buffer washes the sections were treated with biotinylated rabbit anti-goat (1:200; Vector Laboratories; Burlingame, USA for CT) for 1 hour at room temperature. The antigenic sites were visualized with 0.025 % DAB and 0.015 % H_2O_2 in 0.1 M TBS, pH 7.4, for 10 minutes to yield a brown staining of the cells. After several washing, the sections were air-dried, dehydrated, and cover-slipped in Depex (Sigma, St. Louis, MO, USA). Some of the sections were processed for

the tracer CT (goat anti-choleragenoid, 1:5000, List Biological Laboratories, Campbell, CA, USA) and for the marker CR (rabbit anti-CR, 1:1000, Swant, Marly, Swiss), for 48 h at 4°C. The sections were then reacted with Cy^3-anti-rabbit (1:200, Dianova, Jackson Immuno research, Baltimore, USA) and Alexa 488-anti-goat (1:200, Molecular Probes, Oregon, USA) for 2 h, mounted and coverslipped.

Macaque monkeys with tracer injections (CT or WGA) into the MR, and one additional macaque monkey with a tracer injection (CT) into the distal part of the superior rectus muscle (SR) were carefully analyzed with focus on the examination of the peripheral C-group neurons of the MR. We distinguished between C-group neurons in close proximity to the dorsomedial aspect of the oculomotor nucleus (nIII) (after belly injections) and those which extend far rostral to nIII encircling the 'preganglionic neurons of the Edinger-Westphal nucleus (EWpg)' (after distal injections into the myotendinous junction). See also: (Büttner-Ennever et al., 2001).

The sections of a supplementary case, containing nIII and immunostained for the cholinergic marker ChAT, were analyzed for the morphology of the peripheral neurons in the C-group. The cholinergic C-group neurons can be distinguished from preganglionic neurons of the EWpg as another cholinergic neuron group in the perioculomotor region by their different histochemical properties, e.g. the lack of non-phosphorylated neurofilaments and the presence of cytochrome oxidase (Eberhorn et al., 2005a, Horn et al., 2008)

3.4 Analysis

The slides were examined either with a Leica microscope DMRB (Bensheim, Germany) or with a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Germany) under brightfield conditions. Both microscopes are additionally equipped with appropriate filters for red fluorescent Cy^3 (excitation: 550 nm) (Leica: N2.1; excitation filter BP 515-560 nm; Zeiss: excitation filter BP 546-590 nm) and green fluorescent Cy^2 (excitation: 492 nm) or Alexa 488 (excitation: 488 nm) (Leica: I3; excitation filter BP 450-490 nm; Zeiss: excitation filter BP 475-530 nm), which were used to analyze the immunofluorescence. Photographs were taken with a digital camera (Pixera Pro 600 ES;

Klughammer, Markt Indersdorf, Germany), captured on a computer with Pixera Viewfinder software (Klughammer) and processed in Photoshop 11.0 (Adobe Systems, Mountain View, CA). Confocal pictures were taken on a Leica TCS SP 1 confocal laser-scanning microscope (Leica Microsystems, Mannheim) with a 40x oil objective (NA 1.4, resolution 200 nm/pixel), equipped with three lasers at 488 nm, 561 nm and 633 nm. The double immunofluorescence slides with Cy^3 or Alexa 488 dyes were recorded at 543 or 488 excitation wave length. The sharpness, contrast, and brightness were adjusted to reflect the appearance of the labeling seen through the microscope. The pictures were arranged and labeled in CorelDraw (CorelDraw 11.0; Corel, Ottawa, ONT, Canada). The axonal course of retrogradely labeled motor neurons was reconstructed using Neurolucida software (MicroBrightField, Inc., Vermont, USA; Version 6). Only those cells with a clearly visible nucleus were measured with ImageJ software. The mean diameter in µm was calculated in Excel 2007 by [Dmin + Dmax] /2. All histograms were made with Microsoft Excel 2010.

Name	Primary Antibodies	Secondary Antibodies
Choleratoxin Subunit B; Brightfield	goat anti-Choleratoxin; 1:20 000; List Biological Laboratories; California, USA	biotinylated rabbit anti-goat; 1:200; Vector Laboratories; Burlingame; USA
Choleratoxin Subunit B; Fluorescence	goat anti-Choleratoxin 1:5 000; List Biological Laboratories; California, USA	Cy3 donkey anti-goat; 1:200; Jackson Immuno Research; Baltimore, USA
Tetramethylrodamin Dextran, MW 3000; Brightfield	rabbit anti-Tetramethylrhodamine dextran 1:6 000; Molecular Probes; Oregon, USA	biotinylated goat anti-rabbit; 1:200;Vector Laboratories; Burlingame, USA
Tetramethylrodamin Dextran, MW 3000; Fluorescence	rabbit anti-Tetramethylrhodamine dextran; 1:2 000; Molecular Probes; Oregon, USA	Cy3 donkey anti-rabbit; 1:200; Jackson Immuno Research; Baltimore, USA
Wheat-Germ-Agglutinin, pure; Fluorescence	goat anti-Wheat-Germ-Agglutinin, 1:250; Axxora, Lörrach, Germany or rabbit anti-WGA, 1:5000, EY-Lab, CA, USA	Alexa 488 donkey anti-goat, or donkey anti-rabbit; 1:200; Molecular Probes/Invitrogen, Eugen, Oregon, USA
Synaptosomal-associated protein 25 (SNAP-25; or SMI 81); Fluorescence	mouse anti-SNAP-25; 1:1000; Sternberger Monoclonals Incorp.; Maryland, USA	Alexa 488 donkey anti-mouse; 1:200; Molecular Probes/Invitrogen, Eugen, Oregon, USA
Synaptophysin; Fluorescence	mouse anti-Synaptophysin; 1:20; DAKO-M0776; DAKO; Glostrup, Denmark	Alexa 488 donkey anti-mouse; 1:200; Molecular Probes/Invitrogen, Eugen, Oregon, USA
Myosin Heavy Chain (slow); Brightfield	mouse anti-Myosin Heavy Chain (slow); Novocastra Laboratories Ltd; Newcastle upon Tyne,United Kingdom	biotinylated horse anti-mouse; 1:200; Vector Laboratories; Burlingame; USA
Tyrosine Hydroxylase; Fluorescence	rabbit anti-Tyrosine Hydroxylase; 1:100; Chemicon AB152; Chemicon, Billerica, USA	Alexa 488 donkey anti-rabbit; 1:200; Molecular Probes/Invitrogen, Eugen, Oregon, USA; or Cy3 donkey anti-rabbit; 1:200, Jackson Immuno Research; Baltimore, USA
Cholinacetyltransferase; Fluorescence	goat anti-Cholinacetyltransferase; 1:80; Chemicon; Chemicon, Billerica, USA	Alexa 488 donkey anti-goat; 1:200; Molecular Probes/Invitrogen, Eugen, Oregon, USA or Cy3 donkey anti-goat; 1:200; Jackson Immuno Research; Baltimore, USA
Cholinacetyltransferase; Brightfield	goat anti-Cholinacetyltransferase; 1:80; Chemicon; Chemicon, Billerica, USA	biotinylated rabbit anti-goat; 1:200; Vector Laboratories; Burlingame; USA
Calretinin, Fluorescence	rabbit anti-Calretinin; 1:1000; Swant, Marly, Swiss	Cy3 donkey anti-rabbit; 1:200; Jackson Immuno Research; Baltimore, USA
Calretinin, Brightfield	rabbit anti-Calretinin; 1:2500; Swant, Marly, Swiss	biotinylated goat anti-rabbit; 1:200;Vector Laboratories; Burlingame, USA
Parvalbumin, Fluorescence	rabbit anti-Parvalbumin; 1: 1000; Swant, Marly, Swiss	Cy3 donkey anti-rabbit; 1:200; Jackson Immuno Research; Baltimore, USA
Substance P, Fluorescence	rabbit anti-Substance P; 1:100; Zymed, Invitrogen, Damstadt, Germany	Cy3 donkey anti-rabbit; 1:200; Jackson Immuno Research; Baltimore, USA
neuronal Nitric Oxide Synthase; Fluorescence	rabbit anti-NOS; 1: 2000; Chemicon, Millipore, MA, USA	Cy3 donkey anti-rabbit; 1:200; Jackson Immuno Research; Baltimore, USA

Tab. 3 List of antibodies and their sources with the applied methods used in this thesis.

4 Results

4.1 Retrogradely labeled cells in the trigeminal ganglion and their innervation in the extraocular motor neurons

4.1.1 Location of eye muscle injections and their control by retrogradely labeled motor neurons

5 monkeys received a tracer injection into the myotendinous junction or the muscle belly of either the MR or LR (Fig. 7) and one monkey (Monkey 6) received a conjunctiva injection as a comparison. Depending on the location and tracer volume of the injection, the actual tracer uptake after eye muscle and conjunctiva injection was verified from the tracer detection in the flat eye muscle sections.

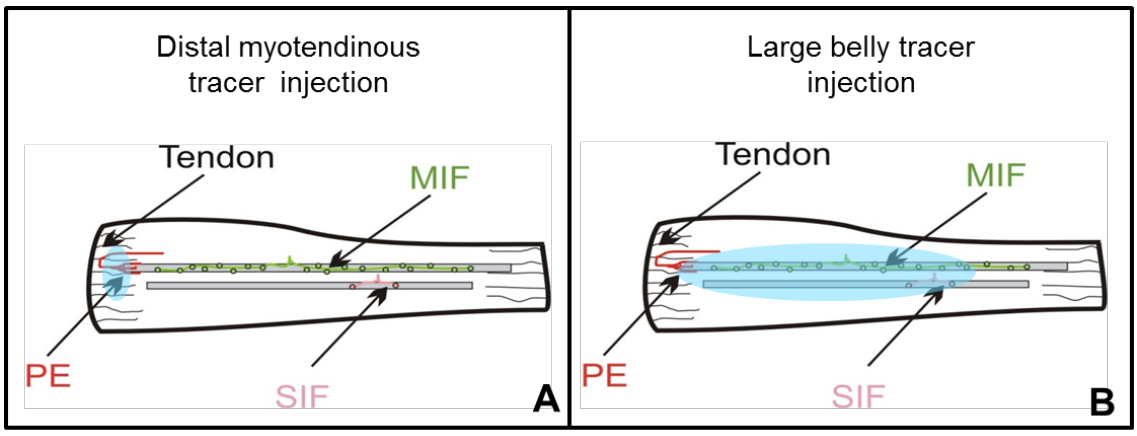

Fig. 7 Different tracer injection sites in eye muscle of two cases (Monkey 1 (A) and 2 (B). (A) Tracer uptake (blue) from a very distal injection into the myotendinous junction that contaminated only very few en plaque endings and only a small fraction of the multiple endings with PEs. (B) Tracer uptake (blue) of a large belly tracer injection that involved en grappe and en plaque endings, but extended also into the myotendinous junctions and contaminated the PEs.

In a next step of analysis, all motor nuclei of extraocular muscles, e.g. oculomotor (nIII), trochlear (nIV) and abducens nucleus (nVI) were analyzed for the presence of retrogradely labeled neurons – with focus on peripheral versus central motor neuronal groups. The complete results of the motor neuronal labeling are listed in table 4. In accordance to earlier studies tracer injections into the muscle belly led to retrogradely labeled motor neurons within and around the respective motor nuclei. A muscle injection into the myotendinous

junction resulted in selective tracer labeling of peripheral motor neurons (Fig. 4) (Büttner-Ennever et al., 2001).

In all cases with a belly injection into the MR, retrograde labeling was found in all MR motor neuronal groups including the peripheral C-group. Additional weaker labeling was found in SR-, and IR- motor neurons, and in one large belly injection case also SO motor neurons (Tab. 4). A tracer injection into the LR often led to additional labeling of the central and or only peripheral IO motor neurons in the S-group. One case (Monkey 4) with a very small CT injection (4.5 µl), very distal into the tendon of the LR muscle, did not reveal any retrograde tracer labeling within the abducens nucleus. However, interestingly light tracer positive afferents in the spinal trigeminal nucleus were noted (below the cuneate nucleus area).

In Monkey 1, with a CT injection into the LR myotendinous junction (9.8 µl), besides the peripheral MIF neurons in the abducens area additional neurons in the S-group were labeled due to contamination of the adjacent myotendinous junction of the IO.

Monkey 2 received a large WGA injection (10 µl) into the belly of the MR muscle. In addition to tracer labeling of the MR motor neurons in the A-, B-, and C-group, all other eye muscles including the LP, but except the IO, were contaminated as judged from retrograde labeling of the corresponding motor neuronal subgroups and the stained muscles (Tab. 4).

Monkey 3, injected with CT (5.3 µl) in the belly of the MR muscle showed selective labeling of the A-, B- and C-group in nIII with no contamination of other muscles.

Monkey 4 had a CT injection into the myotendinous junction of the LR (4.5 µl). Although the muscle showed a tracer uptake, no labeled motor neurons, even in the nVI were found.

Monkey 5 was injected into the myotendinous junction of the SR muscle and showed only labeled motor neurons in the SR area of the nIII contralateral, but also ipsilateral. Furthermore, peripheral motor neurons in the S-group were also tracer positive.

Monkey 6 received an injection into the conjunctiva of the eye to compare the retrogradely labeled TG neurons with eye muscle injections. Next to the injection area, the IO, the peripheral neurons of the S-group and also nVI and peripheral neurons of nVI were contaminated.

In none of the cases did we find any tracer positive cells within the mesencephalic trigeminal nucleus, but in all cases including case 4 without motor neuronal labeling retrogradely labeled ganglion cells were found in the ipsilateral TG. Furthermore, in a few cases (Monkey 1, 3 and 4) tracer labeled neurons in the orbicularis oculi region of the facial nucleus were seen (Tab. 4).

	Tracer	Location of the injection	n III central	n III peripheral	n IV	n VI	TG	Vmes	VII
Monkey 1 (P 181)	CT	LR myotendinous junctions	IO	S-group	No	peripheral	Y	No	Y
Monkey 2 (M 182)	WGA	MR belly	MR (A- and B-group) SR IR	C-group	Y	Y	Y	No	No
Monkey 3 (X183)	CT	MR belly	MR (A- and B-group)	C-group	No	No	Y	No	Y
Monkey 4 (L 184)	CT	LR myotendinous junction	No	No	No	No!	Y	No	Y
Monkey 5 (RZn10 ri)	CT	SR myotendinous junction	SR mostly contralateral, a few ipsilateral	S-group	No	No	Y	No	No
Monkey 6 (32-473)	CT	conjunctiva	IO	S-group	No	central and peripheral	Y	No	No

Table 1 Overview of the tracer injection cases in the EOMs or the conjunctiva, listing the injection site and location of retrogradely labeled neurons.

4.1.2 Location and population of the retrogradely labeled trigeminal ganglion cells after eye muscle injections

Almost all retrogradely labeled ganglion cells in the TG were found in the ophthalmic subdivision. Only a few were at the border to, or in the area of the maxillary subdivision. Irrespective of the injected muscle or the location of the tracer uptake area within the muscle the distribution pattern in the ophthalmic TG subdivision was the same (Fig. 8).

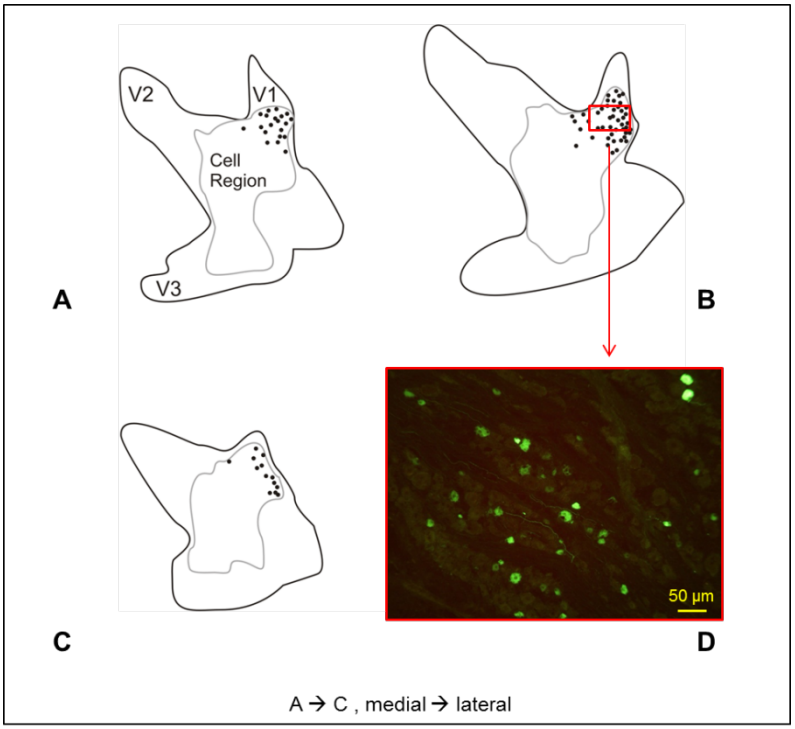

Fig. 8 Drawing of three representative flat sections of a monkey trigeminal ganglion (A-C) from medial to lateral, indicating the location of CT tracer labeled cells after a muscle belly injection into the MR. Almost all retrogradely labeled cells, indicated by black dots, are located in the ophthalmic part of the TG. (A-C) (D) High-power photograph of immunofluorescence (green) of CT-labeled cells in the ophthalmic part of V1 (insert of C).

The morphometric analysis of the tracer labeled cells in TG revealed a large population of small and medium-sized cells with a mean diameter ranging between 16 and 44 µm and a small population of large neurons with a diameter between 48 and 74 µm (Fig. 9). The cell numbers of both populations were more evenly distributed in the case with the belly injection (Monkey 2) or in the case with an injection into the conjunctiva (Monkey 6) (Fig. 9).

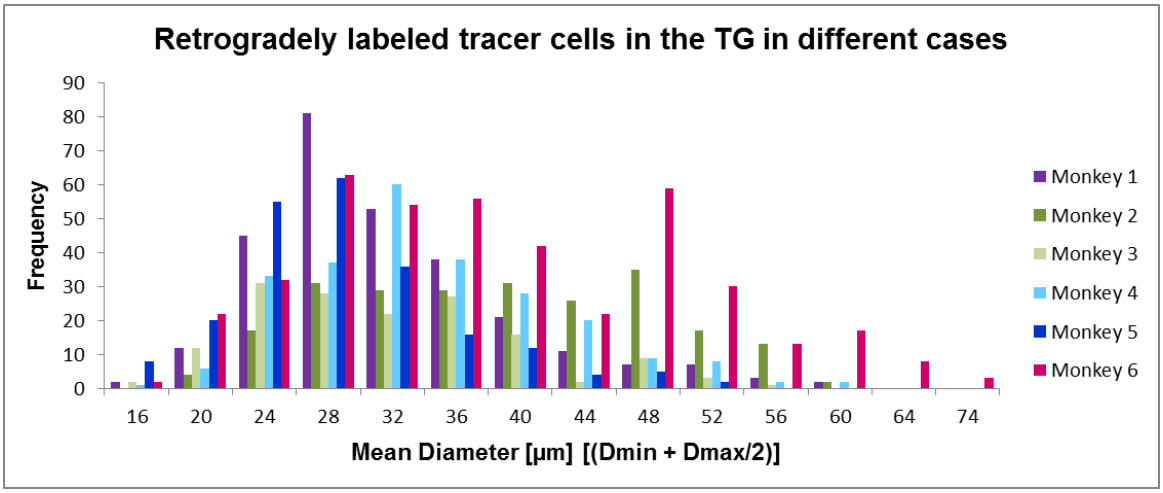

Fig. 9 Cell size profile of retrogradely labeled neurons in the TG after different injections into the eye muscle (cases: P181, M182, X183, L184 and RZn10ri) or conjunctiva (case 32-473). Most of the tracer containing cells have a mean diameter between 24 µm and 44 µm. Only in the belly injection case M182 and in after a conjunctiva injection additional large cells are labeled.

4.1.3 Histochemically characterized trigeminal ganglion populations innervating extraocular muscles .

In order to characterize the tracer labeled TG cells, additional stainings for either substance P, neuronal nitric oxide synthase, calretinin, parvalbumin or cholinacetyltransferase have been performed. All markers were found in the trigeminal ganglion and involved cells of all sizes, small, medium-sized and large neurons (see WGA-labeling in Fig. 10). The analysis of double labeled TG sections revealed that all markers were present in more or less large populations of tracer labeled TG cells (Fig. 10 and 11).

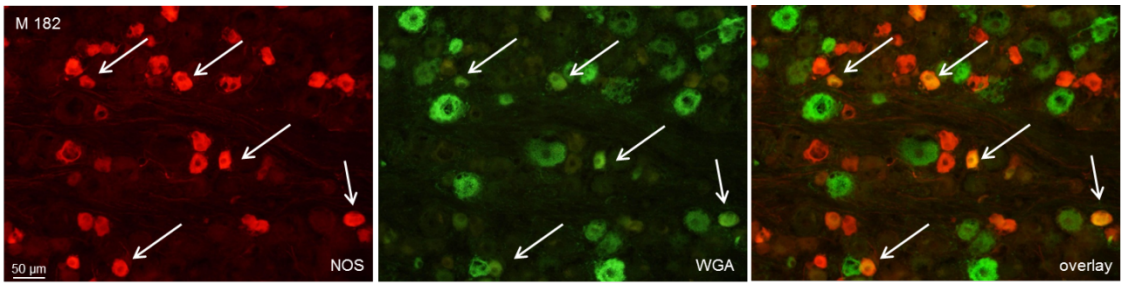

Fig. 10 High-power photograph of a flat section of the trigeminal ganglion (Monkey 2) immunostained for NOS (A) and the tracer wheat germ agglutinin (WGA) (B). Note that tracer labeled population includes all cell sizes (B), whereas NOS positive neurons involve only small cells (A). The expression of both markers in some neurons (C, arrows) indicates that NOS positive TG neurons innervate the extraocular muscle.

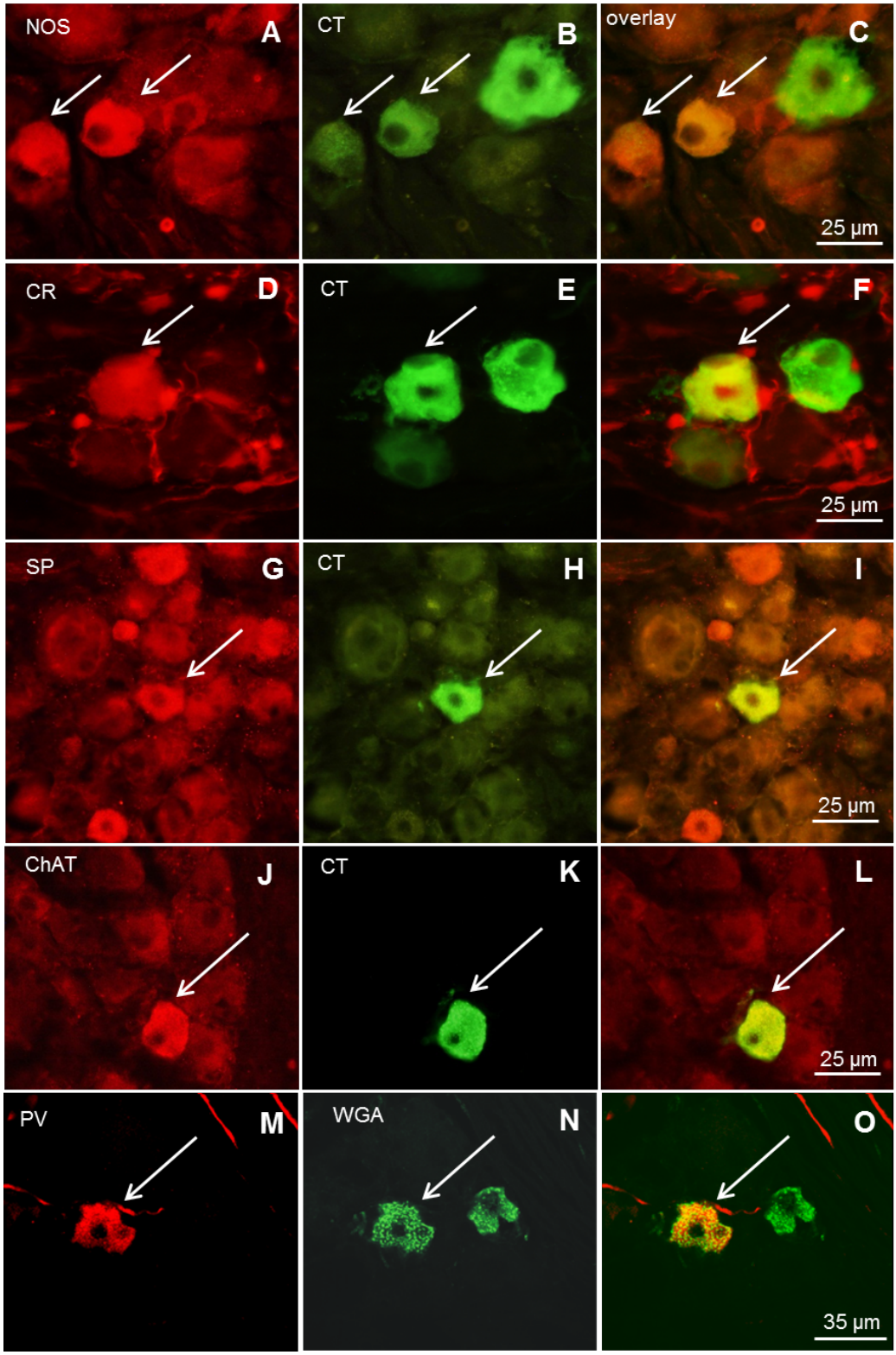

Fig. 11 High-power photographs of double immunofluorescence preparations in monkey trigeminal ganglion demonstrating tracer-labeled cells (middle panel, green cells) projecting to extraocular muscles and the markers nitric oxide synthase (NOS) (A-C), calretinin (CR) (D-F), substance P (SP), (G-I), cholinacetyltransferase (ChAT) (J-L), parvalbumin (PV) (M-O). The right panel shows the overlay of both fluorescent markers. Double labeled cells appear in yellow (arrows). Note the unexpected finding of ChAT positive neurons in the TG that project to extraocular muscles (J, K, L).

Substance P

In all cases, SP double labeled cells were mostly small or medium sized, with a mean diameter between 16 µm and 36 µm (Fig. 11, G-I). The largest population of SP positive and tracer labeled cells were counted after an injection into the conjunctiva (42.4 %), were some of the SP double labeled neurons had a cell size up to 48 µm, followed by injections into the myotendinous junction (34.39 %, 22.08 % and 13.7 %). One of the belly injections (Monkey 3) showed also 22.08 % double labeled SP neurons; in the other case (Monkey 2) only 6.23 % were counted (Fig. 12, I-VI and Tab. 6).

Nitric oxide synthase (NOS)

As for SP the largest populations of NOS positive, tracer labeled trigeminal ganglion cells were found in those cases with tracer injections into the distal region of the eye muscles including the myotendinous junction (Fig. 11, A-C and Tab. 6). This was very obvious for the tendon injection of Monkey 4 with 27.53 %, which had not revealed any motor neuronal labeling, and for Monkey 5 with a myotendinous injection into the SR with 39.7 % NOS double labeled cells. In the distal injection of Monkey 1, 16.57 % were NOS positive and tracer labeled. The central belly injection cases (Monkey 3 and 2) revealed variable populations of double labeled cells with 22.86 % and 6.91 %, respectively (Tab. 6). In all cases the tracer labeled NOS neurons represent a population of small to medium-sized cells with a mean diameter between 16 µm and 40 µm. (Fig. 12, I-V)

Calretinin (CR)

Within the tracer labeled TG neurons, calretinin-expressing cells formed a very small population of small to medium-sized neurons (mean diameter 16-24 µm) (Fig. 11, D-F, Fig. 12 IV, Tab. 6). Again the largest population (1.12 %) was seen for the case with a tendon injection, and only 0.5 % for Monkey 3 and 1. No double labeled neurons were found in Monkey 2 with a muscle belly injection. Only 2 cells of Monkey 4 are included in the histogram, because the

cells size was only measured when the nucleus was visible (Fig. 12 IV and Tab. 6).

Parvalbumin (PV)

PV-immunoreactive neurons make up only a very small population of tracer labeled neurons (8.8 % and 9.7 %) (11, M-O and Fig. 12 V, VI). They were present only after an injection into the myotendinous junction (Monkey 5) or conjunctiva (Monkey 6) and included mean to large sized neurons (mean diameter 22 µm - 52 µm) (Fig. 12, V, VI and Tab. 6)

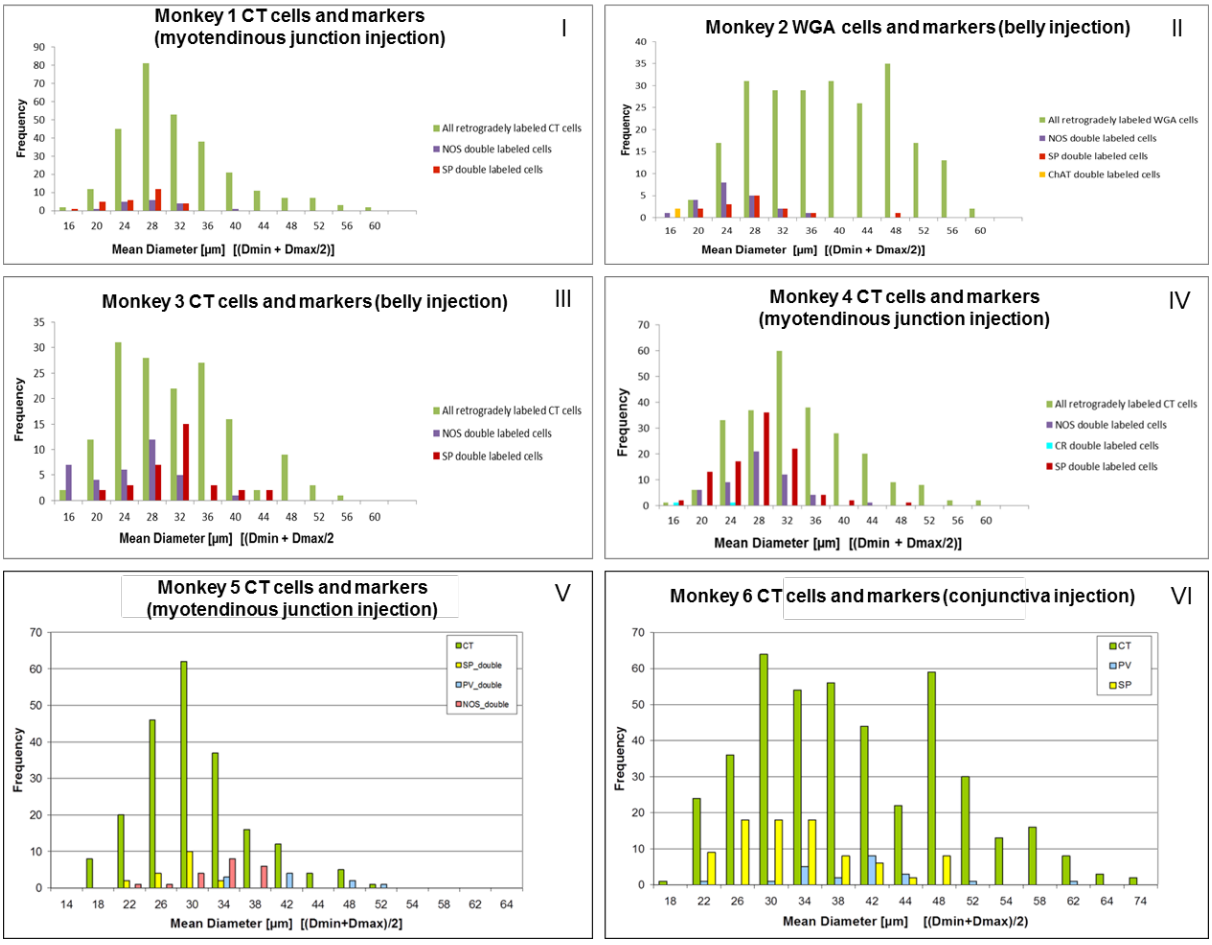

Fig. 12 (I-VI) Cell size profiles of double labeled tracer neurons and the respective marker associated population in the TG after different injection targets in the eye muscles (I-V) or the conjunctiva (VI). (I) The SP positive tracer cells were small or middle sized, the NOS double labeled cells were almost in the same range (II) The two ChAT labeled cells had a very small mean diameter (16 µm), whereas the SP and NOS neurons range between were small to middle sized. A single double labeled SP cell had a mean diameter of 48 µm. (III) The mean diameter for NOS double labeled showed also small and midsize cells with a peak at 28 µm, as well as for SP cells with a peak at 32 µm. (IV) Case L 184 was the only case showing double labeled CR positive tracer cells

with a small mean diameter. NOS or SP positive tracer labeled cells ranged in the middle field with a peak at 28 µm. (V) SP and NOS double labeled cells were small to middle sized, whereas the PV immunostained neurons had large cell bodies. (VI) The SP double labeled cells after a conjunctiva injection were also small and middle sized, the PV stained and tracer positive neurons had middle sized and large cell bodies.

Cholinacetyltransferase (ChAT)

Stimulated by the work of Tooyama and Kimura (Tooyama and Kimura, 2000, Yasuhara et al., 2007) who found a splicing variant of ChAT, which they termed peripheral ChAT (pChAT), in the TG of rat, the presence of pChAT and the central ChAT were investigated in our monkey TGs. The appearance of ChAT positive cells within the TG as a sensory ganglion was an unexpected finding (Fig. 11 J-L and Fig. 12 II). Unlike in rat that served as a control no immunostaining for pChAT was present in the monkey TG (Fig. 13). All TGs investigated for the presence of ChAT (Tab. 5) contained a consistent small population of neurons expressing this cholinergic marker. The majority was found in the ophthalmic subdivision (Fig. 14). The systematic analysis of 5 cases (Monkey 1, 2, 3, 4 and 5) for tracer labeled neurons expressing ChAT, revealed only in 2 cases (Monkey 2 and 5) a few tracer double labeled ChAT positive cells (0.7 %) (only a few cells in Monkey 2 could be measured) (Tab 5).

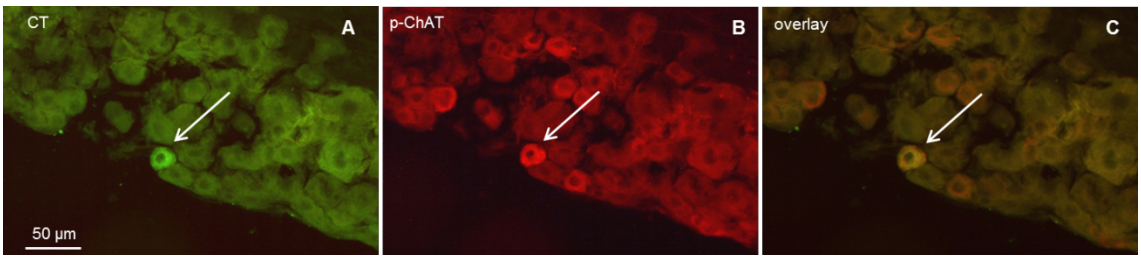

Fig. 13 (A) High-power photograph of rat trigeminal ganglion demonstrating a tracer labeled neuron (green, arrow) after a medial rectus muscle injection expressing immunoreactivity for the peripheral cholinacetyltransferase (p-ChAT) (B) (red, arrow). (C) The overlay of A and B reveals the double labeled neuron in yellow.

TG cells in Case:	Monkey 1 (P181)	Monkey 2 (M182)	Monkey 3 (X 183)	Monkey 4 (L 184)	Monkey 5 (RZn10)
Fluorescence	ChAT pos.	ChAT pos. and tracer labeled	ChAT pos.	ChAT pos.	ChAT pos. and tracer labeled

Tab. 4 Overview of cases with ChAT immunoreactive cells in the trigeminal ganglion, demonstrated in fluorescence. All tested cases revealed ChAT positive cells, two of them (Monkey 2 and 5) showed in addition double labeling with a retrogradely tracer from EOM injections.

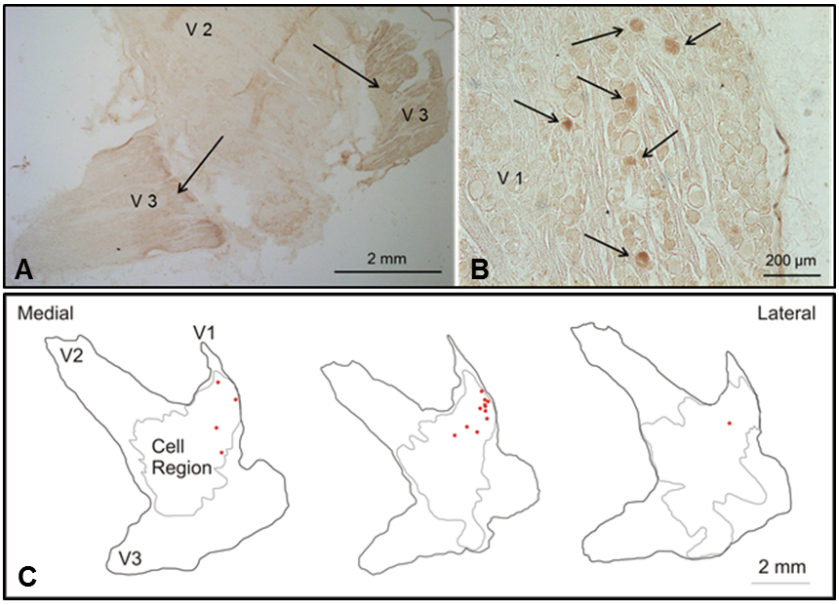

Fig. 14 (A-B) Photographs of flat sections through monkey trigeminal ganglion (TG) immunostained for cholinacetyltransferase (ChAT) in overview (A) and high-power magnification revealing several ChAT positive neurons (B, arrows). The ChAT immunostaining of motor fibers in the mandibular branch (V3) served as positive control. (C) Drawing of three TG sections demonstrating the location of ChAT immunolabeled neurons (red dots). Note that most ChAT positive neurons were found in the ophthalmic division of the TG, and few at the border or in the maxillary division

For verification of the ChAT immunolabeling, selected TG section underwent an incubation in pre absorbed anti-ChAT with its antigen at different dilutions (8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml). A control slide with the ChAT antibody showed a few positive ganglion cells in the ophthalmic division and positive nerve fibers supplying the masticatory muscles in the mandibular division (V3) (Fig. 15 A,B).

The incubation of the 1 and 2 µg/ml antigen solution plus ChAT antibodies revealed no positive labeling of cells, but a lightly stained mandibular branch (Fig. 15 C). In the slides, incubated with the 4 and 8 µg/ml antigen solutions plus ChAT antibodies, there was no immunolabeling of either cells or fibers, indicating that the ChAT antibody detects specially the synthetizing enzyme of acetylcholine (Fig. 15 D).

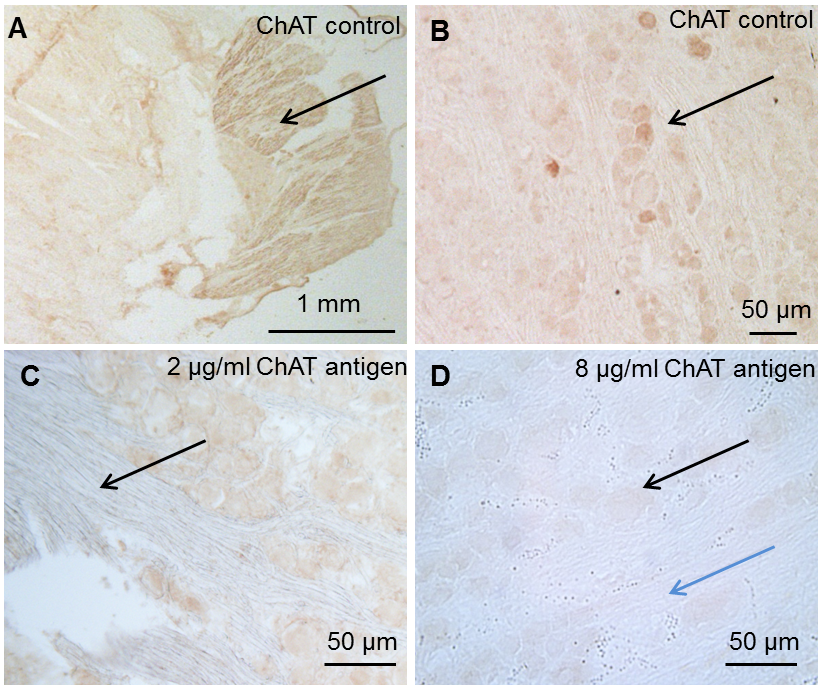

Fig. 15 Preabsorption controls of the ChAT antibody. Monkey TG tissue stained with ChAT antibodies using immunoperoxidase methods before (A and B) and after (C and D) preabsorption of the antibody with its antigen cholinacetyltransferase (ChAT) at different concentrations. In the control stained motor fibers of the mandibular branch (A, arrow) and few cells are immunolabeled (B, arrow), whereas preabsorption with ChAT abolished immunostaining, thereby confirming the specifity of the antibody.

	Injection		SP	NOS	CR	ChAT	PV
Monkey 1 (P 181) **CT** tendon LR	very distal injection (myotendinous juction)	double labeled cells	108 - 34,39 %	56 - 16,57 %	2 - 0,57 %	0 - 0 %	not done (n.d.)
		all tracer labeled cells	314	338	349	630	n.d.
Monkey 2 (M 182) **WGA** belly MR	belly injection (A-, B- and C-group) with big contaminations (SR, IR, SO, LR)	double labeled cells	81 - 6,23 %	67 - 6,91 %	0 - 0 %	2 - 0,7 %	n.d.
		all tracer labeled cells	1301	969	589	285	n.d.
Monkey 3 (X 183) **CT** belly MR	large belly injection (A-, B- and C-group)	double labeled cells	87 - 22,08 %	56 - 22,86 %	1 - 0,50 %	0 - 0 %	n.d.
		all tracer labeled cells	394	245	202	241	n.d.
Monkey 4 (L 184) **CT** tendon LR	very distal injection (myotendinous juction)- no labeled motor neurons!	double labeled cells	101 - 22,80 %	117 - 27,53 %	5 - 1,12 %	0 - 0 %	n.d.
		all tracer labeled cells	443	425	445	569	n.d.
Monkey 5 (RZn10ri) **CT** tendon SR	distal injection (myotendinous junction)	double labeled cells	50 - 13,7 %	127 - 39,7 %	n.d.	n.d.	40 - 8,8 %
		all tracer labeled cells	364	323	n.d.	n.d.	453
Monkey 6 (32-473) **CT** Conjunctiva	conjunctiva injection	double labeled cells	87 - 42,4 %	n.d.	n.d.	n.d.	22 - 9,7 %
		all tracer labeled cells	205	n.d.	n.d.	n.d.	227

Tab. 5 Number and relative amount of cells in the trigeminal ganglia associated with a specific marker projecting to the extraocular muscles or conjunctiva

In addition, the analysis of anterogradely tracer labeled trigeminal afferents in the brain revealed very light staining in all cases with tracer labeled TG cells from EOM injections (Fig. 16). After entering the brainstem at pontine level tracer labeled axons traveled caudally and terminated in the oral and interpolar part of the spinal trigeminal nucleus. Furthermore, there were tracer labeled afferents in the more rostral part of the principal sensory nucleus of the trigeminal complex. No labeling was seen in the mesencephalic trigeminal nucleus.

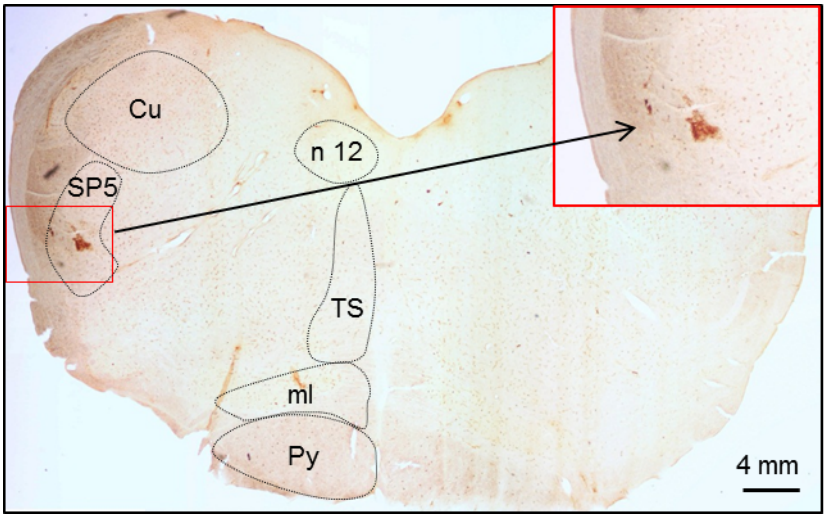

Fig. 16 Transverse section of a monkey (Monkey 4) brainstem at the level of the hypoglossal nucleus (n 12) and cuneate nucleus (Cu) demonstrating tracer (CT) labeled trigeminal afferents in the spinal trigeminal nucleus (SP5), see detailed view (arrow). ml – medial lemniscus; Py – pyramidal tract.

4.1.4 Labeling of eye muscle structures with different markers

In order to study the innervation targets of the different TG cell populations, extraocular muscles of monkey have been immunostained for the markers substance P, nitric oxide synthase, calretinin and cholinacetyltransferase. For a more thorough study of proprioceptive structures flat eye muscle sections of sheep were stained in addition.

Substance P labeled predominantly thin nerve fibers and small terminals, which surround the blood vessels like a net (Fig. 17 A-F). SP was neither expressed in en grappe or en plaque endings nor in annulospiral or flower-spray endings in muscle spindles in sheep.

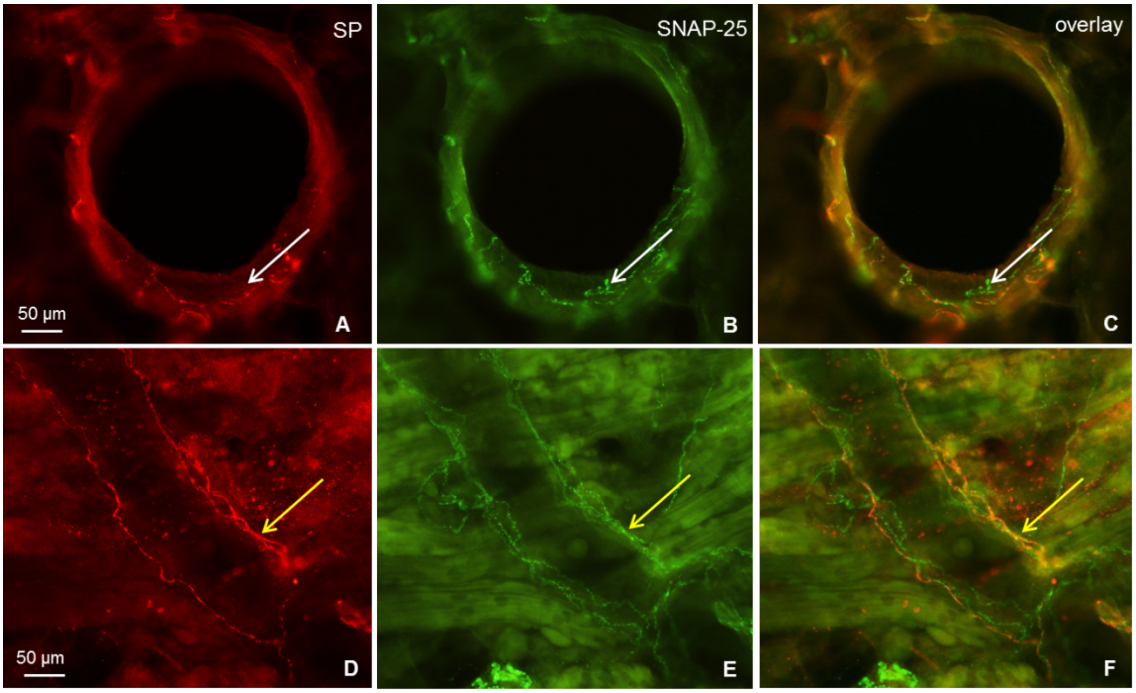

Fig. 17 A-C: High-power photographs of a cross section (A-C) and a longitudinal sections (D-F) through an extraocular muscle. It shows a blood vessel associated with immunoreactive terminals containing substance P (SP) (A, D) confirmed as neural structures by SNAP-25 immunoreactivity (B, E), which reveals the complete innervation. The overlay of both photographs (C, F) demonstrates thin SP- negative (C, arrow) and SP positive nerve fibers in yellow (C, F).

NOS was present in thin nerve fibers around blood vessels (Fig. 18 A-C). In addition the collagen capsule of muscle spindles only in sheep eye muscle was NOS positive (Fig 19).

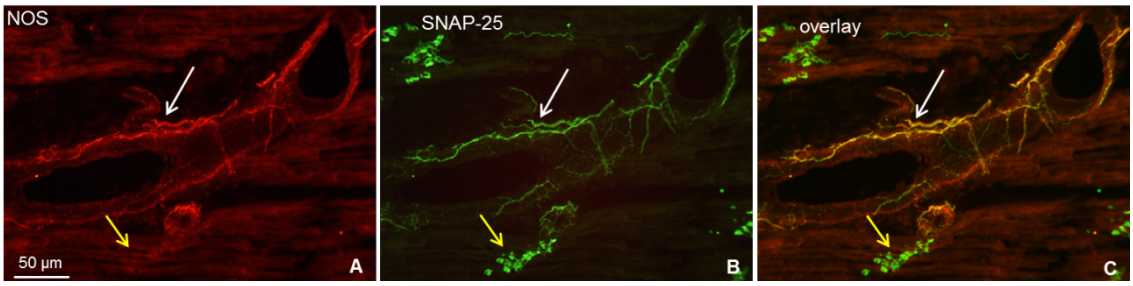

Fig. 18 NOS (A) and SNAP-25 (B) double labeling (C) of monkey (A-C) eye muscles; (A) NOS labeled nerve fibers around blood vessels (C, white arrow) but no other fibers or terminals like en plaque endings (C, yellow arrow).

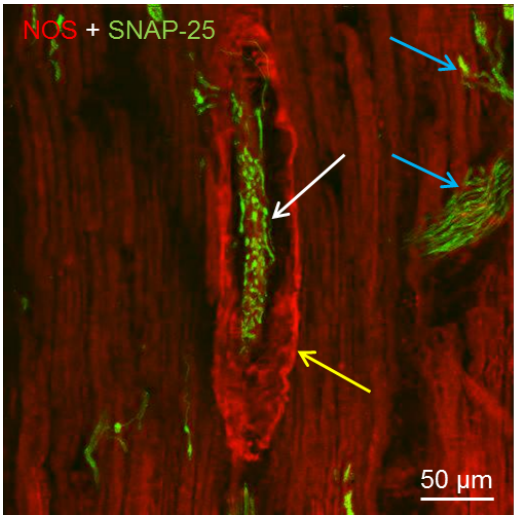

Fig. 19 NOS (red staining) labeled in the sheep muscle tissue the capsules of the muscle spindles (yellow arrow). SNAP-25 (green staining) shows the innervation of the muscle spindle (white arrow) and the innervation of the extrafusal fibers of the eye muscle (blue arrows).

CR was present in palisade endings and associated tendon endings, which have been identified with SNAP-25-immunostaining and outlined them clearly (Fig. 20 A-C). Combined immunostaining for CR and synaptophysin (SYN), a synaptic vesicle protein, showed that the majority of SYN positive terminals were CR positive, but between 16 % and 22 % were not double labeled. These CR-negative terminals were not confined to either tendon or muscle, but were found in both (Fig. 20 D-F). The close inspection of the complete muscle innervation did not reveal any CR positive en plaque ending, however a few en grappe endings (Fig. 20 G-L).

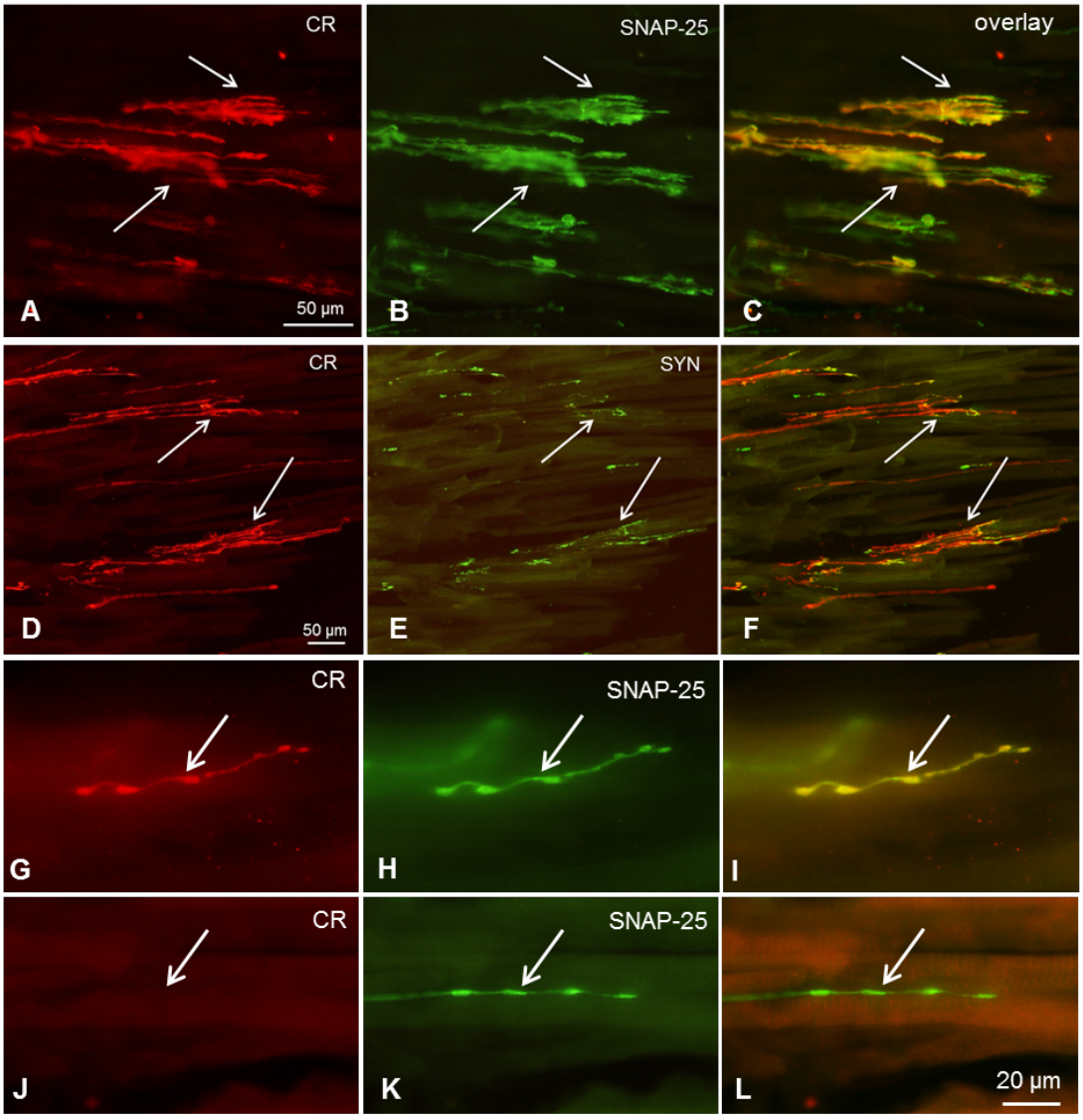

Fig. 20 CR staining in the monkey eye muscle (A, D, G, J) double labeled (yellow color) with either SNAP-25 for the complete innervation (B, H, K) or synaptophysin for the terminals (SYN) (E). CR was only present in palisade endings (C, F, arrows) and few en grappe endings (G-I), but most of them did not contain CR (see J-L).

PV was only present in en plaque endings on the outside margin of the EOMs, which involves the orbital layer (Fig. 21). No PV positive en plaque endings were seen in the middle, innervation zone of the muscles. Furthermore, no en grappe endings or any other terminals were immunoreactive for PV.

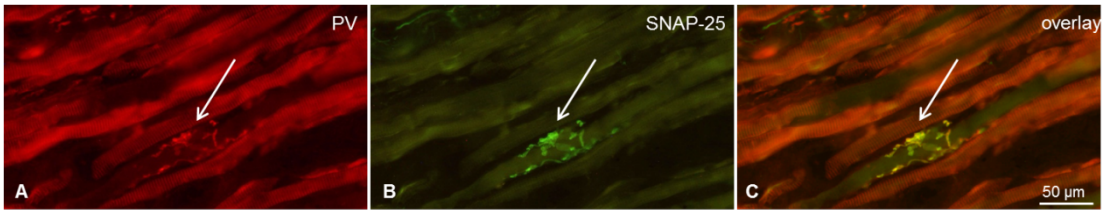

Fig. 21 Immunostaining for parvalbumin (PV) in the extraocular muscles (A) confirmed as neural staining by SNAP-25 expression (B) also shown in yellow in the overlay of A and B (C). PV immunoreactivity was only found in en plaque endings on the outside margin of the EOMs corresponding to the orbital layer.

The cholinergic marker ChAT was present in all putative motor en plaque (Fig. 22 A, B) and en grappe endings (Fig. 22 C, D), but also in PEs including their tendon endings (Fig. 22 E, F). Double immunofluorescence for ChAT and TH revealed that the fine nerve endings around blood vessels represent sympathetic nerve fibers (not shown).

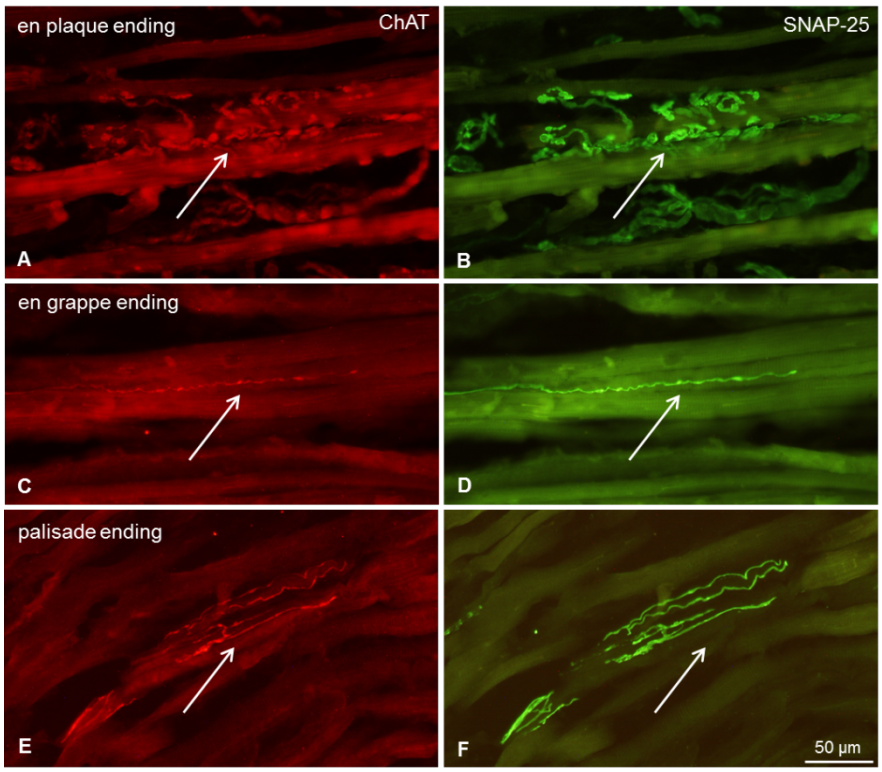

Fig. 22 Examples of ChAT labeled structures the monkey extraocular muscles (A, C, E) confirmed as neural endings revealed by combined immunostaining for SNAP-25 (B, D, F): en plaque ending (A, B), small en grappe endings (C, D) and palisade endings at the myotendinous junction (E, F).

4.2 Anterogradely labeled terminals in the extraocular muscles after tracer uptake within and around the oculomotor nucleus

In order to find out, whether palisade endings can anterogradely be labeled after a tracer injection into the oculomotor nucleus, two monkeys had received tracer injections into the nIII area.

4.2.1 Tracer injections and uptake area

In one monkey with two injections (case 1 and 2), the tracer injection sites were centered in nIII (Fig. 23; Tab. 7). A second monkey with one tracer injection in the nIII axons (case 3) was also analyzed.

In case 1 the injection (TMR-DA) was centered ventrally in the MR A-group of the left nIII (Fig. 23 A-C). The tracer uptake area involved the subgroups of the IR, superior rectus (SR) and inferior oblique (IO) muscles in the left nIII as well as the trochlear nucleus (nIV) and the central caudal nucleus (CCN) containing levator palpebrae (LP) motor neurons (Porter et al., 1989).

In case 2 the tracer injection (CT) was centered more dorsally in the right nIII involving the subdivisions of the IR, MR, SR and IO (Fig. 23 D-F). Additional uptake was noted in the left nIII covering the SR area, the right nIV and CCN. Tracer uptake had also occurred from the C-group and the preganglionic neurons in the Edinger-Westphal nucleus (EW) as indicated by the retrograde labeling in the olivary pretectal nucleus (Büttner-Ennever et al., 1996).

In case 3 the injection site (TMR-DA) was placed ventrolateral to nIII hitting the traversing axons of the third nerve as they leave nIII. Strongly labeled tracer-filled axons within the fascicles of the third nerve indicated that tracer uptake had occurred from the injured axons of the nerve (Fig. 23.G-I). Case 3 was the only case which was not analyzed in detail.

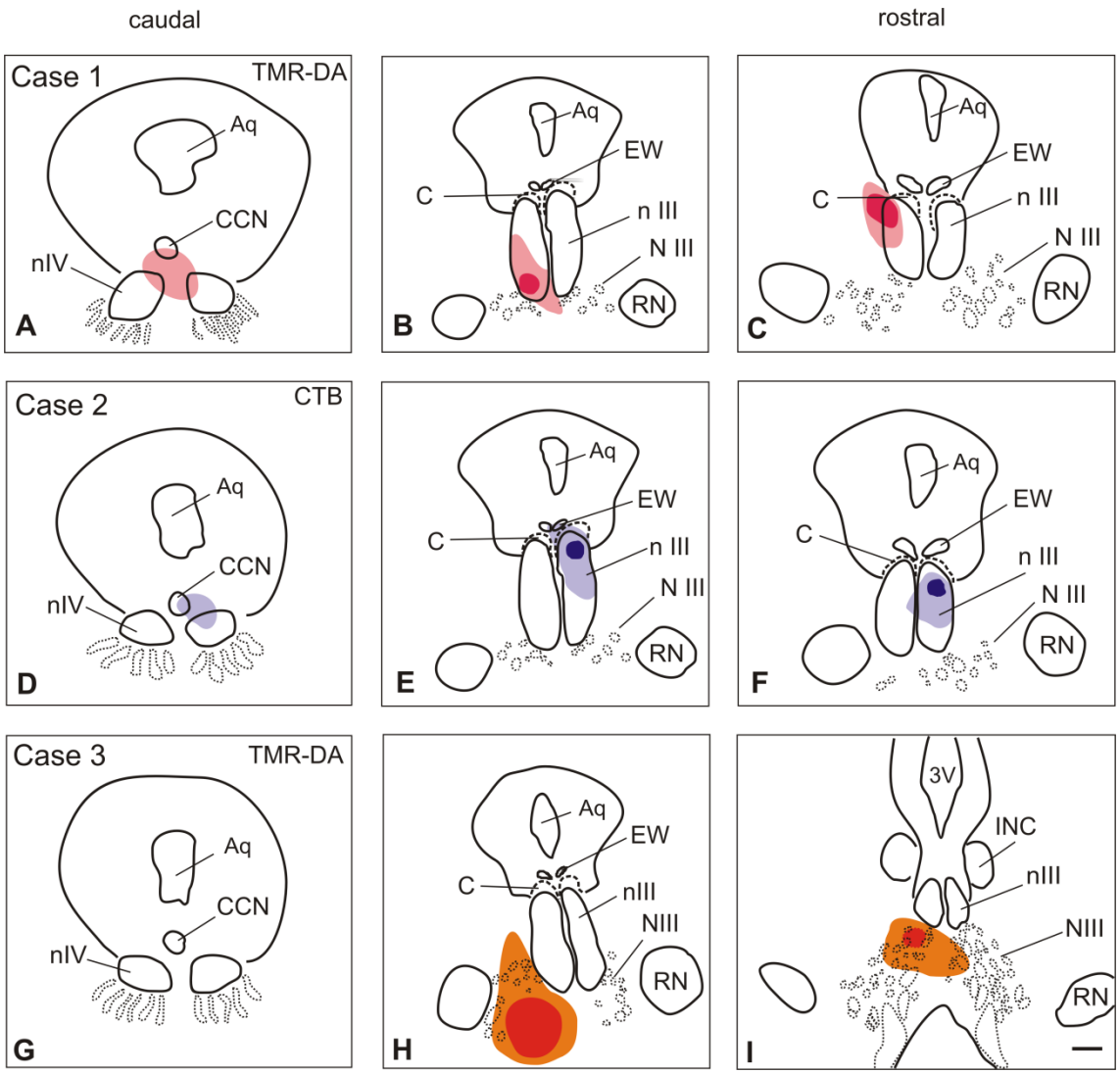

Fig. 23 Reconstructions of transverse brain stem sections (from caudal to rostral) of three monkey cases showing the location (case 1: deep red, case 2: deep blue, case 3: deep orange) of the tracer injections and the extent of the uptake area (case 1:light red , case 2: light blue, case 3: light orange). (A–C) Case 1: The tetramethylrhodamine dextran (TMR-DA) injection involved the oculomotor (nIII) and trochlear nucleus (nIV), but spared the C-group (C). (D–F) Case 2: CTB injection involved nIII, EW and the C-group and, to a minor extent, nIV. (G–I) Case 3: The TMR-DA injection was placed into the oculomotor nerve (NIII), and did not involve the motor neurons in nIII and nIV. Aq, aqueduct; CCN, central caudal nucleus; RN, red nucleus; 3V, third ventricle; INC, interstitial nucleus of Cajal. Scale bar 500 µm. (Figure from Lienbacher et al. 2011b)

4.2.2 Anterograde tracer labeling in extraocular muscles (case 1-3)

All tracer injections involving either nIII (case 1 and 2) or nerve bundles of the oculomotor nerve (NIII) (case 3) revealed distinct anterograde labeling of nerve endings in the respective extraocular muscles, depending on the injection site (Tab. 7)

Accordingly, in all cases the lateral rectus muscles, whose motor neurons lie in the abducens nucleus at pontomedullary level, were tracer negative. The anterogradely labeled terminals comprise en plaque endings concentrated around the middle third of the muscle fibers in the global and in the orbital layer (Fig. 24 A, B), and small en grappe endings, which are distributed along single muscle fibers but concentrated at the distal and proximal ends (Fig. 24 E,F). In addition all extraocular muscles contained numerous anterogradely tracer labeled palisade endings at the myotendinous junction (Fig. 24 C,D; 25 F,G).

The systematic analysis of combined immunostaining for tracer and either SNAP-25 (Fig. 24; 25 A, B; D, E), or SYN (Fig. 25 F,G), permitted an estimation of the percentage of tracer labeled nerve endings and a judgment on their types (Tab. 8). Slides from the orbital and global layer of cases 1 and 2 were used for the semi-quantitative analyze.

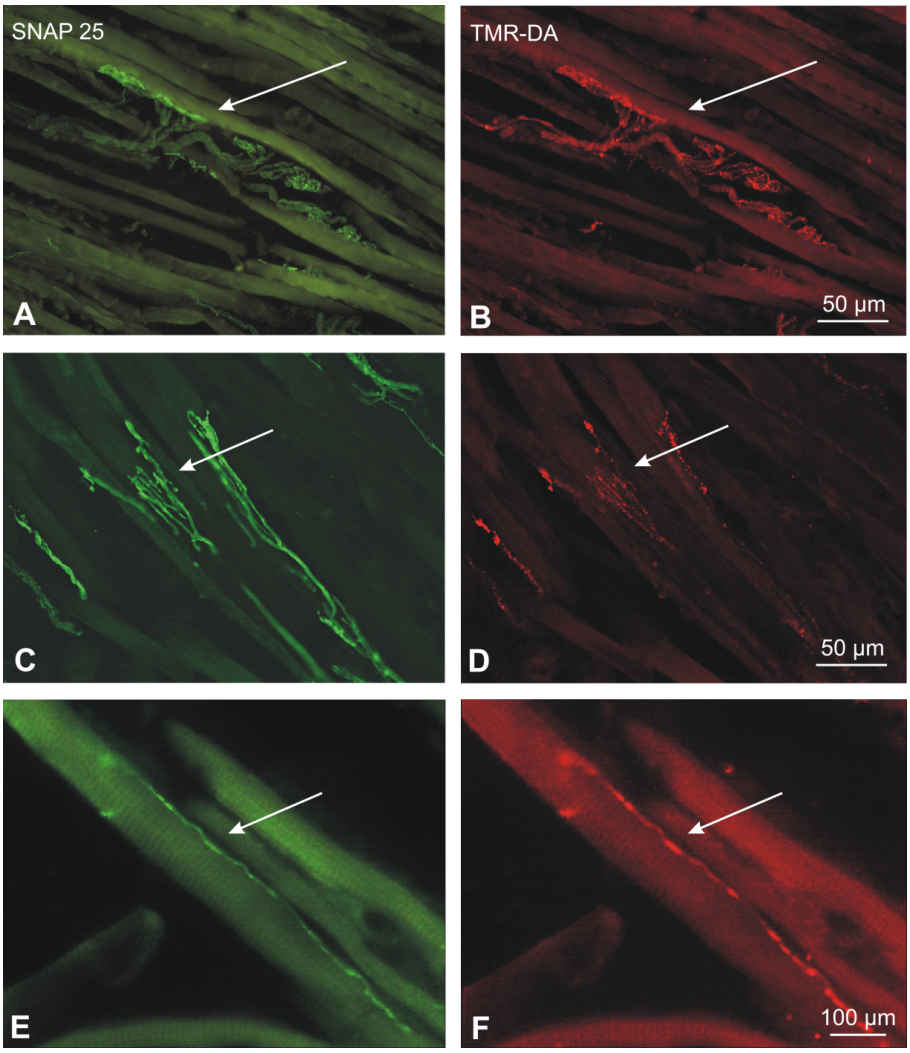

Fig. 24 Combined immunofluorescence of the tracer TMR-DA (red) and SNAP-25 (green) identifying en plaque (A, B), en grappe (E, F), and palisade endings (C, D) in the medial rectus muscle of the

monkey in case 1. Each pair of neighboring photographs shows the same section with different fluorescence filters. (Figure from Lienbacher et al. 2011b)

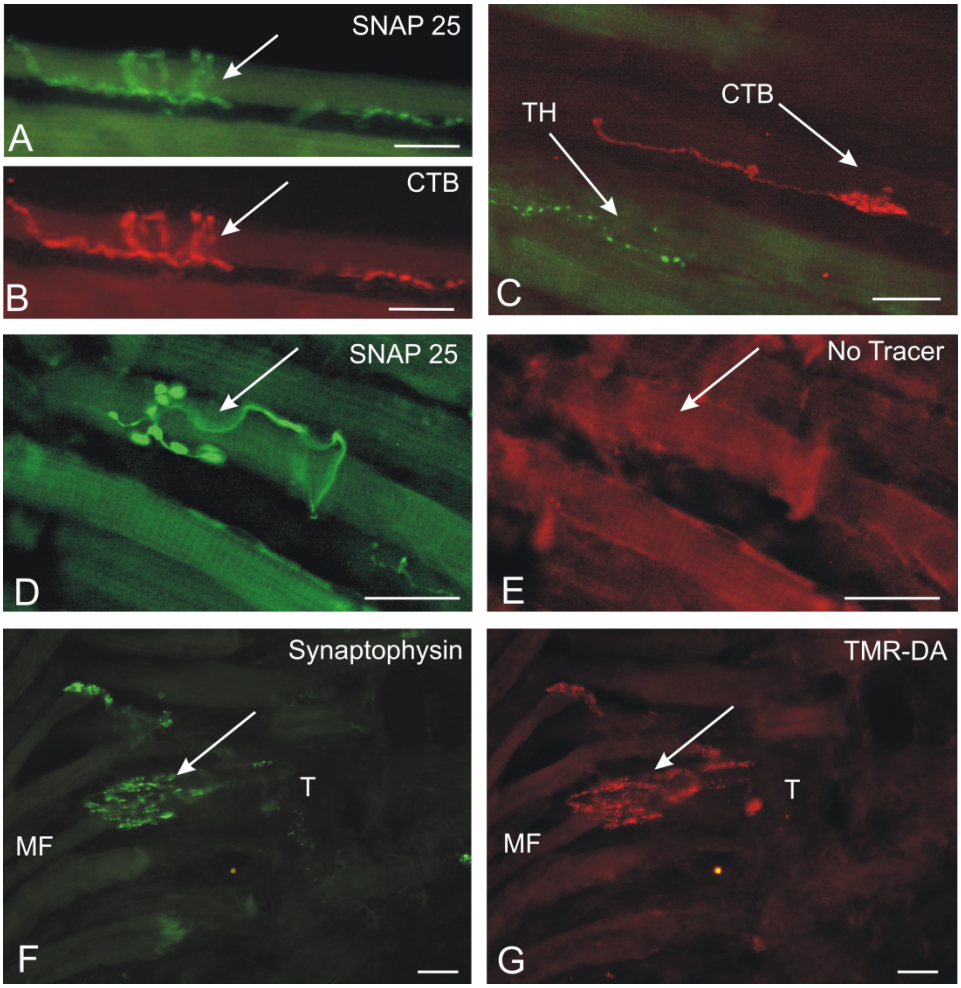

Fig. 25 Different nerve endings in the extraocular muscles. (A, B) An identified SNAP-25 positive spiral ending (A; green) was anterogradely labeled with CTB (B; red) after injection into the oculomotor nucleus tracer (case 2). (C) CTB-labeled en plaque endings (red) and TH positive nerve ending (green), which does not contain tracer (case 2). (D, E) Tracer-negative (D) small nerve ending in the eye muscles stained for SNAP-25 (E) (case 1). (F, G) The synaptic endings of an anterogradely TMR-DA-labeled palisade ending (G; red) are visualized by immunostaining for synaptophysin (F; green) (case 1). (Figure from Lienbacher et al. 2011b)

The TMR-DA injection in case 1 resulted in strong anterograde terminal labeling in the corresponding extraocular muscles, i.e. ipsilateral MR, IR and IO and contralateral SR (Fig. 23 A-C). The detailed analysis revealed that in the left MR almost all (99,6 %) en plaque, en grappe and palisade endings appeared to be tracer labeled (Fig. 23 B,D,F; Tab. 6 and 7). In the ipsilateral IR 20 % were labeled, in the inferior oblique 75 % and in the contralateral SR 85 %. The

strong labeling of all nerve ending types in the superior oblique (SO) of both sides (ipsilateral: 91 %; contralateral: 87 %) was attributed to tracer uptake of the injured trochlear nerve damaged by the injection needle.

In case 2, CT positive nerve terminals were found in the ipsilateral IR (18 %), MR (50 %), IO (46 %) as well as a few in the contralateral SO (1 %), and SR of both sides (ipsilateral: 14 %; contralateral: 15 %) (Fig. 23 D-F, Tab. 7 and 8). The partial tracer labeling of the complete nerve ending population in the respective eye muscles was attributed to the incomplete coverage of the nIII with partial inclusion of the nIV by the CT injection (Fig. 23 D-F).

For both cases it should be pointed out that in all muscles – irrespective of the amount of tracer-labeling – all types of nerve endings, en plaque, en grappe and palisade endings, were labeled. In addition every muscle contained a few tracer positive spiral endings around single muscle fibers (Fig. 25 A,B).

The TMR-DA injection in case 3 (Fig. 23 G-I, Tab. 7) which involved only the oculomotor nerve revealed a similar pattern of anterogradely labeling in the ipsilateral IR, MR and SR as described above. A fraction of all nerve ending types including palisade endings were tracer labeled.

	Tracer Injection	Tracer Uptake
Case 1	Tetramethylrhodamine dextran injection in the left nIII	MR, IR, IO region in left nIII ; SR region right; trochlear nucleus, both sides
Case 2	Choleratoxin subunit B injection in the right nIII	IR, MR, IO in right nIII; SR region in nIII bilateral, C-group, EW; trochlear nucleus right
Case 3	Tetramethylrhodamine dextran injection in the left NIII	Nerve fibres mainly left side, also contamination of a few fibres on the right side
Case 4	WGA-HRP injection in the distal part of IR; Choleratoxin subunit B injection in the distal part of the MR	Myotendinous area of injected IR and MR

Tab. 6 Overview of the central tracer injection cases, indicating the injection side and specifying the involved motor neuronal groups.

	Extraocular Muscles	% of tracer positive en plaque endings	Counted en plaque endings	Tracer positive en plaque endings	% of tracer positive en grappe endings	Counted en grappe endings	Tracer positive en grappe indings	% of tracer positive palisade endings	Counted palisade endings	Tracer positive palisade endings
	MR le	**100**	2497	2487	**100**	20	20	**100**	23	23
	IR le	**20**	1610	325	**6**	18	1	**0**	3	0
Case 1	IO le	**76**	1202	910	**46**	11	5	**100**	1	1
TMR-DA	SO ri	**88**	1248	1096	**71**	51	36	**100**	6	6
	SO le	**90**	1072	968	**94**	36	34	**95**	19	18
	SR ri	**85**	1034	878	**73**	26	19	**100**	4	4
	SR le	**0**	n. c. (not counted)	0	**0**	0	0	**0**	0	0
	LR ri	**0**	n. c.	0	**0**	0	0	**0**	0	0
	MR ri	**50**	1765	875	**29**	14	4	**60**	15	9
	IR ri	**18**	932	167	**13**	8	1	**0**	2	0
Case 2	IO ri	**46**	811	374	**50**	6	3	**100**	2	2
CTB	SR le	**15**	910	137	**5**	20	1	**33**	6	2
	SR ri	**14**	1744	241	**8**	37	3	**25**	4	1
	SO le	**1**	1108	13	**0**	67	0	**0**	4	0
	SO ri	**0**	n. c.	0	**0**	0	0	**0**	0	0
	LR ri	**0**	n. c.	0	**0**	0	0	**0**	0	0

Tab. 7 Number of en plaque, en grappe and palisade endings in different extraocular muscles of case 1 and 2 and their tracer labeling. (Table from Lienbacher et al. 2011b)

A surprising result was the discovery of several anterogradely labeled neurotendinous ending complexes, which correspond to the tendon organs described by Ruskell (Ruskell, 1979) in the myotendinous junction of MR, in both the CT and the TMR-DA tracer cases (case 1 and 2), (Fig. 26 A-C). They were often seen associated with a tracer labeled palisade ending (Fig. 26 A, B), but evidence for a definite neural continuity between a tendon organ and a palisade ending was not visible in these tracer sections. However a neural continuity between tendon organs and palisade endings was apparent from analysis of a complete series of SO sections systematically stained for SNAP-25 to reveal all neural structures.

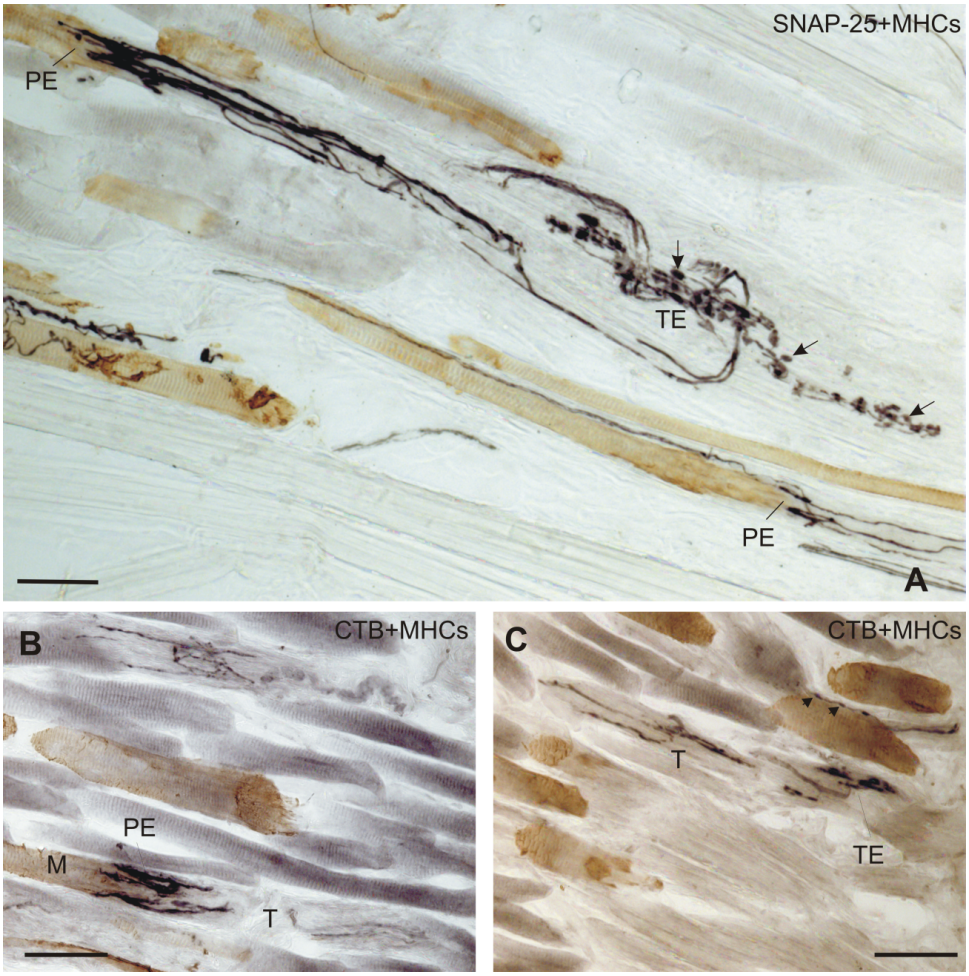

Fig. 26 Brightfield immunoperoxidase staining for MHCs (brown) combined with either SNAP-25 (black) or CTB (black). (A) A SNAP-25 positive palisade ending (PE) (black) contacts an MHCs positive MIF in the top left corner; its axon is seen to be continuous with a tendon ending (TE) in the superior oblique tendon (T). (B, C) CTB labeled palisade endings (black) and labeled tendon ending (black) in the MR muscle, after tracer injection in the oculomotor nucleus (case 2). (Figure from Lienbacher et al. 2011b)

4.2.3 Tracer-negative nerve endings in extraocular muscles

In all central injection cases (1-3) two distinct nerve fiber types remained tracer-negative and were visualized only by SNAP-25 immunoreactivity. One group gave rise to very small terminals, which innervated one muscle fiber via a single contact. These terminals were found throughout the muscle with an incidence of two to three per section (Fig.25, D,E). Another tracer-negative type represents fine fibers with small endings resembling that of en grappe, but meandering across several muscle fibers and often being associated with blood vessels. Combined immunostaining for TH and the tracer indicated that they were sympathetic nerve fibers (Fig. 25, C).

4.2.4 Analysis of the trigeminal ganglia and the mesencephalic trigeminal nucleus

The systematic analysis of the trigeminal ganglia (TG) and the mesencephalic trigeminal nucleus (VMes) of both sides in case 1 and 2, did not disclose any tracer labeled cell bodies or terminals.

4.2.5 Course of axons arising from motor neurons in the C-group (case 4)

Injections into the nIII label axons of passage as well as the motor neurons. In order to investigate how much, and which terminal labeling in the eye muscles in cases 1-3 could be attributed to axons of passage through nIII, injections of retrograde tracer substances were placed in the distal muscle ends of the MR (CT) and IR (WGAHRP) in one monkey (case 4). Retrogradely filled cells were located only in the peripheral oculomotor C-group and not in the classical MR (A- and B-group) and IR motor neuron subgroups within nIII (Fig. 27 B). The course of the C-group axons in nIII is plotted in Fig. 27 A; they take a ventrolateral course through nIII thereby traversing the other motor neuron subgroups before leaving nIII (Fig. 27 A). These axons are involved in the oculomotor injections and contribute to the terminal labeling seen in cases 1-3.

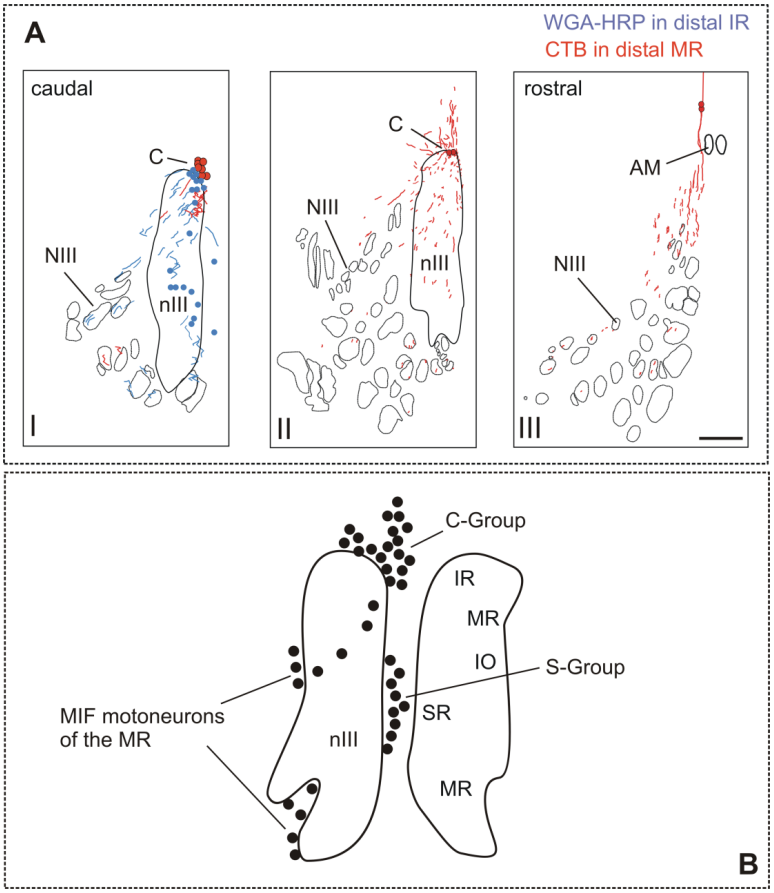

Fig. 27 (A) Reconstruction of caudorostrally arranged transverse brain stem sections demonstrating motor neurons (dots) in the C-group (C) that have been tracer-labeled by injections into the myotendinous junction of the medial rectus (MR; red) and inferior rectus (IR, blue). The respective tracer-labeled axons of both populations travel through the oculomotor nucleus (nIII) and would be labeled by injections into nIII. Scale bar, 500 µm. NIII, oculomotor nerve; AM, anteromedian nucleus. (B) A summary diagram of the total population of neurons around and within the oculomotor nucleus (nIII) after injection into the myotendinous junction of all eye muscles, including the C-group and S-group. Present results suggest that they contain both MIF-motor neurons and palisade ending somata. (Figure from Lienbacher et al. 2011b)

4.3 Differentiation of peripheral oculomotor nuclei cells

4.3.1 Morphologic and morphometric differentiation of peripheral oculomotor nuclei cells

Morphological and morphometric analysis of the nIII peripheral groups revealed a heterogeneous cell population. Belly tracer injections into the MR led to retrogradely labeled twitch motor neurons within nIII and neurons in the periphery of nIII, with the largest accumulation within the C-group adjacent to the dorsomedial aspect of nIII. Very distal tracer injections into the

myotendinous junction mostly sparing muscle fibers resulted in exclusive labeling of the peripheral neurons around nIII, some of them extending dorsally to nIII, encircling the EWpg (Kozicz et al., 2011) and also further rostral to nIII.

After a systematic inspection of the peripheral, either tracer labeled or ChAT labeled neurons, we found numerous multipolar neurons, a morphology typical for motor neurons (Fig. 28 B). We also identified a small but consistent population of neurons that were round or bipolar, a morphology (Fig. 28 A) resembling the sensory ganglion cells of the mesencephalic trigeminal nucleus (*V*Mes; Fig. 28 C). The population lay closer to the EWpg, and distant from nIII; and, importantly, they were only back labeled after very distal tracer injections. These two different morphological cell types were also seen in tracer labeled neurons of the S-group after a tracer injection into the myotendinous junction of the SR (Fig. 28 D).

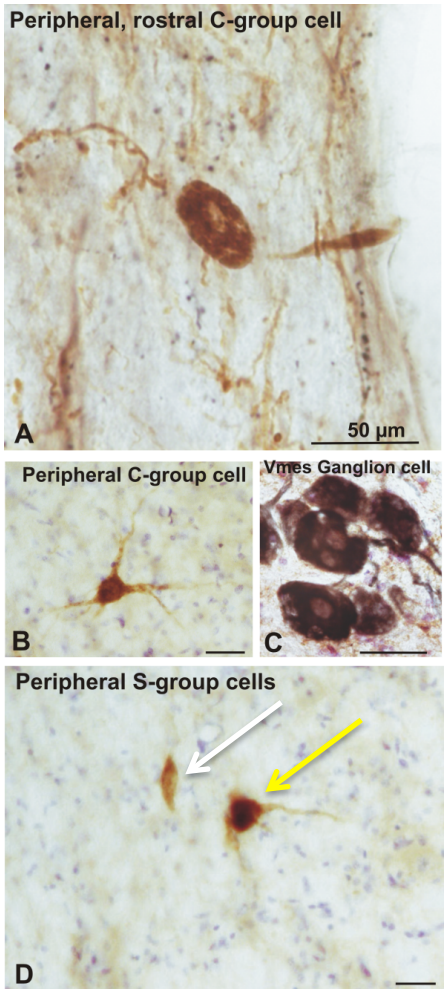

Fig. 28 (A, B) Immunoperoxidase staining for cholinacetyltransferase (ChAT) of peripheral C-group neurons in the monkey. (C) Ganglion cells within the mesencephalic trigeminal nucleus immunostained for non-phosphorylated neurofilament (NP-NF). Note the similarity to the peripheral

C-group neuron in A. (D) Retrograde tracer labeled (WGA-HRP) spindle-shaped (white arrow) and multipolar (yellow arrow) neuron in the S-group after a distal tracer injection into the superior rectus muscle of a monkey. All scale bars: 50 µm. (Figure from Lienbacher et al. 2011a)

4.3.2 Immunohistochemical differentiation of peripheral oculomotor nuclei cells

Based on the finding that, in addition to ChAT, the calcium-binding protein CR is present in PEs and that PEs can anterogradely be labeled after tracer injections into nIII, a systematic study on nIII neurons with emphasis on the peripheral C-group was undertaken for the presence of CR.

As expected all neurons of the C-group dorsomedial to nIII are ChAT positive (Eberhorn et al., 2005a), but a few neurons expressed additional CR-immunoreactivity (Fig. 29). Within nIII all presumed SIF motor neurons express only ChAT but no CR.

The systematic analysis of tracer labeled neurons in the C-group revealed a consistent population (12 % - 15 %) of CR-expressing neurons. This was most obvious in cases with tracer injections into the myotendinous junction, which exclusively label the peripheral neurons around nIII, and also extend further rostral to nIII (Fig. 30).

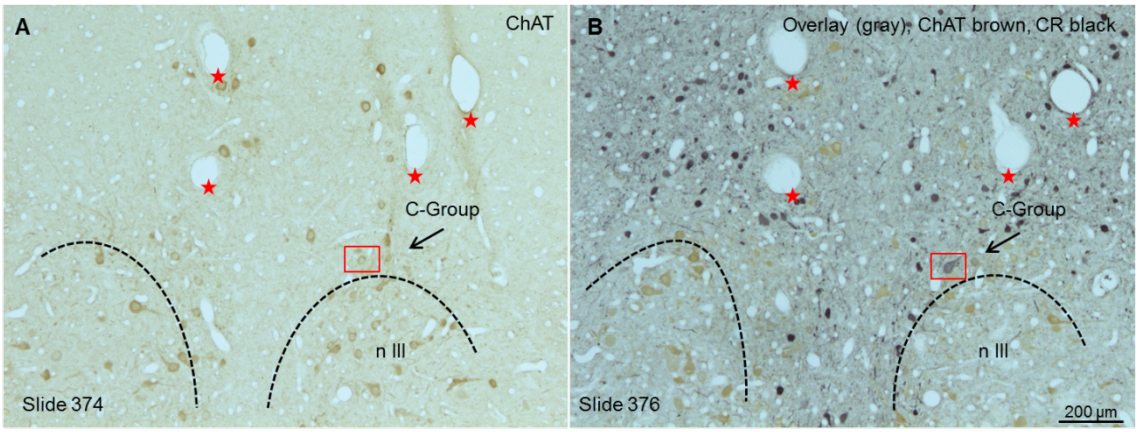

Fig. 29 Neighboring transverse sections through the oculomotor nucleus of monkey showing the C-group immunostained for ChAT (A, brown) and CR (B, black). For clarity the red stars indicate corresponding blood vessels. T(A) Within the ChAT positive population of C-group neurons a small fraction expressed immunoreactivity for calretinin (CR) as well (red rectangle).

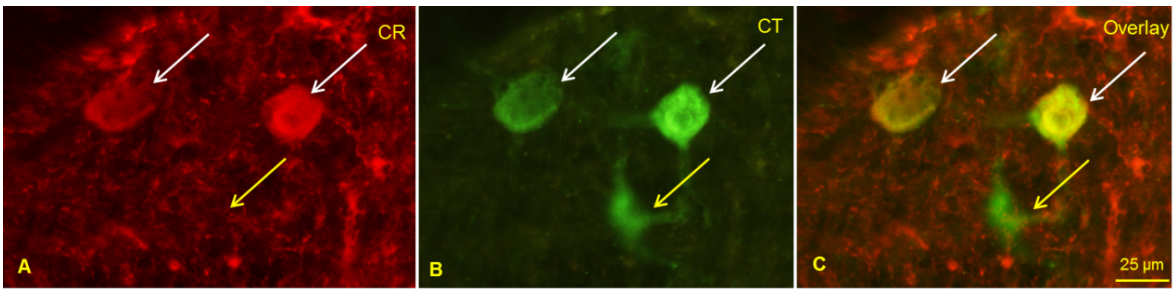

Fig. 30 High-power magnification of tracer labeled neurons in the C-group (B, green) after a CT-injection into the myotendinous junctions, some of which contain calretinin (CR) (A, red, white arrows. In the overlay (C) tracer labeled CR positive neurons appear yellow. These neurons are assumed to present the cell bodies of palisade endings, whereas the CR-negative tracer-labeled neurons (A-C, yellow arrow) may represent motor neurons of non-twitch muscle fibers.

5 Discussion

Since the 19th century there have been debates on whether there is a conscious sense of position of the eye, in correspondence to that of the skeletal system. For example a person cannot detect passive movements of his occluded eye, if the surface of the eye is anaesthetized (Brindley and Merton, 1960). Unlike the skeletal system eyes can use visual information, and two basic theories have evolved for the perception of the eye position in the head (for review: Carpenter, 1988, Donaldson, 2000): 1. In the *outflow theory* the eye position signal is supplied by a feed-forward motor command (Helmholtz, 1866), which was later revived in the concept of efference copy (Holst, 1950). 2. In the *inflow theory* afferent signals are carried from proprioceptors of the eye muscles to the brain, as originally described for the skeletal muscles (Sherrington, 1906). The source of this signal is still unclear for extraocular muscles. One reason for the ongoing debate on the monitoring of eye position is that in contrast to the skeletal muscles, there is no need for regulating posture in the eye muscles. Furthermore, sensory information is projected to the brainstem via the ophthalmic division of the trigeminal nerve, but even if this nerve is cut, there is still a working eye movement control, with an adaptation to novel visual targets (Guthrie et al., 1983, Lewis et al., 2001). This led to the suggestion, that in the absence of the proprioceptive system, the efference copy alone is sufficient for visual localization (Lewis et al., 1998). In addition, most of the tested species show no stretch reflex in their eye muscles, in contrast to skeletal muscles (Keller and Robinson, 1971). On the other hand, proprioceptors in the extraocular muscles do exist and a stretch reflex was indeed demonstrated in the eye muscles of rats and squirrel monkeys, even though they are reputed to lack muscle spindles (Fiorentini and Maffei, 1977, Dancause et al., 2007). In addition, recent studies presented data for proprioceptive representations in the cortex of human and monkey, even in total absence of visual stimulation (Wang et al., 2007, Balslev and Miall, 2008).

In the present study we did not see any muscle spindles in the extraocular muscles in monkey (Macaca mulatta). This is in agreement with other studies on non-human primates (Ruskell, 1999), in which they are not found or described as very few and poorly developed (Greene and Jampel, 1966, Maier

et al., 1974, Ruskell, 1999). A small number may have escaped detection since only a subset of muscle sections, mainly of the global layer, were systematically analyzed, and detection of muscle spindles in the longitudinal plane is more difficult than in transverse sections.

In view of the fact that the EOMs perform highly precise movements mediated by small motor units, which may require a strong sensory control system, it is unsettling to accept that the presence of muscle spindles in eye muscles is highly variable between different species (Harker, 1972, Barker, 1974), or even absent (Cooper and Daniel, 1949, Ruskell, 1999, Donaldson, 2000, Büttner-Ennever et al., 2006). If present in EOMs, they are confined to the orbital layer. In addition they exhibit a simpler morphology with less intrafusal muscle fibers, only of the nuclear chain type (sensitive only to length changes), some of them running through the capsule without innervation and variable in number depending on the species (Donaldson, 2000),(Cooper et al., 1955, Ruskell, 1999, Büttner-Ennever et al., 2006), which raised the question of their functionality by some authors (Ruskell, 1989, Bruenech and Ruskell, 2001). Only the EOMs of ungulates including sheep, pig, and cow possess well developed muscle spindles, very similar to that of skeletal muscles including the presence of nuclear chain and nuclear bag fibers (Blumer et al., 2001a, Blumer et al., 2003). And furthermore, numerous less developed muscle spindles have been found in the orbital layer of human eye muscles (Ruskell, 1989, Blumer et al., 1999, Wicke et al., 2007).

There are only two possible direct routes by which the afferents of EOMs may enter the brainstem: the trigeminal nerve, which is generally seen as projector of sensory information from head structures, or the oculomotor nerves usually considered as efferent nerve. There is still no general agreement as to the one or the other possibility, or maybe both are being used to transmit proprioceptive information, besides the fact that there are enormous differences between species. There are even debates, if extraocular muscles use afferent pathways for proprioception at all, or if other systems are involved (Carpenter, 1977, Guthrie et al., 1983).

In an attempt to obtain more insight into the afferents of the EOMs, with emphasis on proprioception, and to clarify the anatomy of PEs, including the

location of their cell bodies, two cell populations were studied more specifically which are usually retrogradely labeled after tracer injection into the myotendinous junction (Porter et al., 1983, Büttner-Ennever et al., 2001): the brainstem regions around the oculomotor nuclei and the ophthalmic portion of the trigeminal ganglion.

5.1 Trigeminal innervation of extraocular muscles

5.1.1 Trigeminal ganglion

In accordance to earlier studies after tracer injection into the eye muscle the retrogradely labeled ganglion cells were all clustered in the ophthalmic subdivision of the TG (Brodal, 1981, Porter and Donaldson, 1991, Usunoff et al., 1997, Lazarov, 2002). Irrespective of the injected EOM or muscle site (belly or myotendinous junction) no obvious somatotopic arrangement was seen in the macaque monkey as described for the guinea-pig (Aigner et al., 2000). The few neurons found in the maxillary division of the TG, mainly on the border to the ophthalmic subdivision, may still represent neurons whose axons travel within the ophthalmic branch as seen before in human TG (Hüfner et al., 2009). As a general pattern cases with distal myotendinous injections resulted in a more homogeneous population of small to medium-sized tracer labeled TG cells, whereas a muscle belly or conjunctiva injection led to tracer-labeled TG cells with the full range of all sizes small to large sized. Interestingly, in one case (Monkey 4) with a very distal tracer LR injection, labeled cells were found in the TG, despite the absence of tracer labeled neurons in all motor nuclei including the abducens nucleus. Since the same survival time was taken for all experiments, and in light of the fact that CT is transported by a faster axonal mechanism retrogradely than anterogradely (Bobillier et al., 1976), the lack of motor neuronal labeling is not due to insufficient tracer transport. It is more likely, that either the distal myotendinous tracer injection hit only sensory endings in the tendon sparing the motor terminals, or lid or conjunctiva contamination led to the exclusive trigeminal labeling as indicated by a strong labeling of the dorsolateral portion in the ipsilateral facial nucleus containing motor neurons of the orbicularis oculi muscle (Porter et al., 1989).

The finding of anterogradely labeled fine nerve endings in the oral and interpolar part of the spinal trigeminal nucleus as that in the principal sensory nucleus of the trigeminal complex is in accordance with the findings of other authors. In cat transganglionic labeling was mainly found in the oral part of the spinal trigeminal nucleus and few in the principal sensory nucleus (Ogasawara et al., 1987), whereas in monkey similar strong labeling was found in the principal sensory nucleus, the cuneate nucleus and the pars interpolaris of the spinal trigeminal nucleus (Porter, 1986).

The nucleus of the spinal trigeminal tract is composed of three distinct nuclei (Liu, 1998): from rostral to caudal the nucleus pars oralis, nucleus pars interpolaris and nucleus pars caudalis. As a relay station of the protopathic system, it transmits pain and temperature sensation. Therefore the anterogradely labeled terminals in the oral or interpolar part of the spinal trigeminal nucleus may derive from protopathic fibers of the EOMs. The termination areas of the trigeminal afferents in the principal sensory nucleus may transmit tactile or light pressure sensation (Liu, 1998, Miller, 1998).

Theoretically the trigeminal labeling may at least in part be due to unwanted tracer uptake from the cornea and/or the orbicularis oculi muscle after leakage of the tracer between the closed eyelids and the cornea. The cornea has a strong supply from trigeminal afferents, which terminate heavily between the caudal pars interpolaris and rostral pars caudalis and only moderately in the pars oralis (Marfurt and Echtenkamp, 1988).

Stimulation of the supraorbital nerve, a branch of the ophthalmic nerve V1, which supplies the forehead, skin and conjunctiva of the upper eyelid results in the blink reflex by a fast contraction of the orbicularis oculi muscle, which closes the eye (Kugelberg, 1952, Horn and Adamcyzk, 2012). Accordingly tracer injections into the upper eyelid in monkey result in retrogradely labeled neurons within the ophthalmic region of the TG and strong terminal labeling in the ventral part of the spinal trigeminal nucleus pars caudalis and less in the interpolar and oral part as well as in the principal trigeminal nucleus (May and Porter, 1998). Via oligosynaptic pathways the trigeminal nuclei project to the dorsolateral portion of the facial nucleus, which contains the orbicularis oculi muscles in monkey, the efferent limb of the blink reflex (Porter et al., 1989). Since in

several cases some retrogradely labeled motor neurons of the orbicularis oculi subgroup in the ipsilateral facial nucleus were found (Tab 4), trigeminal neurons and afferents from lid and/or cornea may be contained in the investigated populations(Marfurt and Echtenkamp, 1988). However the presence of trigeminal labeling even in the absence of motor neuronal labeling in the facial nucleus in several cases indicates that a trigeminal innervation of the EOM exists.

5.1.2 Mesencephalic trigeminal nucleus

Usually all spinal and cranial sensory ganglia lie in the periphery, but the VMes, located in the rostral rhombencephalon, is a peculiarity (Ramón Y Cajal, 1896, Scharf, 1958, Brodal, 1981, Usunoff et al., 1997, Lazarov, 2000). These ganglion cells derive from mesencephalic neural crest cells, which migrate inward to their final location (Weston, 1970, Narayanan and Narayanan, 1978). The VMes is the only known nucleus containing cell bodies of primary afferent neurons, which is located in the central nervous system (Johnston, 1909, Freeman, 1925).

Confirming the observations by other investigators (Porter and Spencer, 1982, Porter et al., 1983, Daunicht et al., 1985, Bortolami et al., 1987a) no labeling was seen in the sensory VMes after tracer injection into the eye muscles in the present study. The VMes is known as the location for the cell bodies of muscle spindles in the masticatory muscles (Alvarado-Mallart et al., 1975a, Capra et al., 1985). But some authors have found retrogradely labeled neurons in the VMes after eye muscle injections in different species including monkey (Alvarado-Mallart et al., 1975a, Bortolami et al., 1987b, Wang and May, 2008). These retrogradely labeled cells were interpreted as cell bodies from muscle spindles of the EOMs. They explained the very low number of cells by the small number of muscle spindles in the macaque extraocular muscles (Wang and May, 2008). But there is conflicting data about the existence of muscle spindles in the macaque eye muscles: some authors did not find any muscle spindles (Cooper and Daniel, 1949), others report on only a small number of poorly developed spindles in some of the eye muscles but none in others (Greene and Jampel, 1966, Maier et al., 1974). During the thorough analysis of many extraocular

muscles of monkey for nerve endings in the present study no muscle spindles were detected (Büttner-Ennever et al., 2006, Lienbacher et al., 2011b).

In any case, the cells of the VMes can be ruled out as a source for palisade endings on account of their transmitter, which is not acetylcholine (Lazarov, 2002) (Lienbacher, own observations).

5.1.3 Proposed function of trigeminal tracer labeled cells and their afferents in the extraocular muscles

The histochemical properties of tracer labeled TG cells were studied to get more information about the function of the sensory innervation. This was complemented by an investigation of nerve endings immunostained for the respective markers within the extraocular muscles, which involved ChAT, substance P, nitric oxide synthase, calretinin and parvalbumin. The complete innervation of the eye muscles was revealed with combined immunostaining of the synaptosomal associated protein of 25kDA (SNAP-25). It belongs to a family of proteins, which are essential for membrane fusion during exocytosis and is part of the SNARE-complex (soluble N-ethylmaleimide-sensitive-factor attachment receptor) that is associated with the presynaptic plasma membrane. SNAP-25 is located at the plasma membrane in synaptic terminals, but also along the axonal membrane (Hodel, 1998, Rizo and Südhof, 2002). It was established as reliable marker for nerve fibers and all nerve endings, e.g. motor nerves and endplates, sensory fibers and terminals, autonomic innervation of blood vessels, and palisade endings at the myotendinous junction in different species (Eberhorn et al., 2005b). Accordingly all types of nerve fibers and nerve endings were successfully detected by SNAP-25 immunostaining in monkey extraocular muscles (Eberhorn et al., 2005b; Lienbacher et al., 2011).

5.1.4 Substance P

The finding of SP positive neurons in the monkey TG is in accordance with other immunohistochemical studies reporting about small SP sensory neurons in the TG of monkey (Ng et al., 1993), and other mammals (Hokfelt et al., 1976, Tervo et al., 1981, Terenghi et al., 1985, Del Fiacco et al., 1990, Lazarov,

1994), but extended by showing that a considerable population of trigeminal SP positive neurons innervate orbital structures in monkey (Fackelmann et al., 2008). In the present study tracer labeled TG cells expressing SP immunoreactivity, were most frequently found after a conjunctiva injection (42 %), followed by the cases with tracer uptake from the myotendinous junction. The lowest percentage of tracer labeled SP positive TG cells was found in a case where very low tracer uptake was noted from the proximal or distal tendon. These findings suggest a strong SP positive innervation of mainly the myotendinous portions of EOM, whereby a considerable supply may also target the conjunctiva, which has been shown to receive a SP innervation from the TG in rat (Luhtala and Uusitalo, 1991, Elsås et al., 1994). Besides in the conjunctiva (Shimizu et al., 1982, Selbach et al., 2005b) SP has been identified in different parts of the eye: the iris, the ciliary body, the cornea, the episclera, trabecular meshwork (Miller et al., 1981, Gibbins and Morris, 1987). The TG was suggested to be the origin of sensory SP nerve fibers for these targets, because after surgical denervation or chemical treatment with capsaicin, the SP positive structures in the anterior eye segments of different species clearly decreased (Butler et al., 1980, Miller et al., 1981, Keen et al., 1982). So far little information is available from the SP supply in extraocular muscles (Eberhorn et al., 2005c).

The present study revealed that SP containing fibers and terminals as identified by combined immunolabeling for SNAP-25 were primarily found around blood vessels.

Substance P is a part of the neuropeptide family and belongs to the tachykinins (Otsuka and Yoshioka, 1993). It is one of the best investigated and described members of the neuropeptides. Its biological activity is demonstrated in primary sensory neurons and it functions there as neurotransmitters (Hokfelt et al., 1975, Konishi et al., 1985, Lembeck, 1985). SP was discovered in 1931 by Euler and Gaddum (Euler and Gaddum, 1931) and since then localized in different brain areas and ganglions including the TG (Cuello et al., 1978, Paxinos et al., 1980, Terenghi et al., 1985, Kuwayama and Stone, 1986, Murata and Masuko, 2006). One major role of SP is the transfer of nociceptive information in sensory afferents, unmyelinated C-fibers (Hunt and Rossi, 1985, Levine et al., 1993). In addition to its role in sensory transmission, SP takes part in vasodilatation, immune and inflammatory processes (Otsuka and Yoshioka,

1993). SP was already demonstrated in the cerebral blood vessels of the rat (Shimizu et al., 1999), and it often participates in vasodilatation by being mediated by the endothelium derived-relaxing factor (EDRF) for example (Beny and Brunet, 1988), or mediating vasodilatation itself (Lembeck and Holzer, 1979) . SP was also shown to be involved in the blood flow regulation of skeletal muscles (Ohlen et al., 1987). Furthermore, the nerve induced vasodilatation can be reduced by a tachykinin antagonists like a NOS inhibitor (Persson et al., 1991).

SP seems to participate not only in vasodilatation, but takes also part in inflammatory processes. Electrical stimulation, chemical irritations or noxious heat released SP on the skin (White and Helme, 1985, Helme et al., 1986, Yonehara et al., 1987), the dental pulp (Olgart et al., 1977, Brodin et al., 1981), or the eye (Bill et al., 1979, Mandahl et al., 1984). Furthermore, when SP was injected into the skin of humans it causes flare, pain and itch (Hagermark et al., 1978, Foreman et al., 1983, Wallengren and Hakanson, 1987, Pedersen-Bjergaard et al., 1989), and it is also involved in histamine release (Johnson and Erdös, 1973, Fewtrell et al., 1982) which is important for SP-induced flare (Hagermark et al., 1978, Barnes et al., 1986).

Taken together the tracer labeled SP positive neurons in the TG represent nociceptive terminals primarily in the conjunctiva (Selbach et al., 2005a). At least a portion may give rise to vegetative afferents associated with blood vessels participating in vasodilatation of the richly vascularized eye muscles and may also participate in inflammatory processes (Johnson and Erdös, 1973, Lembeck and Holzer, 1979, Foreman et al., 1983, White and Helme, 1985).

5.1.5 Nitric oxide synthase

Similar to SP, tracer labeled NOS positive cell bodies within the TG represent small and medium-sized neurons in the present study. The presence of small to medium-sized NOS positive neurons in the ophthalmic division of the TG has been described for different mammals (Nozaki et al., 1993, Alm et al., 1995, Lohinai et al., 1997, Tajti et al., 1999) (Edvinsson et al., 1998, Lazarov, 1998).

As for SP the most tracer labeled NOS positive neurons in the TG were seen after injections into the myotendinous junction of EOMS (up to 40 %), whereas belly injections back labeled only the half amount of them (Tab. 6). Nitric oxide synthase (NOS) is an enzyme that synthesizes L-Arginin into nitric oxide (NO), which is an important signaling, gaseous molecule broadly found in the nervous system (Garthwaite, 2008, Steinert et al., 2010). The immunohistochemical detection of NOS is regarded as a confirmation of NO as a neuroactive substance (Bredt et al., 1990). There are three isoforms of NOS, found in neurons (nNOS), or endothelial cells (eNOS) and one inducible type, which is expressed in different cell types like macrophages and microglia in the CNS (iNOS). All this isoforms have distinct functional and structural peculiarities (Alderton et al., 2001). In the present study only nNOS was used, which was abbreviated NOS for simplicity. NO, originally identified as endothelium-derived relaxing factor (EDRF), is considered to regulate the relaxation of blood vessels (Furchgott and Zawadzki, 1980, Palmer et al., 1987, Kelm et al., 1988, Whittle et al., 1989, Bredt et al., 1990, Persson et al., 1991). NO regulates cardiovascular tone and blood pressure and acts as a mediator of endogenous endothelium-dependent vasodilators (Whittle et al., 1989). It is controversially discussed if NO is involved in nociception. NO is a second messenger that synthetizes cyclic guanosine monophosphate (cGMP) by activating guanylyl cylase and some data point to the fact that NO and cGMP are involved in the nociceptive system (Meller and Gebhart, 1993, Semos and Headley, 1994, Budzinski et al., 2000, Hoheisel et al., 2005). Latest publications show that neuronal NOS may be involved in the induction of nociception, but not in the maintenance (Isaak and Ellrich, 2011). In addition neuronal NOS participates in learning and memory, synaptic modulation, feeding, sleeping, reproductive behavior as well as neuronal development and neuronal death (Kiss, 2000, Garthwaite, 2008).

The fact that NOS positive fibers were only found around blood vessels of the extraocular muscles is in accordance with the notion that nNOS is involved in blood pressure regulation and vasodilatation. Why the collagen tissue of the sheep muscle spindles of the eye muscles is NOS labeled, is unclear. NOS is indeed sometimes associated with collagen, but always related to damage and repair. NO addition resulted in improved collagen synthesis (Pessanh and

Mandarim-de-Lacerda, 2000, Bokhari and Murrell, 2012). At the moment there is no satisfying answer to the function of NOS positive muscle spindle capsule.

5.1.6 Parvalbumin

The cases investigated for TG cells containing parvalbumin revealed similar small populations of rather large neurons (Fackelmann et al., 2008). It is not clear which terminals in the EOMs are associated with the PV positive neurons in the TG, since only en plaque endings were found to express PV immunoreactivity. The latter is in accordance with the presence of PV in the presumed twitch motor neurons within the eye muscle motor nuclei described in several species (De la Cruz et al., 1998, Eberhorn et al., 2005a, Horn and Adamcyzk, 2012). The specific association of PV with twitch motor neurons is in accordance with the notion that the calcium binding protein PV is primarily found in neurons with high firing rates as shown for many other functional cell groups of the oculomotor system, e.g. saccadic burst neurons (Horn et al., 1995, Horn and Büttner-Ennever, 1998) or omnipause neurons (Horn et al., 1994). The trigeminal PV afferents most likely target the conjunctiva and may transmit pressure or touch information by mechanoreceptors, like shown in orofacial tissue (Ichikawa and Sugimoto, 1997).

5.1.7 Calretinin

The most surprising finding was that the palisade endings (PE) at the myotendinous junction rather selectively expressed the calcium-binding protein calretinin (CR). Since a few en grappe endings were CR positive as well, the important question as to whether PE and the en grappe endings form an anatomical entity, cannot fully be resolved on this basis. However it indicates that the twitch and the non-twitch system is separated by a further histochemical property the en plaque endings associated with PV the PEs, and perhaps few en grappe endings, with CR.

Given the fact that no other CR positive nerve endings were detected in the EOMs and the findings that PEs originate from peripheral cell croups around the eye muscle motor nuclei (Lienbacher et al., 2011; Zimmermann et al., 2011) the

very small population of tracer labeled CR positive neurons in the TG was not surprising. One possible source for these cells is the conjunctiva or the cornea (Felipe et al., 1999).

CR and PV are members of the calcium-binding protein family, which are supposed to be involved in the maintenance of the homeostasis of intracellular calcium ions (Baimbridge et al., 1992). CR probably activates and generates proteins to indirectly influence neurotransmitter release, firing patterns of neurons or development and plasticity (Rogers, 1987, Andressen et al., 1993). It is specifically synthesized in neurons of distinct populations in the CNS (Rogers, 1987, Andressen et al., 1993), but also in peripheral neurons like the dorsal root or trigeminal ganglion cells (Ichikawa et al., 1993a, Ichikawa et al., 1993b, Ichikawa et al., 1994). Furthermore, it is related to proprioceptive terminals in the ciliary muscle (Flügel-Koch et al., 2009) and both are shown to be specific markers for proprioceptive primary neurons in the dorsal root ganglion (Celio, 1990, Ichikawa et al., 1994).

Data from the ciliary muscle showed that CR was the only proprioceptive marker which was present in terminals surrounding the posterior and reticular ciliary muscle tips and their elastic tendons (Flügel-Koch et al., 2009). The receptors at the posterior muscle tips of the ciliary muscle are supposed to measure stretch of the tendon (Flügel-Koch et al., 2009). Because of their location on the myotendinous junction at the distal muscle part, and because of their CR labeling, which is an often described proprioceptive marker, the PEs could have the same function as the CR positive terminals in the ciliary muscle: measuring the stretch of the tendon.

5.1.8 Cholinacetyltransferase

The enzyme cholinacetyltransferase (ChAT), which synthetizes acetylcholine from Acetyl coenzyme A and Choline, is a reliable marker for cholinergic structures. Until now it is still “the most specific indicator for monitoring the functional state of cholinergic neurons in the central and peripheral nervous systems” (Oda, 1999). It is present in nerve terminals of cholinergic neurons in the central and peripheral nervous system (Oda, 1999). Developed in the 1980s, the ChAT antibody enabled more detailed insight into the organization

and distribution of cholinergic neurons in the brain (Mesulam et al., 1989, Mesulam et al., 1992).

In the present study the ChAT antibody labeled the known motor terminals, en plaque and en grappe, but also all PEs, in all eye muscle of monkey, sheep, rat, and prenatal pig EOM tissue confirming other studies (Kupfer, 1960, Sadeh and Stern, 1984, Oda, 1993, Blumer et al., 1998, Blumer et al., 2009). The cholinergic nature of PEs as well as α-bungarotoxin binding to the postsynaptic sites of PEs was taken as an indicator for a motor function of these structures by other groups (Konakci et al., 2005b, Blumer et al., 2009). However the close analysis revealed that only 10 % of the one third of the terminals contacting MIFs, show α-bungarotoxin binding (Konakci et al., 2005a, Konakci et al., 2005b, Blumer et al., 2006, Blumer et al., 2009) indicating postsynaptic acetylcholine receptors (Berg et al., 1972). Even if there are parts of the terminals cholinergic or α-bungarotoxin positive, it has to be checked very keenly if this is enough data to give the PEs a motor function. There is information from different fields, that the presence of cholinergic markers is not necessarily a proof for a motor function (Yasuhara et al., 2004).

This was surprisingly confirmed in our own experiments, where a small but consistent number of ChAT positive neurons in the TG were found, and shown to be most abundant in the ophthalmic division. In few cases, even a projection from ChAT positive neurons from the TG to the eye muscles was demonstrated by few double labeled neurons. The innervation targets and the function of these neurons are unclear, but they are not the source of PEs, since these were found within the brainstem around the motor nuclei (Lienbacher et al., 2011; Zimmermann et al., 2011).

A few years ago a splice variant of ChAT cDNA was cloned, that lacks the exons 6-9 and was named ChAT of the peripheral type (p-ChAT) (Tooyama and Kimura, 2000). P-ChAT was detected in populations of neurons that were until than not known as cholinergic, like trigeminal ganglion cells and the sensory afferents of the ophthalmic nerve (Yasuhara et al., 2004). The ChAT molecule was found in central and peripheral neurons, but not in the TG (Yasuhara et al., 2004). The immunohistochemical characterization of p-ChAT cells in the rat TG with SP or the calbindin (CB) showed different double labeling patterns. There

was almost no overlapping of p-ChAT and CB, a marker for proprioceptive cells, but most of the SP labeled neurons were p-Chat positive. The absence of pChAT labeling in the present monkey study may depend on the lack of this ChAT variant in this species and not on methodic problems, since pChAT positive neurons were identified in rat TG serving as positive control (Yasuhara et al. (Lienbacher et al., 2009).

5.2 Localization of the cell bodies of palisade endings and implications for the function

Central tracer injections into the midbrain targeting nIII, nIV or the oculomotor nerve, led to anterograde labeling of classical motor endings in the eye muscles, “en plaque” and en grappe terminals, as well as palisade endings and nerve terminals within the tendon at the distal myotendinous junction. These results indicated that the cell bodies of PEs are located within the brainstem around the motor nuclei of extraocular muscles.

The only nerve fiber type lacking tracer labeling were tyrosine hydroxylase (TH) positive fibers, which represent postganglionic noradrenergic sympathetic fibers often encircling small blood vessels (Nielsen and Owman, 1967). This finding is in accordance with the fact that TH positive sympathetic nerves do not originate from nIII, but join the extraocular motor nerves only in the orbit on their way from the superior cervical ganglion to supply the arteries to the eye muscles, and controlling the regulation of the blood flow to the muscles (Hayakawa et al., 2000, Thakker et al., 2008).

The extent of the labeling of “en plaque” endings of SIFs in each individual eye muscle depended on the involvement of the motor neuronal subgroups in nIII in the tracer uptake area. Only the relatively small uptake of tracer in the trochlear nucleus of case 1 did not correlate with the high number of labeled terminals in the SO muscles, and the cause remains unclear. A possible explanation could be a polyneuronal innervation of the muscle fibers as described in cat inferior oblique muscle (Dimitrova et al., 2009). Hence the en grappe terminals on the MIFs (which form about 10-20 % of the muscle fibers) were also labeled, even though the motor neurons thought to supply them in the peripheral C- and S-cell

groups were not within the uptake area (see case 1). Injections of retrograde tracers into the myotendinous junctions of MR and IR muscle targeted the en grappe innervated tips of the MIFs (case 4), and retrogradely labeled only the peripheral C-group and their axons, which travelled ventrally through nIII before joining the oculomotor nerve (see case 4, Fig. 27).

A major finding of this study is the anterograde tracer labeling of palisade endings and neurotendinous terminals by nIII injections.

5.2.1 Location of the palisade ending cell bodies in the brainstem

A central brainstem location of the cell bodies of palisade endings was already proposed by Tozer and Sherrington in 1910, who observed degenerated motor endplates and palisade endings after transecting the third, fourth and sixth cranial nerves in monkey (Tozer and Sherrington, 1910). Similar observations were made later by Sas and Scháb (Sas and Schab, 1952) after lesions in the area of the eye muscle motor nuclei in cat. Our data and those of others (Zimmermann et al., 2011) support these previous findings rather than those of more recent studies, which suggest that the palisade ending somata lie in the trigeminal ganglion. In one study, tracer injections into the trigeminal ganglion of cat anterogradely labeled palisade endings, but not after tracer injections into the extraocular motor nuclei (Billig et al., 1997). The reason for the discrepancy between this study and our results remains unclear. It is well established by previous studies that tracer injections into the myotendinous junction of extraocular muscle – the location of palisade endings and tips of MIFs – led to retrogradely labeled neurons around the periphery of the classical motor nuclei within the brainstem, in the C-and S-groups, the dorsal cap of nIV and at the periphery of nVI, and not within the classical motor neurons (Büttner-Ennever et al., 2001).

5.2.2 Morphology of palisade endings

Ever since the description of palisade endings by Dogiel (Dogiel, 1906) there has been evidence that the axons giving rise to palisade endings form additional multiple contacts along the muscle fiber. Richmond et al. (Richmond

et al., 1984) confirmed the finding in humans; and recently in monkey it was shown that only about one third of palisade endings have any contact at all to MIFs, and in this one third only 10 % of the palisade ending terminals had motor properties: more specifically they bound α-bungarotoxin (Blumer et al., 2009), which is also confirmed by our tests. The ultrastructural profile showed that these synapses have no basal lamina in the synaptic junction, and in so far resemble the motor terminals on intrafusal fibers, not those of “en plaque” terminals on SIFs (Kubota, 1988, Ruskell, 1989, Blumer et al., 2003, Konakci et al., 2005a). The vast majority of palisade terminals does not contact muscle fibers, but terminate amongst the collagen bundles of the tendon.

Furthermore Dogiel (Dogiel, 1906) showed that branches from the PE axons forming elaborate terminals in the tendon, often extending deep into the tendon and far from the palisade terminal itself. We have found that these are particularly well developed in the superior oblique muscle, not studied by Dogiel or Ruskell (Dogiel, 1906, Ruskell, 1979) (Fig. 26 A, C). Unlike classical Golgi tendon organs, described by Ruskell (Ruskell, 1979), the labeled neurotendinous ending complexes were not encapsulated and did not form neuromuscular junctions. Our results indicate a unity of tendinous terminals with palisade endings, both deriving from cell bodies around the periphery of the nIII.

5.3 The peripheral groups of the oculomotor nuclei – location of proprioceptive neurons?

Tracer injections into different parts of primate eye muscles have shown the motor neurons giving rise to en plaque endings innervating the twitch SIFs lay within the motor nuclei, whereas the cell bodies of the en grappe endings associated with MIFs are located in the periphery of the motor nuclei (Büttner-Ennever et al., 2001). Accordingly up to now the peripheral cell groups around the motor nuclei have been considered as non-twitch motor neurons and as the source of multiple nerve endings that are distributed along the whole length of muscle fibers characterized by the expression of specific myosin isoforms (Kjellgren et al., 2003). However, these distal tracer injections also involve the PEs and tendon organs at the myotendinous junctions and in the tendon. In light of our recent findings that the cell bodies of PEs are located in the

periphery of the motor nuclei, the peripheral cell groups, back-labeled after tracer injections into the myotendinous junction, it must be considered that some are back-labeled also from PEs. Theoretically, there are two possibilities of organization as already suggested previously (Büttner-Ennever et al., 2001): either the peripheral neurons around the motor nuclei form one homogenous population of neurons that give rise to multiple en grappe endings *and* PEs plus tendon organs, or there are at least two different neuron populations, for example, cell bodies of the motor en grappe endings and cell bodies of possible sensory PEs. Currently the latter hypothesis is supported by the findings of this study showing that the peripheral groups around the motor nuclei contain at least two populations that differ in their histochemical features and morphology. Within the well-established C-group dorsomedial to the oculomotor nucleus, which contains separate populations of neurons innervating IR and MR (Tang, 2007), a consistent population of CR-expressing neurons was identified. This unexpected finding was only confirmed by a systematic detailed analysis of peripheral cell groups based on the fact that PEs express CR-immunoreactivity. Thereby the expression of ChAT and CR of these peripheral neurons is in accordance with the expression of both markers in PEs, supporting the CR positive neurons as source for PEs.

For the MR the CR positive neurons are primarily located rostral to the nIII in proximity to the EWpg nucleus. The findings of this reanalysis are in good accordance with previous data on the cell sizes comparing tracer labeled peripheral and tracer labeled presumed twitch neurons within nIII after a large tracer injection into the muscle belly, where a bimodal distribution of cell sizes was noted, as well (Büttner-Ennever et al., 2001, see Fig. 13A20). As in the previous paper, no difference in cell sizes of retrogradely labeled neurons in the peripheral and central, presumed twitch motor neurons, were noted after a tracer injection in the belly (Büttner-Ennever et al., 2001, see Fig. 2). We extended this morphometric analysis by comparing the cell sizes of peripheral cell groups around the EWpg and those of peripheral cells adjacent to nIII only back-labeled after distal tracer injections and found a bimodal distribution, with the latter group being the larger cells. Only tracer injections into the myotendinous junction—the location of PEs—revealed, in addition, a group of round or spindle-shaped neurons with a morphology resembling that of sensory

ganglion cells (Johnston, 1909) (Fig. 28, A, C). A similar attempt of classification based on morphological cell features of tracer labeled neurons after EOM injections was undertaken in the pig (Kubota et al., 1988). Multipolar large cells were considered as alpha motor neurons corresponding to twitch motor neurons and small cells as γ-motor neurons, which may represent the non-twitch motor neurons providing multiple innervation and their spindle-shaped and round neurons—suggested as sensory cell bodies of spindle afferents—may represent the cell bodies of PEs. Although a possible location of sensory neuronal cell bodies within the brainstem and not in a separate ganglion, is very unusual, there is at least one example of well-known sensory neurons in the brainstem. The large round ganglion-like neurons of the mesencephalic trigeminal nucleus (Vmes) located close to the fourth ventricle at pontine levels and as single neurons at the border of the periaqueductal gray at mesencephalic levels represent well-described sensory neurons innervating the muscle spindles of the jaw muscles (Johnston, 1909, Alvarado-Mallart et al., 1975a). Furthermore, several studies indicate that at least part of them may innervate muscle spindle afferents of the eye muscles (Bortolami et al., 1987b, Wang and May, 2008). Because we never saw tracer labeled Vmes cells after distal injections of the EOM (Lienbacher et al., 2011b), this nucleus was ruled out as a source of PEs. In addition, tracer injections into the medullary part of Vmes did not result in anterogradely labeled PEs (Zimmermann et al., 2011).

Based on the findings that only tracer injections into the myotendinous junction of MR revealed two populations of peripheral neurons—differing in their cell sizes and/or morphology—we propose that the round neurons within the peripheral cell groups represent sensory cell bodies of the PEs. These sensory cells in the periphery of nIII are assumed to give rise to sensory terminals of PEs, and the multipolar neurons in the periphery of nIII to represent MIF motor neurons, innervating the non-twitch muscle fibers via multiple en grappe endings.

Only active MIFs would provide a tension on the tendon thereby transferring the information to sensory palisade ending terminals. A central circuit between sensory and motor pathways would stimulate the MIF motor neurons thereby modulating the MIF activity (Fig. 31).

5.4 Do palisade endings and non-twitch muscle fibers form a specialized proprioceptive apparatus?

Present findings suggest that palisade endings and en grappe endings innervate non-twitch MIFs. Both may derive from neurons in the periphery of the motor nuclei (Lienbacher et al., 2011; Zimmermann et al., 2011), whereas the motor neurons within the motor nuclei, innervating twitch muscle fibers, would generate the eye movement. To date, the peripheral neurons have been considered as a homogenous population of neurons for the MIFs (Büttner-Ennever, 2001). Accordingly, in a first hypothesis one homogenous neuron population gives rise to the multiple nerve endings supplying non-twitch MIFs that become palisade endings at their terminus (Fig. 31B) (Lienbacher et al., 2011a). In such an arrangement, the palisade ending terminals including the tendon organ-like outgrowth lying in the tendon may be activated upon stretching the tendon during an eye movement. The resulting activity would reinforce the action of the motor nerve branch and its associated MIF terminals on non-twitch muscle fibers locally. Such an *intrinsic* stretch reflex similar to that known for classical smooth muscle fibers was suggested for EOMs (Carpenter, 1988). However if assumed that such an arrangement serves for maintaining the fine alignment of the EOM during eye movements, the activation of the PEs by a stretch during an eye movement in the opposite direction should induce a deactivation of the MIFs and vice versa: the deactivation of the PEs during an eye movement in the ipsilateral direction should activate the MIFs. In this case motor and sensory signals would not only coexist in the MIF axon branches and support a motor-sensory function, as suggested previously (Lukas et al., 2000; Tozer and Sherrington, 1910), but would work against each other. Although a co-innervation of sensory and motor endings on MIFs by a single axon is highly unusual, there are some examples of similar unusual innervation patterns, e.g. the intraganglionic laminar endings on striated oesophageal muscle (Wörl and Neuhuber, 2005), where a partial co-localization of cholinergic markers (motor) and vesicular glutamate transporter (sensory) was found within single endings.

A second hypothesis is favored by the present work, and that is that the peripheral motor neurons around the classical oculomotor nuclei may represent two sets of neurons (Lienbacher et al., 2011a): sensory CR positive neurons in

the peripheral groups around the motor nuclei giving rise to sensory PEs - and CR negative neurons represent the MIF motor neurons providing the multiple innervation of non-twitch muscle fibers (Fig. 31 A). A previous hypothesis was based on the assumption that PEs originate from the trigeminal ganglion (Büttner-Ennever et al., 2002). The proposed arrangement would reflect a circuit similar to the muscle spindle. MIF activity would adjust the tension on the tendon, and in response to tension the palisade endings would determine the contraction of non-twitch fibers thereby maintaining a sensitive system. A local circuit in the oculomotor complex between sensory and motor pathways for example by direct collaterals from PE cell bodies would modulate the MIF activity (Fig. 31 A) (Lienbacher et al., 2011a). This would support the idea of a stretch reflex involving only the non-twitch fibers, as suggested by Keller (Keller and Robinson, 1971). Due to their exclusive association with multiple-innervated non-twitch fibers and their location at the myotendinous junction, the palisade endings are well suited to determine the muscle tension. It was suggested by David A. Robinson that the palisade endings, together with the non-twitch fibers, may function as large „inverted muscle spindles“ that provide a sensory input signal to the brainstem. In fact there are some similarities between EOM non-twitch fibers and intrafusal fibers: both are multiply innervated and they show similar myosin expression (Rossi et al., 2010). Assuming the palisade endings are sensory, the non-twitch fibers receive a motor and sensory innervation as do intrafusal fibers (Fig. 32) (Lienbacher and Horn, submitted). With this model the motor neurons of non-twitch fibers would act as gamma motor neurons and through proprioceptive pathways via palisade endings determine the muscle tension, rather than contributing to the eye movement itself.

This concept fits well with the fact that the peripheral neurons around the motor nuclei receive only input from premotor neurons involved in smooth pursuit, vergence and gaze holding, whereas the motor neurons within the motor nuclei – corresponding to alpha-motor neurons – receive input from premotor neurons involved in saccade and VOR generation (Ugolini et al., 2006; Lienbacher and Horn, 2012).

It will have to be clarified by which routes the signals are transferred to the somatosensory cortex. Proprioceptive signals have been found in the superior

colliculus, vestibular nuclei, nucleus prepositus hypoglossi and cerebellum, but the exact connectivity and routes taken in monkey must be further explored (Ruskell, 1999).

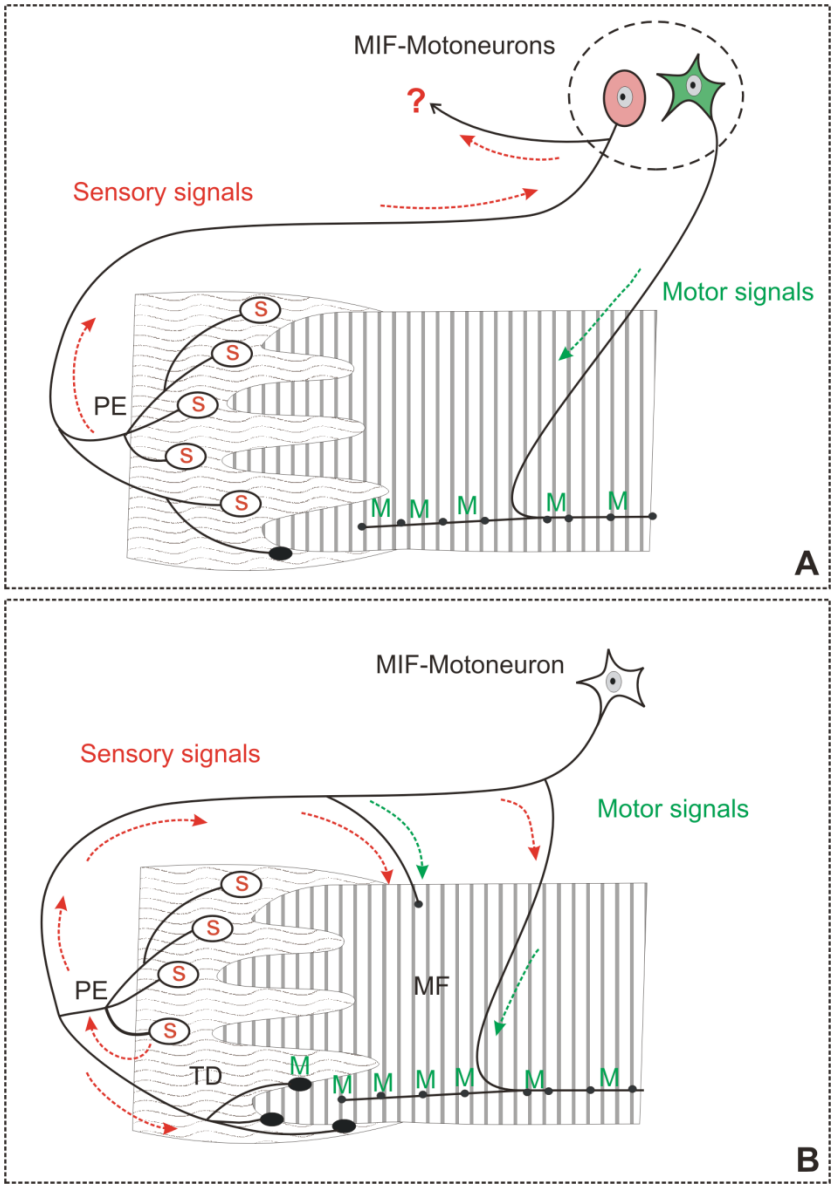

Fig. 31 Schematic drawing of the myotendinous junction and its innervation illustrating two hypotheses for palisade ending (PE) function. (A) Two functionally different sets of neurons within the peripheral neuronal subgroups of nIII provide innervation of the myotendinous junction. Sensory neurons give rise to PEs and motor neurons provide multiple innervation by en grappe endings. A stretch of the myotendinous junction would transmit a sensory signal via the PEs centrally to activate MIF-motor neurons. (B) The cell bodies of peripheral groups around the motor nuclei give rise to palisade endings *and* the multiple en-grappe endings of non-twitch muscle fibers thereby mediating an *intrinsic* stretch reflex in response to tension on the tendon. (Part of this figure from Lienbacher et al. 2011a)

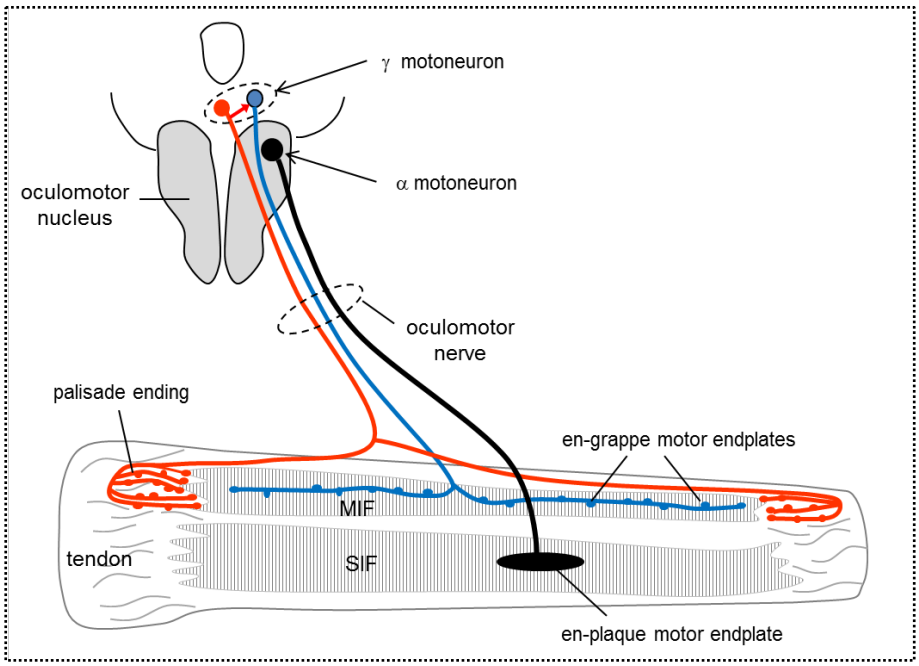

Fig. 32 Scheme of the proposed hypothesis: palisade endings and multiple-innervated non-twitch muscle fibers may act as a large "inverted" muscle spindle, the sensory palisade innervation at the distal parts, the motor innervation centrally along the non-twitch muscle fibers. They are arranged in parallel to the singly-innervated twitch muscle fibers, innervated by "alpha" motor neurons within the motor nuclei providing the contraction of the muscle. Unlike for skeletal muscles the cell bodies of sensory (red) and efferent "gamma" motor neurons (blue) are located together in the periphery of the motor nuclei. (Figure from Lienbacher, K. and Horn, Anja K. E., Biol Cybern, Palisade endings and proprioception in extraocular muscles: a comparison with skeletal muscles (2012) (in press), DOI: 10.1007/s00422-012-0519-1).

5.5 Conclusion

Palisade endings are unique to eye muscles. Their location, histochemistry and fine structure have been well studied, but their exact function is unknown (Ruskell, 1999, Donaldson, 2000).

The debate on the palisade ending function was compounded by the lack of knowledge about the location of their cell bodies. For sensory function they would be expected to lie in the trigeminal ganglion or mesencephalic trigeminal nucleus, for motor function in the motor nuclei. Recent studies of Wang et al. (Wang and May, 2008) have rekindled interest in the presence of proprioception in eye muscles; and as a result palisade endings, which are a constant feature of eye muscles, have been often suggested as possible sensory receptors (Ruskell, 1999, Donaldson, 2000). Some of their properties are typical of sensory endings, such as the numerous terminations in the collagenous tendon,

and the lack of a basal lamina at neuromuscular junctions (Lienbacher, 2011b, Büttner-Ennever et al., 2006). Further properties of palisade endings favor a motor function, such as their transmitter acetylcholine, the location of their cell bodies in the periphery of motor nuclei of extraocular muscles and the α-bungarotoxin binding of 10% of terminals in the myotendinous junction. (Ruskell, 1989, Blumer et al., 1999, Demer et al., 2000, Konakci et al., 2005a).

The TG, location of almost all sensory cell bodies innervating the face together with the eye, seems not to contain the cell bodies of PE afferents. The retrogradely labeled TG cells from tracer injections into the myotendinous junction, which includes the PEs, show many double labeled cells containing SP and NOS. Both, SP and NOS seem to be involved in nociception and vasodilatation rather than in a proprioceptive function. Only very few CR positive and tracer labeled TG cells were found, although all PEs were CR immunoreactive. If there are cell bodies, which are possible proprioceptive afferents of the PEs, it seems more likely that they are situated in the brainstem and not in the TG.

Two recent studies have independently shown in monkey that palisade ending are anterogradely labeled from central tracer injections into the motor nuclei of EOMs (Lienbacher, 2011b; Zimmermann, 2011). Based on the tracing results applying tracer injections into the myotendinous junction, where palisade endings are located, their cell bodies lie in the periphery of the motor nuclei. Furthermore the present study supports a hypothesis that the peripheral cell groups around the motor nuclei contain two populations: motor neurons that provide the multiple innervation of non-twitch muscle fibers and calretinin positive neurons that give rise to palisade endings.

Future experiments are necessary to explore the exact role of PEs, such as recording studies in awake monkeys. Another approach may include a resection of the myotendinous junction, as done in strabismus surgery and analysis of the eye stabilization properties. As an anatomical control the extraocular muscles and motor nuclei must be investigated for the absence of calretinin positive palisade endings and neurons, respectively.

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Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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