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INTERNAL ORGANIZATION OF MEDIAL RECTUS AND INFERIOR RECTUS MUSCLE NEURONS IN THE C-GROUP OF THE OCULOMOTOR NUCLEUS IN MONKEY

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Summary

Mammalian extraocular muscles contain singly-innervated twitch muscle fibers (SIF) and multiply-innervated non-twitch muscle fibers (MIF). In monkey, MIF motoneurons lie around the periphery of oculomotor nuclei and have different premotor inputs from the motoneurons inside the nuclei. The most prominent MIF motoneuron group is the C-group, which innervates the medial rectus (MR) and inferior rectus (IR) muscle. To explore the organization of both cell groups within the C-group we performed small injections of cholera toxin subunit B into the myotendinous junction of MR or IR in monkeys. In three animals the IR and MR myotendinous junction of one eye was injected simultaneously with different tracers (cholera toxin subunit B and wheatgerm agglutinin). It revealed that both muscles were supplied by two different non-overlapping populations in the C-group. The IR neurons lie adjacent to the dorsomedial border of the oculomotor nucleus, whereas MR neurons are located further medially. A striking feature was the differing pattern of dendrite distribution of both cell groups. Whereas the dendrites of IR neurons spread into the supraoculomotor area bilaterally, those of the MR neurons were restricted to the ipsilateral side and sent a focussed bundle dorsally to the preganglionic neurons of the Edinger-Westphal nucleus, which are involved in the 'near response'. In conclusion, MR and IR are innervated by independent neuron populations from the C-group. Their dendritic branching pattern within the supraoculomotor area indicates a participation in the near response providing vergence, but also reflects their differing functional roles.

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Conflict of interest statement.

The authors have no real or potential conflicts of interest that could influence or be perceived to influence the work.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AKH, JAB, Acquisition of data: XT, MM. Analysis and interpretation of data: XT, AKH, JAB. Drafting of the article: XT, AKH. Critical revision of the article for important intellectual content: AKH, JAB, MM. Obtained funding: AKH, MM. Study supervision: JAB, AKH.

Keywords

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INTRODUCTION

The extraocular muscles are the effector organs for voluntary and reflexive movement of the eyes. The presence of six extraocular muscles, four recti (superior, inferior, medial and lateral recti muscles) and two oblique (superior and inferior oblique muscles) is rather constant among gnathostome vertebrate classes, but show variations in arrangement and innervation (Isomura, 1981, Spencer and Porter, 2006; Young, 2008). The eye muscles consist of an outer orbital layer adjacent to the orbital bone, and an inner global layer adjacent to the eye globe. In the recti muscles, the orbital layer contains thinner muscle fibers and is typically C-shaped, encompassing the global layer (Oh et al., 2001). In the oblique muscles, the orbital layer often completely encircles the global layer. The global layer extends over the full muscle length from its origin at the annulus of Zinn to a well-defined tendon at the limbus of the globe (for review see: Spencer and Porter, 2006). In contrast, the orbital layer ends before the muscle becomes tendinous (Oh et al., 2001), and inserts on collagenous pulleys, around the equator of the eyeball, anatomically referred to as the Tenon's capsule (Demer, 2002; Demer et al., 2000). The myofiber composition in mammalian extraocular muscles is highly complex compared to the limb muscles. At least six fiber types can be distinguished in extraocular muscles, and these types can be divided into two categories based on their innervation: the singly-innervated muscle fibers (SIF) and multiply-innervated muscle fiber (MIF) (review: Morgan and Proske, 1984; Spencer and Porter, 2006).

The SIFs are innervated by relatively large nerves (7–11 μm), which terminate as “en plaque” endings in an endplate zone occupying the central third of the muscle. They correspond to the type IIA fibres of the limb muscles (Schiaffino and Reggiani, 2011). To electrical stimulation they respond with an “all-or-nothing” action potential that propagates along the whole fiber length resulting in a twitch (Spencer and Porter, 2006). The multiply-innervated non-twitch muscle fibers (MIFs) are more common in reptilian and avian skeletal muscles, but in mammals they are only found in few small muscles, e.g. the extraocular muscles, vocalis, stapedius and tensor tympani muscles (Schiaffino and Reggiani, 2011). These fibers are fatigue resistant and respond to electrical stimulation with a slow tonic contraction, which is not propagated along the muscle fiber (Bondi and Chiarandini, 1983). They are innervated by a thin (3–5 μm) myelinated nerve fiber, via small motor endplates that are distributed along the whole length of the fiber, with a higher density in the distal half of the muscle. At the myotendinous junction the MIFs of the global layer are capped by a cuff of nerve terminals called palisade endings, or myotendinous cylinders. Palisade endings are unique to extraocular muscles and have been described in several species (for review: Büttner-Ennever et al., 2006; Rungaldier et al., 2009), but their function is still under debate (Lienbacher and Horn, 2012; Lienbacher et al., 2011a; Zimmermann et al., 2013).

Taking advantage of the spatial arrangement of the nerve terminals on SIFs and MIFs the respective populations of motoneurons were identified in monkey by retrograde tract tracing (Büttner-Ennever et al., 2001): A tract-tracer injection into the myotendinous junction affecting only the “en-grappe” endings resulted in the selective back-labelling of neurons in the periphery of the extraocular motonuclei, which were considered to be the MIF-motoneurons. Accordingly, a muscle belly injection involving all nerve endings resulted in the back-labelling of the complete motoneuron population of the eye muscle within the respective motonuclei. Thereby it was found that the SIF motoneurons are located within the boundaries of the motonuclei, whereas the MIF motoneurons are found in the periphery of the motonuclei (Büttner-Ennever, 2006; Büttner-Ennever et al., 2001). The MIF motoneurons of the lateral rectus muscle (LR) are scattered around the medial aspect of the abducens nucleus, those of the superior oblique muscle (SO) form a dorsal cap over the trochlear nucleus. Interestingly, the MIF motoneurons of superior rectus (SR) and inferior oblique (IO) are grouped together, as are those of medial rectus (MR) and inferior rectus muscle (IR). Those of the SR and IO are located bilaterally around the midline between both oculomotor nuclei (nIII) and called the ‘S-group’ (Büttner-Ennever et al., 2001; Wasicky et al., 2004). The most prominent group of MIF motoneurons is formed by those of the IR and MR, termed the ‘C-group’. It lies at the dorsomedial border of nIII and can be identified in several species, such as cat, lesser Galago, marmoset and macaque monkeys (Büttner-Ennever and Akert, 1981; Büttner-Ennever et al., 2001; Clarke et al., 1987; McClung et al., 2001; Spencer and Porter, 1981; Sun and May, 1993). Recent tract-tracing studies in monkey indicated that the peripheral MIF motoneuron groups including the C-group may also contain the cell bodies of palisade endings (Lienbacher et al., 2011b; Zimmermann et al., 2011). The C-group lies adjacent to the preganglionic neurons of the Edinger-Westphal nucleus (EWpg) and receives common inputs for the “near response” and is thought to contribute to the vergence required for eye alignment when fixating an object (Büttner-Ennever et al., 1996; Erichsen et al., 2013). Up to date no recording studies from C-group neurons in behaving primates are available that would elucidate their function. Furthermore, it is not clear, whether neurons in the C-group form a homogeneous group of neurons innervating both, IR and MR. To further study the internal organization of the C-group we performed tracing experiments in the monkey with simultaneous small injections of different tracers into the myotendinous junction of IR and MR. We were able to demonstrate that the IR and MR populations remain strictly separated within the C-group. A brief report in abstract form has been published previously (Büttner-Ennever and Horn, 2001).

MATERIALS AND METHODS

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). They were approved by animal care officers and the Institutional Animal Care and Use Committees at Emory University and University of Washington, where all surgical interventions and perfusion were made. For surgery under aseptic conditions, the animals were anesthetized with isoflurane (1.25% – 2.5%). Blood pressure, heart rate, blood oxygenation, body temperature and CO₂ in expired air were monitored (SurgiVet monitor; Smiths Medical, Dublin OH) and maintained within physiological limits. Under sterile

conditions the extraocular muscles were exposed after retraction of the eyelids and a small conjunctival incision. Small tracer volumes (5–10µl) were injected with a thin cannula attached to a Hamilton syringe positioned in parallel to the myotendinous junction. Three macaque monkeys (*Macaca mulatta*) were injected in the distal part close to the myotendinous junction of the medial rectus (MR) or inferior rectus (IR) muscle of one eye with cholera toxin subunit B (CTB; 1% in distilled water) or wheat germ-agglutinin horseradish peroxidase (WGA-HRP; 2,5% in saline). The sections of one additional animal (B55) from a previous study were reanalyzed (Büttner-Ennever et al., 2001). Three additional macaque monkeys received a simultaneous injection of CTB in the distal MR and WGA-HRP in the distal IR, respectively (see table 1). Postsurgical analgesia (buprenorphine, 0,01 mg/kg, every 6 hours) were administered for several days. After a survival time of 2–4 days, the animals were euthanized with an overdose of sodium-pentobarbital (80mg/kg body weight) and transcardially perfused with 0,9% saline followed by 4% paraformaldehyde in 0,1M phosphate buffer (pH 7,4). The brainstems and the orbits were removed and equilibrated in increasing concentrations of sucrose (10–30% in phosphate buffer, pH 7,4). Flat sections of the eye muscles were cut at 20µm, and transverse sections of the brainstem were cut at 40µm using a cryostat (HM; Thermo Scientific-Microm, Walldorf, Germany).

Tracer detection

Cholera toxin subunit B (CTB)—For the immunocytochemical detection of CTB, on slide mounted eye muscle sections and free floating brainstem sections were treated with a goat polyclonal antibody against cholera toxin subunit B (List Biological Laboratories, Cat# 703, RRID:AB_10013220; 1:40,000) for 2 days at 4°C, after blocking endogenous peroxidase with 1% H₂O₂, and a one hour incubation in 2% normal rabbit serum (NRS) in 0,3% Triton-X-100 in 0,1M Tris buffer, (pH 7,4). Then, sections were washed in Tris buffer and incubated in biotinylated rabbit anti-goat (Vector laboratories, BA 5000, RRID:AB_2336126; 1:200) with 2% bovine serum albumin (BSA) for one hour at room temperature. After rinsing in Tris buffer the sections were treated with Extravidin peroxidase (EAP) (1:1000; Sigma E2886) in 2% BSA for one hour at room temperature. After two rinses in Tris buffer (pH 7,4) and one rinse in 0,05M Tris buffer (pH 8) the antigenic sites were visualized with 0,05% diaminobenzidine (DAB) and 0,01% H₂O₂ in 0,05M Tris buffer for 10–15 minutes, which yields a brown reaction product. Some sections were counterstained with 0,5% cresyl violet to illustrate the cytoarchitecture simultaneously.

Simultaneous detection of WGA-HRP and CTB—To find out whether double-labelled neurons were present in the C-group that would indicate neurons projecting to both, IR and MR, two different approaches were used to detect CTB and WGA-HRP in one section. One method involved the detection of WGA-HRP with a reaction in 0,05% DAB and 0,01% H₂O₂ in phosphate buffer in the presence of 1% cobalt chloride for 10–15 minutes resulting in a black granular staining, followed by the immunoperoxidase detection of CTB with DAB and H₂O₂ yielding a homogeneous brown staining as described above. Alternatively, in two cases (P1, P2), WGA-HRP was first detected either with a reaction in 0,05% DAB in 0,1M phosphate buffer and 0,01% H₂O₂ resulting in a brown granular deposit or with a modified tetramethylbenzidine method and stabilized with DAB and

cobalt-chloride, which yield a black reaction product in retrogradely labelled motoneurons (Horn and Hoffmann, 1987). After thorough washing the sections were preincubated in 5% rabbit normal serum containing 0,3% triton X-100 for 1h at room temperature before treated with goat anti-choleraegenoid (List Biological Laboratories, Cat# 703, RRID:AB_10013220; 1:20.000) for 48 hours. After three rinses with Tris buffered saline the sections were incubated in rabbit anti-goat tagged with Cy3 (Jackson ImmunoResearch Cat# 305-001-003, RRID:AB_2339374) for one hour at room temperature. This resulted in a red fluorescence of the CTB-filled neurons.

Controls

The polyclonal goat antibody against CTB was raised against the purified toxin from *Vibrio cholerae* (manufacturer's datasheet), which was injected only into the medial rectus eye muscle. Since CTB is not physiologically present in the animal organism, the antibodies to CTB did not reveal any immunostaining in the absence of injected CTB as observed for all eye muscles except the medial rectus muscle and their motoneurons.

Analysis of stained sections

The slides were examined with one of two microscopes (DMRB; Leica, Bensheim, Germany, or Axioplan; Carl Zeiss MicroImaging, Oberkochen, Germany), equipped with appropriate filters for red fluorescent Cy3 (DMRB: N2.1; excitation filter, BP 515–560 nm; dichromatic mirror, 580 nm; suppression filter, LP 590 nm; Axioplan: excitation filter, BP 546 nm; dichromatic beam splitter, FT 580 nm; barrier filter, LP 590 nm) and green fluorescent Cy2 or Alexa 488 (DMRB: I3; excitation filter, BP 450–490 nm, dichromatic mirror: 510 nm, suppression filter LP 515 nm; Axioplan: excitation filter, BP 475 nm; dichromatic beam splitter, FT 500 nm; barrier filter, LP 530 nm). Micrographs were taken with a digital camera (Pixera Pro 600 ES; Klughammer, Markt Indersdorf, Germany), captured on a computer (Pixera Viewfinder software; Klughammer), and processed in image analysis software (Photoshop 7.0; Adobe Systems, Mountain View, CA). The sharpness, contrast and brightness were adjusted to reflect the appearance of the labeling seen through the microscope. The dendritic branches of CTB-labeled neurons in the C-group following selective injections into IR or MR were reconstructed using Neurolucida software, V 6 (MicroBrightField, Inc., Williston, VT; RRID: nif-0000-10294). The pictures and graphs were arranged and labelled with drawing software (Coreldraw 11.0; Microsoft).

RESULTS

Location of medial and inferior rectus motoneurons

In two cases (B55, A09057) the CTB-tracer injections into the myotendinous junction of IR led to retrogradely labelled neurons in the C-group adjacent to the dorsomedial border of nIII (Fig. 1 A, B). No IR motoneurons within the dorsal part of the rostral nIII were labelled indicating a selective tracer-uptake from nerve endings in the distal muscle part, but not from “en-plaque” endings from the central muscle part (Fig. 1 A, B). This observation was confirmed by visualizing the tracer uptake site in flat sections of the injected IR, which was confined to the area around the myotendinous junction and adjacent muscle fibers (not shown). In two cases (RDJ7, X183) the tracer injection into the distal part of MR led to

retrogradely labelled neurons only in the C-group (Fig. 1 C, D). This was in accordance with the small localized tracer uptake area in the injected MR (not shown). Whereas at caudal levels retrogradely labelled IR and MR neurons showed a similar location in the C-group, further rostrally the MR neurons of the C-group tend to lie more separated from the dorsomedial border of nIII, most apparent at section planes through the rostral third of nIII (Fig. 1; Fig. 2; Fig. 3).

To find out, whether neurons within the C-group project to both, MR and IR, three cases were analysed, where different tracers had been injected simultaneously into the distal parts of MR and IR, respectively. In sections of these cases, reacted for the combined peroxidase detection of both tracers, the black granular labelling of WGA-HRP could be easily distinguished from the more homogeneously brown labelled neurons back-filled with CTB (Fig. 2 C, D, E, F). Only at sections through the caudal half of nIII both populations appeared overlapping, but even here, they were clearly separated into a lateral IR and medial MR population of the C-group (Fig. 3). The close systematic inspection at high magnification did not reveal any neuron containing both tracers. Since at caudal levels it was more difficult to detect a potential homogeneous brown staining of CTB in strongly labelled neurons filled with black WGA-HRP granules (Fig. 2 D, F), a different approach for simultaneous tracer detection was chosen in two additional cases (P1, P2). Here the WGA-HRP labelling from IR injections was detected with DAB, which yielded a brown granular labelling, and the CTB was detected with immunofluorescence using Cy-3 tagged secondary antibodies. In these cases the sections were viewed and photographed in bright-field and at fluorescent illumination (Leica DMRB; N2.1) (Fig. 2 A, B). Again, the systematic analysis and plotting of IR- and MR-populations within the C-group did not reveal any double-labelled neurons and confirmed the observations made with immunoperoxidase techniques (Fig. 2, Fig. 3). IR and MR neurons form two layers in the C-group, with IR neurons located laterally and MR neurons medially. These layers tend to separate from each other at more rostral levels. In general, MR neurons extended further rostrally. As described previously, a considerable group of MR neurons is located as dorsal as the preganglionic neurons of the Edinger-Westphal nucleus (EWpg) and dorsal to EWpg at rostral planes (Fig. 3 D, K) (Büttner-Ennever et al., 2001). In one case MR neurons were even found at the level of the anteromedian nucleus (AM) rostral to nIII (Fig. 3 E).

Distribution of medial and inferior rectus motoneuron dendrites

Aside from the layered location of IR and MR MIF motoneurons, a striking feature of both neuron populations was the different pattern of their dendritic trees. This was most clearly seen after retrograde tracing with CTB (Fig. 1 B, D). To illustrate this, the dendrites of tracer-labelled MR or IR neurons in the C-group have been reconstructed after a CTB injection in the myotendinous junction. The dendrites of the CTB-labelled IR neurons in the C-group showed a widespread distribution in the supraoculomotor area (SOA) dorsal and lateral to nIII including the SOA of the contralateral side (Fig. 4 A–D). Thereby the dendrites spread through the medial part of the C-group housing the MR neurons ipsilaterally. The dendrites could be followed dorsally traversing through the EWpg and beyond it mainly on the ipsilateral side. However, dendritic branches were also found around the contralateral EWpg (Fig. 4 C,D, Fig. 5 A,C,D). Within the SOA the IR dendrites

covered an area lateral to EWpg and dorsal to nIII on both sides. This region contains smaller neurons that express urocortin immunoreactivity, but lack cholinergic markers. They are referred to as 'the central projecting neurons of the EW' (EWcp) and are distinguished from the cholinergic preganglionic neurons of EWpg (Kozicz et al., 2011). A considerable number of IR dendrites crossed the midline and traversed the C-group of the contralateral side reaching up to the EWpg. Horizontally directed dendrites could be followed as far as to the lateral border of the contralateral nIII (Fig. 4 C). Due to limited tracer filling of the neurons a study of the full extent of distal dendritic branches was not possible. The dendrites of the MR neurons in the C-group also showed a widespread distribution within the dorsal and lateral SOA, but unlike those of IR, they did not cross the midline (Fig. 4 E–H). In fact, long dendrites of MR coursed parallel to the midline dorsally in a focussed projection pathway, and passed in part through the EWpg, the AM and beyond it (Fig. 4 F,G,H; Fig. 5 B,E). At caudal and midlevels tracer-filled MR neuron processes entered the B-group of MR SIF motoneurons, but not the A-group (Büttner-Ennever and Akert, 1981), although the ventrally traversing fibers most probably represent axons that are visible in distinct fascicles of the oculomotor nerve lateral to nIII (not shown).

DISCUSSION

The present work confirms previous observations on the localization and morphology of MR and IR non-twitch (MIF) motoneurons in the C-group of monkey, but extends these studies with two major findings. First, the IR and MR neurons in the C-group form two independent populations that are layered at the dorsomedial border of nIII. Second, the neurons of both groups exhibit a different distribution pattern of their dendrites. Whereas the dendrites of IR neurons spread laterally and dorsally into the supraoculomotor area (SOA) and EW of both sides, the dendrites of the MR neurons remain confined to the ipsilateral SOA and EW, and do not cross the midline.

MR and IR neurons form independent cell groups in the C-group

The selective labelling of the C-group dorsomedial to nIII after tracer injections into the myotendinous junction of IR or MR confirmed earlier reports (Büttner-Ennever et al., 2001). These neurons are considered as non-twitch motoneurons that have taken up the tracer via their multiple '*en-grappe*' endings that are distributed along the whole length of muscle fibres including the distal part towards the myotendinous junction (for review: Spencer and Porter, 2006).

The lack of double-labelled neurons in the C-group after injections of different tracers into the myotendinous junction of MR and IR indicated that neither neuron population provides a common source for the innervation to the IR and MR MIF fibers. Up to date no selective recordings in C-group neurons have been performed in behaving animals but, based on the studies showing different sources of inputs to MIF motoneurons and SIF motoneurons, the following hypothesis was put forward: the SIFs with twitch properties are involved in the generation of (fast) eye movements, whereas the tonic MIFs are thought to mediate fine alignment of the eye muscles during gaze holding; and in interplay with palisade endings (see below) the MIFs may function as gamma-motoneurons (Büttner-Ennever et al., 2003;

Lienbacher et al., 2012). The finding that MR C-group neurons are contacted by only half the number of synaptic boutons compared to MR SIF motoneurons within nIII – similar to the observations of alpha and gamma motoneurons in cat – support this notion (Erichsen et al., 2014). The fact that the pretectum targets specifically neurons in the C- and S-group, but not the SIF-motoneurons within nIII, was taken as the first indication that MIF and SIF motoneurons participate in different functions concerning eye movements (Büttner-Ennever et al., 1996). This hypothesis was later extended for projections from the vestibular nuclei (Ugolini et al., 2006; Wasicky et al., 2004). It was shown that inputs to the MIF motoneurons arise from structures involved in more tonic functions, such as gaze-holding (the parvocellular parts of medial vestibular nucleus) and also areas associated with vergence or object fixation (e.g. pretectum). In contrast, the afferents to the SIF motoneurons arise from areas related to the generation of saccades, e.g. the paramedian pontine reticular formation, or the vestibuloocular reflex, e.g. the magnocellular part of the medial vestibular nucleus (Büttner-Ennever and Gerrits, 2004; Ugolini et al., 2006).

The tracer-labelled C-group neurons are considered as a source of the innervation for MIFs of the global layer. Only the muscle fibers of the global layer extend into the myotendinous junction (Oh et al., 2001), whereas the muscle fibers of the orbital layers insert in the collagenous pulleys (Demer et al., 2000). Furthermore, the distal end of the MIFs of the global layer that insert at the ocular bulbus are associated with special nerve terminals, the palisade endings, whose functional role is still under discussion (Donaldson, 2000; Büttner-Ennever et al., 2006). Previous work in monkey and cat indicated that the palisade endings originate from neurons in the brainstem most probably from the peripheral groups around the oculomotor nuclei (Lienbacher et al., 2011b; Zimmermann et al., 2011; Zimmermann et al., 2013). Currently it is not clear, whether the cell bodies in the periphery of the motonuclei give rise to both, the multiple motor innervation of MIFs *and* the palisade endings at the tips of MIFs, as is suggested from whole mount stainings in cat (Zimmermann et al., 2013); or whether they form two separate cell populations, a sensory and a motor population, that functionally act together (Lienbacher and Horn, 2012; Lienbacher et al., 2011a). In the present work we could not strengthen either hypothesis, but it should be kept in mind that both the IR group of the C-group neurons and the MR group may each consist of two subpopulations: motoneurons of MIFs, and the cell bodies of palisade endings. Irrespective of a further subdivision of C-group populations the presence of independent IR and MR populations is shown by the present work.

Neuronal populations reached by dendrites of C-group neurons

IR and MR C-group neurons showed a widespread dendritic distribution pattern within the SOA that differed significantly in regard to laterality. Whereas the dendrites of both, MR and IR neurons, reached into the ipsilateral dorsal and lateral SOA covering similar territories, the dendrites of IR neurons extended across the midline into the contralateral SOA in addition (Fig.4). Several functional cell groups are located in the SOA that are involved in the pupillary light reflex, accommodation and vergence, which all participate in the ‘near response’, and may share common inputs to the C-group. A projection targeting the SOA of one side may activate MR neurons of one eye, but IR neurons of both eyes.

Edinger-Westphal nucleus: central projecting- and preganglionic neurons—

Immediately dorsal to nIII the cholinergic preganglionic neurons of the ciliary ganglion form a paired compact nucleus in monkey, the Edinger-Westphal nucleus (EWpg), that is distinguished from the cytoarchitecturally less well defined centrally projecting neurons (EWcp) located more laterally (Horn et al., 2008; Kozicz et al., 2011; May et al., 2008). The EWcp is not directly involved in oculomotor function, but in stress reactions, food and fluid intake and alcohol consumption (Giardino et al., 2011; Xu et al., 2012). Its neurons have widespread projections within the brain and contain several peptides, such as urocortin 1 (for review: Kozicz et al., 2011). The recent description of the nitridergic nature of EWcp is in line with its presumed modulatory function, but a specific interaction with the adjacent C-group neurons has not been shown (Erichsen and May, 2012).

In mammals the EWpg contains two groups of preganglionic neurons, one mediating pupillary constriction and one for lens accommodation. In cat, the pupillary preganglionic neurons are located within and around the anteromedian nucleus (AM) rostral to nIII, and those for accommodation in a loose neuron group located at more caudal planes dorsal and ventral to nIII (Erichsen and May, 2002). In monkey, a spatial segregation of both cell groups is likely, since a recording study of preganglionic neurons mediating accommodation did not reveal any pupillary preganglionic neurons at this site (Gamlin et al., 1994). Electrical stimulation in the EWpg area resulted either in accommodation (more rostrally) or pupillary constriction (more caudally) alone, with a considerable overlap of both responses (Jampel and Mindel, 1967). Nevertheless a close association of C-group dendrites with all preganglionic neurons of EWpg and AM is evident from the present study, and has already been noted previously for MR neurons of the C-group (Büttner-Ennever et al., 2001; Lienbacher et al., 2011b; Erichsen et al., 2014). Ultrastructural studies in monkey demonstrated that MR C-group neurons are contacted by less synapses on their somata, or proximal dendrites, than on their distal dendrites in the SOA and EWpg (Erichsen et al., 2014). From all these anatomical findings it is reasonable to assume that the MR of the C-group share synaptic inputs with the preganglionic neurons in the EWpg and AM, providing vergence during accommodation and pupillary constriction for focussing an object, i.e. participating in the ‘near response’ (Erichsen et al., 2014). The fact that the IR neurons of the C-group show a similar, but less focussed, dendritic distribution towards the EWpg may indicate their general participation in this function during object fixation in the lower visual field. This is in line with observations from electrical stimulation experiments in the monkey brainstem (Jampel and Mindel, 1967). When the electrode approached nIII from dorsally bilateral pupillary constriction was observed, followed by bilateral downward ocular movement with an inward component, and most likely to reflect the stimulation of C-group neurons.

Inputs to C-group neurons

‘Near response’ neurons in the SOA—Several areas that participate in the near response have been shown to project to the SOA possibly targeting dendrites of C-group neurons and preganglionic neurons, but unfortunately the exact definition of cell groups within the SOA is still unclear. In the dorsal and lateral perioculomotor region, neurons active during vergence and accommodation, or both, have been identified and referred to as

‘near response neurons’ (Mays, 1984; Mays et al., 1986; Zhang et al., 1992). Increased firing rates are observed during near viewing, but not during vertical or horizontal conjugate eye movements (Gamlin, 1999). Near response neurons have monosynaptic connections with MR motoneurons as shown by their antidromic activation after MR motoneuron stimulation, where only “convergence” neurons were activated, and accordingly assumed to exert an excitatory action on MR motoneurons (Zhang et al., 1991). Not all neurons within the MR subgroup induced an antidromic activation of near response neurons in the SOA, although adducting eye movements were elicited upon stimulation (Zhang et al., 1991). This could be the result of stimulating MR SIF motoneurons within nIII in the A- or B-group, which may not be connected to near response cells in the SOA. Unlike the dendrites of C-group neurons, those of putative SIF motoneuron groups in the A and B nIII do not extend far dorsally in monkey, but reach laterally into the medial longitudinal fascicle (MLF) (Horn, personal observation). The absence of activation of near response neurons from microstimulation of contralateral MR motoneurons indicate an ipsilateral input from near response neurons in the SOA to MR (Zhang et al., 1991), which fits well with the unilateral dendritic branching field of MR neurons in the C-group observed here. Further support for the role of near response neurons in the SOA is provided by the identification of neurons in the SOA of strabismic monkeys that encode eye misalignment (i.e. the difference in position of both eyes), but do not encode eye position per se (Das, 2012).

Pretectum—Tracer injections into the olivary pretectal nucleus (PON) or the adjacent nucleus of the optic tract (NOT) in monkey resulted in anterograde labelling of an area in the SOA termed the lateral visceral cell column (LVC), the C-group and the preganglionic neurons in EWpg and/or anteromedian nucleus (AM) (Büttner-Ennever et al., 1996a, b; May et al., 2008). In cat a direct synaptic input from PON to primarily the rostral presumed pupillary preganglionic neurons in and around the AM was found (Sun and May, 2014a; b). The PON is known as the first relay nucleus in the pupillary light reflex. Its electrical stimulation elicits pupillary constriction (Magoun et al., 1936; Distler and Hoffmann, 1989a; b; Pong and Fuchs, 2000), it responds to luminance changes (Clarke et al., 2003; Pong and Fuchs, 2000; Gamlin et al., 1995) and receives a monosynaptic input from melanopsin-containing intrinsically photosensitive retinal ganglion cells (Gamlin et al., 2007; Sun and May, 2014a; Hannibal et al., 2014).

In monkey only injections into the ventral pretectum including the deep layers of the rostral superior colliculus (SC) gave rise to strong anterograde labelling in the LVC, AM, the SOA with EWpg and the C-group (Büttner-Ennever et al., 1996). The source of this projection may originate from vergence-related neurons in the pretectum (Judge and Cumming, 1986) or from the rostral SC, which is important for eye fixation (Suzuki et al., 2004; Van Horn et al., 2013). In monkey and cat, the rostral SC contains neurons whose activity is linked to vergence (van Horn et al., 2013) and accommodation (Suzuki et al., 2004), indicating a role in the linkage of fixation, accommodation and vergence as required for aligning the eyes during fixation of a visual target.

Central mesencephalic reticular formation—In monkey another monosynaptic bilateral input to the MR C-group neurons and preganglionic neurons arises from the central

mesencephalic reticular formation (CMRF) (May et al., 2011; Horn et al., 2012). The CMRF is considered as part of a circuitry for the control of horizontal saccades presumably providing a feedback signal to the SC (Cohen and Büttner-Ennever, 1984; Wang et al., 2010; Zhou et al., 2008). More recently, recording and stimulation studies in monkey have shown that specific areas in the CMRF encode conjugate and disconjugate saccades, and thereby may carry a vergence signal. Single unit recording revealed that the activity of CMRF neurons encodes conjugate and vergence movement of an individual eye rather than conjugate eye motion (Waitzman et al., 2008). This supports a concept originally put forward by von Helmholtz (1962) that each eye is controlled independently, and receives its own integrated signal for conjugate and vergence eye movements. In contrast, the concept of Hering (1977) is that both eyes are controlled by independent neural pathways for the generation of conjugate and vergence eye movements (for review: Cullen and Van Horn, 2011; King, 2011). Our data adds some support to the concept of Helmholtz, since the C-group MR dendrites, associated with the near response of one eye, remain unilateral.

Hypothalamus and perifornical region—A further common input to cholinergic preganglionic neurons in AM, EWpg and the C-group neurons arises from orexin-positive afferents from the perifornical region and the lateral hypothalamus; and these inputs do not target SIF motoneurons within nIII (Schreyer et al., 2009). Considering the function of orexin-positive neurons in the regulation of sleep-wake cycle (Inutsuka and Yamanaka, 2013), the rather specific orexin input to preganglionic neurons and C-group neurons may stabilize these systems during wakefulness. Pupillary fluctuations are stronger and lens accommodation is abolished in drowsiness (Wilhelm et al., 1998). Interestingly, in the sleep cycle during the transition period and REM sleep an active downward movement with convergence has been observed (Marquez-Ruiz and Escudero, 2008). This movement could be generated by afferents to the C-group.

In conclusion, the C-group was shown to consist of two separated populations of IR and MR neurons which control the slow non-twitch type of muscle fibres with well-developed tonic properties (Bondi and Chiarandini, 1983). Each group of motoneurons send dendrites into the SOA and EW, both regions are known to support the near response and vergence eye movements. Other inputs to the C-group arise from the pretectum, and CMRF, which also have an association with vergence functions. Although this list of C-group afferents is certainly incomplete, it clearly associates the C-group with some functional relevance in the control of convergent eye movements. Furthermore the MR and IR are the two muscles whose pulling directions must act synergistically to move the eyes into the lower-medial quadrant of vision, where vergence is most naturally used for the close inspection of objects (or reading). Convergence is driven by networks in higher neural centers such as the cerebral cortex, where disparity neurons generate eye movements to fuse the images from each eye (Leigh and Zee, 2006), but pure voluntary control of this synergy is also possible, and sometimes used as a party trick by children, ‘crossing their eyes’ to focus on a finger close to the nose. Usually vergence takes place in the horizontal plane involving disconjugate activation of the two MR to determine the angle and plane of vergence; this is accompanied by conjugate activation of both IR to adjust the line of vision. Anatomically this could be achieved by premotor inputs to the C-group, which would encounter the bilateral dendrites

of IR and unilateral dendrites of MR. However, the specific characteristics of the eye movement resulting from activation of the C-group MIF motoneurons is totally unclear. The complex arrangement of the 6 different eye muscle fibre types, as well as their mechanical cross-coupling (Goldberg et al 1997; (Demer, 2014).) and their unknown recruitment order, driven through more than one final common pathway, make the resultant movement through MIF activity impossible to predict at present. Yet it is likely that the MIF activation could provide the solution to various anomalous results revealed in studies on vergence, and summarized by Miller et al. (2002). These authors measured contraction forces in MR and LR during convergence, and paradoxically found the forces in both muscles to decrease.

Finally, the C-group was shown to consist of two separated populations of IR and MR neurons, but further experiments must determine whether both groups represent homogeneous groups of MIF motor neurons, or whether they are each a mixed population of motor and sensory neurons (Lienbacher and Horn, 2012).

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Abbreviations

AM	anteromedian nucleus
BSA	bovine serum albumine
C	C-group
ChAT	choline acetyltransferase
CMRF	central mesencephalic reticular formation
CTB	cholera toxin subunit B
DAB	diaminobenzidine
EAP	extravidin peroxidase
EW	Edinger-Westphal nucleus
EWcp	central projecting Edinger-Westphal nucleus
EWpg	preganglionic Edinger-Westphal nucleus
IO	inferior oblique muscle
IR	inferior rectus muscle
LR	lateral rectus muscle
LVC	lateral visceral cell column
MIF	multiply innervated muscle fiber

MLF	medial longitudinal fasciculus
MR	medial rectus muscle
NOT	nucleus of the optic tract
NRS	normal rabbit serum
nIII	oculomotor nucleus
nIV	trochlear nucleus
nVI	abducens nucleus
PON	olivary pretectal nucleus
SC	superior colliculus
SIF	singly innervated muscle fiber
SO	superior oblique muscle
SOA	supraoculomotor area
SR	superior rectus muscle
WGA-HRP	wheat germ agglutinin conjugated to horseradish peroxidase

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Figure 1.

Retrograde tracer labelling of the C-group in monkey. Brightfield photographs of transverse sections through the oculomotor nucleus (nIII) after a small CTB injection into the myotendinous junction of the right inferior rectus (IR) (A, B) or medial rectus (MR) muscle (C, D). Note that labelled IR neurons lie adjacent to the dorsomedial border of nIII (A, B). Their dendrites extend into the supraoculomotor area (SOA) (arrows). Labelled dendrites can be followed across the midline (asterisk). Tracer-labelled MR neurons lie medially in

the C-group (C). Their dendrites extend into the SOA far dorsally and laterally (arrows, asterisk), but are confined to the ipsilateral side. Scale bar = 500µm in A, C; 200µm in B, D.

Figure 2.

Photographs of transverse sections showing the two independent populations of MR and IR neurons in the C-group after simultaneous injections of WGA-HRP into the right IR and CTB into the right MR with different detection methods. Detailed view of the right C-group demonstrating WGA-HRP labelled IR neurons detected with diaminobenzidine (DAB) in brightfield (A, arrows), and CTB labelled MR neurons detected with immunofluorescence with CY3-tagged secondary antibodies (B, arrowheads). Note that none of the neurons contains both tracers. For easier comparison the same blood vessels are labeled by asterisks.

Detailed view of the C-group of two different cases demonstrating WGA-HRP labelled IR neurons with DAB-Co (C–F, black) and CTB positive neurons with immunoperoxidase method using DAB (C–F, brown). Note the clear separation of both populations within the C-group for case MUS (E, F). Black labelled dendrites of IR neurons can be followed across the midline (arrows), whereas brown labelled dendrites of MR neurons are confined to the ipsilateral side (arrowheads). Scale bar = 100µm in A, B; 200µm in C, E; 50µm in E, F.

Figure 3.

Drawings of transverse sections through different levels of the oculomotor nucleus (nIII) of case P2 (A-E) and MUS (F-K) with plottings of WGA-HRP labelled IR (black) and CTB labelled MR neurons (red). At caudal levels IR and MR neurons within the C-group lie adjacent to each other (A,B,F,G) and separate further rostrally. Note that only MR neurons are present at rostral planes adjacent to the preganglionic neurons of EWpg (D,E,K) and the anteromedian nucleus (AM) (E). Scale bar = 1mm.

Figure 4.

Reconstructions of dendritic distribution pattern for tracer-labelled C-group neurons after injection into the myotendinous junction of the right inferior rectus (IR) (A–D) and right medial rectus muscles (MR) (E–H). Note the IR dendrites show a bilateral distribution pattern within the supraoculomotor area (SOA) also passing through the preganglionic Edinger-Westphal nucleus (EWpg) (see Figure 5), whereas the MR dendrites are confined to the ipsilateral side. Scale bar = 0.5mm.

Figure 5.

Detailed view of the tracer-labelled dendrites of C-group neurons passing through the preganglionic cell population of the Edinger-Westphal nucleus (EWpg) for the inferior rectus muscle (A) and medial rectus muscle (B). Note that the dendrites of IR neurons are found in EWpg of both sides (A, C, D), the dendrites of MR neurons are confined to the ipsilateral side (B, E). Asterisks in the overviews in A and B mark the corresponding structures shown in the magnification in C, D and E. Scale bar = 100 μ m in A, B; 30 μ m in C, D, E.

Table 1

Overview of experimental monkey cases used in the study.

case	Injection		Detection	analysis
X183	CTB in distal MR		IHC DAB	
Jd7	CTB in distal MR		IHC DAB	Reconstruction of dendrites
B55	CTB in distal IR		IHC DAB	Reconstruction of dendrites
A09057	CTB in distal IR		IHC DAB	Reconstruction of dendrites
Mus	CTB in distal MR	WGA-HRP in distal IR	CTB: IHC DAB WGA-HRP: TMB-DAB-Co or DAB-Co	Double-labelling and dendrites
P2	CTB in distal MR	WGA-HRP in distal IR	CTB: IHC DAB or IF WGA-HRP: TMB-DAB-Co or DAB	Double-labelling
P1	CTB in distal MR	WGA-HRP in distal IR	CTB: IHC DAB or IF WGA-HRP: TMB-DAB-Co or DAB	Double labelling

CTB, cholera toxin subunit B; DAB, diaminobenzidine; DAB-Co, cobalt intensified DAB reaction; IF, immunofluorescence; IHC, immunohistochemistry; IR, inferior rectus muscle; MR, medial rectus muscle; WGA-HRP, wheatgerm agglutinin conjugated to horseradish peroxidase.