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A central mesencephalic reticular formation projection to the Edinger–Westphal nuclei

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Abstract

The central mesencephalic reticular formation, a region associated with horizontal gaze control, has recently been shown to project to the supraoculomotor area in primates. The Edinger–Westphal nucleus is found within the supraoculomotor area. It has two functionally and anatomically distinct divisions: (1) the preganglionic division, which contains motoneurons that control both the actions of the ciliary muscle, which focuses the lens, and the sphincter pupillae muscle, which constricts the iris, and (2) the centrally projecting division, which contains peptidergic neurons that play a role in food and fluid intake, and in stress responses. In this study, we used neuroanatomical tracers in conjunction with immunohistochemistry in *Macaca fascicularis* monkeys to examine whether either of these Edinger–Westphal divisions receives synaptic input from the central mesencephalic reticular formation. Anterogradely labeled reticular axons were observed making numerous bouton associations with the cholinergic, preganglionic motoneurons of the Edinger–Westphal nucleus. These associations were confirmed to be synaptic contacts through the use of confocal and electron microscopic analysis. The latter indicated that these terminals generally contained pleomorphic vesicles and displayed symmetric, synaptic densities. Examination of urocortin-1-positive cells in the same cases revealed fewer examples of unambiguous synaptic relationships, suggesting the centrally projecting Edinger–Westphal nucleus is not the primary target of the projection from the central mesencephalic reticular formation. We conclude from these data that the central mesencephalic reticular formation must play a here-to-for

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest pursuant to the publication of this research.

unexpected role in control of the near triad (vergence, lens accommodation and pupillary constriction), which is used to examine objects in near space.

Keywords

Lens; Pupil; Oculomotor; Parasympathetic; Urocortin; Eating disorders; Stress

Introduction

In a series of experiments aimed at obtaining a better understanding of the brainstem connections of the central mesencephalic reticular formation (cMRF), anterograde tracers have been injected into this midbrain structure in cats and monkeys (Warren et al. 2008; Zhou et al. 2008; Perkins et al. 2009; Wang et al. 2010, 2013). Examination of these cases indicated that labeled terminals were present within the confines of the supraoculomotor area (SOA). A recent detailed examination of this SOA projection in a primate model (Bohlen et al. 2015), noted that the cMRF might provide input to either the preganglionic component of the Edinger–Westphal nucleus (EWpg) or the centrally projecting component of the Edinger–Westphal nucleus (EWcp).

In monkeys, these two Edinger–Westphal nuclei (EW) represent two physically separate populations. EWpg contains cholinergic, preganglionic, parasympathetic motoneurons that project to the ciliary ganglion via the oculomotor nerve. They control lens accommodation and pupillary constriction via synapses with postganglionic motoneurons (Warwick 1954; Akert et al. 1980; Burde and Loewy 1980; Clarke et al. 1985; Crawford et al. 1989; Loewenfeld 1993; Gamlin et al. 1994; May et al. 2008b; McDougal and Gamlin 2015). EWcp contains peptidergic neurons that project widely in the central nervous system. They are believed to play a role in stress modulation and the control of eating and drinking behaviors (Maciewicz et al. 1983; Burde 1988; Kozicz et al. 1998; Vasconcelos et al. 2003; Ryabinin and Weitemier 2006; Kozicz 2003). Due to the fact that these two populations display a variety of species specific arrangements and the fact that the term Edinger–Westphal had been applied to both of them, there was initially some confusion as to their organization. However, detailed studies of their location in monkeys (Horn et al. 2008; May et al. 2008a) have made it clear that the primate EWpg occupies a paired cell column that is oriented rostrocaudally, and sits on either side of the midline, dorsal to the oculomotor nucleus (III) within the SOA. At their rostral end, the columns fuse together and form part of the anteromedian nucleus (AM). In contrast, the EWcp of primates includes neurons that lie on the midline between the two oculomotor nuclei and then extend dorsally into the SOA. Rostral to III, they are also found within AM, where they surround the smaller preganglionic population. The neuropeptide most commonly used to characterize EWcp neurons is urocortin-1. Different patterns of EWpg and EWcp organization are found in other species, but the nomenclature used here for the two divisions has been adopted to clarify which population is being described (Kozicz et al. 2011).

The term central mesencephalic reticular formation (cMRF) was coined by Cohen and colleagues (Cohen et al. 1985, 1986) to describe an area in the core of the midbrain

tegmentum in which electrical stimulation produced contraversive, horizontal saccades. Later recordings in the cMRF indicated that it contains a variety of neurophysiological cell types that fire in relationship to different aspects of saccades (Waitzman et al. 1996; Handel and Glimcher 1997; Cromer and Waitzman 2006, 2007) and head movements (Pathmanathan et al. 2006a, b). Anatomical studies have revealed that the cMRF is heavily connected to other gaze-related structures in the brainstem, particularly the superior colliculus, and to the cervical spinal cord (Edwards 1975; Edwards and de Olmos 1976; Cohen and Büttner-Ennever 1984; Chen and May 2000; Warren et al. 2008; Zhou et al. 2008; Wang et al. 2010, 2013; Perkins et al. 2014).

On the face of it, this saccade-related physiological and anatomical profile of the cMRF would seem to argue against projections to either the EWpg, whose activity is primarily related to the autonomic components of the near triad and to the pupillary light reflex, or to the EWcp, whose functions are not related to oculomotor behaviors. However, it should be noted that the initial investigations of the midbrain reticular formation using electrical stimulation indicated that it elicited a variety of behaviors (Bender and Shanzer 1964). With this in mind, we elected to more carefully examine the targets of the cMRF projection by utilizing immunohistochemical approaches to identify the cellular components of the EWpg and EWcp: cholinergic preganglionic motoneurons and urocortin-1-positive neurons, respectively. A brief report of these findings has appeared in abstract form (Horn et al. 2012).

Materials and methods

These experiments were carried out in 4 young adult, male, *Macaca fascicularis* monkeys. The protocols used were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were fully compliant with U.S. federal rules and regulations. All surgeries were performed in a sterile surgery suite in animals that were first tranquilized with ketamine HCl (10 mg/kg, im) and then anesthetized with isoflurane (3 %). Carprofen (4 mg/kg) was administered before the surgery as a preemptive analgesic and Buprenex (0.01 mg/kg, im) was used as a postsurgical analgesic. In addition, the incision edges were infused with Marcaine. Atropine sulfate (0.05 mg/kg, im) administration decreased secretions and Dexamethasone (2.5 mg/kg, iv) was employed to reduce edema during the surgery. Temperature, respiratory rate and heart rate were monitored and maintained within physiologic norms.

To make the cMRF injection, the animal's head was placed in a stereotaxic head holder (David Kopf Instruments, Tujunga, CA, USA). A midline incision was made through the scalp and a craniotomy removed the skull over the medial parietal lobe. The medial bank of the cerebral cortex was aspirated to reveal the anterior edge of the tentorium, the surface of the superior colliculus and the caudal pole of the pulvinar. In 3 animals, the tracer, 10 % biotinylated dextran amine (BDA) (10,000 MW, Molecular Probes) in dH₂O, was pressure injected by use of a 5 µl Hamilton syringe. The syringe was held in a micromanipulator at an 11° angle in the sagittal plane with the needle tip pointed in the rostral direction. The micromanipulator was then rotated 10° clockwise, as seen from above. The needle was advanced through the pulvinar to reach the cMRF. Readings from the surface of the superior

colliculus were used as a baseline for depth. Generally, two tracks were made at slightly different mediolateral and rostrocaudal locations, in order to increase the region of the cMRF receiving tracer. On each track, 0.1 μ l of tracer was placed at two locations 1.0 mm apart. In the fourth animal, a glass micropipette was held by the micromanipulator as described above for the syringe. It was filled with a solution containing 2 % *Phaseolus vulgaris* leucoagglutinin (PhaL) in 0.1 M, pH 8.0 phosphate buffer (PB). The micropipette was advanced through the pulvinar to the target and the tracer was iontophoretically ejected by passing a 7 μ A square-wave positive current with a 50 % duty cycle through the solution for 10 min. In all cases, the aspiration defect was then filled with Gelfoam and the incision closed with suture. See Bohlen et al. (2015) for further details.

After a survival period of 14–21 days, the animals were again tranquilized with ketamine HCl (10 mg/kg, im) and then deeply anesthetized with sodium pentobarbital (50 mg/kg, ip). In the two animals used for immunohistochemistry, the monkey was perfused through the heart with 0.1 M, pH 7.2 phosphate buffered saline (PBS) followed by 4 % paraformaldehyde in 0.1 M, pH 7.2 PB. The brains were then blocked in the frontal plane and postfixed overnight at 4 °C in the same fixative. Next, they were equilibrated in 30 % sucrose in 0.1 M, pH 7.2 PB at 4 °C as a cryoprotectant. The brainstem was sectioned at 50 μ m in the frontal plane on a sliding microtome. In the two animals used for electron microscopy, the monkey was perfused through the heart with 0.1 M, pH 7.2 PBS followed by 1 % paraformaldehyde and 1.5 % glutaraldehyde in 0.1 M, pH 7.2 PB. The brains were then blocked in the frontal plane and postfixed for 2 h at 4 °C in the same fixative. The brainstem was sectioned in the frontal plane using a Vibratome (Leica). The sections thickness for the BDA injection case was 100 μ m and for the PhaL injection case 50 μ m.

Immunohistochemical processing

Immunohistochemistry for light microscopy—To investigate whether anterogradely labeled axons from the BDA injection into the cMRF form bouton associations with choline acetyl transferase (ChAT)-positive, preganglionic motoneurons of the EWpg and/or with urocortin-1-labeled EWcp neurons, selected sections were stained for BDA and ChAT or for BDA and urocortin-1 using a double immunoperoxidase protocol. All the following steps were carried out with gentle agitation. Free floating sections were washed in 0.1 M, pH 7.4 Tris buffered saline (TBS) before and after pretreatment with a mixture containing 3 % hydrogen peroxide (H_2O_2) and 10 % methanol in 0.1 M, pH 7.4 TBS for 15 min to suppress endogenous peroxidase activity. Then, sections were incubated in ExtrAvidin-Peroxidase (1:1000; Sigma, E2886) in 0.3 % Triton X-100 (Sigma) in 0.1 M TBS for 1 h to detect BDA. After three washes in 0.1 M, pH 7.4 TBS, sections were reacted with 0.025 % diaminobenzidine (DAB), 0.2 % nickel ammonium sulfate and 0.015 % H_2O_2 in 0.05 M, pH 8.0 TBS for 10 min to yield a black reaction product in BDA-positive axons and boutons. Next, sections were washed in 0.1 M, pH 7.4 TBS and residual peroxidase was blocked with 1 % H_2O_2 in 0.1 M, pH 7.4 TBS for 30 min. To block unspecific binding sites, the sections were treated with 5 % normal donkey serum in 0.3 % Triton X-100 in 0.1 M, pH 7.4 TBS for 1 h at room temperature. This was followed by an incubation in a mixture of either goat anti-ChAT (1:50; Millipore, AB144P) or rabbit anti-urocortin-1 (1:6000; Sigma, U4757) with 5 % normal donkey serum in 0.3 % Triton X-100 in 0.1 M TBS (pH 7.4) for 48 h at

4 °C. After three washes in 0.1 M TBS (pH 7.4), the sections were incubated either in donkey anti-goat IgG (1:50, Dianova, Jackson ImmunoResearch, 705-005-003) for ChAT or donkey anti-rabbit IgG (1:50, Dianova, Jackson ImmunoResearch, 711-005-152) for urocortin-1 in 0.1 M, pH 7.4 TBS containing 2 % bovine serum albumin (BSA) for 1 h at room temperature. After three washes in 0.1 M, pH 7.4 TBS, the sections were then treated with goat peroxidase-anti-peroxidase (PAP) (1:500, Dianova, 323-005-024) for ChAT or rabbit-PAP (1:500, Dianova 123-005-024) for urocortin-1 for 1 h at room temperature and subsequently reacted with 0.025 % DAB and 0.015 % H₂O₂ in 0.05 M, pH 8.0 TBS for 10 min to detect the antigen binding site, and so stain the immunopositive cells brown. After washing, the sections were mounted on slides, air-dried, dehydrated in ethanols, and coverslipped with DPX (Sigma).

Immunohistochemistry for fluorescence microscopy—For fluorescence or confocal microscopy, free floating sections were pretreated with 0.3 % Triton X-100 in 0.1 M, pH 7.4 TBS for 1 h at room temperature. After three washes in 0.1 M, pH 7.4 TBS for 10 min each at room temperature, sections were incubated in Cy³-tagged Streptavidin (1:200, Dianova, Jackson ImmunoResearch) in 0.3 % Triton X-100 in 0.1 M, pH 7.4 TBS for 2 h at room temperature in the dark. (All further steps were carried out in the dark.) Sections were again washed three times in 0.1 M, pH 7.4 TBS for 10 min each at room temperature. Next, they were pretreated with 5 % normal donkey serum in 0.3 % Triton X-100 in 0.1 M, pH 7.4 TBS for 1 h at room temperature. This was followed by incubation in goat anti-ChAT (1:25, Millipore, AB144P) or rabbit anti-urocortin-1 (1:2000, Sigma, U-4757) in 5 % normal donkey serum with 0.3 % Triton X-100 in 0.1 M, pH 7.4 TBS for 48 h at 4 °C. After three washes in 0.1 M, pH 7.4 TBS, sections were incubated in donkey anti-goat (for ChAT) or donkey anti-rabbit (for urocortin), each tagged with the fluorescent dye Alexa 488 (1:200, Molecular Probes) in 0.1 M, pH 7.4 TBS and 2 % BSA for 1–2 h at room temperature. After several buffer rinses, the sections were mounted on slides, dried at room temperature, and coverslipped with permanent aqueous mounting medium Gel/Mount (Biomed, San Francisco, CA, USA). Coverslipped, fluorochrome-stained sections were stored in the dark at 4 °C.

Light microscopic analysis and confocal imaging of double labeled tissue

The slides were examined with one of two light microscopes (Leica; DMRB, Bensheim, Germany, or Zeiss; Axioplan, MicroImaging, Oberkochen, Germany) under brightfield conditions or by using filters for red fluorescent Cy³ (Leica: N2.1; excitation filter BP 515–560 nm, dichromatic mirror 580 nm, suppression filter LP 590 nm; Zeiss: excitation filter BP 546 nm, dichromatic beam splitter FT 580 nm, barrier filter LP 590 nm) and green fluorescent Alexa 488 (Leica: I3; excitation filter BP 450–490 nm, dichromatic mirror 510 nm, suppression filter LP 515 nm; Zeiss: excitation filter BP 475 nm, dichromatic beam splitter FT 500 nm, barrier filter LP 530 nm). Conventional photomicrographs were taken with a digital camera (Pixera Pro 600 ES, Klughammer, Markt Indersdorf, Germany) mounted on the microscope. Confocal images from selected sections stained for BDA and ChAT or BDA and urocortin-1 immunofluorescence were taken with a laser-scanning confocal microscope (Leica SP5, Mannheim, Germany). Dual-channel imaging of Alexa 488 and Cy³ fluorescence was sequentially recorded at a 543- or 488-nm excitation wave

length. Z series were collected every 0.49 μm through each section. Image stacks were processed by using ImageJ software (Rasband 1997, <http://imagej.nih.gov/ij/>). Sharpness, contrast, and brightness of the final images were adjusted to reflect the appearance of the labeling, seen through the microscope by using Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA).

Quantitative analysis

In selected sections from cases stained for either ChAT or urocortin-1 by use of immunoperoxidase methods, a quantitative estimation of the cMRF input to both EW populations was performed. For this analysis, BDA-labeled boutons attached to either the somata or proximal dendrites of cholinergic preganglionic EWpg neurons and urocortin-positive EWcp neurons were counted, respectively. Only examples where no space was seen between the bouton and the cell membrane were considered as putative contacts. Consequently, bouton endings located above or underneath the EW neurons were not included in the analysis. A simple two tailed student's *t* test was used to establish whether there were significant differences ($p < 0.02$) between the EWpg and EWcp populations.

Electron microscopic processing

Sections from the two brains fixed with mixed aldehydes were processed for electron microscopic analysis of the tracer distribution. Sections from the brain with a BDA injection were equilibrated in 0.05 % Triton-X 100 in 0.1 M, pH 7.2 PB. They were then incubated in a 1:500 solution of Avidin D conjugated to horseradish peroxidase in 0.05 % Triton-X 100 in 0.1 M, pH 7.2 PB overnight at 4 °C. After rinsing in 0.1 M, pH 7.2 PB, the sections were reacted in 0.5 % diaminobenzidine (DAB) in 0.1 M, pH 7.2 PB with 0.01 % nickel ammonium sulfate and 0.005 % cobalt chloride by addition of 0.005 % hydrogen peroxide. Sections from the brain containing a PhaL injection were first equilibrated in 0.1 % Triton-X 100 in 0.1 M, pH 7.2 PB with 0.1 % normal goat serum. Next they were incubated overnight at 4 °C in biotinylated goat anti-PhaL (Vector Labs, BA0224) diluted 1:200 in 0.1 % Triton-X 100 in 0.1 M, pH 7.2 PB with 0.1 % normal goat serum. These sections were then tagged using avidin–biotin–horseradish peroxidase, as per the goat ABC kit instructions (Vector Labs). The label was visualized by reacting the sections in 0.5 % DAB in 0.1 M, pH 7.2 PB with 0.01 % nickel ammonium sulfate and 0.005 % cobalt chloride by addition of 0.005 % hydrogen peroxide. See Wang et al. (2010) and Barnerssoi and May (2015) for further details.

In both cases, once these sections were washed in 0.1 M, pH 7.2 PB, samples were taken that contained the EWpg by microdissection under a Wild M8 stereoscope (Leica). The sections were then mounted, counterstained, dehydrated, cleared and coverslipped to allow confirmation that the appropriate area was sampled. The distribution of labeled axons and sample regions was charted using an Olympus BH-2 microscope equipped with a drawing tube. The samples underwent routine electron microscopic processing to produce embedded blocks (Wang et al. 2010; Barnerssoi and May 2015). Semithin sections stained with toluidine blue were used to confirm that the block contained the EWpg, based on the presence of large motoneuronal cell bodies. Any areas outside the borders of EWpg were then trimmed away. Ultrathin section were then cut and collected on copper mesh grids, and

underwent routine staining before being examined and photographed using Zeiss Leo and Jeol 1400 electron microscopes (Barnerssoi and May 2015).

Results

Preganglionic Edinger–Westphal nucleus (EWpg) connections

Figure 1 shows one of the two cases used for immunohistochemical identification of the preganglionic motoneurons found in EWpg. In this case, a large injection of BDA was centered in the central mesencephalic reticular formation (cMRF) (Fig. 1a, e). It extended ventrally into the pontine reticular formation. As shown in the low magnification views (Fig. 1b, f), the preganglionic Edinger–Westphal nucleus (EWpg) was easily identified as a circumscribed group of immunostained cells located dorsal to the cholinergic somatic motoneurons in the oculomotor nucleus (III) and the C-group (Fig. 1f) that also contained choline acetyl transferase (ChAT). Black, BDA-positive axons were observed within the supraoculomotor area (SOA). As shown in the higher magnification views Fig. 1c, d, g, h, many of these labeled axons displayed bouton enlargements that lay in close association (arrowheads) with the somata and dendrites of the brown, ChAT-positive cells. These close associations were seen with cells of both the ipsilateral (Fig. 1c, g) and contralateral (Fig. 1d, h) EWpg, and were found at both rostral (Fig. 1a – d) and caudal (Fig. 1e – h) levels of the EWpg.

The second case, shown in Fig. 2, had a much more circumscribed injection of BDA located in the middle of the cMRF (Fig. 2i). Even though the injection was smaller, BDA-labeled axons were almost as common within the SOA of this case and more bouton contacts on EWpg neurons were present (see quantification below). As shown in the low magnification views (Fig. 2b, f, j), brown, ChAT-positive motoneurons were observed within III, in the C-group and, most dorsally, in EWpg. The latter extended rostrally into the anteromedian nucleus (AM) (Fig. 2b). As shown in the higher magnification views (Fig. 2c, d, g, h, k, l), the BDA-labeled axons displayed numerous bouton enlargements that had close associations (arrowheads) with the somata and dendrites of ChAT-positive cells in EWpg. This was true throughout the rostrocaudal extent of the nucleus, and on both the ipsilateral (Fig. 2c, g, k) and contralateral (Fig. 2d, h, l) sides.

We analyzed these same cases using double immunofluorescence and confocal microscope imaging. Figure 3 shows the results in the ipsilateral EWpg. In this case, the ChAT-positive cells display a green fluorescent marker. BDA-labeled axons from the cMRF injection shown in Figs. 1 or 2 fluoresced red. Numerous BDA-labeled boutons are present within the confines of EWpg (Fig. 3a). These boutons were often located immediately adjacent to ChAT-positive motoneurons. Generally, several of these close associations (arrowheads) were present along the contour of the somatic (Fig. 3b) and dendritic (Fig. 3c) membranes of these cells. Figure 3d, e shows a rotation of the image of the ChAT-positive cell seen at the top of Fig. 3a. As can be seen in the high magnification inserts, no space is present between the membranes of the red, BDA-labeled boutons and the green, ChAT-positive motoneuron. This is strong evidence for synaptic connection between the two labeled elements.

We also examined the synaptic nature of the cMRF terminals in EWpg at the ultrastructural level. Figure 4a shows an injection site for one of the EM cases. The BDA filled much of the cMRF and spread slightly into the overlying pretectum. BDA-labeled axons extended throughout the SOA, but were particularly prominent within the EWpg (Fig. 4b). At higher magnification, the BDA-labeled boutons could be observed in high concentration, immediately surrounding the counterstained cell bodies within the nucleus (Fig. 4d). We took samples from the region containing the EWpg for electron microscopic analysis (Fig. 4c, arrows) and further trimmed them once embedded, so that our analysis was confined to this division.

Examination of the samples taken for electron microscopy revealed numerous BDA-labeled terminals (At*) throughout the neuropil of the ipsilateral (Fig. 5a – f) and contralateral EWpg (Fig. 5g – l). These could be discriminated from non-labeled terminals (At) (Fig. 5e, h, k) because the labeled terminals contained fine granular electron dense reaction product in their cytoplasm. The vesicles could only be characterized in the more lightly labeled examples (Fig. 5c, k). They appeared to be pleomorphic in form. Postsynaptic densities were modest, if present, so these contacts would be described as symmetric, although there was some heterogeneity within the sample (Fig. 5c, e, f, h, k). With respect to the specific goal of determining whether the cMRF contacts preganglionic motoneurons, terminals were found making synaptic contacts (arrowheads) with large somata (Fig. 5a, j). However, most of the contacts were with dendrites (Den). Postsynaptic targets included very large, proximal dendrites (Fig. 5b, h) that presumably belong to preganglionic motoneurons, as well. In addition, medium diameter dendrites (Fig. 5c, e, i), small dendrites (Fig. 5f, k), and even spines (Sp) (Fig. 5d, l) were targeted by cMRF terminals. These may belong to preganglionic motoneurons or to other cells, such as C-group motoneurons that extend dendrites into the nucleus (Erichsen et al. 2014; Tang et al. 2015). One of the striking features of the ultrastructure of this cMRF projection is that it appeared much the same within the ipsilateral EWpg (Fig. 5a – f) as within the contralateral EWpg (Fig. 5g – l), both in terms of the terminal characteristics and the pattern of postsynaptic targets.

One technical consideration for the use of BDA as a neuroanatomical tracer is its propensity for uptake by axons of passage. Since the axons of the descending tracts from the superior colliculus pass through the cMRF, and the superior colliculus is known to send projections to the SOA (Edwards and Henkel 1978; Harting 1977), this is a particular concern here. To confirm that the terminals observed following BDA injections did indeed originate from cells in the cMRF, we injected this region with PhaL, which exhibits little or no fiber-of-passage uptake (Gerfen and Sawchenko 1984; Wouterlood and Groenewegen 1985). The injection site for this case is indicated in Fig. 6a. It was centered within the cMRF, but the track extended up into the pretectum. This injection produced numerous labeled axonal arbors with bouton enlargements throughout the SOA (Fig. 6b). The labeled boutons were somewhat more densely distributed within the confines of EWpg. The higher magnification drawing (Fig. 6d) indicates that the PhaL-labeled terminals were often observed in close association with the counterstained (gray) somata of these EWpg cells.

Trapezoidal samples including the EWpg were taken from this case for ultrastructural examination (Fig. 6c, arrows). Examples of the PhaL-labeled terminals (At*) found within

EWpg from this case are presented in Fig. 7. The reaction product appears as a granular, electron dense material in the cytoplasm of the labeled terminals, and, in more lightly labeled examples (Fig. 7d – f), outlines the synaptic vesicles. The vesicles, when observable, were pleomorphic. Post-synaptic densities were modest, at best. Synaptic contacts (arrowheads) were observed along the plasma membranes of somata (Fig. 7a – c) and sometimes several labeled terminals could be observed along a single somatic profile (arrows, Fig. 7a). This strongly suggests that preganglionic motoneurons are targeted by the cMRF projection. Labeled axon terminals also contacted large (Fig. 7d), medium (Fig. 7e), and small (Fig. 7g, h) dendrites (Den). Occasionally, labeled terminals contacted spines (Sp) (Fig. 7c). These labeled terminals were present in samples taken from both the ipsilateral EWpg (Fig. 7a – e) and contralateral EWpg (Fig. 7f – h) following this injection.

Centrally projecting Edinger–Westphal (EWcp) connections

We also addressed the issue of whether the other division of the EW, the EWcp, was targeted by the cMRF. Here we used the cases illustrated in Figs. 1 and 2, but stained for urocortin-1, a neuropeptide found in most of this population. In Fig. 8, cells containing urocortin-1 were stained brown via immunohistochemical means. This allows this population of peptidergic neurons, which are a major component of the EWcp, to be clearly seen lying between the oculomotor nuclei and stretching dorsally between III and EWpg into the SOA (Fig. 8b, f). The axons labeled by a BDA injection into the cMRF (Fig. 8e) are stained black in this material. They were observed throughout the EWcp, arborizing among the urocortin-1-positive cells (Fig. 8c, d, g, h), although the density of this projection is much less than that observed in EWpg (Note that EWpg is visibly darker in plates b and f due to the BDA-labeled terminals there). Boutonal enlargements on the BDA-labeled arbors were common in EWcp. The labeled boutons displayed close associations (arrowheads) with some, but not all, of the urocortin-1-positive cells. We rarely saw more than three such associations (Fig. 8h) with an individual neuron.

The close associations observed in Fig. 8 suggest, but do not prove, the presence of synaptic contact between the cMRF axons and the urocortin-1-positive cells in EWcp. Towards this end, we used fluorescent markers to tag the BDA-labeled axons and the urocortin-1 cells, and examined the material using confocal microscopy. Numerous, green fluorescent, urocortin-1-positive cells were present within AM (Fig. 9a) and SOA (Fig. 9f). This region also contained large numbers of axons with terminal boutons that fluoresced red, indicating they contained BDA from the cMRF injection. Examination of this material revealed that only some of the urocortin-1-positive cells maintained close associations (arrowheads) with the cMRF terminals (Fig. 9). Anterogradely labeled terminals were observed adjacent to both the somatic perimeter and proximal dendrites of urocortin-1-positive cells. It should be noted that there were relatively few close associations between labeled boutons and any individual labeled cell. Furthermore, use of the confocal microscope to rotate the image, as shown in Fig. 9b–e, g, h, often failed to confirm the presence of a likely synaptic contact (Fig. 9b, c, and upper right cell in d, e), and only occasionally supported the presence of a synapse (Fig. 9d, e lower left cell, g, h). Thus, our data suggest that the cMRF provides a weaker input to the urocortin-1-positive cells in the EWcp, compared to the projection upon the cholinergic EWpg motoneurons.

In an attempt to quantify the strength of a cMRF input to the EW, we examined somata and proximal dendrites of cells stained with immunoperoxidase techniques in the two cases (Figs. 1, 2, 8). We found that the vast majority of preganglionic neurons in EWpg (case 1: 84 %; case 2: 94 %) was contacted by BDA-labeled bouton endings. Fewer urocortin-positive neurons in the EWcp were contacted by labeled boutons (case 1: 72.5 %; case 2: 72 %). In both cases, a higher number of BDA-labeled boutons was attached to the neuronal surface of the preganglionic neurons, with an average of 6.1 (case 1) and 11.3 (case 2) boutons in close association with their somata and an average of 4.1 (case 1) and 7.7 (case 2) boutons in close association with the proximal dendrites (Fig. 10). The neurons of the EWcp received a far weaker supply of BDA-labeled, cMRF terminals: numbering 3.0 (case 1) or 2.4 (case 2) boutons contacting somata and 0.9 (case 1) and 1.1 (case 2) boutons contacting their proximal dendrites (Fig. 10). For statistical analysis, we combined the values from the two cases. When all presumptive contacts were considered, there were significantly ($p < 0.0001$) more contacts made onto EWpg cells (mean = $14.07 \pm \text{SEM} = 0.613$) cells than EWcp cells (mean = $3.64 \pm \text{SEM} = 0.132$) cells. It was possible that this might have been due to less complete filling of EWcp dendrites, so we considered just the somata. However, we still found that significantly ($p < 0.0001$) more cMRF contacts were made onto EWpg somata (mean = $8.373 \pm \text{SEM} = 0.378$) than EWcp somata (mean = $2.54 \pm \text{SEM} = 0.092$). It should be noted that confidence in this finding must be tempered by the fact only two animals were analyzed. Moreover, since no rotated views were possible in this type of analysis, these values may represent overestimates. On the other hand, the limited dendritic staining in these sections means that most of the dendritic interactions would not have been seen or counted.

Discussion

The data supplied here provide strong evidence that the central mesencephalic reticular formation (cMRF) supplies a direct, bilateral input to the motoneurons in the preganglionic Edinger–Westphal nucleus (EWpg). While neither the confocal nor the ultrastructural evidence is incontrovertible, the combined results from these two lines of evidence strongly support the contention that cells in the cMRF synaptically contact preganglionic motoneurons. Previous work has already shown that injections of retrograde tracers that include the EWpg along with the supraoculomotor area (SOA) and oculomotor nucleus (III) do label cells within the cMRF (Bohlen et al. 2015). These cells occupy a horizontally oriented sublamina within the middle third of the cMRF. The results of the present study indicate that one target of this cMRF population is the EWpg. This finding gains further support from the fact that the same terminal distribution pattern was observed when either BDA or PhaL was used as a neuroanatomical tracer. The prominent arrangement of terminals on the somata and proximal dendrites of EWpg cells suggests that this cMRF input is, in fact, a major drive for these cells.

In contrast, the more limited input to urocortin-1-positive cells demonstrated in the same animals suggests that cMRF input likely provides only a modulatory drive to the cells of the centrally projecting division of EW (EWcp). The urocortin-1-positive cells in the EWcp are believed to play a role in feeding and drinking behaviors (Ryabinin and Weitemier 2006; Weitemier and Ryabinin 2005, 2006). In consideration of the potency of urocortin in

activating corticotropin releasing hormone receptors, it is not surprising that the EWcp is also thought to play a role in stress-related behaviors (Kozicz et al. 2001, 2011; Kozicz 2003). In this context, even the evidence for a modest projection from the cMRF, which has primarily been described in terms of gaze-related activity, may seem puzzling. However, procuring food and drink almost always involves changes in gaze direction. Moreover, defense against predators, which is certainly a stressful event, also can involve turning one's head and eyes towards the source of danger (Kübler et al. 2014). Thus, the gaze signal supplied by the cMRF may help time changes in activity in the urocortin-1-positive population. It is also certainly possible that the gaze-related activity recorded in the cMRF is part of more complex behaviors that are relevant to EWcp actions. Perhaps the presence of this terminal field helps explain the presence of the EWcp within SOA. Even the modest input observed here suggests that the EWcp may be located next to III and within the SOA in order to facilitate sharing inputs received by these adjacent structures.

The presence of a major drive to the EWpg originating in the cMRF is a surprise in light of what is known about the function of this part of the reticular formation. Electrical stimulation of the cMRF produces contraversive, horizontal saccades (Cohen and Büttner-Ennever 1984; Cohen et al. 1985, 1986). While no recordings of lens accommodation or pupil diameter were made in these studies, changes in these two features are not normally expected with conjugate eye movements of the sort described. A similar argument can be made with respect to recordings of cMRF neuron activity (Waitzman et al. 1996; Handel and Glimcher 1997; Cromer and Waitzman 2006, 2007). Since only eye movements were recorded and targets were presented on a screen in a darkened room, activity related to lens accommodation or pupil changes would not have been seen. Due to the fact that saccades and attention have been closely correlated, one might posit that this projection was related to attentional changes in pupil diameter. However, in this study, we saw cMRF terminals spread throughout the rostrocaudal extent of the EWpg with close associations present on nearly all the motoneurons, not just the pupillary component of the EWpg, which is small, and topographically restricted (Warwick 1954; Erichsen and May 2002; May et al. 2008b; Sun and May 2014a, b). This argues against a role for this cMRF projection in independent control of pupillary function, such as would be expected for attention, emotion or the pupillary light reflex.

Instead, the wide and bilateral distribution of cMRF terminals within the EWpg argues for dual action on both the lens and pupillary components. Such combined actions are observed with the near triad (near response) in which convergence through bilateral activation of the medial rectus muscles is combined with bilateral lens accommodation and bilateral pupillary constriction. This is accomplished through simultaneous activation of medial rectus motoneurons and preganglionic motoneurons on both sides of the brainstem when looking at a near target. The premotor neurons thought to be responsible for this action are the so-called "midbrain near response cells". Activity in this population has been correlated with both lens accommodation and convergent eye movements (Mays 1984; Judge and Cumming 1986; Mays et al. 1986). These cells were described as sitting dorsolateral to III. This has sometimes been interpreted as placing them in the SOA (May et al. 1992; Das 2011, 2012). However, this dorsolateral location might also apply to the cMRF itself, as this region sits both lateral and dorsolateral to III. In fact, the location indicated by Fig. 9 of Mays et al.

(1986) suggests the medial part of the cMRF contains vergence cells. If they do lie in the cMRF, then the data presented here represent the first anatomical demonstration of the projection of the midbrain near response neurons.

An alternative possibility has been presented by the most recent recordings done within the cMRF (Waitzman et al. 2008). This study utilized an adaption of the methodology developed by Zhou and King (1998) to test whether an individual cell's activity was specifically correlated with the movements of either the ipsilateral or contralateral eye, or with both eyes. Waitzman reported the presence of a significant population of eye-specific neurons within the cMRF. This would, in theory, allow the cMRF to encode disjunctive eye movements. In fact, these observers noted that, in some cases, the saccades produced by microstimulation within the cMRF were not all conjugate. Some were disjunctive saccades. Normally, a disjunctive saccade is made when the origin and target of the eye movement differ both in direction and distance from the viewer. Such saccades are generally slower than conjugate saccades, although they are faster than pure vergence motions (Enright 1984; Maxwell and King 1992). It is thought that the saccade is slowed to better match the speed of lens accommodation. In this case, each eye might be presumed to be independently focusing on the new target to match its distance from the viewing eye. However, lens and pupil changes were not recorded in the Waitzman et al. (2008) study, so we do not know whether these cells also encode changes in lens curvature. Nevertheless, it is reasonable to hypothesize that one possible interpretation of the present data is that the terminals seen on EWpg cells represent part of a pathway for controlling lens accommodation during disjunctive saccades.

The presence of this new cMRF projection should be considered within the context of the known connections of this structure. The main source of input to the cMRF is the superior colliculus (Harting 1977; Chen and May 2000), and the superior colliculus has not traditionally been viewed as playing a role in vergence eye movements or lens accommodation (Walton and Mays 2003). However, there is some evidence from work in cats that the rostral superior colliculus might modulate lens accommodation (Ohtsuka and Sato 1997; Ohtsuka and Nagasaka 1999). More recently, stimulation of the rostral pole of the superior colliculus was shown to modulate vergence movements in monkeys (Chaturvedi and Van Gisbergen 2000) and cells whose activity is modulated with respect to vergence eye movements have been recorded (Van Horn et al. 2013). In addition, pupillary dilation has been recorded following electrical stimulation of the superior colliculus (Wang et al. 2014). These effects may be transmitted by connections through the cMRF, if tectal terminals are found to terminate on the cells projecting to the EWpg.

The main outflow from the cMRF is back to the superior colliculus (Cohen and Büttner-Ennever 1984; Zhou et al. 2008; Wang et al. 2010). This collicular projection appears to be separate from the downstream projections of the cMRF to the pons, medulla and spinal cord (Moschovakis et al. 1988; Perkins et al. 2014). The collicular projection is also likely to be separate from the projection to EWpg described here, as Moschovakis et al. (1988) did not observe non-tectal axon collaterals for cMRF reticulotectal neurons. The projection to EWpg is probably also separate from the population of cMRF neurons sending axons to the medulla and spinal cord, as these reticulospinal and reticuloreticular neurons display a very

different distribution within the cMRF (Warren et al. 2008; Perkins et al. 2009) than those reported to project to the SOA and EWpg (Bohlen et al. 2015). Certainly, a connection between lens and pupil control and head turning would not be expected. It remains to be determined whether another projection of the cMRF, such as its connection with omnipause neurons (Wang et al. 2013) might be the source of collaterals to EWpg. In considering all the different efferent projections of the cMRF, one must also take into account the variety of physiological cell types that have been described there with respect to how their activity correlates with aspects of gaze changes (Waitzman et al. 1996; Handel and Glimcher 1997; Cromer and Waitzman 2006, 2007). At present, we have no clear correlation between the different physiological classes and their possible axonal targets, to which we have now added the EWpg. In order to make progress in this regard, it may be necessary to record cMRF activity in antidromically identified cells, while tracking lens accommodation.

The present study also provided evidence with respect to the ultrastructural characteristics of the cMRF terminals contacting motoneurons in the EWpg. For the most part, these terminals contained pleomorphic vesicles and made symmetric contacts on their targets, suggesting that they are inhibitory in nature. They closely resemble the type At₂ terminals described as contacting preganglionic motoneurons in the feline EWpg (Sun and May 2014b). In fact, the cMRF is known to contain GABAergic neurons (Appell and Behan 1990) and GABAergic cMRF projections to both the superior colliculus and the nucleus raphe interpositus have been described (Wang et al. 2010, 2013). However, one would expect that midbrain near response neuron inputs to preganglionic motoneurons would be excitatory, not inhibitory, as the pupil is constricted and the ciliary muscle contracts when looking at a near target. With this in mind, some caution in interpreting the data may be warranted. The transmitters and receptors present at these synapses remain to be determined. Furthermore, all the cases analyzed had injection sites centered in a similar place within the middle of the cMRF. Injection sites located, for example, more medially, might produce a different result. In light of the present results, it would certainly be of interest to stimulate at different sites within the cMRF, while recording from preganglionic motoneurons in EW.

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References

- Akert K, Glicksman MA, Lang W, Grob P, Huber A. The Edinger–Westphal nucleus in the monkey. A retrograde tracer study. *Brain Res.* 1980; 184:491–498. [PubMed: 6153290]
- Appell PP, Behan M. Sources of subcortical GABAergic projections to the superior colliculus in the cat. *J Comp Neurol.* 1990; 302:143–158. [PubMed: 2086611]
- Barnerssoi, M.; May, PJ. Postembedding immunohistochemistry for inhibitory neurotransmitters in conjunction with neuroanatomical tracers. In: Van Boekstaele, EJ., editor. *Transmission electron microscopy methods for understanding the brain.* New York: Springer; 2015. in Press

- Bender, MB.; Shanzer, S. Oculomotor pathways defined by electrical stimulation and lesions in the brainstem of monkey. In: Bender, MB., editor. oculomotor The system. New York: Hoeber Medical Division, Harper & Row; 1964. p. 81-140.
- Bohlen MO, Warren S, May PJ. A central mesencephalic reticular formation projection to the supraoculomotor area in macaque monkeys. *Brain Struct Funct*. 2015
- Burde RM. Disparate visceral neuronal pools subserve spinal cord and ciliary ganglion in the monkey: a double labeling approach. *Brain Res*. 1988; 440:177–180. [PubMed: 2451977]
- Burde RM, Loewy AD. Central origin of oculomotor parasympathetic neurons in the monkey. *Brain Res*. 1980; 198:434–439. [PubMed: 7407608]
- Chaturvedi V, Van Gisbergen JA. Stimulation in the rostral pole of monkey superior colliculus: effects on vergence eye movements. *Exp Brain Res*. 2000; 132:72–78. [PubMed: 10836637]
- Chen B, May PJ. The feedback circuit connecting the superior colliculus and central mesencephalic reticular formation: a direct morphological demonstration. *Exp Brain Res*. 2000; 131:10–21. [PubMed: 10759167]
- Clarke RJ, Coimbra CJ, Ale'ssio ML. Distribution of parasympathetic motoneurons in the oculomotor complex innervating the ciliary ganglion in the marmoset (*Callithrix jacchus*). *Acta Anat (Basel)*. 1985; 121:53–58. [PubMed: 3918402]
- Cohen B, Büttner-Ennever JA. Projections from the superior colliculus to a region of the central mesencephalic reticular formation (cMRF) associated with horizontal saccadic eye movements. *Exp Brain Res*. 1984; 57:167–176. [PubMed: 6519224]
- Cohen B, Matsuo V, Fradin J, Raphan T. Horizontal saccades induced by stimulation of the central mesencephalic reticular formation. *Exp Brain Res*. 1985; 57:605–616. [PubMed: 3979501]
- Cohen B, Waitzman DM, Büttner-Ennever JA, Matsuo V. Horizontal saccades and the central mesencephalic reticular formation. *Prog Brain Res*. 1986; 64:243–256. [PubMed: 3726146]
- Crawford K, Terasawa E, Kaufman PL. Reproducible stimulation of ciliary muscle contraction in the cynomolgus monkey via a permanent indwelling midbrain electrode. *Brain Res*. 1989; 503:265–272. [PubMed: 2605519]
- Cromer JA, Waitzman DM. Neurones associated with saccade metrics in the monkey central mesencephalic reticular formation. *J Physiol*. 2006; 570:507–523. [PubMed: 16308353]
- Cromer JA, Waitzman DM. Comparison of saccade-associated neuronal activity in the primate central mesencephalic and paramedian pontine reticular formations. *J Neurophysiol*. 2007; 98:835–885. [PubMed: 17537904]
- Das VE. Cells in the supraoculomotor area in monkeys with strabismus show activity related to the strabismus angle. *Ann NY Acad Sci*. 2011; 1233:85–90. [PubMed: 21950980]
- Das VE. Responses of cells in the midbrain near-response area in monkeys with strabismus. *IOVS*. 2012; 53:3858–3864.
- Edwards SB. Autoradiographic studies of the projections of the midbrain reticular formation: descending projections of nucleus cuneiformis. *J Comp Neurol*. 1975; 161:341–358. [PubMed: 50329]
- Edwards SB, de Olmos JS. Autoradiographic studies of the projections of the midbrain reticular formation: ascending projections of nucleus cuneiformis. *J Comp Neurol*. 1976; 165:417–431. [PubMed: 1262539]
- Edwards SB, Henkel CK. Superior colliculus connections with the extraocular motor nuclei in the cat. *J Comp Neurol*. 1978; 179:451–467. [PubMed: 641226]
- Enright JT. Changes in vergence mediated by saccades. *J Physiol*. 1984; 350:9–31. [PubMed: 6747862]
- Erichsen JT, May PJ. The pupillary and ciliary components of the cat Edinger–Westphal nucleus: a transsynaptic transport investigation. *Vis Neurosci*. 2002; 19:15–29. [PubMed: 12180856]
- Erichsen JT, Wright NF, May PJ. Morphology and ultrastructure of the medial rectus subgroups motoneurons in the macaque monkey. *J Comp Neurol*. 2014; 522:626–641. [PubMed: 23897455]
- Gamlin PDR, Zhang Y, Clendaniel RA, Mays LE. Behavior of identified Edinger–Westphal neurons during ocular accommodation. *J Neurophysiol*. 1994; 72:2368–2382. [PubMed: 7884465]

- Gerfen CR, Sawchenko PE. An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L). *Brain Res.* 1984; 290:219–238. [PubMed: 6198041]
- Handel A, Glimcher PW. Response properties of saccade-related burst neurons in the central mesencephalic reticular formation. *J Neurophysiol.* 1997; 78:2164–2175. [PubMed: 9325383]
- Harting JK. Descending pathways from the superior colliculus: an autoradiographic analysis in the rhesus monkey (*Macaca mulatta*). *J Comp Neurol.* 1977; 173:583–612. [PubMed: 404340]
- Horn AK, Eberhorn A, Ha`rtig W, Ardeleanu P, Messoudi A, Büttner-Ennever JA. Periocular motor cell groups in monkey and man defined by their histochemical and functional properties: reappraisal of the Edinger–Westphal nucleus. *J Comp Neurol.* 2008; 507:1317–1335. [PubMed: 18186030]
- Horn AKE, Bohlen MO, Warren S, May PJ. Evidence for the central mesencephalic reticular formation playing a role in the near triad. *Soc Neurosci Abst.* 2012; 38(371):02.
- Judge SJ, Cumming BG. Neurons in the monkey midbrain with activity related to vergence eye movement and accommodation. *J Neurophysiol.* 1986; 55:915–930. [PubMed: 3711972]
- Kozicz T. Neurons colocalizing urocortin and cocaine and amphetamine-regulated transcript immunoreactivities are induced by acute lipopolysaccharide stress in the Edinger–Westphal nucleus in the rat. *Neuroscience.* 2003; 116:315–320. [PubMed: 12559087]
- Kozicz T, Yanaihara H, Arimura A. Distribution of urocortin-like immunoreactivity in the central nervous system of the rat. *J Comp Neurol.* 1998; 391:1–10. [PubMed: 9527535]
- Kozicz T, Li M, Arimura A. The activation of urocortin immunoreactive neurons in the Edinger–Westphal nucleus following stress in rats. *Stress.* 2001; 4:85–90. [PubMed: 22432129]
- Kozicz T, Bittencourt JC, May PJ, Reiner A, Gamlin PD, Palkovits M, Horn AK, Toledo CA, Ryabinin AE. The Edinger–Westphal nucleus: a historical, structural, and functional perspective on a dichotomous terminology. *J Comp Neurol.* 2011; 519:1413–1434. [PubMed: 21452224]
- Kübler TC, Kasneci E, Rosensteil W, Schiefer U, Nagel K, Papegeorgiou E. Stress-indicators and exploratory gaze for the analysis of hazard perception in patients with visual field loss. *Transport Res (F).* 2014; 24:231–243.
- Loewenfeld, IE. The light reflex. The pupil, anatomy, physiology, and clinical applications. Ames: Iowa State University Press; 1993. p. 83-273.
- Maciewicz R, Phipps BS, Foote WE, Aronin N, Difiglia M. The distribution of substance P-containing neurons in the cat Edinger–Westphal nucleus: relationship to efferent projection systems. *Brain Res.* 1983; 270:217–230. [PubMed: 6192874]
- Maxwell JS, King WM. Dynamics and efficacy of saccade-facilitated vergence eye movements in monkeys. *J Neurophysiol.* 1992; 68:1248–1260. [PubMed: 1432082]
- May PJ, Porter JD, Gamlin PD. Interconnections between the primate cerebellum and midbrain near-response regions. *J Comp Neurol.* 1992; 315:98–116. [PubMed: 1371782]
- May PJ, Reiner AJ, Ryabinin AE. Comparison of the distributions of urocortin-containing and cholinergic neurons in the periocular motor midbrain of the cat and macaque. *J Comp Neurol.* 2008a; 507:1300–1316. [PubMed: 18186029]
- May, PJ.; Sun, W.; Erichsen, JT. Defining the pupillary component of the periocular motor preganglionic population within a unitary primate Edinger–Westphal nucleus. In: Kennard, C.; Leigh, RJ., editors. Using eye movements as an experimental probe of brain function, *Prog Brain Res.* Vol. 171. 2008b. p. 97-106.
- Mays LE. Neural control of vergence eye movements: convergence and divergence neurons in midbrain. *J Neurophysiol.* 1984; 51:1091–1108. [PubMed: 6726313]
- Mays LE, Porter JD, Gamlin PD, Tello CA. Neural control of vergence eye movements: neurons encoding vergence velocity. *J Neurophysiol.* 1986; 56:1007–1021. [PubMed: 3783225]
- McDougal DH, Gamlin PD. Autonomic control of the eye. *Compr Physiol.* 2015; 5:439–473. [PubMed: 25589275]
- Moschovakis AK, Karabelas AB, Highstein SM. Structure-function relationships in the primate superior colliculus. II. Morphological identity of presaccadic neurons. *J Neurophysiol.* 1988; 60:263–302. [PubMed: 3404220]

- Ohtsuka K, Nagasaka Y. Divergent axon collaterals from the rostral superior colliculus to the pretectal accommodation-related areas and the omnipause neuron area in the cat. *J Comp Neurol.* 1999; 413:68–76. [PubMed: 10464370]
- Ohtsuka K, Sato A. Retinal projections to the accommodation-related area in the rostral superior colliculus of the cat. *Exp Brain Res.* 1997; 113:169–173. [PubMed: 9028787]
- Pathmanathan JS, Cromer JA, Cullen KE, Waitzman DM. Temporal characteristics of neurons in the central mesencephalic reticular formation of head unrestrained monkeys. *Exp Brain Res.* 2006a; 168:471–492. [PubMed: 16292574]
- Pathmanathan JS, Presnell R, Cromer JA, Cullen KE, Waitzman DM. Spatial characteristics of neurons in the central mesencephalic reticular formation (cMRF) of head-unrestrained monkeys. *Exp Brain Res.* 2006b; 168:455–470. [PubMed: 16292575]
- Perkins E, Warren S, May PJ. The mesencephalic reticular formation as a conduit for primate collicular gaze control: tectal inputs to neurons targeting the spinal cord and medulla. *Anat Rec.* 2009; 292:1162–1181.
- Perkins E, May PJ, Warren S. Feed-forward and feedback projections of midbrain reticular formation neurons in the cat. *Front Neuroanat.* 2014; 7:55. [PubMed: 24454280]
- Rasband, WS. ImageJ, U. S. National Institutes of Health. Bethesda, Maryland, USA: 1997. <http://imagej.nih.gov/ij/>
- Ryabinin AE, Weitemier AZ. The urocortin 1 neurocircuit: ethanol-sensitivity and potential involvement in alcohol consumption. *Brain Res Rev.* 2006; 52:368–380. [PubMed: 16766036]
- Sun W, May PJ. Central pupillary light reflex circuits in the cat: the olivary pretectal nucleus. *J Comp Neurol.* 2014a; 522:3960–3977. [PubMed: 24706328]
- Sun W, May PJ. Central pupillary light reflex circuits in the cat: morphology, ultrastructure and inputs of preganglionic motoneurons. *J Comp Neurol.* 2014b; 522:3978–4002. [PubMed: 24706263]
- Tang X, Büttner-Ennever J, Mustari MJ, Horn AKE. Internal organization of medial rectus and inferior rectus muscle neurons in the C group of the oculomotor nucleus in monkey. *J Comp Neurol.* 2015; 523:1809–1823. [PubMed: 25684641]
- Van Horn MR, Waitzman DM, Cullen KE. Vergence neurons identified in the rostral superior colliculus code smooth eye movements in 3D space. *J Neurosci.* 2013; 33:7274–7284. [PubMed: 23616536]
- Vasconcelos LA, Donaldson C, Sita LV, Casatti CA, Lotfi CF, Wang L, Cadinouche MZ, Frigo L, Elias CF, Lovejoy DA, Bittencourt JC. Urocortin in the central nervous system of a primate (*Cebus apella*): sequencing, immunohistochemical, and hybridization histochemical characterization. *J Comp Neurol.* 2003; 463:157–175. [PubMed: 12815753]
- Waitzman DM, Silakov VL, Cohen B. Central mesencephalic reticular formation (cMRF) neurons discharging before and during eye movements. *J Neurophysiol.* 1996; 75:1546–1572. [PubMed: 8727396]
- Waitzman DM, Van Horn MR, Cullen KE. Neuronal evidence for individual eye control in the primate cMRF. *Prog Brain Res.* 2008; 171:143–150. [PubMed: 18718293]
- Walton MM, Mays LE. Discharge of saccade-related superior colliculus neurons during saccades accompanied by vergence. *J Neurophysiol.* 2003; 90:1124–1139. [PubMed: 12904503]
- Wang N, Warren S, May PJ. The macaque midbrain reticular formation sends side-specific feedback to the superior colliculus. *Exp Brain Res.* 2010; 201:701–717. [PubMed: 19940983]
- Wang N, Perkins E, Zhou L, Warren S, May PJ. Anatomical evidence that the superior colliculus controls saccades through central mesencephalic reticular formation gating of omnipause neuron activity. *J Neurosci.* 2013; 33:16285–16296. [PubMed: 24107960]
- Wang CA, Boehnke SE, Itti L, Munoz DP. Transient pupil response is modulated by contrast-based saliency. *J Neurosci.* 2014; 34:408–417. [PubMed: 24403141]
- Warren S, Waitzman DM, May PJ. Anatomical evidence for interconnections between the central mesencephalic reticular formation and cervical spinal cord in the cat and macaque. *Anat Rec.* 2008; 291:141–160.
- Warwick R. The ocular parasympathetic nerve supply and its mesencephalic sources. *J Anat.* 1954; 88:71–93. [PubMed: 13129172]

- Weitemier AZ, Ryabinin AE. Lesions of the Edinger–Westphal nucleus alter food and water consumption. *Behav Neurosci*. 2005; 119:1235–1243. [PubMed: 16300431]
- Weitemier AZ, Ryabinin AE. Urocortin 1 in the dorsal raphe regulates food and fluid consumption, but not ethanol preference in C57BL/6J mice. *Neuroscience*. 2006; 137:1439–1445. [PubMed: 16338088]
- Wouterlood FG, Groenewegen HJ. Neuroanatomical tracing by use of *Phaseolus vulgaris*-leucoagglutinin (PHA-L): electron microscopy of PHA-L filled neuronal somata, dendrites, axons and axon terminals. *Brain Res*. 1985; 326:188–191. [PubMed: 3882193]
- Zhou W, King WM. Premotor commands encode monocular eye movements. *Nature*. 1998; 393:692–695. [PubMed: 9641680]
- Zhou L, Warren S, May PJ. The feedback circuit connecting the central mesencephalic reticular formation and the superior colliculus in the macaque monkey: tectal connections. *Exp Brain Res*. 2008; 189:485–496. [PubMed: 18553075]

Fig. 1.

Central mesencephalic reticular formation (cMRF) projections to the preganglionic Edinger–Westphal nucleus (EWpg) labeled by a large injection of biotinylated dextran amine (BDA). The extent of the BDA injection site is illustrated by rostral (a) and caudal (e) chartings on the left. **b, f** Low magnification photomicrographs of the region of interest (*green boxes* in **a, e**) are used to indicate the location (*black boxes*) of the higher magnification samples (**c, d, g, h**, respectively). Numerous black, BDA-labeled axons arborized within the EWpg on both the side ipsilateral to the injection (**c, g**) and contralateral to the injection (**d, h**) at both rostral (**b–d**) and caudal (**f–h**) levels. These axons displayed bouton enlargements, many of which lay in close association (*arrowheads*) with the somata and dendrites of *brown*, choline acetyl transferase (ChAT)-positive motoneurons. *III* oculomotor nucleus, *SOA* supraoculomotor area. *Scale* in **b = f**, scale in **h = c, d, g**

Fig. 2.

Central mesencephalic reticular formation (cMRF) projections to the preganglionic Edinger–Westphal nucleus (EWpg) labeled by a small injection of biotinylated dextran amine (BDA). The extent of the BDA injection site is illustrated in the caudalmost (i) of three section chartings (a, e, i). Low magnification photomicrographs (b, f, j) of the regions of interest (green boxes in a, e, i) are used to indicate the location (black boxes) of the higher magnification samples (c, d, g, h, k, l, respectively). Numerous black, BDA-labeled axons arborized within the EWpg on both the side ipsilateral to the injection (c, g, k) and contralateral to the injection (d, h, l) at all levels. These axons displayed bouton enlargements, many of which lay in close association (arrowheads) with the somata and dendrites of brown, choline acetyl transferase (ChAT)-positive motoneurons. AM anteromedian nucleus, III oculomotor nucleus, SOA supraoculomotor area. Scale in b = f, j, scale in l = c, d, g, h, k

Fig. 3. Confocal images of contacts made by labeled terminals on motoneurons in the preganglionic Edinger–Westphal nucleus (EWpg) ipsilateral to the injection site. Central mesencephalic reticular formation (cMRF) axons labeled by biotinylated dextran amine (BDA) are pseudocolored *red*. Choline acetyl transferase (ChAT) containing motoneurons labeled via immunohistochemistry are pseudocolored *green*. The lower magnification view (**a**) reveals the large number of BDA-labeled axons in the nucleus. *Boxes* indicate the regions shown at higher magnification in **b–e**. Numerous close associations (*arrowheads*) were present

between the labeled boutons and labeled motoneurons. *Inset* in **b** shows a closer view of three terminals lined up along the edge of a soma suggesting synaptic contact between cMRF boutons and preganglionic motoneurons. This point is further investigated in the confocal image (**d, e**) rotated in space to provide two different views. *Insets* show examples of the fact that space could not be visualized between the bouton membrane and that of the preganglionic motoneuron when viewed in any plane, suggesting the presence of a synaptic contact. *Scale* in **e** = **b–d**

Fig. 4.

Distribution of biotinylated dextran amine (BDA)-labeled central mesencephalic reticular formation (cMRF) terminals within the supraoculomotor area (SOA). A third example of a BDA injection site is shown in the charting (**a**). The terminal fields that resulted in SOA are shown in a higher magnification view of the same section (**b**). Terminal arbors are distributed bilaterally with a slight ipsilateral predominance. The *box* shows the region containing the preganglionic Edinger–Westphal nucleus (EWpg) that is illustrated at even higher magnification in **d**. Large numbers of anterogradely labeled terminals are associated with the counterstained somata (*shaded gray*) present in this EW division. Samples of this region containing EWpg were taken for ultrastructural analysis, as shown by the trapezoids in **c** (*arrows*). *III* oculomotor nucleus, *PAG* periaqueductal gray

Fig. 5.

Biotinylated dextran amine (BDA)-labeled terminals in the preganglionic Edinger– Westphal nucleus (EWpg). Electron dense, BDA-labeled, axon terminals (At*) were present in both the ipsilateral EWpg (a–f) and contralateral EWpg (g–l). They made synaptic contacts (arrowheads) onto both the large somata (Soma) in the nucleus and dendrites (Den) of all sizes. Even spines (Sp) were contacted. Boxes in g indicate areas sampled in i, j. At unlabeled axon terminal, Ax axon, Nu nucleus. Scale in c = d–f, h = k and i = j, l

Fig. 6.

Distribution of *Phaseolus vulgaris* leucoagglutinin (PhaL)-labeled central mesencephalic reticular formation (cMRF) terminals within the supraoculomotor area (SOA). The PhaL injection site is shown in the charting (a). The terminal fields that resulted in SOA are shown in a higher magnification view of the same section (b). Terminal arbors are distributed bilaterally, with a slight ipsilateral predominance. The *box* shows the region containing the preganglionic Edinger–Westphal nucleus (EWpg) that is shown at even higher magnification in d. Large numbers of anterogradely labeled terminals are associated with the counterstained somata (*shaded gray*) present in and around the nucleus. Samples of this region containing EWpg were taken for ultrastructural analysis, as shown by the trapezoids in c (*arrows*). *III* oculomotor nucleus, *PAG* periaqueductal gray

Fig. 7.

Terminals in the preganglionic Edinger–Westphal nucleus (EWpg) labeled following a *Phaseolus vulgaris* leucoagglutinin (PhaL) injection into the central mesencephalic reticular formation (cMRF). Electron dense, PhaL-labeled, axon terminals (At*) were present in both the ipsilateral EWpg (a–e) and contralateral EWpg (f–h). They made synaptic contacts (arrowheads) onto both the large somata (Soma) (a–c) and dendrites (Den) (d–h) of all sizes, as well as spines (Sp) (c). In some cases multiple terminals could be seen on the plasma

membranes of somata (**a**, *arrows*) or dendrites (**d**). *Ax* axon, *At* unlabeled axon terminal.
Scale in **h** = **b–g**

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Fig. 8. Central mesencephalic reticular formation (cMRF) projections to the centrally projecting Edinger–Westphal nucleus (EWcp) labeled by a small injection of biotinylated dextran amine (BDA). The extent of the BDA injection site is illustrated by the caudal (e) charting on the left. **b, f** Low magnification photomicrographs of the region of interest (see green boxes in **a, e**) are used to indicate the location (black boxes) of the higher magnification samples (**c, d, g, h**, respectively). Numerous black, BDA-labeled axons arborized within the EWcp, both in the region that occupies the supraoculomotor area (SOA) (**c, g**) and in the

area that sits between the two oculomotor nuclei (III) (**d, h**) at both rostral (**a–d**) and caudal (**e–h**) levels. These axons displayed bouton-like enlargements, many of which lay in close association (*arrowheads*) with the somata and dendrites of brown, urocortin-1-positive neurons. Numerous BDA-labeled terminals make the region containing the preganglionic Edinger–Westphal nucleus (EWpg) slightly darker (**b, f**). *Scale* in **f** = **b**, and **h** = **c, d, g**

Fig. 9. Confocal images showing the relationship of central mesencephalic reticular formation (cMRF) terminals and urocortin-1-positive cells in the centrally projecting Edinger–Westphal nucleus (EWcp) (**f–h**) and its rostral extension into the anteromedian nucleus (AM) (**a–e**). Biotinylated dextran amine (BDA) containing fibers from the injection site shown in Fig. 1 are pseudocolored red. Immunohistochemically stained cells containing urocortin-1 are pseudocolored green. Close associations between cMRF terminals and these peptidergic cells are indicated by *arrowheads*. The area in the *boxes* in plates **a** and **f** are

shown at higher magnification in **b–e, g, h**, respectively. These plates show a computer generated rotation of the images which indicate that the labeled terminals are in synaptic contact with the labeled cells in some cases (**d left cell, e, g, h**), but in most cases are not actually in contact (**b, c, d right cell**). *Scale in f = a; h = b–e, g*

Fig. 10.

A bar graph demonstrating the mean number of boutons in close association with cholinergic preganglionic neurons in EWpg and urocortin-1-positive neurons in EWcp for the two cases illustrated in Figs. 1, 2, 3, 8, 9. The analysis of terminal location included only those EWpg neurons (case 1: 42; case 2: 33) and EWcp neurons (case 1: 29; case 2: 98) that have close associations with BDA-positive cMRF afferents. For both populations, more cMRF afferents were found in association with the somata (*blue*) compared to the proximal dendrites (*red*). In the individual cases, the number of terminals contacting EWpg neurons (*green bars*) was greater than those contacting EWcp neurons. These results were also apparent when the results from the two cases were combined (*bars on the right*)