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The macaque midbrain reticular formation sends side-specific feedback to the superior colliculus

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Abstract

The central mesencephalic reticular formation (cMRF) likely plays a role in gaze control, as cMRF neurons receive tectal input and provide a bilateral projection back to the superior colliculus (SC). We examined the important question of whether this feedback is excitatory or inhibitory. Biotinylated dextran amine (BDA) was injected into the cMRF of *M. fascicularis* monkeys to anterogradely label reticulotectal terminals and retrogradely label tectoreticular neurons. BDA labeled profiles in the ipsi- and contralateral intermediate gray layer (SGI) were examined electron microscopically. Postembedding GABA immunocytochemistry was used to identify putative inhibitory profiles. Nearly all (94.7%) of the ipsilateral BDA labeled terminals were GABA positive, but profiles postsynaptic to these labeled terminals were exclusively GABA negative. In addition, BDA labeled terminals were observed to contact BDA labeled dendrites, indicating the presence of a monosynaptic feedback loop connecting the cMRF and ipsilateral SC. In contrast, within the contralateral SGI, half of the BDA labeled terminals were GABA positive, while more than a third were GABA negative. All the postsynaptic profiles were GABA negative. These results indicate the cMRF provides inhibitory feedback to the ipsilateral side of the SC, but it has more complex effects on the contralateral side. The ipsilateral projection may help tune the “winner-take-all” mechanism that produces a unified saccade signal, while the contralateral projections may contribute to the coordination of activity between the two colliculi.

Keywords

Oculomotor; Saccades; GABAergic; Central mesencephalic reticular formation; Ultrastructure

Introduction

The superior colliculus (SC) integrates multiple incoming sensory signals, and then sends motor signals for the next saccade to the brainstem gaze centers. The superficial layer (stratum griseum superficiale, SGS) contains a map in which neuronal activity encodes everything the retina sees, while the intermediate gray layer (stratum griseum intermediale, SGI) contains a map in which the activity is centered at a single location that specifies the next saccade target (Goldberg and Wurtz 1972; Robinson 1972; Sparks et al. 1976; Sparks 1978; Wurtz and Goldberg 1972). Although numerous SC studies have confirmed its role in eye movements, not much is known about how the SC transforms the complex pattern of visual signals found in SGS into a unitary, saccade-related burst in SGI (Sparks 1986; Optican 2005). Signal transmission through interlaminar projections from SGS to SGI is present (Isa et al. 1998; Hall and Lee 1993, 1997; Lee and Hall 1995; Moschovakis et al. 1988a), but this sensory signal is not necessarily sophisticated enough to produce the correct presaccadic discharge in SGI under all conditions (Mays and Sparks 1980; Isa and Kobayashi 2004; Hafed and Krauzlis 2008; Van Horn 2009). Therefore, understanding the extrinsic inputs to SGI becomes particularly important for solving the puzzle of signal transformation in the SC. In the present study, we concentrated on a major source of input to SGI, the central mesencephalic reticular formation (cMRF) (Cohen and Büttner-Ennever 1984; Chen and May 2000; Moschovakis et al. 1988b; Zhou et al. 2008).

Physiological investigations of the cMRF suggest that it is part of the circuitry guiding horizontal saccades. In fact, the term cMRF was coined to describe an area of the primate midbrain tegmentum, which, when electrically stimulated, produces contraversive horizontal saccades (Cohen and Büttner-Ennever 1984; Cohen et al. 1985). Single unit recordings from the cMRF also support a role in saccade production. Waitzman et al. (1996) found that 76% of the cMRF cells encountered discharged before and/or during contraversive, visually guided and rapid eye movements. When the cMRF was reversibly inactivated with microinjections of muscimol, a GABA_A agonist, contraversive, upward saccade hypermetria and instability in fixation were the result (Waitzman et al. 2000). Based on these findings, it was suggested that the cMRF plays a role in saccade triggering and participates in control of saccade accuracy.

Anatomical investigations support the concept that the cMRF is part of a collicular feedback circuit. The chief input to the cMRF is from collaterals of predorsal bundle fibers originating from the ipsilateral SC (Cohen et al. 1985; Cohen and Büttner-Ennever 1984; Grantyn and Grantyn 1982; Moschovakis et al. 1988a, b). Moschovakis et al. (1988b) demonstrated that the axons of some cMRF neurons project bilaterally to the SC, and terminate primarily in SGI. Subsequently, cat and monkey studies revealed that labeled tectoreticular axons show numerous close bouton associations with labeled reticulotectal cells in cMRF, suggesting extensive synaptic contact (Chen and May 2000; May et al. 2002; Warren et al. 2008a). In fact, Moschovakis et al. (1988b) suggested that tectoreticular neurons projecting to cMRF are the primary drive for cMRF reticulotectal cells based on the similarity of their saccade-related firing patterns. Recently, Zhou et al. (2008) examined the pattern of connections established by the cMRF projection onto the SC. Their results confirm that the ipsilateral SGI projection is stronger than the contralateral, and suggest that the cMRF feedback signal targets the predorsal bundle neurons that provide the main saccade-related SC output.

Taken together, these studies suggest the existence of a feedback loop in which the cMRF receives input from SGI and sends a feedback signal back to SGI. However, the specific function of the ipsilateral feedback loop is currently a matter of conjecture, and the role of the contralateral projection has not been addressed. With respect to the ipsilateral connections, Moschovakis et al. (1988b) raised the possibilities of both negative feedback and positive feedback circuits, with negative feedback gating SC bursts and positive feedback contributing

to the production of bursts. This was extended to suggest that this projection could provide the efference copy signal necessary to redirect SC activity in a two-step saccade paradigm (Bozis and Moschovakis 1998). Others have suggested that excitatory feedback from the cMRF maintains the signal produced in the SC for the duration of the saccades (Soetedjo et al. 2002). More specifically, Cromer and Waitzman (2006) proposed that excitatory input from cMRF cells coding for duration could sustain the firing of those SC neurons bursting for a specific saccade vector. Evidence from studies combining retrograde tracers with immunohistochemistry indicates that a portion of the cMRF's reticulotectal neurons may be GABAergic (Araki et al. 1984; Appell and Behan 1990).

Clearly, to advance our understanding of the role of this cMRF feedback loop, it is necessary to determine whether cMRF feedback is excitatory or inhibitory. Furthermore, to understand how cMRF input affects collicular circuits, we need to know whether it targets excitatory output cells and/or inhibitory interneurons on each side of the tectum. In the present study, we investigated these points, by injecting biotinylated dextran amine (BDA) into the cMRF of macaque monkeys (*M. fascicularis*) to anterogradely label reticulotectal terminals and retrogradely label tectoreticular neurons. The material was then prepared for electron microscopic (EM) examination. Finally, postembedding, gamma-aminobutyric acid (GABA) immunocytochemistry was used to identify putative inhibitory presynaptic and postsynaptic elements in ipsilateral and contralateral SGI.

Materials and methods

Surgery

All animal procedures were undertaken in accordance with the animal care and use guidelines of the NIH, including the Principles of Laboratory Care, and with the approval of the University of Mississippi Medical Center IACUC. Three male *Macaca fascicularis* monkeys underwent surgeries performed with sterile technique under isoflurane anesthesia (1–3%). Animals were pre-anesthetized with ketamine HCl (10 mg/kg, IM). Atropine sulfate (0.05 mg/kg, IV) was administered to control secretions, and dexamethasone (0.4 mg, IV) was given to minimize cerebral edema. Core temperature, respiration, heart rate, and blood O₂ saturation, were monitored and maintained at physiological levels. After placement in a stereotaxic apparatus, cortex overlying the midbrain was aspirated to allow direct visualization of the surface of the SC, and the caudal pole of the pulvinar. Pressure injections were made with a 1.0 µl Hamilton microsyringe attached to a micromanipulator. The micromanipulator was set at an angle of 10° (tip rostral) in the rostrocaudal plane, and rotated 10° counterclockwise (from above in the horizontal plane). To avoid the SC, the needle was inserted through the dorsal surface of the pulvinar, with the injection depth adjusted with respect to the SC surface. The coordinates used were based on the previous anatomical (Chen and May 2000) and physiological descriptions (Cohen et al. 1985), and atlas information (Paxinos et al. 2000). A 10.0% solution of BDA (BDA, Molecular Probes) measuring between 0.1 and 0.2 µl was delivered into the left cMRF along each of one or two penetrations. The aspirated cortical cavity was filled with hydrated gel foam. The overlying muscles and skin were re-approximated, and stabilized with vicryl suture. The incision was closed and the wound edges were infused with Sensorcaine. Buprenex (0.005 mg/kg, IM) was administered as a postsurgical analgesic.

Perfusion and sampling

After a survival time of 3 weeks, the animals were sedated with ketamine HCl and deeply anesthetized with sodium pentobarbital (50–70 mg/kg, IP). They were then perfused transcardially with buffered saline, followed by fixative containing 1.0% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M, pH 7.2 phosphate buffer (PB). Each brain was blocked in

the frontal plane, post-fixed for 1 h, and stored in cold PB. Brain blocks were cut on a Vibratome (Leica) and arranged into three ordered series of 100- μ m thick frontal sections.

To demonstrate the BDA labeling, a procedure similar to that described in Chen and May (2000) was followed. Two series of sections were rinsed and transferred into a solution containing 0.05% Triton X-100, in 0.1 M, pH 7.2 PB. Then, they were incubated in a 1:500 avidin–HRP (Vector) solution in 0.05% Triton X-100 PB for 24 h at 4°C. Next, they were reacted in a 0.5% diaminobenzidine (DAB) solution containing 0.01% each of cobalt chloride and nickel ammonium sulfate. Hydrogen peroxide (0.005%) was added to catalyze the reaction, and reveal the BDA with a black reaction product. After rinsing in PB, one series was mounted, counterstained with cresyl violet, dehydrated and cover slipped. It was used to guide the selection of EM samples in the second series. Small tissue blocks containing labeled cells and terminals were cut out of free-floating sections from the second series. Specifically, samples were cut from SGI of SC under a stereomicroscope (Leica Wild M8), and collected in 0.1 M, pH 7.2 PB. These sections were then mounted and treated as the first series to provide a record of the sampled sites.

Tissue processing for EM analysis

The tissue samples were post-fixed with 1.0% osmium tetroxide in 0.1 M, pH 7.0 PB for 1 h, and stained en bloc with 2.0% uranyl acetate in deionized H₂O for 5 min. Next, they were dehydrated in a graded series of acetones, before being placed in 1:3 Durcupan/acetone overnight, 3:1 Durcupan/acetone for 4 h, and then 100% Durcupan for 1 h. Finally, they were flat embedded in fresh Durcupan resin, which was polymerized overnight at 60°C. After embedding, 1- μ m thick sections were cut with glass knives on an ultramicrotome (Reichert Ultracut E), counterstained with toluidine blue, and coverslipped for inspection. Using information from the semithin sections, the blocks were retrimmed to exclude the areas with less labeling. Ultrathin silver/gold sections were then cut with a diamond knife on an ultramicrotome (Leica Ultracut UCT). For normal EM procedures on single (BDA) label material, sections were placed on copper-mesh grids. The copper grids were stained with calcinated 0.4% lead citrate for 30 s.

For double label material, GABA postembedding immunohistochemical staining was performed on sections collected onto formvar-coated nickel 1 $\times$ 2 mm slot grids. They were etched by 3.0% H₂O₂ for 3 min, and conditioned by Tris-buffered saline (TBS, with 0.85% NaCl and 0.76% Trizma, pH 7.4) and then TBS + 1.0% bovine serum albumin (BSA). Sections were then incubated in a 1:250 dilution of rabbit anti-GABA (Sigma) in TBS + BSA + 0.05% Tween 20 overnight. The next day, after being rinsed in TBS + BSA + Tween 20, the sections were incubated in a 1:40 dilution of goat-anti-rabbit IgG conjugated to 15-nm gold particles (Amersham) in TBS + BSA + Tween 20. The tissue was rinsed again with TBS + BSA + Tween 20, followed by distilled H₂O. Finally, 2.0% glutaraldehyde in 0.1 M, pH 7.0 PB was used to fix the samples, before staining with calcinated lead citrate.

Data analysis

Sections analyzed with the light microscope (LM), were drawn with an Olympus BH-2 microscope, equipped with a drawing tube. Photographic evidence was acquired with a Nikon Eclipse 80i microscope equipped with a Nikon DXM1200F digital camera. Images were obtained using Metamorph software, and were adjusted in Photoshop to match their appearance to the eye. Ultrathin sections were examined, and labeled profiles were photographed by the use of an electron microscope (Zeiss 906). EM photographs of terminals were generally taken at magnifications of 21,560 $\times$. For sections stained for GABA, the number of gold particles in a 0.25 μ m² square sampled from three or more regions over axon myelin sheaths, per grid, was counted to provide a background particle density for use as a baseline. We reasoned that myelin

sheaths should not contain more GABA than is normally present for metabolic needs, and so would provide an excellent, in tissue measurement of background labeling. We set a density greater than three times baseline as a conservative threshold for classifying terminals as GABA positive (GABA⁺). Once the number of particles per unit area in selected profiles was counted, those with counts less than or equal to baseline were judged to be GABA negative (GABA⁻). Those falling between the baseline and three times baseline were placed in an intermediate, non-classified category. Different criteria were used for somata and dendrites as the latter are believed to contain less transmitter. Those profiles containing more than two times baseline were classified as GABA⁺ and those with baseline or fewer particles were classified as GABA⁻. Those containing between one and two times baseline were placed in an intermediate category. In the present study, baseline varied among samples from three to six particles. Consequently, depending on the specific grid's baseline, those terminals containing more than 9–18 gold particles per 0.25 μm^2 ($3 \times$ baseline) were classified as GABA⁺. Similarly, the somata and dendrites containing 6–12 gold particles per 0.25 μm^2 ($2 \times$ baseline) were classified as GABA⁺.

Results

LM findings

Following BDA injections into cMRF, BDA labeled axons were observed in numerous brainstem regions, with an ipsilateral predominance. Figure 1 shows an example from the cases we analyzed. In this case, a small BDA injection site was centered within cMRF (Fig. 1a). In the rostral midbrain, BDA labeled terminals (stipple) were distributed within the SC on both sides, with an ipsilateral predominance (Fig. 1b–i). The terminal fields were densest in the intermediate gray layer (SGI). The labeled terminals were also observed in the contralateral cMRF, the deep gray layer (SGP) and the periaqueductal gray (PAG) (Fig. 1).

The BDA also retrogradely labeled neurons (dots) in the SC. These labeled tectoreticular cells, which presumably send their main axons via the predorsal bundle (Moschovakis et al. 1988a) were mainly located in the intermediate gray layer (SGI) (Fig. 1b–i). The vast majority of labeled cells were found ipsilaterally. The field of anterogradely labeled terminals overlay retrogradely labeled cells on both sides of SC. This is further illustrated in Fig. 2. Numerous BDA labeled axonal boutons were distributed in the ipsilateral SC, some of which were closely associated (arrowheads) with BDA labeled dendrites (Fig. 2a, b) and somata. BDA labeled neurons in the ipsilateral SC were multipolar, and measured about 20–25 μm with respect to their somatic long axes (Fig. 2a). The density of labeled boutons was lower in contralateral SC (compare Fig. 2a, c), and far fewer cells were labeled. Nevertheless, BDA labeled terminals and lightly labeled neurons were occasionally seen in close association with the contralateral SC (Fig. 2d).

The small windows in Fig. 1 demonstrate the areas where samples were cut from SGI for EM. These samples were taken from both sides of the SC. The ultrastructural data that follow come from samples such as these.

EM findings: ipsilateral SC

cMRF terminals in BDA labeled material—BDA labeled reticulotectal terminal and pre-terminal profiles were identified under the EM by the greater electron density of the cytoplasm that they displayed, particularly in the vicinity of vesicular and other membranes (Fig. 3a–d). The terminal profiles were roughly round or oval in shape, and ranged in size between 0.46–2.90 μm along their long axis. They were moderately packed with small, clear vesicles that were pleomorphic in shape. The vesicle density was higher adjacent to synaptic densities. A few (2–7) mitochondria were usually seen in these labeled terminals. Most labeled terminals

contacted (arrowheads) dendrites (Fig. 3c, d), but examples contacting putative spines (Fig. 3a) or somata (Fig. 3b), were also observed. The postsynaptic densities of contacts between the BDA labeled terminals and other cell profiles were modest, and most were judged to be symmetric (Fig. 3a, c).

cMRF terminals in BDA/GABA double labeled material—In some cases, the samples containing BDA labeled profiles were processed by GABA postembedding immunostaining to reveal GABAergic profiles (Fig. 4). BDA labeled, vesicle-containing profiles were still evident in this tissue (Fig. 4a–d) and most were GABA⁺ (Fig. 4a–c). Double labeled, reticulotectal terminal profiles have greater electron density in their cytoplasm due to the reagents marking the presence of BDA, as described above for single labeled terminals. In addition, they were overlain by numerous gold particles (black dots) produced by the postembedding procedure. GABA positive terminals without BDA labeling were also commonly observed in the neuropil. For example, in Fig. 4a, a BDA and GABA double labeled terminal (At⁺) opposes a GABA[−] dendrite. The dendrite was also apposed by a GABA⁺ terminal that did not contain BDA (At⁺). However, no synaptic contacts were evident. Figure 4b and c shows possible synapses (arrowheads) between double labeled terminals and small GABA[−] dendrites. Note the additional GABA⁺ profiles (At⁺) in the adjacent neuropil. They have scattered, flattened vesicles and may represent presynaptic dendrites (PsDs), but no synapse is evident. In Fig. 4c, note the adjacent GABA[−] profile (At) with clear, flattened vesicles and occasional dense-cored vesicles. These profiles may contain another inhibitory transmitter such as glycine. In Fig. 4d, a rare BDA labeled terminal, which is considered to fall into the intermediate GABA category, is presented. It contacts a GABA[−] profile that is a presumptive dendritic spine. Some BDA labeled terminals contained irregular, unstained vacuoles (Fig. 4b, d). These may be artifacts due to the tracer, histochemical procedures or perfusion.

Semiserial sections through BDA/GABA double labeled cMRF terminals were collected to confirm the quantitative findings. Figure 5a, b shows two semiserial sections through a BDA labeled GABA⁺ cMRF terminal in the ipsilateral SGI. Double labeling of the terminal indicates the presence of the gold particles was not due to background variations.

EM results: contralateral SC

cMRF terminals in BDA labeled material—When compared with those in the ipsilateral SC, contralateral BDA labeled reticulotectal terminals, displayed more heterogeneity in vesicle shape and the extent of the postsynaptic density. In Fig. 6a, b, BDA labeled terminals (At*) with increased electron density synaptically contact (arrowheads) adjacent dendritic profiles. In Fig. 6a, the postsynaptic density was modest and the synaptic contact was judged to be symmetric. The vesicles in this profile were distinctly pleomorphic. In contrast, in Fig. 6b, where the connection to the pre-terminal axon is observable, a postsynaptic density was clearly present. Consequently, the synaptic contact was judged to be asymmetric. The vesicles contained in this profile are more spherical in nature.

cMRF terminals in BDA/GABA double labeled material—BDA labeled reticulotectal terminals that were judged to be either GABA⁺ or GABA[−] were both found in the contralateral SC. Examples of BDA labeled, GABA⁺ terminals are shown in Fig. 7. The BDA labeled terminal and pre-terminal profiles displayed the characteristic greater electron density in comparison to unlabeled profiles. They were also peppered with numerous gold particles, indicating that they fall into GABA⁺ category (At⁺). Examples of terminals that were associated with both somata (Fig. 7a), and dendrites (Fig. 7b, c) were seen. Symmetric synapses (arrowheads) between a BDA labeled, GABA⁺ terminal and GABA[−] dendrites are shown in Fig. 7b. For comparison, note that one of these postsynaptic profile was also contacted by a

GABA⁻ terminal (At) with an asymmetric synapse. Figure 7d shows a BDA labeled, GABA⁺ terminal found adjacent to two GABA⁺ presumed “presynaptic dendrites” (PsD⁺), but no contact was observed.

Figure 8 shows examples of BDA labeled, GABA⁻ reticulotectal terminals (At*) found in contralateral SGI. These electron dense terminals were overlain by few, if any, gold particles. They were also associated with both somata (Fig. 8b) and dendrites (Fig. 8a, b, d). A clearly asymmetric synapse between the BDA labeled, GABA⁻ terminal and a GABA⁻ dendrite is evident in Fig. 8a. The BDA labeled, GABA⁻ terminal shown in Fig. 8b, which is adjacent to a GABA⁻ soma and its dendrite, is among the largest we observed. The vesicle filled profile shown in Fig. 8c falls into the intermediate category, as it had higher than baseline levels of gold particles, but less than three times baseline.

Contacts between BDA labeled reticulotectal terminals and tectoreticular cells

Both BDA labeled dendrites (Den*) (Fig. 9a–d) and somata (not illustrated) displayed moderately electron dense cytoplasm that could be differentiated from the pale, unlabeled cell profiles in the neuropil. BDA labeled dendrites were distinguished from BDA labeled terminals by the fact that the former contained microtubules and endoplasmic reticulum, and lacked vesicles. BDA labeled neurons were distinguished from glia, which also have electron dense cytoplasm, by the absence of glial filaments and inclusions in the neurons, and their lack of the heterochromatin pattern characteristic of glial nuclei.

In the ipsilateral SC, BDA labeled terminals and dendrites were seen in synaptic contact. For example, Fig. 9a, b shows terminals that are BDA labeled (At⁺), as indicated by comparison to an adjacent unlabeled terminal (At). They make symmetric contacts with BDA labeled dendrites (Den*). Possible contacts of this type were also encountered in contralateral SGI. Figure 9c, d shows semiserial sections taken from contralateral SGI. The terminal contacting (arrowhead) the dendrite is clearly BDA labeled. The postsynaptic element appears to be lightly labeled with BDA reaction product, which would correlate with LM findings that the small number of cells labeled with BDA on the contralateral side had a lighter appearance (Fig. 2d). In this case, the contact was judged to be symmetric in nature. This example is from BDA/GABA double labeled material. The BDA labeled terminal is GABA⁺, while BDA labeled post-synaptic element is GABA⁻ (Fig. 9c, d). The presence of gold particles over GABA⁺ terminals (At*⁺) in semiserial sections provides added confidence that our designation of their GABAergic nature was not due to random variations in the immunohistochemistry.

Quantitative analysis

A total of 119 BDA labeled terminals in SGI of the ipsilateral SC were photographed from both the single labeled (only BDA labeled) and double labeled (both BDA and GABA labeled) material obtained from three cases. Thirty one of 119 had evident synapses. Only one (3.2%) was observed to synaptically contact a somatic profile. The sizes (short axes) of the dendritic profiles postsynaptic to these synaptic terminals varied between 0.4 and 5.1 μm . They can be divided into two categories. The large dendrite profiles, presumably the proximal dendrites, had short axes longer than 4.0 μm . The profiles with short axes <4.0 μm were presumed to be more distal dendrites or spines. In our samples ($n = 30$), 16.7% (5) of BDA labeled terminals contacted proximal dendritic profiles, and 80.3% (25) of terminals synapsed on smaller profiles, either distal dendrites or spines.

Following GABA postembedding immunostaining, a total of 76 BDA labeled terminals in SGI of the ipsilateral SC were collected from the micrographs. The histogram in Fig. 10a illustrates the percentage of the terminals in the three categories described above (GABA⁺, intermediate and GABA⁻). Of these, 94.7% (72) were found to be GABA⁺. Just 5.3% (4) fell into the

intermediate category. None was GABA⁻. There was some diversity in the ipsilateral results from the three cases (see Table 1). All of the profiles that were clearly postsynaptic to BDA labeled terminals were exclusively GABA⁻. Most, but not all, of the cellular profiles observed adjacent to, but not in contact with the ipsilateral BDA labeled terminals, were GABA⁻.

A total of 63 BDA labeled terminals were collected from the contralateral SGI. Among them, 50 were from the double labeled tissue. As the Fig. 10b histogram clearly illustrates, the percentage of the BDA labeled terminals in the three categories was quite different from that seen ipsilaterally. Half (25) were judged to be GABA⁺, 14.0% (7) fell into the intermediate category, and 36.0% (18) were judged to be GABA⁻. BDA labeled terminals judged to be both GABA⁺ and GABA⁻ were observed in each of three cases, but there was some diversity in the contralateral results (see Table 1). Contralaterally, all the profiles synaptically contacted by BDA labeled terminals were GABA⁻, and most, but not all, of those found adjacent to, but not contacted by BDA labeled terminals, were GABA⁻.

Discussion

The study's results indicate that the vast majority of cMRF projections to the ipsilateral intermediate gray layer of the SC are GABAergic. These ipsilateral cMRF projections contact non-GABAergic targets, some of which are the tectoreticular neurons supplying the cMRF with input. In contrast, the cMRF sends both GABAergic and non-GABAergic projections to the contralateral SGI. The crossed projections also generally contact non-GABAergic targets. SGI receives major excitatory inputs from the frontal and parietal eye fields (Fries 1984; Leichnetz 1982; Stanton et al. 1988; Harting et al. 1992), and the cerebellum (May et al. 1990), as well as inhibitory ones from the substantia nigra (Beckstead 1983; May and Hall 1986) and zona incerta (May et al. 1997; May 2006), that likely play important roles in defining its pattern of activity. As Fig. 11 illustrates, SGI also receives a major input from the cMRF. It is inhibitory, ipsilaterally, and mixed, contralaterally. These findings were surprising, as we had expected that the cMRF, which plays a role in contraversive horizontal saccades (Cohen et al. 1985; Waitzman et al. 1996, 2000), would excite the ipsilateral SC and either directly or indirectly inhibit the contralateral SC.

Technical considerations

For the sake of brevity, we refer the reader to Zhou et al. (2008), where the possibility of artifactual labeling of terminals in the SC by BDA injections of the cMRF is discussed in detail. The possibility of false-positive or false-negative GABA labeling exists. Appropriate antibody concentrations were obtained from multiple trials, to avoid false-positive results from too high a concentration, and false negatives due to insufficient labeling. Because GABA is an intermediate metabolic product of glutamate, and serves as an energy source through the Krebs cycle, it can also be found in non-GABAergic neurons. However, far greater amounts are present in GABAergic neurons. In addition, due to the close relationship of the GABA shunt and Krebs cycle, GABA is prone to be accumulated in the mitochondria, but this is again more prominent in cells that utilized GABA as a neurotransmitter (Gibson and Danel 2007). Previous studies have used retinotectal or corticothalamic terminals known to be GABA negative to establish baseline values (Mize and Butler 1996; Patel and Bickford 1997; Boka et al. 2006). Because we did not have that option, in that corticotectal terminals in SGI cannot be specified on ultrastructural grounds, we measured the gold particle density over myelin sheaths, which should not be GABA⁺, for use as a baseline.

Sampling bias cannot be completely excluded. First, sampling areas were not distributed evenly in SGI. The areas with denser BDA labeling were usually sampled to improve chances of finding BDA labeled terminals under EM. Thus, we may have missed any heterogeneity that is present in less densely labeled parts of SGI. In addition, samples were not obtained from the

rostral end of SGI, to avoid the pretectum. Consequently, this study does not address the question of whether the cMRF projections to the vertical meridian and fixation zone exhibit different features (Munoz and Wurtz 1993; Olivier et al. 1998; Sparks et al. 1976). The sample size ($n = 3$) was relatively small, which may weaken our quantitative results, but is unlikely to change the overall features observed. There was some inter-animal variation in the results, e.g. the larger number of intermediate class reticulotectal terminals on the ipsilateral side in case 3. These variations might be due to differences in the injection placement, extent, or spread outside the cMRF. Furthermore, if the cMRF displays regional differences (Zhou et al. 2008; Warren et al. 2008a) then it is possible that injections at cMRF sites that are not centrally located could result in findings different from those shown here.

Ultrastructure of reticulotectal terminals

The ultrastructure of axonal terminals in the SC has been studied in rat (Boka et al. 2006), cat (Norita 1980; Behan et al. 1987) and monkey (Mize et al. 1991). Using cats, Norita (1980) divided the terminals in the neuropil of the deep SC into seven categories, based on the shape of their vesicles and their postsynaptic profiles. BDA labeled terminals found in our study were similar to Norita's type III terminals, which were most numerous in the deep layers of the SC. Behan et al. (1987) observed the ultrastructure of nigrotectal terminals and their postsynaptic targets in cat SGI. Most of the nigrotectal terminals were packed with pleomorphic vesicles and formed symmetrical synaptic contacts. These nigrotectal terminals predominately (more than 90%) contacted small profiles, presumably distal dendrites. The cMRF reticulotectal terminals we observed in monkey SGI have many similarities to the cat nigrotectal terminals: pleomorphic vesicles, symmetrical synaptic contacts and distal dendrite target preference.

Mize et al. (1991) investigated the distribution, morphology and ultrastructure of GABA immunoreactive elements in the SC of the rhesus monkey (*Macaca mulatta*). They found that GABAergic profiles in both SGS and SGI contained numerous flattened vesicles, and formed symmetric synapses, but GABAergic terminals in deeper layers were more variable in morphology, and included more large terminals, many of which made synapses onto somata or large proximal dendrites. Our findings showed that most (67.7%) of the cMRF's reticulotectal terminals contact distal dendritic profiles with smaller diameters. This suggests that the reticulotectal projection targets more peripheral parts of the tectal neuron dendritic tree than other GABA⁺ inputs. This distribution of cMRF terminals implies that its GABA⁺ input may be of a modulatory, as opposed to driving, nature (Reichova and Sherman 2004).

Mize et al. (1994) also examined the ultrastructure of GABAergic synapses in the rabbit SC via postembedding immunocytochemistry. Three distinct types of vesicle-containing profiles (PSD, H and F) were labeled by the GABA antibody, with each type containing different concentrations of GABA. In the present study, the BDA labeled, GABA⁺ terminals were moderately packed with small, clear vesicles that were pleomorphic in shape. They also displayed a high density of gold particles, reflecting their GABA concentration. We believe these reticulotectal terminals are very similar to Mize's F-type profiles, based on the density of GABA staining and the packing density of the vesicles. The vesicles in our material were not as flattened as their F-type profiles, perhaps because they used a higher osmolarity perfusion fixative (5% glutaraldehyde). It is noteworthy that Mize et al. suggest that F-type profiles are morphologically similar to Guillery's F1 profiles in the cat dorsal lateral geniculate (Wang et al. 2001), which are believed to provide a recurrent, feedback inhibition, the same function these cMRF terminals appear to subserve.

GABAergic inputs to SC

The neurotransmitter, GABA, is believed to play a role in tectal eye movement control, since GABA agonists and antagonists can alter the firing pattern of neurons in the deep collicular

layers. Specifically, Hikosaka and Wurtz (1983) found that injection of muscimol, a GABA_A agonist, into the SC of awake, behaving monkeys altered saccades to visual targets, resulting in longer latencies and decreased amplitudes. In contrast, when a GABA antagonist, bicuculline, was used, irrepressible saccades toward the movement field of the injection loci were induced (Hikosaka and Wurtz 1985a, b). They also found that iontophoresis of muscimol into the substantia nigra has an effect similar to bicuculline in the SC (Hikosaka and Wurtz 1985b). Presumably, this disrupted the two-neuron inhibitory basal ganglia pathway, in which the second leg is the nigrotectal projection (Ficalora and Mize 1989; Behan et al. 1987). However, saccades induced by bicuculline placed in the SC differ subtly from those induced by muscimol injections in the substantia nigra. This difference suggests that other sources of GABAergic synapses in SC, in addition to nigral ones, influence saccadic activity. Studies combining immunohistochemistry and retrograde tracer staining indicate that zona incerta and the midbrain reticular formation are also extrinsic sources of GABAergic input to the feline SC (Araki et al. 1984; Ficalora and Mize 1989; Appell and Behan 1990). Furthermore, small GABAergic intrinsic neurons are present within the deeper layers of the SC (Mize 1988). Each of these GABAergic inputs to SGI neurons is likely to serve a different function with respect to tectal eye movement control.

In the present study of SGI, virtually all the terminals in the SC arising from ipsilateral cMRF were GABAergic. Their postsynaptic targets were exclusively non-GABAergic, and among these GABA⁻ postsynaptic profiles a portion were shown to be predorsal bundle neurons, the output of the SC to brainstem gaze centers (Fig. 11). The latter finding confirms the suggestions of Zhou et al. (2008). Interestingly, this pattern differs from that of the substantia nigra and cortex. Not only do nigral neurons suppress predorsal bundle neurons (May and Hall 1984; Karabelas and Moschovakis 1985; Bickford and Hall 1992), they also inhibit the local GABAergic interneurons in the SC (Kaneda et al. 2008). Similarly, cortical inputs access both output neurons and interneurons (Fuentes-Santamaria et al. 2008). In contrast, the cMRF does not appear to modulate inhibitory local circuits in SGI, since we did not observe BDA labeled cMRF terminals contacting GABA⁺ postsynaptic dendrites (PsDs). However, we cannot rule out the possibility that this happens on a limited basis.

Role of the ipsilateral tecto-reticulo-tectal loop

Because SGI contains most of the monkey's gazed-related output neurons, it is probable that inputs to SGI are key factors in promoting the transformation of sensory signal patterns into a gaze-related output (May and Hall 1984; May 2006; Moschovakis et al. 1998). Furthermore, during each saccade, the current motor signal is compared with the desired one and, if necessary, refined so that future movements are more accurate. Owing to the cMRF's inputs to SGI (Cohen and Büttner-Ennever 1984; Chen and May 2000; May et al. 2002) and possible afferent inputs from downstream regions like raphe interpositus (Langer and Kaneko 1983) and the paramedian pontine reticular formation (Warren et al. 2008b), it has been suggested that it provides an ascending pathway for modulating tectal outputs with respect to saccade accuracy (Waitzman et al. 1991). However, the presence of numerous close appositions between tectal axons and cMRF reticulotectal neurons (Chen and May 2000) and their similar latencies for saccadic activity (Moschovakis et al. 1988b) suggests that the primary drive for cMRF reticulotectal cells is the SC. In the present study, a GABAergic, presumably inhibitory, projection was found to the ipsilateral side, which indicates that cMRF neurons driven by predorsal bundle input influence saccadic movements by inhibiting the burst of SGI neurons. This ipsilateral feedback loop suggests that SGI works through the cMRF to inhibit its own pattern of spatial activity, and/or terminate presaccadic bursts.

Although we have observed BDA labeled terminals contacting BDA labeled cells (Figs. 2, 8), the constraints of the techniques used in the present study preclude confirming whether an

individual cMRF neuron sends feedback to those specific SGI cells that activated it (Fig. 11, arrow 1). However, predorsal bundle neuron bursts generally last for 200 ms, and the cMRF reticulotectal inhibition would be turned on within a few milliseconds. When considering the timing of cMRF activity, it seems more likely that cMRF neurons suppress the activity of predorsal bundle neurons in SGI that do not provide them with input (Fig. 11, arrow 2). This would explain why not all of the BDA labeled SGI cells were associated with BDA labeled terminals, and synaptic contacts between labeled elements were challenging to find at the ultrastructural level. It has been proposed that when multiple visual stimuli are present (Fig. 11, SGS), SGI neurons within different loci of the motor map compete for control of the circuitry that initiates saccades (Sparks et al. 1987; Basso and Wurtz 1998; Moschovakis et al. 1998). Presumably inputs to SGI and local recurrent excitation increase the activity of neurons in the “winner” locus to a threshold for a command, while the other inputs and inhibitory interneurons suppress more distant cells that command undesired movements (Van Opstal and Van Gisbergen 1989; Arai et al. 1999). If it is true that individual cMRF neurons turn off those SGI tectoreticular neurons which do not drive them, the cMRF feedback could contribute to the winner-take-all system by which the SGI regulates the locus of activity in the collicular motor map, preventing spurious activation by SGS (Fig. 11, left X).

Finally, it is possible that the cMRF input to the SC acts as a switch between orienting and avoidance behaviors. There is evidence that the SC participates in both activities (Buckingham and Yeomans 1993; Redgrave et al. 1986). Only orienting-related activity has been recorded in the cMRF, but avoidance has not been strictly tested. Furthermore, because the cMRF projection is inhibitory, it is possible that it serves to inhibit avoidance circuits when an orienting response is to be made. However, the presence of predorsal bundle cells among the targets of the cMRF projections indicates that inhibiting avoidance circuits could not be its sole function.

Role of the crossed reticulotectal projections

Both of the previous monkey studies (Moschovakis et al. 1988b; Zhou et al. 2008) and our present data showed that the crossed reticulotectal projections were sparser than the ipsilateral ones, but nevertheless present. Retrograde data from injecting the SC on each side with different fluorescent tracers (Perkins et al. 2009) indicates that ipsilateral, crossed and bilaterally projecting cMRF cells are also present in cats. Similarly, intra-axonal staining shows that most cMRF reticulotectal axons (80%) issue contralateral collaterals, which cross in the intertectal commissure and ramify in SGI (Moschovakis et al. 1988b). Based on our ipsilateral data, we presume that the bilaterally projecting cells are GABAergic. Given that the two sides of SC act as antagonists for the horizontal component of gaze, when one side is activated for a saccade, the other side should be suppressed (Fig. 11, shading). Under these conditions, it would be expected that the cMRF neurons, which seem to primarily code for horizontal components, should have opposing effects on each side. Thus, we expected that the bilaterally projecting axons would target inhibitory interneurons on one side of the SC. However, our data indicate that GABAergic interneurons are not an important target of either projection. Instead, the heterogeneity of the crossed cMRF projection found here suggests that it has a more complex function.

Tectotectal commissural connections also contain both excitatory and inhibitory elements (Behan 1985; Olivier et al. 2000). Inhibitory tectotectal projections mediate mutual suppression between two SCs, to prevent competing presaccadic activity during horizontal conjugate gaze, while excitatory commissural fibers may yoke activity in the two SCs for vertical saccades or fixation (Behan 1985; Moschovakis and Karabelas 1985; Peck 1990; Munoz and Istvan 1998). In cats, commissural excitation and inhibition display mirror-symmetric fields in the rostral SC (Takahashi et al. 2007), with the excitatory and inhibitory axons projecting to

function-specific areas, the fixation zone and saccadic zones, respectively (Takahashi et al. 2005). However, in the present study, both GABAergic and non-GABAergic terminals were found within the grids cut from each tissue sample. This suggests that a simple dichotomy of excitation and inhibition with respect to saccades versus fixation or horizontal versus vertical gaze is not present, and that the crossed cMRF projection has a different role. Presumably, the cMRF's crossed GABAergic projections, such as the crossed tectal (above) and nigral projection (Jiang et al. 2003), suppress predorsal bundle cells in contralateral SGI (Fig. 11, arrow 3), precluding access to SGI by activity in the overlying SGS (Fig. 11, right X).

Although there is no direct evidence which shows how the cMRF affects the contralateral SC, the results of physiological studies (Mays and Sparks 1980) imply that crossed projections may be needed to generate visually guided saccadic discharges in SC. In double-saccade trials, Mays and Sparks (1980) used two visual targets on a horizontal line in the monkey's left visual field to activate two different neuron populations, corresponding to the targets' positions, within the visual map of right SGS. However, since the first saccade was of greater amplitude, the second saccade was to the right, not the left. Saccade-related neurons in the motor map of the right SGI discharged before the first (leftward) saccade, while saccade-related neurons in the left SGI fired before the second (rightward) saccade. Their results suggest that in visually guided saccades, the linkage between the superficial layers and the deeper layers of SC is not necessary for the activation of saccade-related neurons, and a crossed signal combining retinal error and eye position information is needed. The frontal eye field (FEF) could be a possible origin of the signals in the other SC, since the two FEFs are tightly coupled via the corpus callosum (Pierrot-Deseilligny et al. 1987). However, based on our findings in the present study, the right cMRF could also contribute to the above task by suppressing the right SC and exciting components of the left SC (Fig. 11, arrow 4) based on the pattern of activity produced in it by the first saccade; such a cMRF input to collicular quasivisual cells has, in fact, been suggested in models of this system (Bozis and Moschovakis 1998; Mays and Sparks 1980).

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Fig. 1. Distribution of anterogradely labeled reticulotectal axonal terminals (*stipple*) and retrogradely labeled tectoreticular neurons (*dots*) following a BDA injection into monkey cMRF. **a** A small BDA injection site is shown within cMRF. **b-i** In the midbrain, BDA labeled terminals were distributed the superior colliculus (SC), especially the intermediate gray layer (SGI), and the periaqueductal gray (PAG). BDA labeled neurons are seen bilaterally in the SC (**b-i**). Small windows in figure **c, e, g** and **i** demonstrate the areas where samples were taken for EM. IC inferior colliculus, SGP stratum griseum profundum, SO stratum opticum, SGS stratum griseum superficiale, LGB lateral geniculate body, MD medial dorsal nucleus, PN pontine nuclei. Scale bar 2 mm

Fig. 2.

Photomicrographs of BDA labeled terminals and retrogradely labeled tectoreticular cells within the ipsilateral and contralateral superior colliculi in the macaque monkey. **a** Labeled tectoreticular neuron (*arrow*) in ipsilateral SGI. Note the homogeneous labeling of its cytoplasm. The BDA labeled reticulotectal axons exhibit numerous en passant boutons along their course. Close associations (*arrowheads*) were present between the labeled tectoreticular dendrites and the labeled reticulotectal axon terminals. **b** Higher magnification of close associations indicated by frame in **a**. **c** When compared with the ipsilateral SGI, the neuropil in the contralateral SC exhibits fewer labeled reticulotectal axons and boutons (*arrowheads*). **d** A BDA labeled tectoreticular neuron (*arrow*) in contralateral SGI shows less reaction product in its cytoplasm. BDA labeled boutons (*arrowheads*) of passing reticulotectal axons are arranged in close association with its dendrites. Scale bar in **a** for **a**, **c**, **d** = 20 μ m, scale bar in **b** = 5 μ m

Fig. 3. Electron micrographs of BDA labeled axon terminals in ipsilateral SGI. **a–d** All from material processed for BDA only. BDA labeled terminals (*At**) make symmetric synapses (*arrowheads*) with non-labeled dendrites (*Den*) (**c, d**) or putative spines (*Sp*) (**a**) or non-labeled somata (**b**). *At* unlabeled axon terminal in this and following figures. Scale bar 0.5 μ m

Fig. 4. GABA labeling in electron micrographs showing BDA labeled axon terminals in the ipsilateral SGI. BDA labeled terminals (At^*) are predominately GABA⁺ (At^{*+}), as shown in **a–c**. They contact (*arrowhead*) GABA[−] dendrites (*Den*). **d** A BDA labeled terminal contacting a small profile which is a putative dendritic spines (*Sp*). Based on the gold particle density, this terminal is categorized in the intermediate, non-classified category ($At^{*+/-}$). Ax axon. Scale bar 0.5 μ m

Fig. 5. Electron micrographs of semiserial sections through a BDA labeled, GABA⁺ cMRF terminal. **a, b** Two semiserial sections (spaced 100 nm apart) through a terminal in ipsilateral SGI. The terminal has a dark cytoplasm, due to BDA labeling, and numerous gold particles, indicating it is GABA⁺. It lies adjacent to an unlabeled soma. *Scale bar 0.5 μ m*

Fig. 6. BDA labeled axon terminals in contralateral SGI from material processed for BDA only. **a** A BDA labeled terminal (*At**) containing pleomorphic vesicles making a symmetric synapse (*arrowhead*) onto a dendritic profile (*Den*). **b** A BDA labeled preterminal axonal profile (*Ax**) continuing into a BDA labeled terminal (*At**). This terminal contains spherical vesicles and forms an asymmetric synapse (*arrowhead*) onto a dendritic profile (*Den*). Scale bar 0.5 μ m

Fig. 7.

BDA labeled, GABA⁺ axon terminals in contralateral SGI. Most BDA labeled, crossed reticulotectal terminals were GABA⁺ (*At*⁺), like those shown in **a–d**. The BDA labeled reticulotectal terminal in **b** contacts (*arrowhead*) two distal dendrites, one of which is also contacted by a BDA[−]/GABA[−] terminal (*At*). The other examples of BDA labeled, GABAergic terminals appose a GABA[−] soma (**a**) and dendrite (*Den* **c**). In **d**, the BDA labeled, GABA⁺ terminal lies adjacent to GABA⁺ profiles that appear to be presynaptic dendrites (*PsD*⁺), but no synaptic contacts are observable. *Ax* axon, *At*⁺ GABA⁺ terminal. *Scale bar* 0.5 μ m

Fig. 8. BDA labeled, GABA⁻ axon terminals in the contralateral SGI. A minority of the BDA labeled terminals were GABA⁻ (*At**), like those shown in **a–d**. **a** An asymmetric synapse (arrowheads) between a BDA labeled, GABA⁻ terminal and a GABA⁻ dendrite. The BDA labeled, GABA⁻ terminal in **b** apposes a GABA⁻ soma and contacts its dendrite (*Den*). The BDA labeled, GABA⁻ terminal in **d** is only associated with a GABA⁻ dendrite (*Den*), no contact is visible. **c** An example of a BDA labeled terminal that fell into the intermediate category (*At+/-*). *Ax* axon. Scale bar 0.5 μ m

Fig. 9. BDA labeled terminals contact (*arrowheads a–d*) BDA labeled dendrites. **a, b** BDA single label material in ipsilateral SGI. The BDA labeled terminals (*At**) contact the BDA labeled dendrites (*Den**) with symmetric synapses (*arrowheads*), **c, d** are from double label material cut from contralateral SGI. These examples are semiserial sections through a BDA labeled, GABA⁺ cMRF terminal (*At*+*), which contacts a dendritic profile with a symmetric synapse. The dendrite appears to be lightly labeled with BDA reaction product (*Den**), and it is contacted by other GABA⁺ terminals (*At+*), **c, d** are spaced 200 nm apart. *Scale bar* 0.5 μ m

Fig. 10.

Histogram of percentage of reticulotectal axonal terminals that are GABA⁺ or GABA⁻ in SGI ($n = 3$); **a** greater than 90% of the BDA labeled reticulotectal axonal terminal profiles within the ipsilateral SGI are GABA⁺, and about 5% of the terminal profiles fall into the intermediate category, but none are GABA⁻. **b** Half of the BDA labeled reticulotectal axonal terminal profiles within the contralateral SGI are GABA⁺, while 36% of them are GABA⁻. The rest fall into the intermediate category

Fig. 11.

Schematic diagram of reciprocal connections of central mesencephalic reticular formation (cMRF) and intermediate gray layer (SGI) of superior colliculi. The predorsal bundle sends excitatory projections (*thick solid arrow*) to the horizontal gaze center (paramedian pontine reticular formation—PPRF) to activate horizontal saccades. Collaterals emitted before the predorsal bundle crosses the midline, provide excitatory input to the ipsilateral cMRF neurons. Inhibitory cMRF neurons (*gray hexagons*) issue reticulotectal axons (*dash arrow*) to the ipsilateral SC to either turn off the predorsal bundle cells that directly activated them (1), or suppress other predorsal bundle neurons (2), and keep them from being excited (X) by activity in the superficial gray layer (SGS). The cMRF neurons send both inhibitory (*dashed arrow*) and excitatory (*continuous arrow*) projections to the contralateral SGI. The inhibitory projection (3) presumably helps keep the contralateral SGS from exciting the contralateral SGI (X). The role of excitatory reticulotectal neurons (*pentagon*) that send a crossed projection to SGI (4) is unclear. *Gray shading* in SGI and cMRF indicates this side is suppressed during rightward saccades

Table 1

Observed classification of terminals in each of the three cases

	GABA-positive terminals	Non-classified terminals	GABA-negative terminals
Ipsilateral SGI			
Case 1	98.3% (57)	1.7% (1)	0
Case 2	100.0% (9)	0	0
Case 3	66.7% (6)	33.3% (3)	0
Contralateral SGI			
Case 1	42.3% (11)	15.4% (4)	42.3% (11)
Case 2	50.0% (3)	0	50.0% (3)
Case 3	61.1% (11)	16.7% (3)	22.2% (4)

Actual numbers indicated in parentheses