Two Distinct Populations of Tectal Neurons Have Unique Connections Within the Retinotectorotundal Pathway of the Pigeon (Columba livia)

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ABSTRACT

The tectofugal pathway is a massive ascending polysynaptic pathway from the tectum to the thalamus and then to the telencephalon. In birds, the initial component of this pathway is known as the tectorotundal pathway; in mammals, it is known as the tectopulvinar pathway. The avian tectorotundal pathway is highly developed; thus, it provides a particularly appropriate model for exploring the fundamental properties of this system in all amniotes. To further define the connectivity of the tectorotundal projections of the tectofugal pathway, we injected cholera toxin B fragment into various rotundal divisions, the tectobulbar projection, and the ventral supraoptic decussation of the pigeon. We found intense bilateral retrograde labeling of neurons that stratified within layer 13 and, in certain cases, granular staining in layer 5b of the optic tectum. Based on these results, we propose that there are two distinct types of layer 13 neurons that project to the rotundus: 1) type I neurons, which are found in the outer sublamina of layer 13 (closer to layer 12) and which project to the anterior and centralis rotundal divisions, and 2) type II neurons, which are found in the inner sublamina of layer 13 (closer to layer 14) and which project to the posterior and triangularis rotundal divisions. Only the labeling of type I neurons produced the granular dendritic staining in layer 5b. An additional type of tectal neuron was also found that projected to the tectobulbar system. We then injected Phaseolus vulgaris-leucoagglutinin in the optic tract and found that the retinal axons terminating within tectal layer 5b formed narrow radial arbors (7–10 µm in diameter) that were confined to layer 5b. Based on these results, we propose that these axons are derived from a population of small retinal ganglion cells (4.5–6.0 µm in diameter) that terminate on the distal dendrites of type I neurons.

This study strongly indicated the presence of a major bilateral oligosynaptic retinotectorotundal pathway arising from small retinal ganglion cells projecting to the rotundus with only a single intervening tectal neuron, the proposed type I neuron. We suggest that a similar organization of retinotectopulvinar connections exist in reptiles and in many mammals.

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The tectofugal pathway, which is a massive ascending polysynaptic pathway to the telencephalon, is first described in birds by Karten and Revzin (1966). A similar pathway is described by Diamond in tree shrews, insectivorous rodents, and primates (Diamond, 1973). Subsequent studies by Hall and Ebner (1970) and Pritz (1975) in reptiles as well as by Northcutt and Butler (1980) in teleost fishes indicate that this major pathway is an ancient route for conducting visual information into the vertebrate telencephalon. The highly developed avian tectofugal pathway provides a particularly appropriate model for exploring the fundamental properties of this system in all amniotes. The central origin of this pathway and the main retinorecipient structure in the brain of most nonmammalian vertebrates is the optic tectum.
In birds, the optic tectum is a complex, topographically organized structure that receives approximately 90% of the retinal ganglion cell projections from the contralateral retina. The retinotectal projection is formed by the axons of various subpopulations of retinal ganglion cells, distinguishable by conduction velocities (Mpodozis et al., 1995), morphology, neurotransmitters, and neural-related molecular contents (Karten et al., 1990). These retinal axons arborize at five different levels, in layers 2, 3, 4, 5, and 7, of the retinorecipient zone (Ramón y Cajal, 1911). Based on the morphology of single axonal arborizations, Ramón y Cajal (1911) suggested that each of the retinorecipient layers contains terminals of a single specific type of ganglion cell, a suggestion that has been partially confirmed by modern studies (Lettvin et al., 1959; Ehrlich et al., 1987; Britto et al., 1994). In addition to the retinal inputs, the tectum also receives massive inputs from other structures located in the forebrain, the thalamus, the pretectal region, and the midbrain, each exhibiting a definite pattern of arborization within specific tectal layers (Ramón y Cajal, 1911; Brecha, 1978).

Efferent tectal projections arise from neurons in layers 9–14 and target such structures as the isthmic nuclei, the lateral pons, some of the nuclei of the dorsal and ventral pretectal complex, and various visual nuclei of the thalamus (Ramón y Cajal, 1911), including the nucleus rotundus. Various studies have demonstrated that the tectotectal projections are bilateral, are much denser ipsilaterally than contralaterally (Hunt and Kü nzle, 1976), and have no obvious topographical organization (Benowitz and Karten, 1976). Benowitz and Karten also demonstrated that these projections arise from neurons located in layer 13, the stratum griseum centrale (SGC), of the optic tectum.

The SGC is a distinctive, multistratified structure formed by neurons of roughly homogeneous size (ca. 20 µm cell body diameter). Dendrites of SGC neurons extend broadly to more superficial tectal layers and have been seen to reach the retinorecipient layer 5b (Ramón y Cajal, 1911; Hunt and Künzle, 1976). Physiological studies using intracellular recordings (Hardy et al., 1984, 1985) have shown that some SGC neurons receive a monosynaptic input, whereas others receive a polysynaptic input from the retina. The axons of SGC neurons run ventrally to form the tectothalamic tract and decussate at the level of the dorsal supraoptic decussation. These axons also provide ipsilateral inputs to the intrinsic nuclei of the tectothalamic tract (Tomboł et al., 1994; Mpodozis et al., 1996). Furthermore, the SGC is the sole tectal source of inputs to the rotundus (Mpodozis et al., 1996).

In addition to the projections from the SGC, the rotundus, which is a large, discrete group of neurons located in the thalamus of the pigeon, also receives γ-aminobutyric acid (GABA) inputs from both the intrinsic nuclei of the tectothalamic tract and the thalamic reticular formation (Benowitz and Karten, 1976; Mpodozis et al., 1996). The rotundus projects exclusively to the ectostriatum of the ipsilateral telencephalon (Karten and Hodos, 1970). In our previous report (Mpodozis et al., 1996), we outline a simplified scheme of four divisions within the rotundus: anterior, centralis, posterior, and triangularis (Fig. 1). The anterior and centralis divisions can be subdivided further (Benowitz and Karten, 1976; Martinez-de-la-Torre et al., 1990; Mpodozis and Karten, unpublished observations). For the purpose of the present study, however, we will concentrate only on the four main divisions for our analysis of the tectofugal pathway.

To further define the functional organization of the tectofugal pathway, we began a series of studies of the anatomical organization of this pathway by using more sensitive techniques than were available previously, including use of the tracers Phaseolus vulgaris-leucoagglutinin (PHA-L) and cholera toxin B subunit (CTb). In our previous report (Mpodozis et al., 1996), we demonstrated that the tectotectal projection is composed of two different pathways: one direct, bilateral, and presumably excitatory and the other indirect, ipsilateral, and presumably inhibitory. The direct pathway arises from tectal neurons of...
layer 13 and provides inputs to all divisions of the rotundus, both ipsilaterally and contralaterally. The indirect pathway arises from collaterals of the layer 13 neurons en route to the rotundus. These collaterals end ipsilaterally in the intrinsic GABAergic nuclei of the tectothalamic tract. The neurons of these nuclei, in turn, terminate in distinct divisions of the ipsilateral rotundus, such that different divisions of the rotundus receive inputs from different components of the intrinsic nuclear complex of the tectothalamic tract.

The present study demonstrates that these tectorotundal projections arise from what we propose are two distinct classes of layer 13 tectal neurons. Each class of layer 13 neuron is the origin of a direct and an indirect projection to different divisions of the rotundus. Type I neurons, which lie within the outer sublamina of layer 13 (the sublamina closer to layer 12), project bilaterally to the anterior and central divisions of the rotundus and are associated with dendritic staining in layer 5b. Type II neurons lie within the inner sublamina of layer 13 (near layer 14), project bilaterally to the posterior and triangularis divisions of the rotundus, and lack any associated dendritic staining in layer 5b.

Our study also provides a preliminary characterization of a specific category of retinal ganglion cells that terminate in layer 5b and their morphological pattern of axonal termination. These studies indicated that there is a far more direct impact of retinal afferentation on rotundal neurons than was appreciated previously. Some preliminary results from this study have appeared in abstract form (Karten et al., 1993).

**MATERIALS AND METHODS**

White Carneau pigeons of either sex were used in this study and were obtained from either the Palmetto Pigeon Plant (Sumter, SC), or Bowman Gray School of Medicine (Winston Salem, NC). All surgical procedures performed on these animals were approved by the Animal Care Committee of the University of California, San Diego, and conformed to the guidelines of the National Institutes of Health on Use of Experimental Animals in Research.

Pigeons were anesthetized with a combination of ketamine (40 mg/kg) and xylazine (12 mg/kg) injected intramuscularly. A single dose usually proved satisfactory for the duration of the surgical procedure. If necessary, a supplementary injection of anesthetic was administered.

Once the animal was anesthetized, the feathers over the skull were removed, and the scalp was washed with an antiseptic solution. Animals were placed in a stereotaxic holder, the skull was exposed, and a hole was made through the skull above the approximate location of the proposed injection. Tracer injections into various divisions of the rotundus, the tectobulbar projection, the ventral supraoptic decussation, and the optic tract were achieved by using the stereotaxic coordinates of Karten and Hodos (1967). Intracocular injections as well as those into the optic tectum were made under direct visual control. To enhance the accuracy of some of the placements of the tracers, electrophysiological responses were monitored. Either a 1% solution (by weight) of CTb (List Laboratories, Campbell, CA) was injected by using manual direct pressure or a PicoSpritzer II pressure system (General Valve, Fairfield, NJ), or a 2.5% solution of PHA-L (Vector Laboratories, Burlingame, CA) was deposited iontophoretically by using a constant-current device (Midgard Electronics, Cambridge, MA; Gerfen and Sawchenko, 1983; for a detailed review of these procedures, see Mpodozis et al., 1996).

In 4–10 days, depending on the tracer employed, the animals were anesthetized with an overdose of ketamine and xylazine and were perfused through the aorta with 500–800 ml of a 0.9% saline solution, followed by 1,000 ml of an ice-cold solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), employing a peristaltic perfusion pump to maintain constant perfusion pressure. Following the perfusion, the brains were removed from the skull and postfixed for 6–14 hours in the paraformaldehyde solution and were then transferred to a 30% sucrose solution made in PB for cryoprotection before cutting on a sliding microscope. The brains were cut into 30-µm-thick sections in either the horizontal, sagittal, or coronal plane.

Following a 30-minute wash in phosphate-buffered saline, the tissue was incubated in either goat anti-CTb (1:15,000; List Laboratories) or rabbit anti-PHA-L (1:10,000; Vector Laboratories), depending on the tracer injected. The tissue was then processed by using the avidin-biotin-peroxidase method (ABC Elite kit; Vector Laboratories; for a more detailed description of these immunohistochemical procedures, see Mpodozis et al., 1996). The tissue was mounted on gelatinized slides and allowed to dry. The tissue was then either stained with 0.04% osmium tetroxide for 10–20 seconds or counterstained with Giemsa stain (Iñiguez et al., 1985), dehydrated, cleared, and coverslipped by using Permount.

Data analysis was performed by using a matched series of sections immunoreacted against CTb. One series was Osmicmed, and the other series was counterstained with Giemsa. The two series were compared, and selected sections from each were drawn by using a macroprojector. The distribution of axonal processes was evaluated frequently by using darkfield microscopy. Labeled neurons and axons were plotted on these drawings, and the resultant drawings were entered directly into a Mac IIx data file using a Hewlett-Packard desk-top scanner. The scanned drawing was traced by using Canvas (Deneba Corp., Miami, FL). Additional sections were charted by using Neurulcida, a computer-controlled, motorized stage and software system (MicroBrightField Inc., Colchester, VT).

Video images of neurons with nonoverlapping fields of adjacent neuronal bodies were captured by using a Dage-MTI 725 monochrom CCD camera (Dage-MTI, Inc., Michigan City, IN), digitized by using a Scion LG-3 (Scion Corp., Frederick, MD), and displayed on a color monitor. The dimensions of labeled neurons in layer 13 were measured by using the processing program by Wayne Rasband, NIH Image, on a Macintosh PowerPC.

Photographs for figures are digital images that were captured by using a Leaf Lumina scanning CCD camera (Leaf System Inc., Southborough, MA) mounted on a Nikon Microphot FX photomicroscope and collected directly into Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) by using a Macintosh PowerPC. Images were collected as RGB files, converted to gray scale images, and brightness and contrast were adjusted to provide the resultant final printed images. No additional digital filtration or image modification was performed. Final
prints were prepared by using a Kodak (Rochester, NY) XLS 8600 PS printer.

RESULTS

Tectal afferents to rotundus

To study the tectorotundal projections, CTb was injected into various divisions of the rotundus of ten pigeons: anterior (two cases), centralis (dorsalis and ventralis; two cases), posterior (one case), and triangularis (one case). The remaining cases involved injections into more than one division: dorsal anterior and centralis (one case), centralis and posterior (one case), posterior and triangularis (one case), and posterior and caudal surround (one case). Tracers were also injected into the tectobulbar projection (two cases), the ventral supraoptic decussation (one case), the optic tract (three cases), the optic tectum (four cases), and the eye (two cases). Some representative injection sites are shown in Figure 2.

The nucleus rotundus lies in a region of the thalamus surrounded by other cell groups that are also in direct receipt of tectal projections, including the nucleus opticus principalis thalami (OPT; the avian homologue of the mammalian nucleus geniculatus lateralis pars dorsalis or GLd), which is located immediately dorsal to the rotundus; the nucleus ventralis thalami (VLT), which is located posterior to the rotundus; the intergeniculate leaflet (IGL), which is located on the rostral and lateral aspects of the rotundus; and the nucleus geniculatus lateralis ventralis (GLv), which is located ventral to...
the rotenus. Therefore, we made CTb injections into each of these regions. We concluded that the tectal neurons projecting to the rotenus did not project to any of these regions. The laminar origins of tectal projections to each of these cell groups will be described in a subsequent report.

Following injections of CTb into either the anterior division or the centralis division of the rotenus, there was extensive bilateral retrograde CTb labeling of tectal neurons only within the outer sublamina of layer 13. Conversely, when the tracer was placed in the posterior or triangularis divisions of the rotenus, retrogradely CTb labeled neurons were confined to the inner sublamina of layer 13. Despite the regional variation in the relative thickness of each of these sublaminae (see below), there was no substantial overlap of the distribution of the layer 13 neurons projecting to the anterior or centralis divisions vs. the posterior or triangularis divisions of the rotenus (Figs. 3, 4). Labeled neurons in the outer sublamina of layer 13 were predominantly round or polygonal in shape (18 µm mean major axis, 13 µm mean minor axis), with their major axis running radially to the tectal surface. CTb-labeled neurons in the inner sublamina were mostly fusiform shape (19 µm mean major axis, 9 µm mean minor axis), with their major axis running parallel to the tectal surface. In all of the experimental cases, the number of ipsilateral vs. contralateral CTb-labeled neurons in layer 13 was approximately 2:1 (Fig. 3).

Fig. 3. Retrograde bilateral labeling of the optic tectum following unilateral injections of cholera toxin B subunit (CTb) into the centralis (A,B) or the triangularis (C,D) divisions of the rotenus. Digital images of the dorsolateral aspect of the optic tectum were taken at the level of the commissura tectalis of CTb-labeled, Giemsa-counterstained transverse sections. Compare the location of the retrogradely labeled neurons: CTb injections into the centralis (A,B) labeled neurons that were restricted to the outer sublamina of layer 13, whereas CTb injections into the triangularis (C,D) labeled neurons that were mostly confined to the inner sublamina of layer 13. An approximate 2:1 ratio of ipsilateral:contralateral labeled neurons can be noted in both instances. In the centralis injection, a fine granular dendritic staining was found in the full extent of layer 5b. Such staining in the ipsilateral side is more dense than in the contralateral side. There is no evidence of CTb labeling of layer 5 following CTb injections into the triangularis. The injection sites of each case are shown in Figure 2. For abbreviations, see list. Scale bar = 180 µm.
Dendritic immunoreactivity in layer 5b of the optic tectum

Intense dendritic staining of the processes of layer 13 neurons labeled following rotundal CTb injections was seen extending superficially to form a dense matrix in layers 9–12 (Figs. 3, 5). This was associated with the presence of retrograde CTb labeling of the inner and outer sublamina of layer 13. However, a band of granular material was seen in layer 5b of the optic tectum only following injections confined to the anterior or centralis divisions of the rotundus (Figs. 2–5). Although it was notably evident on the ipsilateral side, a somewhat lighter pattern of staining within layer 5b was also observed in the contralateral optic tectum (Fig. 3). In clear contrast, a CTb injection within the posterior or triangularis rotundus resulted in dense CTb labeling of the inner sublamina of layer 13 but failed to produce evidence of such granular staining in layer 5b (Figs. 2, 3).

Thus, the granular staining was associated only with retrogradely CTb-labeled neurons in the outer sublamina of layer 13. This did not represent a projection of the rotundal neurons to the tectum. This study repeatedly indicated that the rotundal neurons projected only to the ipsilateral telencephalon and not to the optic tectum. Similarly, injections of tracers in the various control sites, including OPT/GLd, GLv, VLT, and IGL, did not result in projections to layer 5b.

This was further supported by our finding that injections in the ventral supraoptic decussation resulted in an equal, bilateral CTb labeling of many neurons in both the inner and outer sublamina of layer 13 as well as an equally dense, bilateral, granular staining in layer 5b (Fig. 2). We found no evidence of retrograde CTb labeling of neurons in the rotundus or in the intrinsic nuclei of the tectothalamic tract. Thus, the granular staining in layer 5b is presumed to represent the distal dendrites of layer 13 neurons, as originally described by Ramon (1899), Ramón y Cajal (1911), and Hunt and Künzle (1976).

Regional variation of layer 5b and layer 13

The pattern of CTb labeling found in layer 5b following injections of CTb into the anterior or centralis rotundus showed a clear regional variation (Fig. 5). The overall width of layer 5b in the ventral tectum was threefold thicker than in the dorsal tectum (ca. 150 µm vs. 50 µm).
The transition point between the thinnest and thickest tectal regions was very abrupt and was located at the lateral margin of the tectum. This distribution matched exactly the pattern of retinal terminals in layer 5b that have been described previously (Gamlin et al., 1996) and that we observed following intraocular injections of CTb (Fig. 6).

Layer 13 also showed a clear ventrodorsal differentiation. The overall width of layer 13 in the ventral tectum was 1.3–1.6 times thicker than the dorsal tectum, but the width of the labeled zone diminished abruptly from the ventral to the dorsal tectum. Tectal layers located superficially to layer 5 showed no evidence of CTb labeling. Note that the CTb-labeled neurons in the outer sublamina of layer 13 form a dense plexus of dendritic processes that extend superficially. Note also the higher density of CTb-labeled neurons in the ventral tectum. Scale bar = 100 µm.

The transition point between the thinnest and thickest tectal regions was very abrupt and was located at the lateral margin of the tectum. This distribution matched exactly the pattern of retinal terminals in layer 5b that have been described previously (Gamlin et al., 1996) and that we observed following intraocular injections of CTb (Fig. 6).

Layer 13 also showed a clear ventrodorsal differentiation. The overall width of layer 13 in the ventral tectum was 1.3–1.6 times thicker than the dorsal tectum, depending on the rostrocaudal level of the analyzed section. This variation matched the differential distribution of the rotundal-projecting neurons located in the outer sublamina of layer 13 (Fig. 7). Following injections of CTb into the anterior or centralis divisions of the rotundus, CTb-labeled neurons were restricted to the outer lamina of layer 13, but CTb-labeled neurons in the ventral tectum were notably
more abundant and densely packed than those found in the dorsal tectum (Fig. 7). The outer sublamina of layer 13 in the ventral tectum was 2.0–2.5 times thicker than in the dorsal tectum, depending on the rostrocaudal level of the section. The inner sublamina of layer 13 showed an opposite but less marked dorsoventral polarity, with, at most, the dorsal tectum 1.4 times wider than the ventral tectum. CTb-labeled neurons in layer 13 following injections of CTb into the posterior or triangularis rotundus were distributed along the entire extent of the inner sublamina of layer 13 and showed a relatively higher density in the dorsal tectum.

In Fig. 6, regional variation of the contralateral retinorecipient layers of the optic tectum labeled following an intraocular injection of cholera toxin B subunit (CTb). Digital images of the dorsal (A), dorsolateral (B), and ventral (C) aspects of the optic tectum were taken at the level of the commissura tectalis of CTb-labeled, Giemsa-counterstained sections. Note that the width of layer 5b diminishes abruptly from the ventral to the dorsal tectum, whereas the width of layers 2, 3, and 4 show an opposite variation. The width of layer 7 remains constant in all tectal regions. Note also that, in the ventral tectum, layer 5b occupies the major proportion of the retinorecipient zone. StOp, stratum opticum. Scale bar = 50 µm.
µm, with outer and inner layer 13 sublamina widths of 260 µm and 140 µm, respectively. We concluded from these observations that the overall distribution of tectal neurons projecting to anterior and central rotundus (the proposed type I neurons) exhibited a close correlation with the distribution of layer 5b retinal terminals.

**Tectal neurons projecting to the anterior or central rotundus also collateralize on the nucleus pretectalis**

The nucleus pretectalis is found in the lateral pretectal region, lying on the dorso medial margin of the rostral optic tectum. The pretectalis contains a compact central region of neurons and a surrounding cell-free region (Gamlin et al., 1996). The pretectalis projects to the ipsilateral and contralateral tectum, with a dense field of terminals in layer 5b. Injections of CTb into the anterior or centralis rotundus resulted in axonal projections to the outer shell of the pretectalis (Figs. 2, 8). A similar but less dense projection was also found in the outer shell of the contralateral pretectalis. In contrast, injections of CTb into the posterior or triangularis rotundal divisions resulted in no evidence of connections to the pretectalis (Fig. 2). There was no indication of retrograde labeling of rotundal neurons. We therefore concluded that the axonal projections of the proposed type I neurons to the dorsal anterior or centralis rotundus also collateralized on the outer shell of the pretectalis.

**Tectorotundal neurons are distinct from tectobulbar neurons**

In the present study, we were readily able to distinguish subpopulations of tectal neurons projecting to the rotundus from those projecting downstream to the bulbar reticular formation and the tectobulbar projection. Following CTb injections into the tectobulbar projection (predorsal bundle) at the level of the pontine nuclei, retrogradely CTb-labeled tectal neurons were occasionally found at the margin between layers 13 and 14 but were more generally found in layer 14 and the underlying tegmentum (Fig. 4). These neurons were polygonal in shape and were consistently smaller than the neurons projecting to the rotundus (14 µm mean major axis). Furthermore, these neurons did not form a dense matrix of dendritic arborizations extending superficially and only rarely showed evidence of den-
Fig. 8. Retrograde labeling of the nucleus pretectalis (nPT) following unilateral cholera toxin B subunit (CTb) injections into the centralis or the triangularis divisions of the rotundus. Digital images were taken from CTb-labeled, Giemsa-counterstained transverse sections. The centralis case shows a bilateral bundle of CTb-labeled axons that surround and extend into the pretectalis. The density of such CTb labeling in the ipsilateral side (A) is higher than in the contralateral side (B). In contrast, the CTb injection into the triangularis resulted in no label of axonal processes either inside or around the pretectalis (C, D). Neurons of the pretectalis were not retrogradely CTb labeled in any of these cases. Injection sites of each of these cases are shown in Figure 2. SpL, nucleus spiriformis lateralis. Scale bar = 300 µm.
dritic branches in the retinorecipient layers of the tectum. There was no evidence of axonal projections of these neurons to the rotundus, the intrinsic nuclei of the tectothalamic tract, or the pretectalis. Further support of this interpretation was derived from our analysis of the CTb injections into the ventral supraoptic decussation. Injections into the ventral supraoptic decussation resulted in retrograde CTb labeling of the neurons of layer 13 projecting to the rotundus as well as the labeling of the axonal projections of these neurons, as described above. In this instance, however, there was no indication of collateral axonal projections to the tectobulbar pathway or to the ipsilateral tectoreticular pathway. On the basis of this set of observations, we concluded that the output of the tectum to the rotundus is independent of that to the brainstem.

**Retinal inputs to layer 5b**

The observation of the many dendritic arborizations of the proposed layer 13 type I neurons in tectal layer 5b prompted us to investigate the nature of the retinal inputs to layer 5b. Therefore, we 1) injected the anterograde tracer PHA-L into the optic tract to examine the morphology of retinal axonal terminals in layer 5b and 2) injected CTb into either the dorsal or the ventral tectum to compare the features of the resulting retrogradely CTb-labeled retinal ganglion cell populations. The rationale for this latter experiment was that the marked increase in the thickness of layer 5b in the ventral tectum, accompanied by a corresponding reduction in the thickness of retinorecipient layers 2, 3, and 4 (Fig. 6), indicated that the ratio of retinal ganglion cells projecting to layer 5b vs. retinal ganglion cells projecting to the other layers was markedly increased in the portion of the retina projecting to the ventral tectum.

Figure 9 shows a representative selection of PHA-L-labeled retinal terminals arborizing in layer 5b in the ventral optic tectum following injections of PHA-L into the optic tract. These retinal terminals formed finely branching, radial processes that were confined to the radial extent of layer 5b. The total length of these terminal arbors was ca. 125 µm, and their width ranged from 7 µm to 15 µm. The individual branches were extremely fine, were often less than 1 µm, and showed several terminal bouton expansions of 2.0–2.2 µm in diameter. Retinal axons arborizing in layer 5b did not form branches or terminal processes in the superficial retinorecipient layers. Furthermore, the retinal terminals in tectal layers 3, 4, and 7 were of distinctively different morphology and were readily distinguishable from the terminals in layer 5b (Fig. 9). Therefore, we concluded that the retinal ganglion cells arborizing in layer 5b did not have endings in other tectal layers.

Injections of CTb restricted to the ventral optic tectum resulted in a well-defined cluster of retrogradely labeled retinal ganglion cells that were confined mainly to the dorsotemporal quadrant of the retina, or red field (Fig. 10). The resulting CTb-labeled cell population was composed almost exclusively of extremely small, round cells with somal diameters of 4.5–6.0 µm. These cells were densely packed and were placed one on top of another in a multilayered array. The point of origin of the dendritic arborizations of these cells was not evident. Larger (8–12 µm in diameter), CTb-labeled retinal ganglion cells were also found, but they were rare. We estimated that the total number of these small cells in the red field was about 1.5 million, i.e., 65% of the total retinal population of ganglion cells.

In contrast, CTb injections restricted to the dorsolateral aspect of the optic tectum resulted in the retrograde labeling of a more heterogeneous and remarkably less dense population of ganglion cells located at the inferior nasal quadrant of the retina (Fig. 10). The CTb-labeled cells were mainly polygonal, and most of them clearly exhibited dendritic branches. The somal size of these cells ranged from 5 µm to 15 µm, but the greater proportion was 7–10 µm in diameter (ca. 75%). The small, round cells were also present but in a lesser proportion (ca. 20%). These cells were evenly distributed and did not show a multilayered array.

A remarkable feature of the pattern of CTb labeling of the retina following the ventral tectum CTb injections was found in the inner plexiform layer. The inner plexiform layer showed a dense, confluent pattern of staining for CTb. However, at the margin of the region containing retrogradely labeled neurons in the retina, the pattern of staining appeared increasingly patchy. This patchy appearance was made up of circular domains of roughly homogeneous size, ranging from 25 µm to 40 µm in diameter (Fig. 11). We interpret this patch as representing the individual dendritic domains of the small retinal ganglion cells. Similarly sized patches were also present, even within regions of very dense staining, presumably reflecting overlapping dendritic domains of these ganglion cells. From these observations, we concluded that tectal layer 5b receives retinal input mainly, if not exclusively, from these extremely small retinal ganglion cells that possess small dendritic fields in the retina and narrow radial axonal arbors in the tectum.

**DISCUSSION**

The present study provided new insights into some of the major features of the tectorotundal pathways of birds. Previous studies (Karten and Revzin, 1966; Revzin and Karten, 1967) have reported that layer 13 neurons are the major source of tectal projections to the nucleus rotundus. These tectal projections, Benowitz and Karten (1976) suggested, project to the different divisions of the rotundus and derive from different sublaminae of layer 13. By using CTb, which is a much more sensitive tracer than the previously used horseradish peroxidase, the present study demonstrates that this differential sublaminar origin is far more prominent than Benowitz and Karten indicated.

Specifically, our study showed 1) the existence of two discrete subpopulations of tectorotundal projecting neurons with different afferent and efferent relationships, 2) the nature of the retinal ganglion cells that provide a major (presumably monosynaptic) input to one of these subpopulations, and 3) the pattern of direct and indirect projections of these two subpopulations to different divisions of the rotundus.

**Two subpopulations of tectorotundal-projecting neurons**

One of the most prominent findings of the present study was the intense pattern of granular staining found bilaterally in layer 5b of the optic tectum following injections of CTb into the anterior or centralis divisions of the rotundus. This staining was not observed in those cases with
injections into either the posterior or triangularis divisions.

The intense layer 5b staining found on the contralateral side appeared to be proportional to the number of contralateral CTb-labeled layer 13 neurons. Furthermore, injections into the ventral supraoptic decussation that resulted in retrogradely labeled neurons in layer 13 of the tectum also resulted in a similar pattern of layer 5b staining, suggesting that this staining represented labeled processes of the layer 13 neurons. We found no indication that this staining was due to projections from the nucleus rotundus or from any other nearby nuclei that might become involved by the spread of tracer.

Ramón y Cajal (1911) repeatedly illustrated the external arbors of the neurons of tectal layer 13 and drew them to indicate a unique expansion as the dendrites entered the

Fig. 9. Representative retinal axonal terminals in the optic tectum labeled following a Phaseolus vulgaris-leucaegglutinin (PHA-L) injection into the optic chiasm. Digital images were taken from PHA-L-labeled transverse sections. Note that the terminals in layer 5 (L.5) form fine branchings that are confined to the extent of the layer and that are oriented radially to the tectal surface. In comparison, terminals in layers 2 (L.2), 4 (L.4), and 7 (L.7) are more coarse and run parallel to the tectal surface. Scale bars = 30 µm.
region of layer 5. However, he did not specifically label this lamina as layer 5. Hunt and Künzle (1976) suggested that the dendrites of layer 13 (SGC) are a major component of the neuropil of layer 5 (designated as layer 1d in the nomenclature of Cowan et al., 1961), as reflected in the dense autoradiographic labeling of layer 5 following injections of radioactive tracers into layer 13. However, the limit of deposit of the injected isotope could not be assessed accurately. Hunt and Künzle then compared their radioactive amino acid uptake and transport pattern to the morphology of layer 13 neurons observed in their Golgi-stained material. They demonstrated dendritic arbors of layer 13 neurons in layer 5, although the density of such arbors was not evident in their Golgi studies.

In our CTb-labeled preparations, the granular staining in layer 5b appeared to lie in a radial orientation, but the connecting processes were so fine in caliber that their morphology could not be discerned readily. However, based both on the specific morphological characteristics of the neurons and on the studies of Ramon (1899), Ramón y Cajal (1911), and Hunt and Künzle (1976), we concluded that the granular staining was contained in distal dendritic terminals of neurons in the outer sublamina of layer 13.

Thus, we propose the presence of two major morphological types of neurons within layer 13 of the avian optic tectum: 1) type I neurons, which lie in the outer sublamina of layer 13, project to the anterior and centralis divisions of the rotundus, and possess extensive, fine dendritic arbors in layer 5b of the optic tectum; and 2) type II neurons, which lie in the inner sublamina of layer 13 and project to the posterior and triangularis divisions of the rotundus but do not show dendritic arbor staining in layer 5b.

Fig. 10. Retinal ganglion cells labeled following cholera toxin B subunit (CTb) injections into the ventral or the dorsal contralateral optic tectum. Digital images were taken at the zone of highest density of CTb-labeled cells in horizontal sections of the retina. Note the small size, homogeneous shape, and high density of the ganglion cell population labeled following the ventral tectum injection (A). Compare this with the lower density and heterogeneity of the CTb-labeled ganglion cell population following the CTb injection into the dorsal tectum (B). Scale bar = 15 µm.

Fig. 11. Contralateral inner plexiform layer of the retina labeled following a cholera toxin B subunit (CTb) injection into the ventral tectum. This darkfield digital image was taken from a CTb-labeled horizontal section of the retina that passed through the inner plexiform layer. Note the circular patches of CTb-labeled processes (arrows). Scale bar = 40 µm.
Retinal inputs to layer 5b

The pattern of retinal terminals within different layers of the superficial laminae of the optic tectum (layers 2, 3, 4, 5, and 7) have been examined by many authors over the past 100 years, including Ramon (1899), Ramón y Cajal (1911), LaVail and Cowan (1971), and Stone and Freeman (1971). The most notable feature is the differing morphologies of retinal terminals found in each layer of the tectum. This is clearly reflected in the illustrations of Ramón y Cajal (1911), who also suggested that these different types of axonal terminals arise from different retinal ganglion cell types.

In the present study, we found that retinal terminals in layer 5b had a notably different morphology than the axons ending in layers 4 and 7 (Fig. 9). The retinal terminals in layers 4 and 7 were more coarse than those found in layer 5b and generally ran parallel to the surface of the tectum. In contrast, layer 5b terminals formed fine, radial processes with numerous small terminal expansions. In our PHA-L-labeled material, the axons that gave rise to these layer 5b terminals did not show branching processes in any other tectal layer. This strongly suggested that the retinal ganglion cells that arborized in layer 5b did not have synaptic endings in other tectal layers.

A striking feature of layer 5b is the marked variation in its thickness in different regions of the optic tectum (Reiner et al., 1982b; Gamlin et al., 1996). Layer 5b, the area of termination of the retinal ganglion cells from the red field (ventral tectum), was as much as three times thicker than the terminal area of the yellow field (dorsal tectum). Layers 2, 3, and 4 showed a complementary opposite variation. We also found that retinal terminals span the full thickness of layer 5b in all areas, but the width of the arborizations varies markedly depending on the area, with the yellow field area three times wider than the red field area (Karten and Mpodozis, unpublished observations). Thus, the overall density of the retinal endings per square µm of horizontal plane in layer 5b of the ventral tectum was much greater than in layer 5b of the dorsal tectum.

This correlates directly with the high density of retinal ganglion cells labeled following our CTb injections into the ventral tectum (Fig. 10). The diameter of the majority of these labeled cells ranged from 4.5 µm to 6.0 µm (with only an occasional cell up to 12 µm) and represented a population of the very smallest ganglion cells in the pigeon retina. The small size of these cells was not a consequence of their relative retinal eccentricity, because they showed similar sizes across the entire extent of the red field, from pericentral to peripheral regions of the red field. In comparison, the retinal ganglion cells labeled following injections into the dorsal tectum formed a remarkably less dense, more heterogeneous population, with cell diameters ranging from 5.0 µm to 15.0 µm. In this population, the small, round cells were also present, but only as a minor fraction.

Thus, based on our results, we propose that 1) in the pigeon, the majority of the retinal ganglion cells terminating in layer 5b was composed of small, round cells with somal diameters of 4.5–6.0 µm and dendritic fields from 25.0 µm to 40.0 µm in diameter; 2) retinal ganglion cells that terminated in layer 5b were distributed across the entire retina and clearly showed a peak in density in the red field area; 3) the tectal projections of these cells ended exclusively in layer 5b, and the larger retinal ganglion cells in this region terminate in layers 3, 4, and 7; and 4) the small cells were the main, if not the only, source of retinal afferents to layer 5b. We designated these retinal ganglion cells as “w-5b”, reflecting their small size and their tectal target.

Oligo- and polysynaptic retinotectorotundal pathways

Our results further support the original suggestion of Ramon (1899) and the recent suggestions and observations of Hardy et al. (1984, 1985) and Catsicas et al. (1992) that the neurons of layer 13 are in direct receipt of retinal input. The specific dendritic arborizations of type I neurons in layer 5b suggest that these neurons may receive a monosynaptic input from the terminals of the w-5b ganglion cells. However, in view of the complexity of inputs to layer 5b (see below), confirmation of such an input requires direct electron microscopic evidence.

The present study revealed that the anterior and centralis divisions of the rotundus may receive a bisynaptic input from the retina, a much more direct and tightly linked input than was previously appreciated. A striking feature of this presumptive oligosynaptic pathway was that it appeared to arise from the smallest retinal ganglion cells, the w-5bs, which, in turn, converged on the large type I neurons of layer 13. This can account for both the large receptive fields and the fine sensitivity to the motion of small objects that the neurons of the tectofugal pathway have been reported to have at the tectal and rotundal levels (Rezvî, 1979; Wang et al., 1993; Letvin, personal communication).

The polysynaptic retinotectorotundal pathway and the nature of the retinal inputs to the type II neurons were less clearly defined. Type II neurons showed a dense pattern of dendritic arborizations within the deep layers of the tectum (layers 10–13), but we found no evidence of the extension of these processes to the retinorecipient layers. We suggest, then, that type II neurons may receive polysynaptic inputs from the retina, derived from some intervening retinorecipient tectal neurons. Our suggestion is consistent with the electrophysiological studies of Hardy et al. (1984, 1985) that demonstrate a population of layer 13 neurons in receipt of polysynaptic inputs from the retina.

Thus, the anterior and centralis divisions of the rotundus presumably receive bisynaptic inputs from the retina, whereas the posterior and triangularis divisions appear to receive polysynaptic inputs. This difference, in turn, may be related to the different visual sensory properties that the divisions of rotundus exhibit (Rezvî, 1979, Granda and Yazulla, 1971; Wang et al., 1993).

Projections of type I neurons to the pretectum

This study also found the presence of a tectal projection to the nucleus pretectalis of the dorsal pretectal region. The pretectalis is a nucleus that projects heavily to both the ipsilateral and the contralateral optic tectae, terminating specifically within layer 5b (Gamlin et al., 1996). Neurons of the pretectalis are rich in both neuropeptide Y (NPY)- (Gamlin et al., 1996) and GABA-like (Veeman and Reiner, 1994) immunoreactivity. The layer 13 projection to the pretectalis was apparently due solely to the axon collaterals of type I neurons. Type II neurons did not appear to project to the pretectalis. The implications of this difference were most interesting, because the type I neu-
rons possessed dense dendritic arbors within layer 5b. This may suggest that the type I neurons are involved in a unique feedback loop whereby they send presumably excitatory, glutamatergic inputs to the pretectalis (Dye and Karten, 1993), which, in turn, send bilateral inhibitory projections to layer 5b. The exact synaptic target of the pretectalis projections, however, is still unknown. Do the pretectalis axons with NPY- and GABA-like immunoreactivity terminate on the type I dendrites, the retinal terminals, other afferent axons (see below), or dendrites of other tectal neurons within layer 5b?

Summary of the tectorotundal connections

Combining the data from previous reports and our present study (Dye and Karten, 1993; Gamlin et al., 1996; Mpodozis et al., 1996), we find that the neurons of layer 13 of the tectum influence a number of pathways that affect rotundal function. 1) Type I neurons project bilaterally to the anterior and centralis divisions of the rotundus. 2) Type II neurons project bilaterally to the posterior and triangularis divisions. 3) Both type I and type II neurons project to the ipsilateral intrinsic nuclei of the tectothalamic tract, with collaterals of type I neurons ending in the nucleus pretectalis and type II neurons ending in the PV and the SP, pars caudalis (SPcd). 4) The GABAergic neurons of the SP and IPS terminate in the anterior and centralis divisions of the ipsilateral rotundus. Neurons of the SPcd terminate in the posterior and triangularis divisions of the ipsilateral rotundus. 5) Type I neurons also project to the nucleus pretectalis, terminating mainly ipsilaterally. The pretectalis neurons with NPY- and GABA-like immunoreactivity project bilaterally to layer 5b of the tectum, the tectal layer in which w-5b retinal terminals and type I dendrites are found in close, possibly synaptic, association. This closed loop potentially provides a profound inhibitory effect on throughput processing of the tectum on the rotundus. 6) Both types of layer 13 neurons presumably operate through a glutamatergic, excitatory pathway.

Tectorotundal vs. tectobulbar pathways

A previous study of the tectal efferents reported that individual neurons of layer 13 as well as neurons from deeper layers are the source of the descending projections to the tectobulbar system of the brainstem (Reiner and Karten, 1982). This could imply a direct output of those neurons to both premotor targets and the tectorotundal system. The present study clearly revealed that layer 13 contained at least three different populations of neurons: type I and II neurons that projected to the rotundus and an additional type of neuron that projected to the tectobulbar system. Neurons of this additional type were found occasionally at the border of layers 13 and 14 (where they were found intermingled with type I or II neurons), although they were located mainly in layer 14 and the underlying tegmentum. Some of these neurons, however, were found to have dendrites that extended into the retinoreceptive layers of the tectum. These neurons were smaller than those that projected to the rotundus. A future study will address the issue of the identity of tectal neurons that contribute to the tectopontine projections to the lateral pontine nucleus.

Nonvisual afferents to layer 13

The nature of the inputs to the layer 13 neurons will obviously determine the type of information conducted rostrally within the tectofugal visual pathway. A variety of projections to the layer 13 neurons have been reported, including those directly from the telencephalon (Edinger et al., 1903; Zeier and Karten, 1971; Karten et al., 1973; Miceli and Reperant, 1983), indirectly from the paleostriatum primitivum of the telencephalon via the nucleus spiriformis lateralis (Reiner et al., 1982a), possible inputs of the nucleus semiluminaris, inputs from the locus coeruleus, possible inputs from raphe neurons (Brecha, 1978), auditory inputs via the inferior colliculus external nucleus (Knudsen and Knudsen, 1983), possible somatosensory inputs (Cotter, 1976), and inputs from the reticular formation (Brecha, 1978). We have been unable to find any evidence of projections to layer 13 from the contralateral optic tectum. The demonstration that layer 13 consists of a clear inner and outer sublamina may prompt reconsideration of the different inputs to each of these regions. This clarification is required to determine where the auditory and somatosensory inputs actually terminate (Knudsen and Knudsen, 1983; Stein and Meredith, 1993). Thus, there may well be a convergent polymodal input to the rotundus. However, there may be intrinsic segregation of connections by independent pathways within and without the tectum.

Diversity of inputs to layer 5b

The present study further revealed the complex nature of inputs to layer 5b of the avian optic tectum. Layer 5b is in receipt of 1) massive retinal glutamatergic (Dye and Karten, 1996) inputs, presumably from the small w-5b neurons; 2) rich dendritic arbors of type I neurons; 3) dense bilateral GABA/NPY inputs from the nucleus pretectalis (Gamlin et al., 1996); 4) massive inputs from the nucleus isthmi parvocellularis (Ramon, 1899) that were found subsequently to be cholinergic (Medina and Reiner, 1994); and 5) distinctive enkephalin-immunoreactive radial processes, possibly arising from the enkephalin-immunoreactive neurons of tectal layer 10 (Reiner et al., 1982b). Layer 5b is also prominently calbindin immunoreactive, which is derived from a distinct population of horizontal neurons lying within layer 5b (Mpodozis and Karten, unpublished observations). However, in the absence of details regarding the specific synaptic relationships of the terminals of the retina, the nucleus pretectalis, the nucleus isthmi parvocellularis, and the type I dendrites, we cannot assess the consequences of these interactions upon the nature of the information being conveyed to the nucleus rotundus (Fig. 12).

Retinotectorotundal topography

Despite the seeming lack of a precise retinal topography within the tectorotundal system, we have confirmed and extended the existence of a regional topographic organization in this system, i.e., neurons located in different sublaminae of the tectum projected to different divisions of the rotundus. But another notable feature emerged in these studies—these tectal sublaminae showed regional variation. The number of type I neurons varied markedly between the dorsal and ventral tectum in a way that follows the regionalization of the retinal inputs to layer 5b. The number of type II neurons, however, was relatively

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similar in all tectal areas, although the numbers were somewhat reduced in the ventral tectum. The consequences of this would be manifest in the predominance of a red field representation in the anterior and centralis divisions of the rotundus, because they were in specific receipt of type I tectal neurons. In contrast, the posterior and triangularis divisions of rotundus that received their inputs from the type II neurons should possess a more uniform representation of all portions of the visual field.

However, the absence of a correspondingly precise retinal topography in the tectorotundal system continues to present a problem: How does this system deal with the precise localization of stimuli within the global receptive field? We hope to address this issue in our subsequent studies, which will describe in more detail the morphology of layer 13 neurons as well as the topography of the retinotectorotundal connectivity.

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