Target-independent diversification and target-specific projection of chemically defined retinal ganglion cell subsets

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SUMMARY

In diverse vertebrate species, defined subsets of retinal ganglion cells (RGCs, the neurons that project from retina to brain) are distinguishable on the basis of their dendritic morphology, physiological properties, neurotransmitter content and synaptic targets. Little is known about when this diversity arises, whether diversification requires target-derived signals, and how subtype-specific projection patterns are established. Here, we have used markers for two chemically defined RGC subsets in chick retina to address these issues. Antibodies to substance P (SP) and the nicotinic acetylcholine receptor (AChR) β2 subunit label two small (<10%), mutually exclusive groups of RGCs in mature retina. SP and AChRs accumulate in distinct RGCs before retinotectal synapses have formed. Moreover, both populations of RGCs form in retinae that develop following tectal ablation or transplantation to the coelomic cavity. Thus, RGC subsets acquire distinct neurotransmitter phenotypes in the absence of extraretinal cues.

In the mature optic tectum, SP- and AChR-positive RGC axonal arbors are confined to distinct retinorecipient (synaptic) laminae. In the developing tectum, SP- and AChR-positive axons are initially intermingled in a superficial fiber layer, but then enter and arborize in appropriate laminae soon after those laminae form. Importantly, SP-positive axons, which synapse in a superficial lamina, never extend into the deeper, AChR-positive lamina. Tectal interneurons rich in SP receptors are concentrated in the lamina to which SP-positive RGC axons project, and a set of cholinergic (choline acetyltransferase-positive) tectal projection neurons elaborate dendrites in the lamina to which AChR-positive RGC axons project. These populations of tectal neurons, which are likely targets of the RGC subsets, form in tecta that develop following enucleation. Thus, RGCs and their targets can diversify in each others absence. Accordingly, we propose that the lamina-selective connectivity we observe reflects the presence of complementary cues on RGC subsets and their laminar targets.

Key words: acetylcholine receptors, choline acetyltransferase, neurotransmitter, optic tectum, retinal ganglion cells, retinotectal projection, substance P, chick

INTRODUCTION

Retinal ganglion cells (RGCs), the sole output neurons of the vertebrate retina, send axons along the inner retinal surface to the optic nerve head, through the optic nerve to the optic chiasm, and then through the optic tract to the brain. It seems likely that all RGC axons follow a common set of cues during this portion of their outgrowth, differing principally in whether they cross or remain ipsilateral at the optic chiasm (Hynes and Lander, 1992). Subsequently, however, retinal axons display diverse behaviors. Best studied in this respect is the positional coding of RGCs, which leads to formation of orderly retinotopic maps in retinorecipient nuclei of the di- and mesencephalon (Sperry, 1963; Mey and Thanos, 1992; Holt and Harris, 1993). In addition, RGCs are heterogeneous in their electrophysiological properties and neurotransmitter phenotype, and these forms of diversity are also correlated with projection pattern. In the mammalian retina, for example, W-, X-, and Y-type RGCs, categorized by their responses to visual stimuli, send axons to different laminae within the lateral geniculate nucleus and superior colliculus (optic tectum; Stone, 1983; Shapley and Perry, 1986; Wassle and Boycott, 1991). In lower vertebrates, physiologically defined heterogeneity has also been noted (Maturana et al., 1963; Mpodozis et al., 1993), but more attention has been paid to biochemical heterogeneity: in frogs, reptiles, and birds, defined subsets of RGCs accumulate distinct neuropeptides, and at least some of the subsets project to specific laminae within the optic tectum (Kuljis and Karten, 1988; Karten et al., 1990).

The existence of defined RGC subsets raises two questions: first, how is heterogeneity among RGCs generated? One possibility is that intraretinal influences lead to diversification of RGCs. In positing target-independent diversification, this model resembles the ‘chemoaffinity’ hypothesis, proposed by Sperry (1963) and generally accepted today (Sanes, 1993) as an explanation for topographic matching. At the other extreme, RGCs might initially be equivalent, and then diversify as a...
result of signals received from their targets. This mechanism has been demonstrated in the autonomic nervous system, where target-derived factors play key roles in determining which neurotransmitter a particular neuron synthesizes (Landis, 1990; Nishi, 1994). One simple way to distinguish these possibilities is to ask whether RGC subsets acquire their distinctive properties in the absence of their targets, but few studies of this sort have been reported.

The second question is: how do RGC axons of a particular type choose appropriate targets? If RGCs are initially uniform, they might make stochastic choices among nuclei or laminae, then acquire specific properties secondarily. If, on the other hand, RGC categories are determined prior to outgrowth, some form of target recognition is likely, but this might involve either orderly projections from the outset or refinement of initially diffuse connections. Again, precedents for several alternatives have been found. For example, individual retinal axons initially innervate several laminae in the geniculate, then lose branches in inappropriate laminae and acquire branches in appropriate laminae, until their lamina-specific arbors emerge (Shatz, 1990). Conversely, geniculate axons invading the cerebral cortex appear to arborize in their appropriate target lamina (IV) as soon as they invade the cortical plate (Katz and Callaway, 1992).

To address these questions, molecular markers for RGC subsets are needed. Unfortunately, none have been defined for the W-_, X-, and Y-type RGC cells in mammals. In birds (pigeon and chicken), however, work by Karten, Britto and colleagues has shown that RGCs differ in the neurotransmitters and neurotransmitter receptors they express. Thus, although all RGCs appear to use glutamate as a primary transmitter (Beart, 1976; Canzek et al., 1981; Kalloniatis and Fletcher, 1993), defined subsets also accumulate particular neuropeptides or catecholamines (Britto and Hamassaki-Britto, 1992; Ehrlich et al., 1987; Gunturkun and Karten, 1991; Karten et al., 1990; Keyser et al., 1990; Reiner, 1992). Moreover, some RGCs bear neurotransmitter receptors – specifically nicotinic acetylcholine receptors (AChRs) – that are absent from others (Britto et al., 1992a; Hamassaki-Britto et al., 1994; Keyser et al., 1988, 1993). Importantly, RGCs that share a neuropeptide or transmitter receptor appear to project to the same target structure (tectum, lateral geniculate or nucleus of the basal optic root) or to the same lamina within the tectum (Hunt and Brecha, 1984; Britto et al., 1988, 1989, 1992b; Ehrlich et al., 1987; Karten et al., 1982; Bagnoli et al., 1992). Here, we have used two transmitter-related antigens as molecular markers of RGC subsets: a neuropeptide, substance P (SP), which is restricted to RGCs that project to lamina SGFSb of the tectum (Ehrlich et al., 1987), and an AChR subunit, β2, which is restricted to RGCs that project to tectal lamina SGFSF (Britto et al., 1992b). Using these and other markers, we show that RGC heterogeneity is detectable soon after RGCs are born, that RGC subclasses differ in the absence of their synaptic targets, that presumptive synaptic targets diversify in the absence of RGCs, and that projections of RGC subclasses to appropriate laminae are accurate from the outset.

MATERIALS AND METHODS

Antibodies
A rat monoclonal antibody to the β2 subunit of the neuronal nicotinic acetylcholine receptor (mAb 270; Whiting and Lindstrom, 1987), and a mouse monoclonal antibody to tyrosine hydroxylase (Fauquet and Ziller, 1989) were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Rabbit and guinea pig polyclonal antisera to SP (Reiner et al., 1985) were obtained through J. Krause (Washington University School of Medicine, St. Louis, MO). Rabbit antisera to the chicken neurofilament M subunit (Bennett et al., 1984), rat substance P receptor (Shigemoto et al., 1993) and chicken choline acetyltransferase (ChAT; Johnson and Epstein, 1986) were kind gifts from G. Bennett (University of Florida, St. Louis, MO). Rabbit antisera to the chicken neurofilament M subunit (Bennett et al., 1984), rat substance P receptor (Shigemoto et al., 1993) and chicken choline acetyltransferase (ChAT; Johnson and Epstein, 1986) were kind gifts from G. Bennett (University of Florida, St. Louis, MO). Rabbit antisera to the chicken neurofilament M subunit (Bennett et al., 1984), rat substance P receptor (Shigemoto et al., 1993) and chicken choline acetyltransferase (ChAT; Johnson and Epstein, 1986) were kind gifts from G. Bennett (University of Florida, St. Louis, MO).

Surgery
Fertilized White Leghorn chicken eggs from SPAFAS (Roanoke, IL), were incubated at 37.5°C. Embryos that were developing abnormally, according to the Hamburger and Hamilton (1951) stage series, were discarded. To ablate the tectum, the mesencephalic lobe was removed at embryonic day (E) 4 with ophthalmological scissors, and the stubbed portion was slightly cauterized (Hughes and LaVelle, 1975; Cohen et al., 1989) using an electrocautery device (Model 285101, Abo Inc., Milwaukee, WI). Embryos were enucleated unilaterally or bilaterally on E3 by electrocautery as described previously (Yamagata and Sanes, 1995). In most cases, unilateral enucleation was chosen, to permit comparison of deprived and control tecta from a single animal. However, similar results were obtained from bilaterally enucleated embryos. For transplantation, optic vesicles were dissected at E3 and placed into the coelomic cavity of another embryo at the same stage of development (Coulombre, 1967). Following surgery, eggs were sealed with tape and returned to the incubator.

Retinal organ culture
Eyes were removed from E7 embryos, and retinae were dissected free of pigment epithelium and vitreous body. A single retina was spread onto a Nuclepore filter (8 μm pores, 13 mm diameter), vitreal side up, and floated on 2 ml of medium in a 35-mm diameter culture dish (Yamagata and Sanes, 1995). Medium was either a 1:1 mixture of Dulbecco modified Eagle and Ham F12, supplemented with 10% fetal calf serum and 15% chick embryonic extract, or Neurobasal (Gibco-BRL, Gaithesberg, MD), supplemented with 10 ng/ml brain-derived neurotrophic factor (BDNF; generous gift from Regeneron Pharmaceuticals, Tarrytown, NY). Cultures were incubated in a 5% CO2 incubator at 37°C, and the medium was changed every 2 days.

Immunohistochemistry
Eyes and tecta were dissected from staged embryos, fixed by immersion in 3.5% paraformaldehyde in phosphate-buffered saline (PBS), cryoprotected in 5% and 15% sucrose in PBS, and frozen in liquid nitrogen-cooled isopentane. Sections were cut at 15 μm in a cryostat, mounted on subbed slides, and stained as described previously (Yamagata et al., 1995). Mounted sections were treated with 0.1% Triton X-100 in PBS for 5 minutes at room temperature before staining with mAb270. For staining with anti-SP, sections were pretreated either with Triton as above, or with methanol for 30 min at –20°C. To stain SP receptor, sections were treated as described by Shigemoto et al. (1993). After permeabilization, sections were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min, incubated with the primary antibodies for 12 hours at 4°C, washed and incubated with secondary antibodies plus the nuclear counterstain, DAPI (Molecular Probes) for 2 hours at room temperature. Finally, the sections were mounted in para-phenylenediamine containing glycerol, and observed with epifluorescence illumination.
using ultraviolet-, fluorescein-, and rhodamine-specific filter sets. Our nomenclature for laminae of developing tecta is discussed in Yamagata et al. (1995).

**Histology**

We used Neurobiotin (Vector Laboratories Inc., Burlingame, CA) as an anterograde tracer (Lapper and Bolam, 1991) to label retinal arbors in optic tecta. The egg shell was windowed, and 10 µl of Neurobiotin (50 mg/ml in 50 mM TrisHCl, pH 7.4) was injected into the vitreous body of the eye, using a 50 µl Hamilton syringe fitted with a 22 gauge needle. After allowing 15 hours for transport, the embryos were perfused through the heart with 4% (w/v) paraformaldehyde plus 0.25% glutaraldehyde in PBS. Tecta were sectioned at 80 µm with a Vibratome (Ted Pella, Inc., Redding, CA). The sections were treated with 1% NaBH₄ in PBS for 30 minutes at room temperature, then incubated with 1% BSA in PBS for 1 hour at room temperature, and with avidin-conjugated horseradish peroxidase (Vector Laboratories Inc.; 1:500 dilution in BSA/PBS) overnight at 4°C. After rinsing in PBS, the sections were developed with 1 mg/ml diaminobenzidine plus 0.01% hydrogen peroxide in 50 mM Tris-HCl, pH 7.4. The sections were mounted in Gel/Mount (Biomeda, Foster City, CA) for observation.

Rapid Golgi silver-impregnation was done as described by Rager and von Oeyhhausen (1979). The silver-stained cells were traced using a camera lucida drawing apparatus.

**RESULTS**

**Markers for retinal ganglion cells**

The three cellular layers of the mature retina are the outer nuclear layer, the inner nuclear layer (INL), and the retinal ganglion cell layer (GCL; Fig. 1a). Although RGCs are the major cell type of the GCL, they are neither the only cell type in that lamina, nor are they confined to the GCL. Thus, although most amacrine cells reside in the INL, some are present in the GCL; these are called displaced amacrine cells. Conversely, some displaced RGCs reside in the INL (Fig. 1f). Because of this complexity, we needed cell type-specific markers for RGCs and cells of the GCL.

Sections of E18 retina were stained with: (a) DAPI, to label all nuclei and reveal laminae; (b) anti-neurofilament M, to label RGCs; (c) mAb 270, to label a subset of RGCs in the GCL; (d) anti-SP, to label a nonoverlapping subset of RGCs in the GCL; (e) anti-ChAT, to label a majority of displaced amacrine cells in the GCL, as well as a subset of amacrine cells in the INL; and (f) anti-tyrosine hydroxylase, to label a subset of displaced RGCs in the INL. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Parts a, c and d are micrographs of a triple-labeled section, viewed with UV, fluorescein and rhodamine optics, respectively. Corresponding points in these three panels are marked by arrows. Bar is 25 µm.

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**Fig. 1.** Molecular markers for RGCs and cells of the GCL.
markers that would distinguish RGCs from amacrine cells. For RGCs, we used antibodies to neurofilaments (Bennett et al., 1985), which have been shown to be RGC-specific within the retinae of several species, including chickens (Drager et al., 1984; Jasoni et al., 1994). In mature retina, anti-neurofilament antibodies stained the majority of cells in the GCL (Fig. 1b), as well as presumptive displaced ganglion cells in the INL (not shown). No marker that stains all amacrine cells was available, but ≥90% of displaced amacrines contain choline acetyltransferase (ChAT; Spira et al., 1987). We therefore used an antiserum to ChAT to mark the displaced amacrine cells of the GCL (Fig. 1e).

As markers for RGC subsets, we used antibodies to two neurotransmitter-related molecules that Karten and colleagues have previously shown to be contained in avian RGCs: SP and AChRs (references in Introduction). We used polyclonal antiserum to detect SP (Reiner et al., 1985) and a monoclonal antibody, mAb 270, to stain neuronal nicotinic AChRs (Whiting and Lindstrom, 1987). mAb 270 recognizes the AChRβ2 subunit (Sargent, 1993), but because it has not been tested on all subunits, we refer to its antigen here as 270-immunoreactive material. Consistent with results of Karten and colleagues on posthatching chickens and adult pigeons, we found that anti-SP and mAb 270 both stained distinct subsets of cells in GCL at E18 (Fig. 1c,d). Double-labeling experiments showed that most if not all of the clearly SP-immunoreactive and 270-positive cells in the GCL were neurofilament-positive, indicating that these cells were RGCs (not shown). All of the SP-positive cells and most of the 270-positive cells were ChAT-negative. In contrast to Keyser et al. (1988) who reported that all 270-positive cells were ChAT-negative, we saw some weakly 270-positive cells that were ChAT-positive. These presumptive amacrine cells were, however, distinguishable from the strongly 270-positive RGCs.

**Appearance of RGC subsets**

Retinal ganglion cells are born (become postmitotic) between E3 and E9, in a central-to-peripheral gradient (Kahn, 1974; Spence and Robson, 1989; Prada et al., 1993). In central retina, all of the RGCs are postmitotic by E7 and lie near the vitreous surface, but plexiform (synaptic) layers have not yet formed, so the GCL remains indistinct at E8 (Fig. 2a). A clear GCL is visible centrally by E10 (Fig. 2b). Neurogenesis in peripheral retina lags by about 2 days: RGCs are all postmitotic by E9, and the GCL is distinct by E12. As the GCL forms, a thin nuclear layer appeared transiently between it and the INL. This layer, which contains the somata of displaced amacrine cells, was distinct in central retina at E10, began to merge with the GCL by E12, and was obliterated by E14 (Fig. 2b,c). Again, peripheral retina lagged by about 2 days: the layer of displaced amacrine cells was most distinct peripherally at E12 and still visible at E13-14. RGCs acquired their cell type-specific antigen, neurofilaments, by the time they were distinguishable by position (Fig. 2d). Initially, a few neurofilament-positive processes were present in apical layers, presumably reflecting the presence of RGCs that were still migrating to their definitive positions; however, neurofilament-like immunoreactivity became confined to RGCs in the GCL and displaced RGCs in the INL by E10. Displaced amacrine cells were ChAT-positive by E10, as soon as they acquired a distinct laminar position (Fig. 2e,f), and ChAT-positive cells represented approx. 10-15% of the nuclei in the GCL by E12 (Fig. 3c).
To determine when RGC subsets acquire their distinctive properties, retinae were dissected from chick embryos, fixed, sectioned and stained with anti-SP and mAb 270. A small number of SP- and 270-reactive cells were present in the retina at E8, the earliest stage examined (Fig. 2g,j). Both cell types were readily detectable in the GCL of central retina by E10 (Fig. 2f,k), and throughout the retina by E12 (Fig. 2i and l). All of these cells were within the GCL per se, not in the transient lamina of displaced amacranies. The fraction of cells in the GCL that were SP- and 270-positive cells was determined separately in central and peripheral retina. SP-positive cells made up about 4% of all cells in the GCL, and 270-positive cells, about 8%. These values are similar to those reported for adults by Ehrlich et al. (1987) and Keyser et al. (1988). SP cells were intensely immunoreactive by E10, whereas 270 cells were only faintly immunoreactive at E10, and accumulated 270 material gradually over the subsequent days. Importantly, however, both subpopulations reached their final proportions as soon as they could be counted reliably (i.e. as soon as the GCL was distinct) (Fig. 3a,b).

Naturally occurring cell death leads to the elimination of about 40% of all RGCs between E11 and E15 (Rager and Rager, 1978; Hughes and McLoon, 1979; Vanselow et al., 1990; Kobayashi, 1993). It is noteworthy that correct proportions of SP- and 270-positive RGCs were present before cell death was underway, and that their proportions changed little during the period of cell death (Fig. 3a,b). This constancy indicates that the RGC subsets we have studied are about as likely to die as RGCs in general.

**Differentiation of RGCs in the absence of synaptic targets**

Retinal axons reach the optic tectum between E6 and E10. In central tectum, on which we focus here, few axons leave the superficial layer to invade the neuropil until E10-E12 (McLoon, 1985; Yamagata and Sanes, 1995). The appearance of appropriate numbers of SP- and 270-positive RGCs by E10-12 suggested that these subsets acquired their identities prior to and therefore independent of contact with their synaptic targets. To test this possibility, we ablated the dorsal mesencephalon, from which the tectum arises, on E4, then examined the development of RGCs in these tecta-less embryos.

When examined at E12, tecta-less retinae were remarkably normal. A GCL formed in an appropriate position (Fig. 4a,b) and the majority of cells within it differentiated into neurofilament-positive RGCs. Both RGCs (neurofilament-positive cells) and displaced amacranies (ChAT-positive cells) were present in large numbers. Numbers of displaced amacranies were approximately normal (Fig. 4i), as were numbers of RGCs (not shown). Fractions of cells that were SP- and 270-positive differed little between normal and tecta-less retinae at E12 (Fig. 4c,d,i). Thus, the chemically marked RGC subsets appear in the absence of their synaptic targets.

We also examined target-deprived retinae at E15, following the period of naturally occurring cell death. As in many other systems, cell survival is target-dependent, so removal of the tectum increases the amount of RGC death (Hughes and McLoon, 1979). As expected, the GCL was depleted in tecta-less retinae examined at E15, and the number of RGCs was substantially decreased (Fig. 4e,f). As a result, the fraction of cells in the GCL that were displaced amacranies was approx. 3-fold greater in target-deprived than in normal retinae (approx. 15% vs. approx. 50%, consistent with a 2-3-fold increase in RGC death; Fig. 4j). When RGC subsets were quantitated in these tecta, the proportion of cells in the GCL that were SP-positive declined by about 70%, whereas the fraction of cells that were 270-positive changed little (Fig. 4g,j). As in normal retinae, the SP- and 270-positive cells were neurofilament-positive and ChAT-negative (Fig. 4g,h). A simple interpretation of these results is that SP-positive RGCs were more likely to die than RGCs in general, following target deprivation.

**Differentiation of RGCs in cultured retinae**

About 90% of all RGCs project to the optic tectum (Hayes, 1981). The remainder send axons to other di- and mesencephalic centers, including the nucleus lateralis geniculatus pars ventralis and the accessory optic system (Britto et al., 1988, 1989; Keyser et al., 1988). Thus, although tectal ablation removes the main target of RGCs, minor targets remain; moreover, some deprived RGCs could send aberrant projections to novel targets (see, for example, Roe et al., 1993). To circumvent this limitation, we studied the differentiation of RGCs in retinae that had been grafted to the coelomic cavity (Coulombre, 1967) at E3 (Hamburger-Hamilton stage 17), then allowed to develop until E15. During this period, an eyeball formed that was fairly normal in shape but about half the normal size. A GCL containing numerous neurofilament-positive RGCs appeared in these retinae (Fig. 5a and b), but no optic nerve formed. SP-, 270-, and ChAT-positive cells were present in the GCL (Fig. 5a-c), and their proportions approximated those seen in E15 tecta-less retinae (Fig. 5g). As in normal and tecta-less retinae, most of the SP- and 270-positive RGCs were neurofilament positive and ChAT negative. Thus, SP- and 270-positive RGC subsets appeared despite the absence of extraretinal targets.

Based on these results, we assayed RGC differentiation in...
retinae that developed in vitro. E7 retinae were spread on Nuclepore filters, which were then floated on medium to provide the explants with abundant access to both nutrients and air (Yamagata and Sanes, 1995). Two media were used: one was supplemented with fetal calf serum and chick embryo extract, while the other was serum free but contained BDNF, which is a survival factor for chick RGCs (Rodriguez-Tebar et al., 1989). In both media, a GCL formed during 8 days in vitro (which brought the retinae to E15), and its cells acquired either neurofilaments (RGCs) or ChAT (displaced amacrines; Fig. 5d and e). As in E15 tecta-less retinae and coelomic grafts, neurofilament- and ChAT-positive cells were present in approximately equal numbers, suggesting that cell survival was compromised by target removal, but was not additionally affected by culture conditions. Moreover, some 270-positive cells were present in the GCL of such explants (Fig. 5f), although fewer such cells were seen in the explants than in the coelomic grafts (Fig. 5g). However, no SP-positive cells were ever seen in the GCL of explanted retinae, whether they had been cultured in serum- and extract-containing medium or in BDNF-supplemented medium. Thus, SP-positive cells failed to survive or differentiate in vitro, suggesting that some factors extrinsic to both retina and target (e.g. humoral factors) are required for the health of this RGC subclass. In addition, the differential response of SP- and 270-positive subsets suggests that the survival or differentiation of these two cell types is controlled in different ways.

Lamina-specific arborization of RGC axons

Retinal axons enter the optic tectum through a superficial lamina, the stratum opticum, then send terminal branches or collaterals to form arbors in one of three retinorecipient laminae, SGFS B, D, and F (Fig. 6a; Ramon y Cajal, 1892; LaVail and Cowan, 1971; Acheson et al., 1980; Mey and Thanos, 1992; Yamagata and Sanes, 1995; as discussed by Yamagata et al., 1995, we use lower case letters to designate definitive retinorecipient laminae but upper case letters to designate their embryonic counterparts). Our choice of anti-SP and mAb 270 as labels was motivated in large part by reports that the RGCs they mark terminate in discrete laminae in adults: SP-positive RGCs in SGFS b (Ehrlich et al., 1987) and 270-positive RGCs in SGFS f (Keyser et al., 1988; Britto et al., 1992b). We confirmed these lamina-selective patterns in E18 chick retina (Fig. 6b and c). Both SP-positive fibers in SGFS b and the 270-positive fibers in SGFS f were absent from enucleated tecta (Fig. 6d-f), demonstrating their retinal origin.

Knowing that SP- and 270-positive RGCs acquire their identities in the absence of synaptic targets, we asked whether their axons innervated separate laminae from the outset, or whether laminar specificity arose by refinement of a diffuse pattern.
Initially, we bulk labeled retinal axons with an anterograde tracer, neurobiotin, to establish a chronology of axonal ingrowth. Most RGC axons were confined to the SO at E10 (not shown). By E12-13, many axons had left the SO, and formed transitory arbors in SGFS A/B (Fig. 7a). Further growth led to invasion of SGFS D by E14 (Fig. 7b) and SGFS F by E15-16 (Fig. 7c). Previous studies of individually (DiI) labeled axons showed that most arbors were confined to a single one of the three retinorecipient laminae throughout this period (Yamagata and Sanes, 1995). By E18, the definitive pattern was clear: the majority of retinal terminals occupied SGFS D, smaller subsets were present in SGFS B and F, and few were present in SGFS C and E (Fig. 7d). At no time did any axons extend beyond SGFS F.

How do SP- and 270-positive retinal axons innervate their appropriate laminae? Staining of tectal sections with anti-SP showed fibers in the SO and some fibers extending into SGFS A/B at E12 (Fig. 8a). At E14, SP-positive fibers were confined to the SO and SGFS B (Fig. 8b), even though the majority of RGC axons had extended into SGFS D (Fig. 7b). Staining of the SO decreased at E16 (Fig. 8c) and was barely detectable at E18 (Fig. 6c), presumably reflecting the concentration of the neuropeptide in synaptic varicosities. Throughout this period, the SP-positive terminal arbors remained restricted to the upper laminae of SGFS, and failed to extend in significant numbers into deeper laminae. Thus, SP-positive fibers arborize in their appropriate target lamina from the outset.

A similarly selective pattern was observed for 270-positive fibers. Few 270-positive fibers were detectable at E12 (Fig. 8d), although this may reflect high levels of background staining as well as (or instead of) lack of positive structure. By E14, however, 270-positive fibers had accumulated at the base of SGFS D (Fig. 8e), which represented the region of deepest penetration of retinal axons at this stage (Fig. 7b). On E16, as the retinal projection to SGFS F formed, 270-positive fibers were present within it (Fig. 8f). Thus, fibers destined to end in SGFS F arborized in that layer directly.

Neither SP- nor 270-positive fibers were detectable in the SO or SGFS of enucleated embryos at any stage tested (not shown).

**RGC-independent development of putative tectal targets**

Results presented above demonstrate that RGCs diversify in the absence of their tectal targets. It was also of interest to ask whether differentiation of tectal subpopulations requires retinal innervation. Unfortunately, of the numerous tectal cell types likely to receive retinal input, few have been identified with certainty (Hunt and Brecha, 1984). However, we reasoned that targets of SP-bearing RGCs would be SP-responsive, and that targets of AChR-positive RGCs would be cholinergic. The rationale for seeking cholinergic targets of cholinceptive axons is that AChRs of the B2 class frequently mediate presynaptic inhibition (Sargent, 1993); thus, retinotectal synapses may be reciprocal, involving excitatory glutamatergic antero-
grade (from RGC axon to tectal dendrite) and inhibitory cholinergic retrograde signalling (H. Karten, personal communication). Accordingly, we used antisera to a SP receptor and to ChAT to seek targets of RGC subsets.

Both at E14 (Fig. 9a) and at E18 (not shown), SP receptor-positive neurons were confined to three laminae: SGFS B, SGFS I, and SGC. Importantly, immunoreactivity within the retinorecipient laminae was confined to SGFS B, which is the lamina in which SP-positive RGC axons arborize. Within this lamina, a subset of small neuronal somata and their short processes were labeled (Fig. 9b). These cells may be horizontal interneurons that receive input from SP-positive axons (Hunt and Brecha, 1984). SP receptor-positive cells were also found in SGFS B in tecta from embryos that had been enucleated before any RGC axons left the retina (Fig. 9c). Thus, putative targets of SP-positive axons differentiate neurochemically in the absence of retinal input.

Likely targets of AChR-positive RGC axons are ‘r2’ cells, which have cell bodies in SGFS I, extend processes that ramify in SGFS F, and project to the ventral thalamus (Reiner and Karten, 1982; Hunt and Brecha, 1984). Previous studies have shown that cells with this morphology are ChAT-positive in adult birds (Sorensen et al., 1989; Bagnoli et al., 1992; Medina and Reiner, 1994). Analysis of Golgi-impregnated tissue revealed that ‘r2’-like cells were present in SGFS I by E14 (Fig. 9g), and immunohistochemical analysis showed that a subset of radial neurons in SGFS I was ChAT-positive at this time (Fig. 9d,e). Our methods did not permit us to definitively identify the ChAT-positive cells as ‘r2’-type or tecto-thalamic at E14, but this seems likely based on the previous studies of adult birds. Cells of similar size, shape, and laminar position were ChAT-positive in tecta from bilaterally enucleated embryos (Fig. 9f). Thus, putative targets of AChR-positive RGC axons differentiate neurochemically in the absence of retinal input.

**DISCUSSION**

The retina has been intensively studied, as a particularly accessible portion of the vertebrate central nervous system. Recently, interest in retinal development has intensified, reflecting the availability of new molecular and cellular methods for elucidating the lineage relationships of cells and the intercellular factors that influence phenotypic choices (Altschuler and Lillien, 1992). In parallel, numerous groups have studied the growth of retinal axons to topographically appropriate regions of the tectum (Mey and Thanos, 1992; Goodman and Shatz, 1993; Sanes, 1993). We have undertaken to study a distinct but related issue: how RGC axons select targets in specific laminae and form synapses on them. In previous studies we obtained evidence that the tectum provides a series of distinct, localized cues to guide ingrowing RGC axons to appropriate laminae (Yamagata and Sanes, 1995), and we identified a set of adhesive macromolecules that might play roles in guiding these axonal choices (Yamagata et al., 1995). Here, we have used neurotransmitter-related markers to determine when subsets of RGCs diversify and how they find their targets. Our three main conclusions are: (a) that neuronal subsets acquire distinct characteristics independent of their synaptic targets, (b) that presumptive tectal targets diversify independently of retinal input, and (c) that axons of each RGC class are prespecified to find their targets.

**Markers**

Our results depend on the markers we used, so it is important to consider their reliability. First, we used antibodies to neurofilaments and ChAT to distinguish the two main classes of cells in the GCL, RGCs and displaced amacrines. Neurofila-
Development of ganglion cell subsets

ments are widely distributed in the nervous system, but have been shown to selectively mark RGCs within the inner retina in several species (Drager et al., 1984; Jasoni et al., 1994). This intraretinal selectivity probably reflects the fact that RGCs are the only projection (long-axon) neurons of the retina; in general, neurons with long axons bear neurofilaments, whereas local interneurons bear other intermediate filaments. No completely satisfactory cell-type specific marker was available for amacrines. However, a large subset of amacrines are cholinergic, and luckily this ChAT-positive subset includes about 90% of the displaced amacrines with somata in the GCL (Spira et al., 1987; Millar et al., 1987). Some glia are also present in the GCL, but they represent <5% of the nuclei in this layer, because the intrinsic glial cells of the retina, Müller cells, have their somata in the INL (Ehrlich, 1981).

To identify subsets of RGCs, we used antibodies to SP and to the AChR β2 subunit (mAb 270). Double-staining with the antibodies that distinguished RGCs from displaced amacrines

Fig. 7. Development of retinal arbors in the optic tectum. Retinae were labeled with neurobiotin at E13.5 (a-c) or E17.5 (d), and tecta were fixed, sectioned, and stained with HRP-avidin 12 hours later (E14 and E18). a-c show dorsal, rostral (anterior), and ventral portions from a single section. Retinal axons are initially confined to the SO and SGFS A/B, but invade SGFS D by E14 and SGFS F by E16. Note the paucity of staining in SGFS C and E at E18. Because of the rostrocaudal and dorsoventral gradients of development, a and c correspond to levels of maturity characteristic of anterior tectum at E13 and E15, respectively. As described in Yamagata et al. (1995), laminae SGFS E and F have not formed in anterior tectum at E14, and A/B are indistinct in dorsal tectum. Note that sections in Fig. 8 are all from anterior tectum. Bar is 50 μm.

Fig. 8. Chemically defined subsets of RGC axons selectively innervate appropriate laminae. Sections of E12 (a,b), E14 (c,d), and E16 (e,f) tecta were stained with anti-SP (a,c,e) or mAb 270 (b,d,f). Laminae rich in stained fibers are arrowed. Bar is 50 μm.
provided evidence that within the GCL, both SP and 270 are confined to the RGCs. Direct evidence that adult avian RGCs contain both antigens has been obtained by others, using retrograde labelling from retinorecipient target areas (Ehrlich et al., 1987; Keyser et al., 1988). Interestingly, SP and nicotinic AChRs are expressed by subsets of RGCs in retinae of diverse

Fig. 9. Putative targets of RGC subsets: laminar position in normal and enucleated tecta. Sections of E14 tecta from normal (a,b,d,e) or enucleated (c,f) embryos were stained with antisera to SP-receptor (a-c) or ChAT (d-f), to mark presumptive targets of SP- and AChR-positive RGCs, respectively. (a-c) SP receptor-positive cells are confined to SGFS A/B, SFGS I, and SGC. Only some of the cells in each lamina are stained. Staining of retinorecipient laminae is concentrated in SGFS A/B, which is where SP-positive axons arborize at this stage. This lamina (c) as well as SFGS I and SGC (not shown) also bear SP receptor-positive cells in enucleated tecta. (d-f) ChAT-positive cells are concentrated in SFGS I, in both normal and enucleated tecta. These cells send long tangential processes toward the retinorecipient laminae, and are thought to correspond to the ‘r2’-type tecto-geniculate neurons that arborize in SGFS F (Hunt and Brecha, 1984; Medina and Reiner, 1994), which is where AChR-positive RGCs arborize. g shows an r2-like cell in E14 tectum, impregnated by a Golgi method. Bar is 50 µm for a, d and g; 25 µm for b, c, e and f.
vertebrate species, including mammals (Brecha et al., 1987; Watt et al., 1994; Caruso et al., 1990; Swanson et al., 1987; Hoover and Goldman, 1992), suggesting that our results may be generally applicable.

We also used anti-SP and mAb 270 to identify terminals of RGCs in the tectum. Four lines of evidence indicate that the immunoreactive fibers observed in the tectum do in fact arise from the immunoreactive RGCs in the retina. First, their laminar distributions (Fig. 6) correspond to those of retinal terminals determined electron microscopically (Acheson et al., 1980). Second, their patterns of development (Fig. 8) correspond to those of retinal fibers marked with an anterograde label (Fig. 7). Third, they are absent from tecta that develop in enucleated embryos (Fig. 6). Fourth, Karten and colleagues showed that small retinal lesions in mature animals lead to loss of immunoreactivity from retinorecipient laminae of topographically appropriate regions of the tectum (Ehrlich et al., 1987; Britto et al., 1992a).

Finally, we used antisera to SP receptors and ChAT to label putative targets of the SP- and AChR-positive RGCs. No previous reports on the structure or distribution of avian SP receptors have appeared, but fortunately a polyclonal antiserum to a cloned rat SP-receptor (Shigemoto et al., 1993) cross-reacted strongly with the avian antigen. The rationale for expecting AChR-positive RGC axons to innervate cholinergic targets is given above, and the use of ChAT as a marker for cholinergic cells in the avian brain is widely accepted (Sorensen et al., 1989; Bagnoli et al., 1992; Medina and Reiner, 1994).

Independent diversification of RGC subsets and their tectal targets

Three observations indicated that SP- and 270-positive RGCs did not acquire their distinct properties as a consequence of interactions with their synaptic targets. First, both RGC subtypes appeared in appropriate numbers at a time when most retinal axons were still confined to the tectal SO, and had yet to penetrate the retinorecipient laminae (Fig. 3). Second, both cell types appeared in appropriate numbers in retinae that developed in tecta-less embryos; these RGCs never had an opportunity to contact appropriate synaptic targets (Fig. 4). Finally, both cell types appeared (although not in appropriate numbers, but see below) in retinae that formed within the coelomic cavity; these RGCs never contacted any extraretinal targets at all. Thus, intraretinal influences are sufficient to specify particular RGC subsets as peptidergic or cholinceptive.

Such target-independent specification of phenotype resembles that seen for several other major neuronal classes. For example, spinal motoneurons, cerebellar granule and Purkinje cells, and hippocampal pyramidal cells all acquire their characteristic morphologies when cultured in isolation, and several also acquire appropriate neurotransmitter synthetic or receptive machinery (Banker and Goslin, 1991). However, in several cases, subsets of cells within a major type have been shown to acquire their identifying features as a result of interactions with their targets. Perhaps best studied in this respect is a cholinergic subset of sympathetic neurons, which receive instructive cues from targets in the sweat glands (Landis, 1990). Target-dependent diversification has also been shown for avian parasympathetic neurons (Nishi, 1994) and leech Retzius cells (French and Kristan, 1992).

It has long been clear that RGCs can acquire their general features when they develop without targets or in vitro (e.g. Spence and Robson, 1989), but little has been known about their diversification into subsets prior to this study. A study by Sakaguchi (1989) demonstrated that distinct populations of large and small RGCs develop in isolated Xenopus retinae. Recently Hankin et al. (1993) showed that nicotinic AchRs appear on schedule in fish retinae that develop following tectal ablation; however, the cellular location of the receptors was not reported. In mammals, the question of whether initial diversification is target-independent in mammals remains open. There is evidence that interactions with targets differentially influence maturation of RGC subsets (Ault and Leventhal, 1994), and that target-deprived RGCs of one subset can form novel connections, characteristic of another subset (Roe et al., 1993), raising the possibility that subset identity is target-dependent. However, the finding that mammalian RGCs of different sizes are born at distinct times has led to the speculation that their diversification depends on intraretinal cues (Rapaport et al., 1992).

Our studies of tectal development provide suggestive evidence that targets of RGC subsets acquire their specialized properties in the absence of retinal input: SP receptor-positive cells are concentrated in SGFS B, which is where SP-positive RGC axons arborize; cholinergic cells project to SGFS F, which is where AChR-positive RGC axons arborize; and both populations of tectal neurons appear in appropriate laminae of enucleated tecta. This complementarity bolsters the idea, discussed below, that laminar selectivity involves chemical matching of pre- and postsynaptic partners. However, our conclusions about the tectal neurons must remain tentative, for two reasons. First, we have yet to show directly that the chemically coded tectal neurons are direct synaptic targets of RGC axons. Second, tectal neurons receive inputs from many sources. Given the known effects of synaptic input on neuronal differentiation (see for example, Baptista et al., 1994), it would not be surprising if nonretinal inputs contributed to the diversification of tectal subsets.

The mechanisms that underly the diversification of neuronal subsets, which may include both intercellular signals and lineage-based predilections, should be particularly accessible to study in the retina. For example, the possibility that progenitors generate particular types or combinations of RGCs could be tested by the method of retrovirus-mediated gene transfer, which has been used to study cell lineage in several portions of the chick nervous system (reviewed by Leber et al., 1995). Intercellular interactions could be sought by assays the differentiation of dissociated RGCs, in isolation or in combination with other cells. Promising starting points are provided by reports that some transcription factors, such as the LIM-type homeodomain protein islet-1 (Thor et al., 1991) and the POU-type homeodomain protein brn-3b (Xiang et al., 1993), are expressed by subsets of RGCs. In several other sensory and motor systems, combinatorial expression of LIM and POU class transcription factors have been hypothesized to be determinants of neuronal fate (Tsuchida et al., 1994). Accordingly, it will be interesting to learn whether the RGCs that express these factors correspond to the chemically defined subsets that we have described here.

Subclass-specific projections

Axons use multiple mechanisms to find their targets in the
developing nervous system. In some areas, connectivity is specific from the outset; in others, initially diffuse projections are later refined (Goodman and Shatz, 1993). Within the visual system, numerous cases of remodeling have been documented. In birds and mammals, for example, retinotopic maps are orderly from the outset, but are sharpened considerably as development proceeds (Mey and Thanos, 1992; Holt and Harris, 1993). Similarly, RGCs that project to the lateral geniculate nucleus in mammals initially innervate several laminae, then rearrange their arbors to limit them to a single, eye-specific lamina (Shatz, 1990). Likewise, many β-class RGCs in the cat, which project only to the geniculate in adulthood, send a transient collateral to the superior colliculus during development (Ramoja et al., 1989). In some of these cases, competitive interactions among inputs seem to be crucial to the refinement process, and patterns of electrical activity are critical determinants of the timing and outcome of the competition.

In apparent contrast to these cases, projections of RGCs to the upper portions of SGFS (A-F) appear to be precise from the outset, in that axons never extend beyond the area they will ultimately occupy (McLoon, 1985: Fig. 7). Here, we have extended this conclusion to SP- and 270-positive subsets of RGCs: the former are restricted to the upper third of the retinorecipient zone and the latter extend into SGFS F as soon as this lamina forms (Fig. 8). Specificity is not likely to be absolute, for we have found occasional retinal axons that arborize in two separate laminae at E14, whereas this pattern is not seen in maturity (Yamagata and Sanes, 1995). It may be, therefore, that activity-dependent and/or competitive interactions shape laminar patterns in this system as well. Nonetheless, the selectivity we have documented makes it likely that individual subsets of RGCs bear chemical labels that interact with laminar-specific labels in the tectum. The findings that RGC subsets and their presumptive targets differentiate chemically in each other’s absence provides further support for a ‘chemoaffinity’ (Sperry, 1963) model in which complementary pre- and postsynaptic labels are independently determined. The neurotransmitter ligand-receptor systems we have described may themselves play a role in matching RGC axons to their targets. In addition, we recently showed that several known cell adhesion and neurite outgrowth-promoting molecules are selectively associated with retinorecipient laminae in developing tectum (Yamagata et al., 1995). Some of these are particularly concentrated in one or another of the sublaminae, and some appear on schedule in enucleated tecta. These molecules, therefore, have several of the features predicted for recognition molecules that mediate selective innervation of tectal sublaminae by RGC subsets.

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