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Lamina–specific cues guide outgrowth and arborization of retinal axons in the optic tectum

Masahito Yamagata and Joshua R. Sanes*

Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA

*Author for correspondence

SUMMARY

In the chick, retinal axons enter the optic tectum through a superficial lamina, then branch into distinct deeper retinorecipient laminae, where they arborize and form synapses. To study factors that guide this laminar selectivity, we devised an organotypic culture system in which a transverse tectal section is overlaid with a retinal explant large enough to allow unimpeded access to all tectal laminae. Outgrowth, branching, and arborization patterns of retinal axons on tectal slices were lamina-selective, indicating the existence of localized cues that guide retinal axons. Further studies suggested that some of these cues are: (1) associated with cell membranes or extracellular matrix (because axons grew selectively on chemically fixed tectal sections); (2) intrinsic to the tectum (because axons grew selectively on tectal sections prepared from enucleated embryos); (3) distinct from topographic cues (because axons from nasal and temporal retina behaved similarly on anterior tectal slices); and (4) selective for retinal axons (because axons growing from other explants exhibited different laminar preferences).

Key words: axon guidance, retinotectal, synaptogenesis, chick

INTRODUCTION

A prominent feature of neural organization in many parts of the vertebrate central nervous system (CNS) is the arrangement of cells into discrete laminae, parallel to the pial and ventricular surfaces. Each lamina bears a distinct array of neuronal types and, of particular interest here, each receives a specific set of synaptic inputs. Indeed, the laminar selectivity of axonal growth and arborization may be a major determinant of synaptic specificity in large portions of the telencephalon (cerebral cortex and hippocampus), diencephalon (lateral geniculate) and mesencephalon (optic tectum). In some of these areas, axons appear to subdivide dendrites into discrete territories. In the hippocampus, for example, inputs from entorhinal cortex, commissural/associational fibers and dentate granule cells are stratified such that they synapse on distal, middle, and proximal portions, respectively, of pyramidal cell dendrites (Bayer and Altman, 1987). In other cases, such as the cerebral cortex, laminar selectivity may largely reflect axonal choices among distinct cell types that populate the different laminae (Katz and Callaway, 1992). In no case, however, are the mechanisms that underlie laminar selectivity understood.

We have chosen to study laminar selectivity in the optic tectum of the chick embryo. The avian tectum has long been favored for developmental studies of specific connectivity in the vertebrate CNS. This is in part because it shares several structural features with the mammalian cerebral cortex, but is considerably more accessible to experimental manipulation at early developmental stages (Mey and Thanos, 1992; Galileo et al., 1992). Most intensively studied in tectum has been the retinotopic map, whereby ganglion cells from various parts of the retina project systematically onto the tectal surface. Comparably striking, but less well studied, is a laminar selectivity in the orthogonal direction. Retinal axons enter the tectum through the most superficial of its 15 discrete laminae, the stratum opticum (SO). Once axons reach defined loci on the tectal surface, they branch inwards and form arbors in only three to four of the 15 laminae (Acheson et al., 1980; Cajal, 1911; Hunt and Brecha, 1984; van Gehuchten, 1892). Moreover, it seems likely that any single retinal axon confines most of its synapses to a single one of these ‘retinoreceptive’ laminae (LaVail and Cowan, 1971; Repérant and Angaut, 1977). Within the target lamina, the axon forms synapses on cells of specific classes, many of which have somata in deeper laminae and receive input from other sources as well (Hunt and Brecha, 1984). This arrangement implies that the retinal axon confines its inputs to a defined segment of a dendritic arbor that extends over several laminae and receives many types of inputs. Thus, laminar selectivity in tectum may require an axon to recognize particular target cells (cellular specificity) and particular portions of the target cell’s surface (subcellular specificity).

How might these laminar patterns arise? One possibility is that cells or dendritic segments within individual laminae bear cell surface labels that axons recognize. It is also possible, however, that patterns seen as lamina-specific in adulthood arise from refinement of a diffuse pattern by activity-

dependent interactions. A third alternative is that laminar selectivity arises from temporally and spatially coordinated growth of pre- and postsynaptic elements. All of these possibilities have been proposed to explain laminar patterns in various systems (Bayer and Altman, 1987; Bolz et al., 1993; Frotscher and Heimrich, 1993; Katz and Callaway, 1992; Shatz, 1990). As a first step in analyzing laminar selectivity in the tectum, we have tried to distinguish among these alternatives. To this end, we devised a retina-tectum coculture system in which growing retinal axons have unimpeded and simultaneous access to all tectal laminae, and in which the retinal and tectal explants can be manipulated independently. The patterns of outgrowth documented here lead us to conclude that the tectum bears a series of distinct, localized, intrinsic, membrane- and/or matrix-associated cues that guide the lamina-specific outgrowth and arborization of retinal axons.

MATERIALS AND METHODS

Anterograde labeling of retinal axons with DiI

Fertilized chicken eggs were purchased from SPAFAS (Roanoke, IL), and incubated at 37.5°C. Only embryos that were developing normally according to the Hamburger and Hamilton (1951) staging system were used. To analyze the intratectal course of retinal axons, we used a lipophilic carbocyanine dye DiI (Molecular Probes, Eugene, OR). At appropriate stages, tecta were dissected out, and a few crystals of DiI were placed on the optic tract near the anterior-ventral pole as described by Senut and Alvarado-Mallart (1986). The tecta were then immersed in 3.5% formaldehyde in Hanks' solution buffered with 20 mM Hepes, pH 7.4, at room temperature. After 1 week, tecta were embedded in agarose, sectioned at 80 μm in a ventral-dorsal plate using a Vibratome 1000 (Ted Pella, Inc., Redding, CA), and mounted in 150 mM NaCl, 20 mM sodium phosphate, pH 7.4 (PBS) containing 0.005% Hoechst 33342 (Molecular Probes, Eugene, OR). The anterior tectum was examined under epifluorescence illumination, and individual arbors were traced with a camera-lucida apparatus, using a white pencil on black paper.

Preparation of explants

Retinal explants were prepared from E7 embryos by the method of Halfter et al. (1983) as modified by Drazba and Lemmon (1990). Briefly, a dissected retina was spread, ganglion cell layer up, onto a black nitrocellulose membrane precoated with concanavalin A (Sigma, St. Louis, MO). Tectobulbar explants were prepared similarly, by spreading E5-6 tecta, ventricular side down, onto the nitrocellulose membrane (Kröger and Schwartz, 1990). The explants were then labeled and cut into sections. Initially, we used rhodamine for this purpose (Walter et al., 1987). Later, however, we found that PKH26 (Sigma) was better retained and less toxic than rhodamine; we therefore used this dye in most of the experiments reported here. Each explant incubated at 25°C for 7 minutes in 1 ml of 20 μM PKH26 in the labeling solution provided in the kit. Then, the explant was rinsed in a 1:1 mixture of fetal calf serum and F12 medium for 1 minutes, and then in the organotypic culture medium (see below) for 30 minutes. Finally, 350 μm strips of retina or tectum were prepared using a Sorvall TC-2 tissue sectioner.

Tectal slices were prepared from E14 embryos. The meninges were carefully removed, the tecta were placed immediately into oxygenated Hanks' solution, and 200 μm thick slices were cut with a Sorvall TC-2 tissue sectioner. For fixation in methanol, the slices were placed in 100% methanol at −20°C overnight, then transferred to Hanks' solution for 3 hours at 4°C. For fixation in formaldehyde, the slices were immersed in 3.5% (w/v) paraformaldehyde in Hanks' solution supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM Hepes for 5 hours at 4°C followed by washing in Hanks' solution for 3 hours. In some cases, embryos were enucleated bilaterally at E3 as described by Kelly and Cowan (1972) using an electrocautery device (Model 285101, Abco Inc., Milwaukee, WI).

Coculture of retina and tectum

To assemble a coculture, a tectal slice was flattened on a multipore polycarbonate filter (Nuclepore, 8.0 μm pore size, 13 mm diameter; Costar, Cambridge, MA) and floated on 2 ml of medium in a 35 mm tissue culture dish or a 6-well cluster plate. The medium was a 1:1 mixture of Dulbecco modified Eagle and F12 media, supplemented with 10% (v/v) fetal calf serum, 15% (v/v) E10 chick embryo extract, 0.5% (v/v) chick optic tectum extract, 10 mM Hepes, 10 μg/ml penicillin, and 10 μg/ml streptomycin. The chick optic tectum extract was prepared from E14-15 embryos by homogenizing tecta in Hanks' solution at a ratio of 1 tectum per 50 μl, then centrifuging at 2,500 ***g*** for 15 minutes to remove debris. The medium was oxygenated in advance with 95% O_2 and 5% CO_2.

A strip of fluorescent dye-labeled retinal explant was then laid on the slice, and a stainless steel bar (400 μm diameter × 5 mm length) was placed atop the explant to improve contact with the underlying tectal slice. The culture was maintained in a humidified incubator with 5% CO_2 at 37°C. After 15 hours in culture, cytosine arabinoside was added to a final concentration of 5 μM. After 32 hours more, the filter containing the culture was placed on 3.5% paraformaldehyde in PBS containing 0.005% Hoechst 33342 overnight. Fixed cultures were mounted in Gel/Mount (Biomeda, Foster City, CA), observed with a Nikon epifluorescence microscope, and drawn with a camera-lucida apparatus.

Labeling live axons with calcein AM

Retinal explants prelabeled with PKH26 could only be cultured for 2-3 days before the dye became too dim to be useful. For longer-term cultures on fixed tectal slices, we used a fluorogenic esterase substrate, calcein AM (Molecular Probes), as a vital stain. These cultures were prepared with explants that had not been prelabeled. After 3-7 days, the culture was rinsed with PBS (plus calcium and magnesium) for 5 minutes at room temperature, then transferred onto 10 μM calcein AM in PBS for 60 minutes at room temperature. Cultures were rinsed with PBS supplemented with Hoechst dye 33342 (0.005%) for nuclear counterstaining, then immediately observed and photographed using an epifluorescence microscope. Axons remained fluorescent for up to 3 hours.

Nomenclature for tectal laminae

For animals E18 or older we use the nomenclature for tectal laminae proposed by Huber and Crosby (1933), and modified by LaVail and Cowan (1971; see Fig. 1d). LaVail and Cowan (1971) proposed an alternative nomenclature for developing laminae. However, most of the definitive laminae can be recognized by E12, so we refer to them accordingly, but with uppercase letters. The correspondence between these terms and those of LaVail and Cowan at E12-14 is: stratum fibrosum periventriculare (SFP) = I; stratum griseum periventriculare (SGP) = II; stratum album centrale (SAC) = III; stratum griseum centrale (SGC) = IV; stratum griseum et fibrosum superficiale (SGFS)-J = V; SGFS-I = VI; SGFS-H = VII; SGFS-G = VIII; SGFS-D = IX; C=X; A/B = XI; and stratum opticum (SO) = XII. From E16 until hatching, I-VII are as above, SGFS-G = VIII; SGFS-F = IX; SGFS-E = X; SGFS-D = XI; SGFS-C = XII; SGFS-B = XIII; SGFS-A = XIV; and SO = XV. Students of Latin may be helped to remember this cumbersome nomenclature by the realization that SGFS is a superficial layer, SAC and SGC are centrally located, and SFP and SGP are deep (profound).

RESULTS

Normal development of retinal arbors in the optic tectum

We began this study by asking when and how retinal axons reach the retinorecipient laminae of the tectum in vivo. We used DiI as an anterograde tracer to label individual axons in fixed tissue and focused on anterior tectum because the laminar segregation of retinal inputs is clearest in this area. Our results confirm and extend previous studies (see Discussion) and lead to the following picture of retinotectal development:

The first retinal axons arrived at the anterior-ventral pole of the contralateral tectum at embryonic day (E) 6. Until E10, most retinal axons remained in the SO (Fig. 1a). By E12, however, many axons had extended into deeper layers. At this stage, two modes of ingrowth from the SO were observed: perpendicular branching of collaterals (Fig. 1b, left), and oblique curving of axonal termini (Fig. 1b, middle). Both types of axons formed rudimentary arbors just under the SO that appeared to be different from the arbors that were observed in later stages both in form (more widespread) and in laminar position (more superficial; Fig. 1b, right). It may be, therefore, that these arbors are part of a transitory neuropil, rather than being direct precursors of the mature arbors. At E13-14, the definitive retinorecipient laminae were recognizable for the first time, and definitive arbors began to form within them (Fig. 1c). At this stage, most arbors were confined to a single lamina, but a minority spread across multiple laminae. At no time, however, did retinal axons extend beyond the retinorecipient zone (SGFS A-F). (Arrangement of laminae is shown in Fig. 1d and nomenclature is explained in Materials and Methods) By E16, nearly all arbors were confined to a single lamina. We are uncertain whether the bilaminar arbors present initially were eliminated in their entirety, or remodeled to eliminate some branches. In any event, three distinct types of arbors were observed by E18, and these were confined to retinorecipient laminae B, D and F, respectively (Fig. 1d). The largest number of arbors (well over half) was in SGFS-D; fewer were present in SGFS-B and still fewer in SGFS-F. (Some arbors were seen in SGFS-C in posterior tectum, but this class was seldom encountered in anterior tectum.) A further increase in the number of branches per arbor was observed between E18 and 2 days posthatching (not shown; hatching is usually at E21), indicating that retinotectal connections continue to mature for several days after lamina-specific arbors are established.

Cocultures of tectal slices and retinal strips

To ask whether local tectal cues guide lamina-specific behaviors of retinal axons, we devised the culture system diagrammed in Fig. 2a. A slice of tectum was supported on a Nuclepore filter and overlaid with a strip of retina that was large enough to contact all the tectal laminae. Retinae were dissected from E7 embryos, labeled with a fluorescent dye, and cut into strips. Previous studies have shown that retinal explants generate abundant outgrowth at E5-E7, times at which ganglion cell axons are leaving the retina in vivo (Halfter et al., 1983). We used E7 because younger retinae were not large enough to cover all tectal laminae. Tectal slices were prepared from E14 embryos, the earliest stage at which definitive retinal arbors can be recognized (see above). Because the tectum develops in an anterior-posterior gradient, we minimized variation by focusing primarily on its anterior part. The filter was floated on culture medium, providing the explant with good access to both medium and air. Lamination was well preserved in most cultures for 2-3 days (Fig. 2b,c), and >90% of the cells appeared viable when stained with a nuclear stain, Hoechst 33342, after 2 days in vitro. However, laminae did not continue to develop during this period and by 4-5 days in vitro, many tectal cells had died and stratification of tectal slices was disrupted. We therefore routinely examined these cultures after 2 days.

Five main features of retinal outgrowth in this culture system are illustrated in the camera lucida drawings shown in Figs 2d, 3a. First, retinal axons were longest and most numerous in the SO. Second, some axons in the SO turned and extended into deeper layers. Third, when these ingrowing axons reached the retinorecipient lamina, they often branched and sometimes showed a varicose morphology. Fourth, few axons grew into deeper portions of SGFS (G-J), either from the retinorecipient laminae or directly from the retinal explant. Finally, some outgrowth was observed along the deepest layers (SGC, SAC, SGP, and SFP).

Patterns of the sort shown in Fig. 2d were usually but not invariably observed. In a set of 5 experiments, for example, results similar to those described above were seen in 69 out of 100 retinotectal juxtapositions from 50 cultures. (Outgrowth from each side of the retinal explant onto anterior tectum was rated separately, giving 2 ‘positions’ from each culture.) In 21/100 positions, no neurites extended from the explant onto the slice. In the remaining 10 positions, many neurites were present, but no lamina-selective outgrowth was evident. We believe that two factors accounted for much of the nonselective behavior in some cultures. First, when cell death was extensive in the tectal slices, debris accumulated and axonal guidance appeared to be perturbed by it. Second, when a very large number of axons extended from the retinal slice, they fasciculated, and the fascicles showed less responsiveness to tectal cues than did individual axons.

To quantitate lamina-selective outgrowth, the tectum was divided into 5 zones, as shown in Fig. 2d: the SO; retinorecipient and intervening laminae (SGFS A-D); retinononrecipient portions of SGFS (G-J); central laminae (SGC and SAC); and deep laminae (SGP and SFP). Within each zone, we measured the maximum length that a neurite or fascicle had grown, and the number of neurites or fascicles that had extended at least one-half of the maximum distance from any zone in that culture. Results from one typical set of cultures (n=8) are shown in Fig. 4a,b. Neurites were longest and most numerous in the SO. Indeed, the figure greatly underestimates the preferential outgrowth we saw because multiple neurites were usually counted together as a single fascicle in the SO, whereas we could generally distinguish individual neurites growing on deep layers. The fewest and shortest neurites grew on the deep (retinononrecipient) portions of SGFS. Thus, measurements confirmed our subjective impression that the SO is a favorable substrate and the retinononrecipient laminae a poor substrate for outgrowth of retinal axons.

Retinal axon behavior on chemically fixed tectal slices

Cues that guide retinal axons might depend on processes that require ongoing metabolism in tectal cells, such as neural

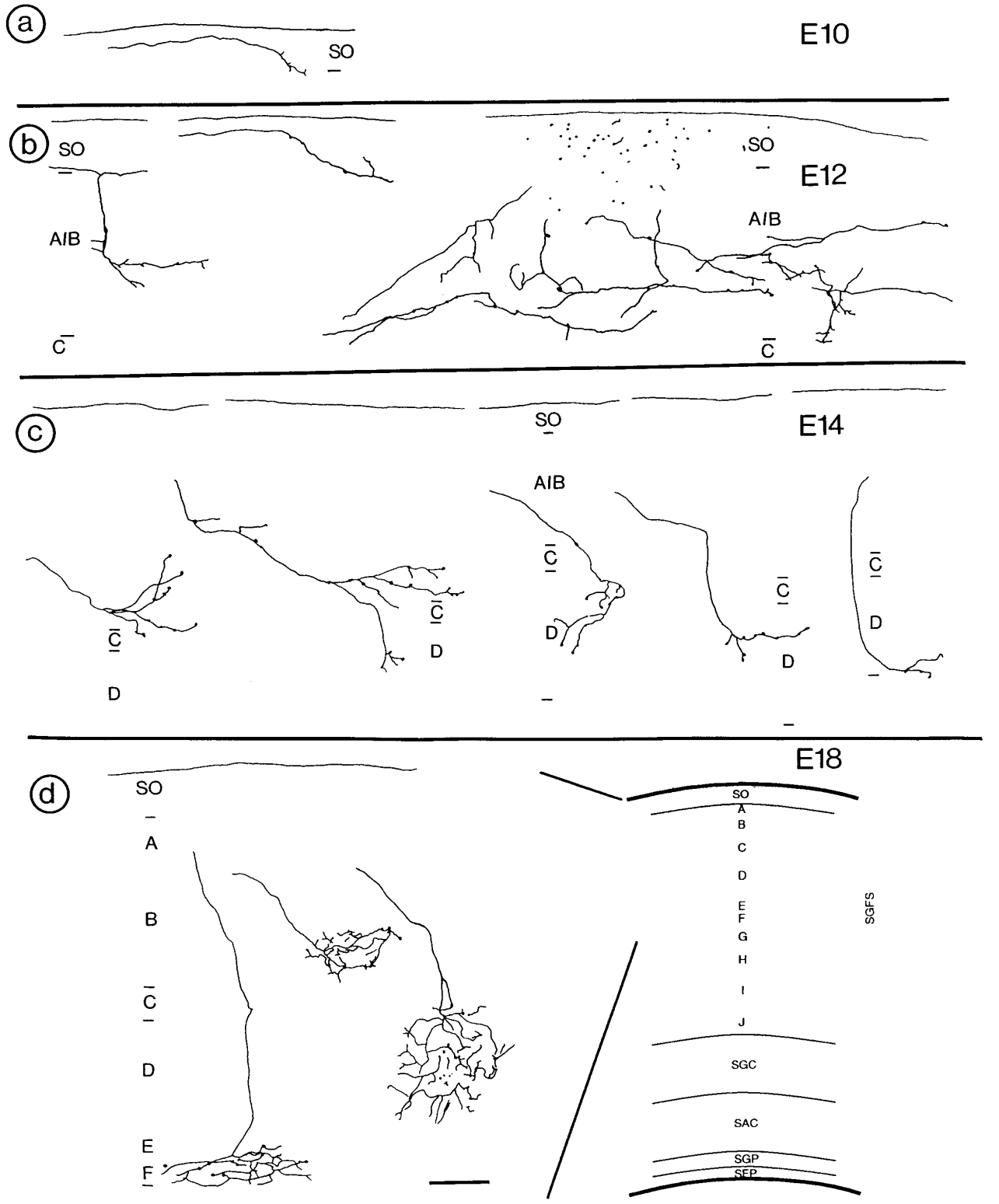

Fig. 1. Development of retinal axonal arbors in the optic tectum. Axons were labeled with DiI in fixed tissue, and traced with a camera lucida. Positions of tectal laminae, revealed with a nuclear counterstain (see Fig. 2b, for example), are indicated. (a) E10: Most retinal axons are confined to the SO. (b) E12: Axons leave the SO obliquely or by collateralization, and form a transient neuropil just beneath the SO. (c) E14: Rudimentary arbors have formed in the retinorecipient laminae. (d) E18: Each retinal axon has an elaborate arbor that is confined to one of the retinorecipient laminae (SGFS B, D, or F). Sketch on the right in d shows all tectal laminae, and indicates the region shown in the camera lucida drawings. Tecta were sectioned parallel to the trajectories of retinal axons in all parts but b (right), in which retinal axons were cross-sectioned. See Materials and Methods for full names of laminae. All parts are at the same magnification. Bar is 50 μm.

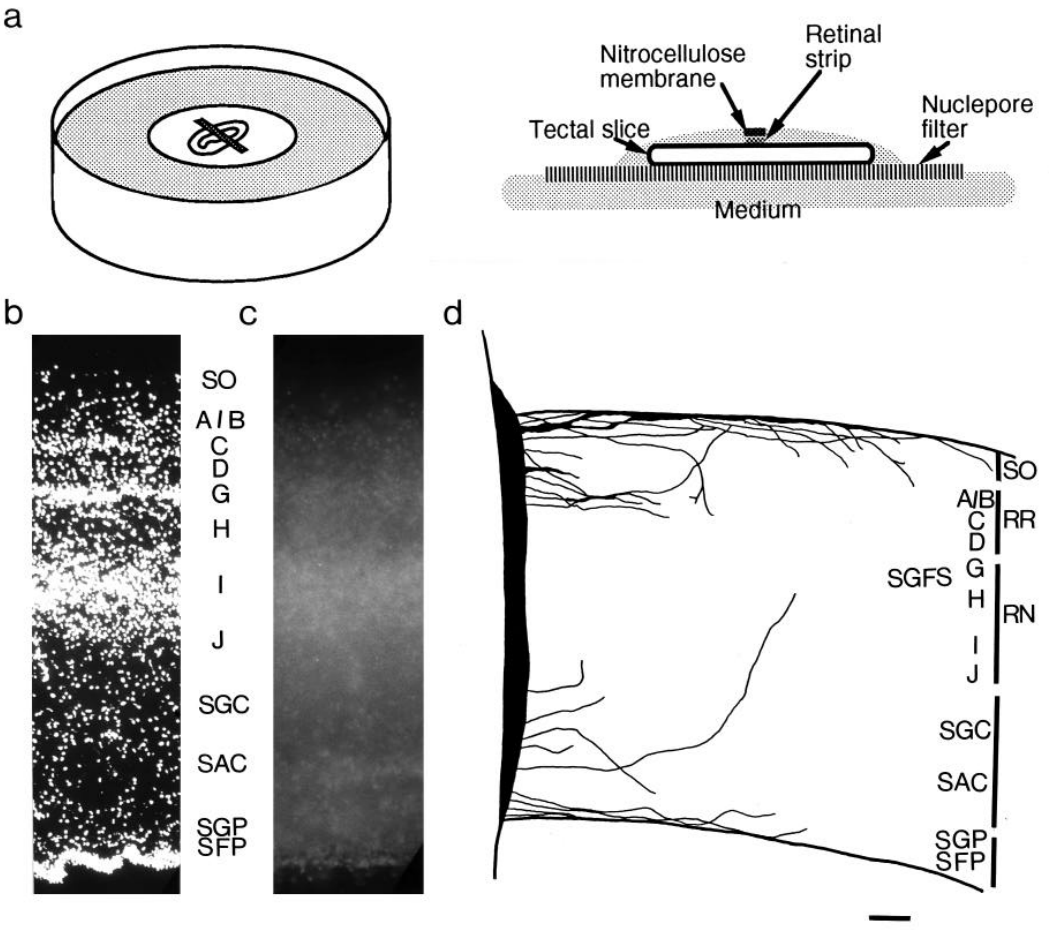

Fig. 2. Outgrowth of retinal axons on tectal slices. (a) Cocultures are prepared by laying a retinal strip across a tectal slice, such that retinal axons have equal access to all tectal laminae. See text for details. (b) Laminae in a cryostat section of E14 tectum, revealed with a nuclear counterstain. (c) Laminae are still distinguishable after 2 days in culture. Although laminae are less clear after incubation, the difference is exaggerated in these micrographs because the section in c is much thicker than that in b (200 μm versus. 10 μm). (d) Retinal axons on a tectal slice, after 2 days in vitro; camera lucida drawing. Neurites are most numerous and longest on the SO. Some neurites branch inward from the SO to form rudimentary arbors in the retinorecipient laminae. Few neurites extend on retinononrecipient laminae (SGFS G–J). Bars at right indicate the zones in which outgrowth was measured; see Fig. 4. Bar in d indicates 80 μm for b and c, and 100 μm for d.

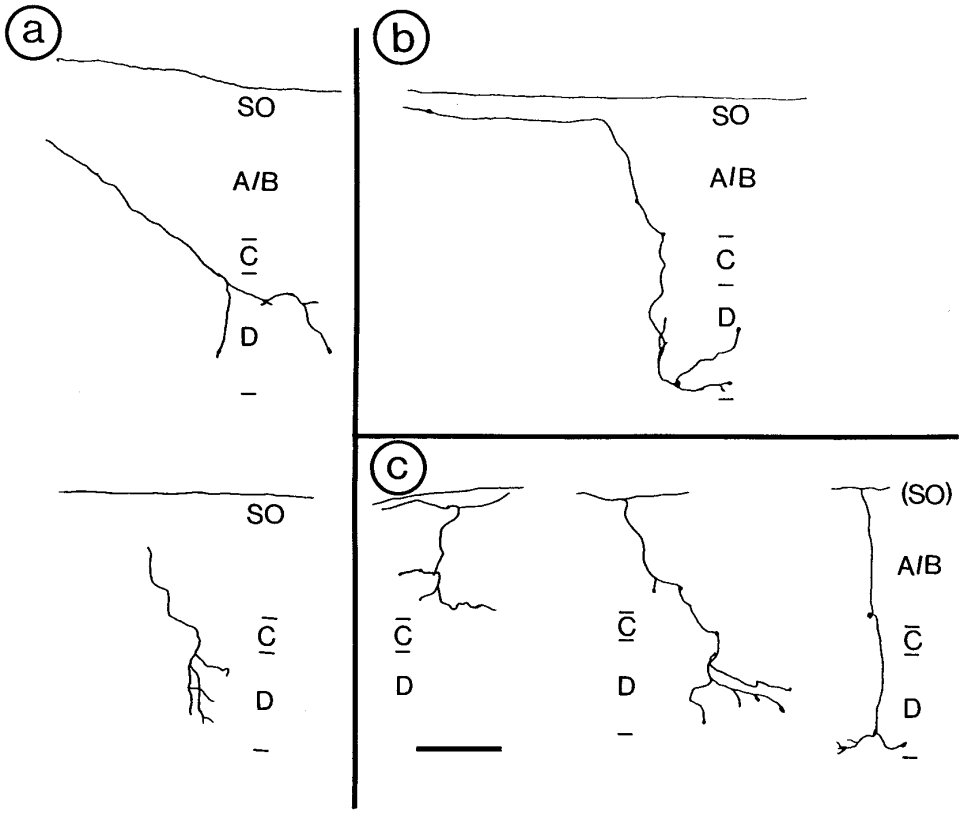

Fig. 3. Arbors formed by retinal neurites growing on tectal slices. The arbors are simple, and generally confined to one of the retinorecipient laminae. (a) Live tectal slices, 2 days in vitro. (b) Formaldehyde–fixed tectal slice, 7 days in vitro. (c) Formaldehyde–fixed tectal slices from a bilaterally enucleated embryo, 7 days in vitro. Bar is 50 μm.

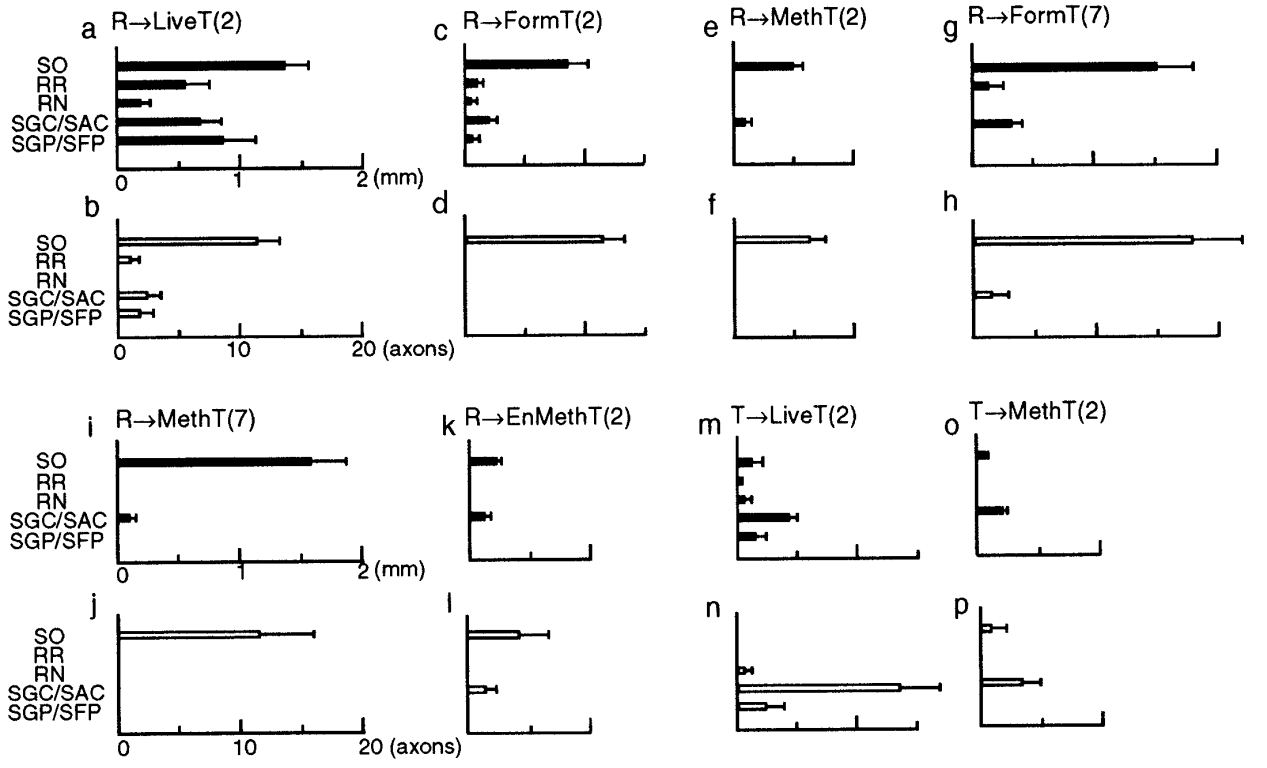

Fig. 4. Lamina–selective axon outgrowth on tectal slices. Measurements were made in each of five zones, as indicated in Fig. 2d: the SO, the retinorecipient laminae (RR; SGFS– A–F), the retinononrecipient laminae (RN; SGFS G–I), the intermediate laminae (SGC and SAC), and the deep laminae (SGP and SFP). In each pair of graphs, the upper one (a,c,e,g,i,k,m,o) shows the distance from the retinal explant to the tip of the longest axon. The lower graph (b,d,f,h,j,l,m,p) shows the number of axons or fascicles per zone at half the maximum distance that growth extended from the explant. (a,b) E7 retinal explants (R) cultured for 2 days on live E14 tectal (T) slices. (c,d) Retinal explants cultured for 2 days on formaldehyde–fixed tectal slices. (e,f) Retinal explants cultured for 2 days on methanol–fixed tectal slices. (g,h) Retinal explants cultured for 7 days on formaldehyde–fixed tectal slices. (i,j) Retinal explants cultured for 7 days on methanol–fixed tectal slices. (k,l) Retinal explants cultured for 2 days on methanol–fixed tectal slices from enucleated embryos. (m,n) E6 tectal explants cultured for 2 days on live E14 tectal slices. (o,p) Tectal explants cultured for 2 days on methanol–fixed tectal slices. Each value is mean ±s.e.m., n=4–8.

activity or secretion of tropic factors. Alternatively, cues might initially be produced by tectal cells, but then maintained in stable form on cell membranes or extracellular matrix. To distinguish these alternatives, we tested the ability of chemically fixed tectal slices to influence the behavior of retinal neurites.

Tectal slices were fixed with either 3.5% formaldehyde or 100% methanol, then washed exhaustively and overlaid with a retinal explant. In both cases, retinal axons grew preferentially along the SO, often forming fascicles. As in live cultures, some neurites turned inward and terminated in retinorecipient laminae. Very few neurites entered deeper layers, either via the retinorecipient laminae or directly from the explant. Thus, the major features of lamina-selective outgrowth and arborization seen on live tectum were also seen on fixed tectal slices (Fig. 5a,c). In fact, laminar selectivity of outgrowth was consistently more pronounced on fixed than on live slices (Fig. 4c-f). These results suggest that some tectal cues are stably associated with cell surfaces.

An unanticipated advantage of using fixed tectal slices was that they allowed us to study cultures for up to a week, and thus follow the development of the arbors in the retinorecipient laminae. First, we were able to vitally stain neurites (with calcein AM; see Materials and Methods) at the end of the experiment without interference from live cells in the underlying tectal slice. In contrast, prelabeling methods provided sufficient intensity for only 2-3 days in vitro. Second, tectal laminae were distinguishable indefinitely in fixed slices, whereas laminar structure in live slices frequently became disrupted after 2-3 days in vitro. Laminar preferences for outgrowth were maintained for at least 1 week in vitro (Fig. 4g-j). By this time, many axons growing along the SO had branched into deeper laminae. Most of these branches were confined to the retinorecipient laminae, and some of them had developed rudimentary arbors (Fig. 3b). Importantly, arbors were restricted to retinorecipient laminae throughout the period that cultures were observed, from 2 to 10 days in vitro (Fig. 5b,d).

Patterns of neurite outgrowth were similar on formaldehyde- and methanol-fixed slices, but three differences were consistently observed (Fig. 5). First, the amount of arborization in the retinorecipient laminae was lower on methanol-fixed than on formaldehyde-fixed slices. Second, those neurites that entered the retinorecipient laminae of methanol-fixed slices generally did so by collateral branching, whereas oblique entry predominated on formaldehyde-fixed slices. It seems unlikely that radial branching was stimulated by methanol-fixation;

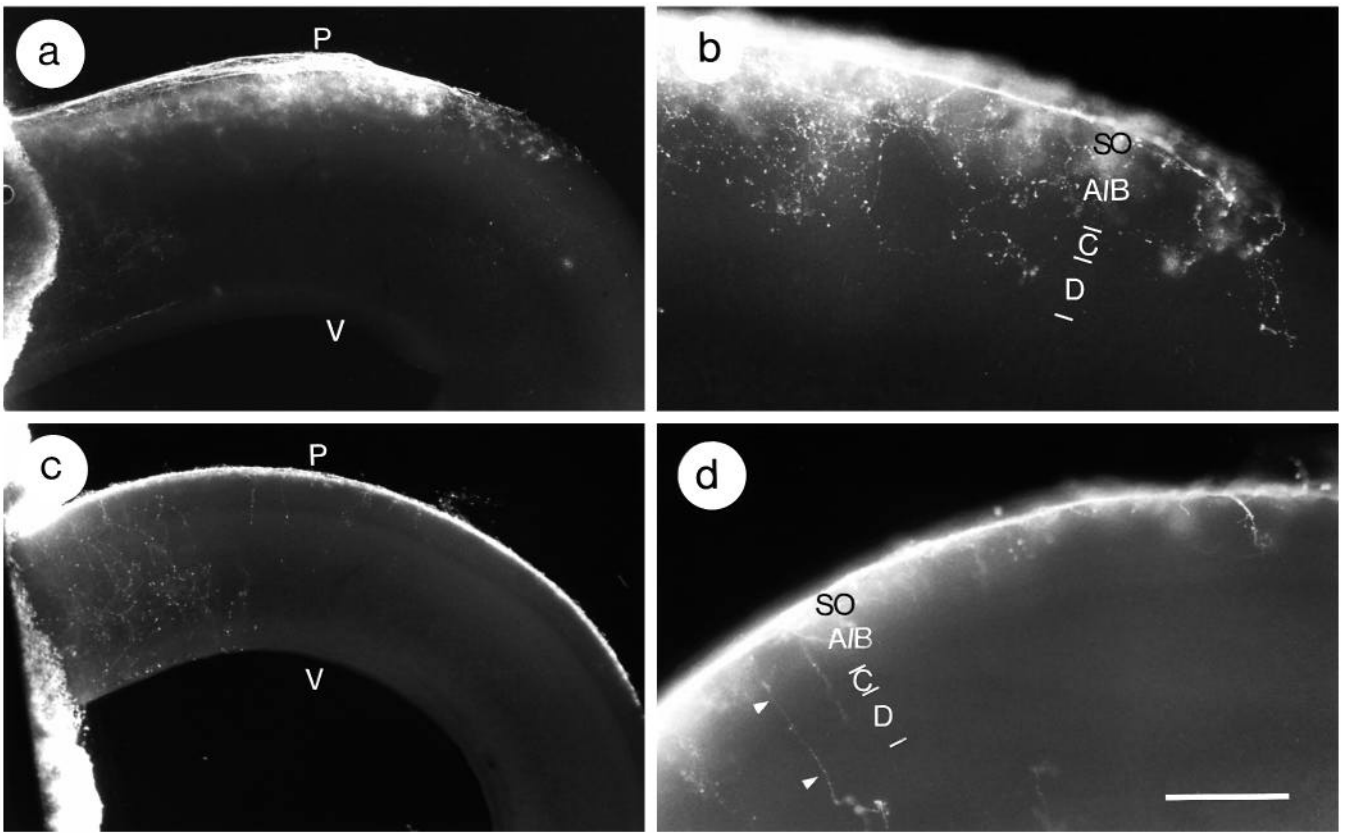

Fig. 5. Outgrowth of retinal axons on fixed tectal slices. Slices were fixed with formaldehyde (a,b) or methanol (c,d), then washed extensively before retinal explants were added. Cocultures were labeled with a vital dye after 7 days in vitro. Outgrowth was lamina–selective with both fixatives, but subtle differences between them, described in the text, were noted. Bright fluorescence at the left of a and c (also seen in Figs 6 and 7) is from nonneuronal cells and debris associated with the retinal explant. Bar is 500 μm for a and c, 125 μm for b and d.

instead, we suspect that it became apparent when oblique growth was suppressed. Finally, some neurites extended past the retinorecipient laminae in methanol-fixed slices (arrowheads in Fig. 5d), whereas this boundary was almost always respected in formaldehyde-fixed slices. Thus, some cues in the SGFS may be more stable to formaldehyde than to methanol.

Retinal axon behavior on tectal slices from enucleated embryos

Tecta have already received retinal inputs by E14 (Fig. 1), the stage we used for preparation of slices. Thus, the tectal slices contained numerous severed retinal axons, and it seemed possible that neurites growing from the retinal explant were being guided by axonal debris in the tectal slice. To test this possibility, we prepared tectal slices from chick embryos whose eye anlagen had been surgically removed at E3, before any retinal axons had contacted the tectum. Tecta in such eyeless (enucleated) embryos develop almost normally up to E14, although the SO, which is formed largely from retinal axons, is understandably attenuated (Kelly and Cowan, 1972; Whitehead, 1979). We enucleated embryos bilaterally because a normally transient projection from the intact ipsilateral retina is maintained following unilateral enucleation (Thanos and Dütting, 1988). The main axonal behaviors documented above for normal tectal slices were also observed on slices of enucleated tecta: preferential outgrowth along the remnant of the SO, turning toward the retinorecipient laminae and little outgrowth onto deeper laminae (Figs 4k,l, 6). Moreover, rudimentary arbors developed in the retinorecipient laminae of both live and formaldehyde-fixed slices from enucleated embryos (Fig. 3c). Overall, fewer neurites grew on slices of enucleated SO than on live or fixed slices from normal tecta, and no outgrowth at all was seen in a substantial fraction of cultures. Nonetheless, the behavior of neurites on tecta that lack endogenous retinal inputs suggests that some of the cues that guide retinal axon lamina-selective outgrowth and arborization are either intrinsic to the tectum or derived from non-retinal inputs.

Behavior of tectobulbar axons on tectal slices

Are the cues that direct the behavior of retinal axons on tectal slices specific for retinal axons? To address this question, we assayed the outgrowth of nonretinal axons on tectal slices. The major output cells of the tectum are multipolar ganglion cells, whose somata reside in the SGC and whose axons extend through the SAC to form the tectobulbar tract. Kröger and Schwartz (1990) have shown that multipolar cells give rise to the majority of neurites that grow from E6 tectal explants, just as the retinal ganglion cells are the source of neurites from retinal explants. Accordingly, we assessed patterns of outgrowth from E6 tectal explants plated atop E14 tectal slices. On both live and methanol-fixed tectal slices, the tectobulbar

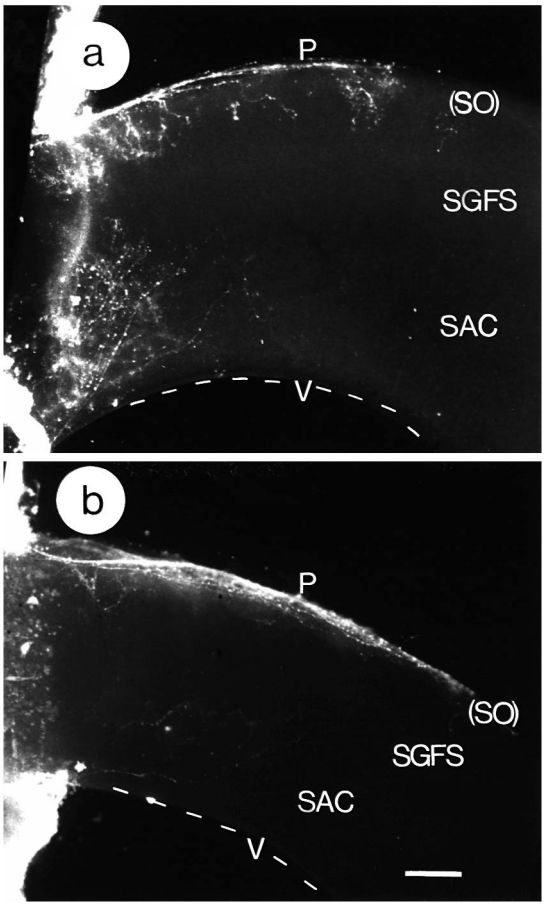

Fig. 6. Outgrowth of retinal axons on tectal slices from embryos that had been bilaterally enucleated. (a) Formaldehyde–fixed slice. (b) Methanol–fixed slice. Outgrowth was lamina–selective on tecta that had never received retinal input in ovo. Bar is 200 μm.

axons grew preferentially along the SAC and SGC, just as they would do in vivo (Figs 4m-p, 7). Laminar selectivity was generally more striking on live than on fixed slices, but little outgrowth was seen along the SO in either type of culture. Moreover, even when tectobulbar axons did extend along the SO, they seldom arborized in the retinorecipient laminae, and when they did extend into retinorecipient laminae they did not regularly stop growing at SGFS-G (not shown). These results suggest that some of the cues in the SO and SGFS are selective for retinal axons, and that the SAC and SGC contain cues selective for tectobulbar axons.

Relationship of tectal topography to laminar-selective outgrowth

The retinotectal projection is topographically organized: temporal axons project to the anterior portion of the tectum and nasal axons project to posterior tectum (Mey and Thanos, 1992). In the experiments described so far, we followed neurite outgrowth onto anterior tectum, without regard to retinal position. To determine whether lamina-selective outgrowth was influenced by retinotopic matching, we systematically varied the relationship between retinal and tectal explants. Five distinct retinotectal combinations were compared. In one set of cultures, temporal retinal axons were apposed to anterior tectum, and nasal axons contacted posterior tectum (Fig. 8b). In a second set, the retinal explant was rotated 180° so that temporal axons contacted posterior tectum and nasal axons contacted anterior tectum (Fig. 8c). In a third, the explant was rotated 90° so that nasal or temporal axons contacted dorsal and ventral tectum (Fig. 8d). Thus, two of the pairings were topographically appropriate (Fig. 8b) and three were inappropriate (Fig. 8c,d). However, we did not detect any significant differences among pairings in the length or density of outgrowth, either in the SO (Fig. 8e and f), or in any other lamina (not shown). Likewise, the form and number of arbors in the retinorecipient laminae was indistinguishable

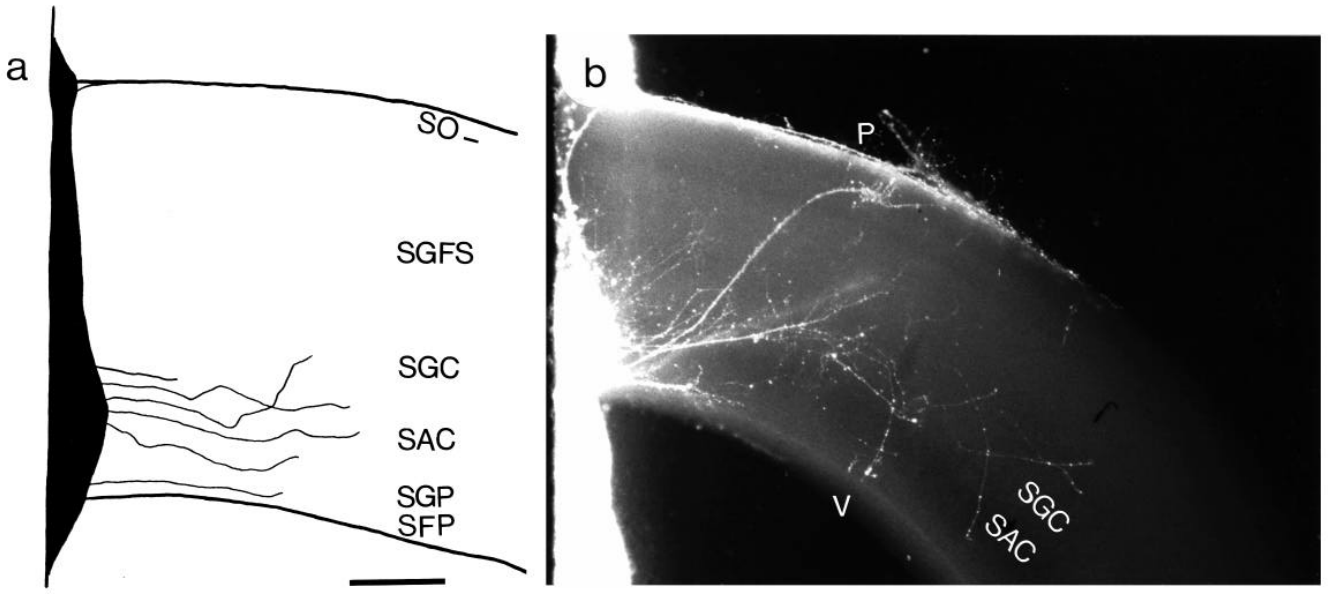

Fig. 7. Outgrowth of axons from E6 tectal explants onto E14 tectal slices. (a) Camera lucida of outgrowth on a live tectal slice. (b) Micrograph of outgrowth on a methanol–fixed slice. Most of the neurites extending from E6 slices arise from tectobulbar cells (Kröger and Schwarz, 1990); they extend preferentially along the SAC and SGC instead of along the SO. Bar is 200 μm in a and 400 μm in b.

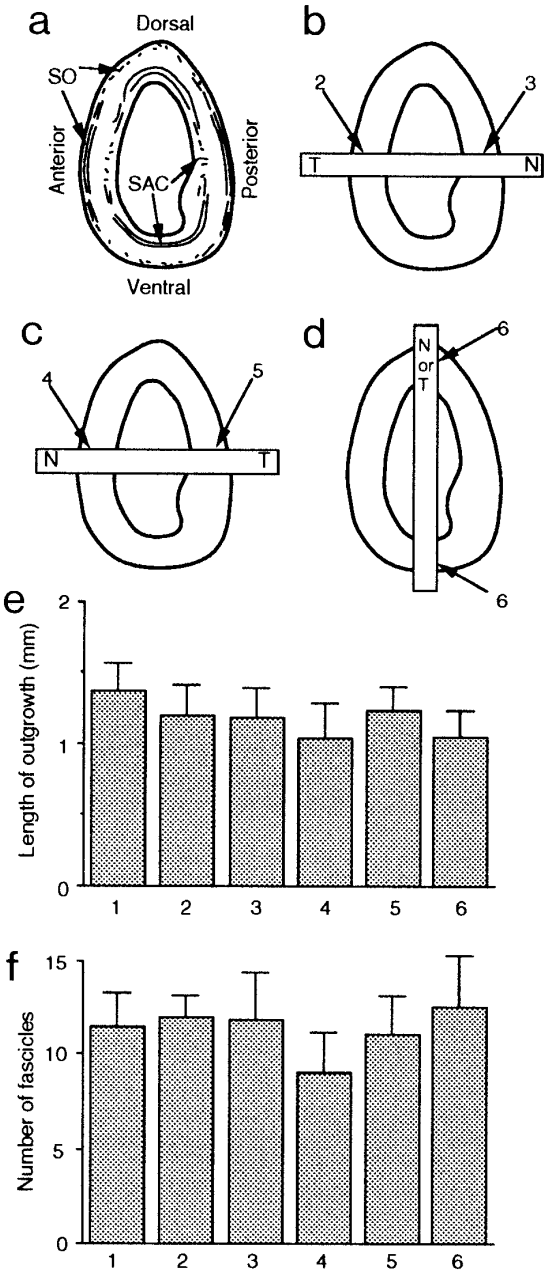

Fig. 8. Lamina–selective outgrowth is independent of retinotopy. (a) Sketch of tectal slice. Note that retinal axons in the SO are cut longitudinally at the anterior and posterior poles but cross–sectioned at the dorsal and ventral poles, whereas the opposite is true for the tectobulbar axons in the SAC. This pattern reflects the fact that these two tracts run perpendicular to each other (Goldberg, 1974). (b–d) Varied orientation of retinal strips on tectal slices, to test the influence of retinotopic matching on the extent and laminar selectivity of neurite outgrowth. (e,f) Maximum length (e), and number of fascicles (f), on the SO, measured as described in Fig. 4. Bars labeled 2–6 show pairings illustrated in parts b–d. Bars labeled 1 show values from Fig. 4; in these cultures, outgrowth on anterior tectum was measured, but nasotemporal orientation of the retinal strip was not noted. Only data for the SO are shown here, but a similar pattern – that is, no influence of retinotopy – was also noted for outgrowth on other laminae and for arborization. Values show mean ± s.e.m. for 6 cultures.

among pairings (not shown). Thus, the laminar cues revealed in our coculture system are apparently distinct from the cues responsible for formation of the retinotopic map. Consistent with this idea are results of Bonhoeffer and colleagues, who have used cultures to demonstrate the existence of membrane-associated cues on posterior tectal cells that inhibit outgrowth of temporal retinal axons: these cues are present in E6-E10 tecta, but can no longer be detected by E14 (Walter et al., 1987).

These same pairings also provided an opportunity to test the possibility that preferential outgrowth of retinal neurites along the SO was influenced by the orientation of processes within the SO. As shown in Fig. 8a, retinal and tectobulbar axons run in orthogonal directions (Goldberg, 1974; Kröger and Walter, 1990). When we studied neurite outgrowth onto anterior tectum, retinal neurites contacted longitudinally oriented processes in the SO but cross-sectioned processes in the SAC (Fig. 8b,c). The preference of these neurites for the SO might, therefore, have been enhanced by the well-documented tendency of neurites to grow along oriented mechanical guides (stereotropism; Purves and Lichtman, 1985). However, neurite outgrowth along the SO, and the preference for SO over SAC, were equally striking when retinal neurites were forced to contact cross-sectioned processes in the SO and longitudinally-oriented processes in the SAC (Fig. 8d-f). Based on this result, we concluded that mechanical factors were not responsible for promoting axon outgrowth along the SO.

DISCUSSION

Cues for outgrowth in the SO

Retinal axons grow exclusively through the SO in ovo, and show a striking preference for outgrowth along the SO in vitro. This preference is maintained in fixed tectal slices, and is seen on both cross-sectioned and longitudinally sectioned SO, but is not seen when the tectal slice is confronted with tectobulbar neurites. Based on these results, we hypothesize that the SO contains matrix- or membrane-associated molecules that selectively promote outgrowth of retinal axons.

Where might these molecules reside? One possibility is the basal lamina of the external limiting membrane or the pia. Several components of basal laminae have been shown to be potent promoters of neurite outgrowth from cultured retinal ganglion cells (Cohen et al., 1990; Halfler et al., 1983; Hall et al., 1987; Kröger and Schwarz, 1990), and may help to guide retinal axons from the eye to the tectum in ovo. Within the tectum, however, axons are generally separated from the basal lamina by a layer of glial end feet (Vanselow et al., 1989); similarly, much of the outgrowth observed in the SO in our cocultures is clearly deep to the overlying basal laminae. Moreover, we routinely removed the pia from tecta before preparing slices, thereby stripping away most of the basal lamina, as shown by staining with antibodies to laminin (M. Y. and J. R. S., unpublished data). Thus, although the basal laminae overlying the SO may contribute to the outgrowth we see, additional outgrowth promoting components are present within the SO itself.

A second possibility is that neurites extending from retinal

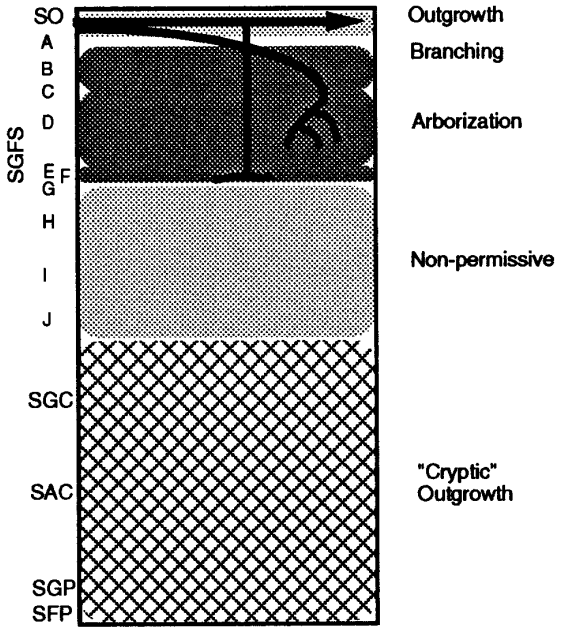

Fig. 9. Location of cues that influence lamina–selective outgrowth and arborization of retinal axons in optic tectum. We hypothesize the existence of 5 sets of localized, membrane– or matrix–associated tectal cues: (1) Promoters of retinal axonal outgrowth in the SO. (2) Stimuli to branching at the base of the SO. (3) Cues that cause axons to stop growing and/or arborize in the retinorecipient laminae. (4) Non–permissive or inhibitory substrates in the retinononrecipient portions of SGFS. (5) Physiologically irrelevant (cryptic) promoters of neurite outgrowth in intermediate and deep laminae. See text for details.

explants might grow along retinal axons already in the tectal slice. Indeed, it seems likely that in ovo, late arriving retinal axons grow along retinal axons that arrived earlier, perhaps using the homophilic promoter of neurite outgrowth, NgCAM/L1 to do so (Lemmon et al., 1989). On the other hand, neurites grow preferentially along the SO of enucleated tecta, indicating that some of the cues they recognize are intrinsic to the tectum.

The cellular source of these intrinsic cues remains to be determined. One intriguing possibility is that they reside on or are produced by a population of non-retinal axons that we have recently found to be present just beneath the pial surface in enucleated tecta (M. Y. and J. R. S., unpublished data; see also Goldberg, 1974). Once the cells from which these axons originate have been identified, it should be possible to test this hypothesis directly.

Cues that cause axons to leave the SO

Many of the retinal neurites growing through the SO eventually extend into deeper layers in vitro, as they do in vivo. Moreover, the same two modes of exit from the SO are discernible in vivo and in vitro: some neurites turn obliquely to penetrate the retinorecipient laminae, while others extend collateral branches (Senut and Alvarado-Mallart, 1986; Nakamura and O'Leary, 1989). It is unclear whether these modes reflect a distinct choice that each neurite makes, or whether there are two discrete categories of neurites. It might be, for example, that the two modes of exit are an early manifestation of differences between axons destined to end in different retinorecipient laminae (Repérant and Angaut, 1977).

In several systems, it has been proposed that soluble chemotropic factors promote the entry or branching of axons into appropriate target areas (Heffner et al., 1990). This seems unlikely to provide a complete explanation in the tectum, however, in that neurites enter the retinorecipient laminae of tectal slices that have been fixed. For those neurites that extend collateral branches, some cues may reside on the radial glial cells. Vanselow et al. (1989) have suggested that radial glia guide ingrowing retinal axons in vivo, based on studies in which they observed labeled axonal and glial processes in close proximity. Similarly, Norris and Kalil (1991) suggest that some callosal axons enter the mammalian cerebral cortex along radial glial guides. Whether specific guides exist for those neurites that leave the SO obliquely remains to be determined.

Cues for arborization in retinorecipient laminae

Neurites that exit the SO sometimes formed rudimentary arbors in the retinorecipient laminae of tectal slices. Similar behaviors were observed on slices that had been chemically fixed and/or prepared from enucleated embryos. We therefore hypothesize the existence of intrinsic, membrane- or matrix-bound cues that are localized to the retinorecipient laminae. These cues are of paramount interest, because they may be crucial to the process of lamina-selective synaptogenesis. The consequences to retinal axons of encountering these cues remains, however, to be determined. For example, the cues may include 'stop' signals (Baird et al., 1992), promoters of axonal branching, and/or factors that trigger the differentiation of growth cones into presynaptic nerve terminals. The coculture system that we have established should be well suited for immunohistochemical, electrophysiological, and time-lapse imaging studies that will help to distinguish among these possibilities.

Several recent studies of cocultured mammalian thalamic and cortical explants have also provided evidence for cues that guide lamina-selective arborization (Bolz et al., 1992; Götz et al., 1992; Yamamoto et al., 1992). Axons growing from thalamic explants invade the cortical explants, and terminate preferentially in a region that corresponds roughly to layer 4, in which such afferents synapse in vivo. Intracellular recording has provided clear evidence for the formation of functional thalamocortical synapses in such cocultures, although the laminar pattern of the synaptic connections has not yet been reported. A combination of tropic and membrane-bound cues have been postulated to account for these axonal behaviors (Bolz et al., 1993). It will be interesting to test whether any aspects of the thalamocortical projection occur in cocultures that use fixed cortical explants.

Although our assay clearly demonstrates that some aspects of terminal arborization occur in the absence of postsynaptic electrical activity, we do not conclude that activity is unimportant. There is excellent evidence that formation of arbors is regulated by neural activity in many parts of the brain (Shatz, 1990), including the tectum (Constantine-Paton et al., 1990; Kobayashi et al., 1990). In this regard, it is important to note that the arbors we observe are immature in form - they resemble some seen in vivo at E14, but are far simpler than those seen just a few days later (Fig. 1). We suspect that more complex arbors will develop in vitro when culture conditions

are refined, or retinae are plated on slices of more mature tectum. Another possibility, though, is that important steps in the maturation of the arbors require synaptic interactions to proceed.

Inhibitory cues in retinononrecipient portions of SGFS

During normal development, few if any retinal axons extend into portions of SGFS deeper than SGFS-F. The observation that this border is also respected in our cocultures suggests the presence of an unattractive substrate for retinal axons, either concentrated at the border or distributed within the deeper layers. The further observation that few neurites extend directly from the explant onto SGFS G-J favors the idea that this cue is distributed through several layers, rather than being confined to a border.

An unattractive terrain could either fail to support or actively inhibit neurite outgrowth. Several examples of each class of substrate, termed 'non-permissive' and 'repulsive', respectively, have been described recently (Schwab et al., 1993). Walter et al. (1987) described a simple method of distinguishing these classes: treatments that inactivate the substrate should have no effect on a nonpermissive factor, but can disinhibit outgrowth on a repulsive substrate. It should be possible to apply this test to tectal slices.

Cues for outgrowth in deep laminae

During normal development, retinal axons do not have access to tectal layers that are deep to the SGFS, i.e., the SGC, SAC, SFP and SGP (Fig. 1). The observation that some retinal explants extended axons onto these laminae is therefore of little biological significance. Nonetheless, two features of this outgrowth are worth mentioning. First, tectobulbar axons grow through the SGC and SAC, both in vivo (Kröger and Schwarz, 1990) and in cocultures (Fig. 7). That some retinal axons extend in the same area in vitro might indicate that cues for outgrowth in this area are nonspecific to some extent. Second, the laminae nearest the ependyma are already known to express high levels of the cell adhesion molecule N-cadherin (Redies et al., 1992), which can promote neurite outgrowth from retinal cells in vitro (Matsunaga et al., 1988). Thus, molecules used to promote neurite outgrowth in one context may be deployed for other purposes in other areas. However, retinal outgrowth on deep layers is abolished by chemical fixation, whereas outgrowth along the SO is not, indicating that there are chemical differences between the cues that promote outgrowth in these two regions.

Conclusions

Numerous factors influence the behavior of growing axons, including membrane molecules, extracellular matrix components, mechanical heterogeneities, diffusible tropic and trophic factors, electrical fields, and neural activity (Purves and Lichtman, 1985). We set out to seek cues that account for the laminar selectivity of retinal innervation in the optic tectum. Our aim in this study was cellular rather than molecular - it is crucial to know what classes of cues exist and where they are located before seeking their molecular identity. Here, we provide evidence for a set of discrete, localized, membrane- or matrix-associated cues within the tectum. Although it is possible (indeed, likely) that soluble factors and neural activity also regulate the behavior of retinal axons, it is noteworthy that relatively stable, immobilized cues are sufficient to generate a major part of the laminar selectivity seen in this system. Thus, our results set the stage for future molecular studies, by suggesting hypotheses about the nature and location of these cues, and by providing a promising bioassay for testing candidate molecules. Important questions that remain to be addressed include: (1) what are the cell surface molecules that guide axonal behavior? (2) how do these cues come to be arrayed in a laminar pattern? (3) what other factors are required for the formation of functional retinotectal synapses? (4) how do cues for laminar selectivity interact with factors that generate the retinotopic map? Finally, in considering tectal organization more generally, it will be important to consider how its many other inputs and outputs respond to cues that we have so far considered from a largely 'retinocentric' perspective.

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