

## Retinal axon guidance by region-specific cues in diencephalon

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### SUMMARY

**Retinal axons show region-specific patterning along the dorsal-ventral axis of diencephalon: retinal axons grow in a compact bundle over hypothalamus, dramatically splay out over thalamus, and circumvent epithalamus as they continue toward the dorsal midbrain. In vitro, retinal axons are repulsed by substrate-bound and soluble activities in hypothalamus and epithalamus, but invade thalamus. The repulsion is mimicked by a soluble floor plate activity. Tenascin and neurocan, extracellular matrix molecules that inhibit retinal axon growth in vitro, are**

**enriched in hypothalamus and epithalamus. Within thalamus, a stimulatory activity is specifically upregulated in target nuclei at the time that retinal axons invade them. These findings suggest that region-specific, axon repulsive and stimulatory activities control retinal axon patterning in the embryonic diencephalon.**

Key words: Chemorepulsion, Chemoattraction, Epithalamus, Floor plate, Hypothalamus, Lateral geniculate nucleus, Neurocan, Tenascin, Visual system development

### INTRODUCTION

The most intensively studied forebrain axonal system is the retinal ganglion cell axon projection. During development retinal axons extend from the eye to the optic chiasm at the ventral surface of the diencephalon. They then extend in a dorsocaudal direction over the surface of the diencephalon, reaching the rostral superior colliculus (SC or 'tectum' in lower vertebrates) on embryonic day (E) 16 in rats (Lund and Bunt, 1976; Bunt et al., 1983). Beginning around E18-19, retinal axons extend branches into their targets (Simon and O'Leary, 1992; R. Tuttle and D. O'Leary, unpublished observations).

Numerous studies have contributed to our understanding of cues that control retinal axon patterning at the optic chiasm (for a review, see Guillery et al., 1995), and in their target, the SC/tectum (for reviews, see Roskies et al., 1995; Friedman and O'Leary, 1996; Drescher et al., 1997). Several substrate-bound activities have been identified in the retinal pathway of embryonic rodents that repulse retinal axons: activities at the optic chiasm specific for ventrotemporal retinal axons (Wizenmann et al., 1993; Wang et al., 1995), a molecule on neurons adjacent to the optic chiasm (Sretavan et al., 1994), and activities in the caudal SC/tectum specific for temporal retinal axons (Godement and Bonhoeffer, 1989; Simon and O'Leary, 1992). Much less is known of stimulatory activities that might affect retinal axon patterning, although the neurotrophin, BDNF, applied in vivo stimulates retinal axons to branch in the frog tectum (Cohen-Cory and Fraser, 1995). In addition, an activity associated with membranes prepared from caudal chick tectum enhances the growth and maintenance of nasal retinal axons (Boxberg et al., 1993).

In contrast to the SC/tectum and the optic chiasm, little is

known of activities that guide retinal axons over the diencephalon, even though a major retinal axon target, the lateral geniculate nucleus (LG), is located in the dorsal diencephalon; roughly a third of retinal axons project to the LG in adult rodents (Martin, 1986), and in higher mammals, nearly all retinal axons do so (Illing and Wassle, 1981; Perry et al., 1984). In this paper we provide evidence for region-specific activities in diencephalon that repulse retinal axons and for a target-specific stimulatory activity in the LG. The distribution of these activities along the dorsal-ventral and rostral-caudal axes correlates with the pattern of retinal axon growth in vivo.

### MATERIALS AND METHODS

#### Animals

Embryos were taken from timed pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) or C57BL mice. The day of insemination is designated E0. White Leghorn chick embryos were also used.

#### In utero DiI injections

Cesarean sections were performed on pregnant rats anesthetized with chloral hydrate (10 mg/100 g). A 2% solution of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; Molecular Probes) in dimethylformamide was pressure-injected into one retina of each embryo of the litter. After 16-22 hours the rats were reanesthetized and the embryos perfused with 4% paraformaldehyde in phosphate buffer (PB), followed by immersion-fixation in the same solution. Fixed brains were cut midsagittally and laid on their cut, medial surface. The lateral surface of the diencephalon was revealed by removing the overlying cortex and pia. A weighted glass coverslip was placed on top of this preparation to reduce the natural curvature

of the diencephalic surface. The preparation was then photographed using rhodamine optics.

### Tissue dissections

Dissections were done in cold L15 medium supplemented with 0.6% glucose (L15-glucose). E15-16 rat or E14-15 mouse retinas were either cut into eight radially symmetric pieces without regard to the retinal axes, or into nasal and temporal thirds with the middle third removed. In the latter case, each nasal or temporal piece was further cut into 3 or 4 explants. E6 chick retinas (Hamburger and Hamilton stage 28-29) were similarly cut into nasal and temporal thirds. These nasal and temporal pieces were cut into 300  $\mu$ m square explants using a tissue chopper. The ages of the retinal explants represent stages when a majority of retinal ganglion cells have just undergone their final mitotic division, and axon growth *in vitro* is most robust (Morest, 1970; Prada et al., 1991; Snow and Robson, 1994).

To prepare coronal sections of diencephalon, E14-19 brains, embedded in 3% low-melting temperature agarose (FMC BioProducts), were sectioned at 200-300  $\mu$ m with a vibratome (the E14-15 brains were sectioned at 200  $\mu$ m). Telencephalic tissue was generally discarded from the sections by cutting through the internal capsule. Diencephalic explants were dissected from serial sections cut in this way (Fig. 2, insets).

Neocortex was dissected from E15 rats and then cut into roughly 300  $\mu$ m square explants. E13 rat floor plate was dissected as described in detail elsewhere (Richards et al., 1997), taking care to remove most, if not all, of the adjacent ventral spinal cord tissue. The long strips of floor plate were then cut into small explants.

### Collagen gel cocultures

Coculture experiments were in three-dimensional collagen gels, which allow one to study both substrate-bound and soluble activities. Collagen solutions were made by mixing 1 part 10 $\times$  MEM (Sigma), 9 parts immature rat tail collagen and a volume of 7.5% sodium bicarbonate solution, to achieve a physiological pH. In the center of each well of 4-well plates (Nunc), 25  $\mu$ l of the collagen solution were spread and allowed to gel in a 37°C incubator. Explants were placed onto this collagen base, covered with 75  $\mu$ l of collagen solution, and quickly positioned. In all experiments, cocultured explants were separated by 100-300  $\mu$ m; the orientation of retinal (and cortical) explants relative to the cocultured tissue was random. When the collagen had gelled, the cultures were fed with 500  $\mu$ l of culture medium consisting of DMEM/F12 (ICN) supplemented with 2 mM glutamine, 0.6% glucose, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 5% heat-inactivated rat serum and 10% heat-inactivated fetal bovine serum. All cultures were maintained in a humidified, 37°C, CO<sub>2</sub> incubator for 1-3 days before being fixed overnight with 4% paraformaldehyde in PB. The retinodiencephalic cocultures were supplemented with 10  $\mu$ M cytosine arabinoside (AraC) for the first 18-24 hours; retinal axon growth is greater and more radially symmetric, and nonneuronal cell emigration from explants is reduced in cultures exposed briefly to AraC. As used here, AraC seems not to have detrimental effects on neurons (Martin et al., 1990).

### Immunolabeling

Brains were immersion-fixed with 4% paraformaldehyde in phosphate buffer (PB), cryoprotected and frozen. Coronal sections, 12-15  $\mu$ m thick, were dried onto gelatin-coated slides and fixed with 4% paraformaldehyde for 10 minutes. To quench endogenous peroxidase activity, sections were incubated in 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 30 minutes, rinsed in PBS, and then incubated in PBS containing 3-5% goat serum (PBS-goat). Sections were exposed overnight at 4°C to antibodies diluted in PBS-goat. Polyclonal antibodies to neurocan (generously provided by Reinhard Fässler) and tenascin (Gibco, A107) were used at 1:4000 and 1:500, respectively. Sections were rinsed in PBS-goat, exposed for 1 hour to biotinylated secondary antibody, and rinsed again in PBS-goat. Sections were then exposed to avidin-biotin

complex (Vector Laboratories), rinsed in PBS, and preincubated in 0.05% DAB in PB containing 0.005% nickel ammonium sulfate. To develop the staining, sections were incubated in the same DAB solution containing 0.009% H<sub>2</sub>O<sub>2</sub>.

### Analysis and quantitation

For most retinodiencephalic cocultures, fixed retinal explants were injected with a DiI solution and then left at 25-30°C for 3 or more days, until the dye transported to the tips of the axons. Axons extending from retinal explants were presumed to be from retinal ganglion cells (Halfter et al., 1983). Labeled retinal axon growth in diencephalic tissue was photographed by first focusing on a bright-field image of the section or explant and then, maintaining the focus, switching to fluorescence optics. In this way, retinal axons that are in focus are extending in or on the diencephalic section, not within the diencephalic tissue. When no diI-labeled axons were visible within the diencephalic tissue, the plane of focus was adjusted up and down to make certain that none were missed. The amount of retinal axon growth within the cocultured diencephalic tissue was assessed using a semiquantitative scale: a value of 0 was assigned when there were no axons in the cocultured tissue; a value of 1 if few axons were present; 2 if there were many axons; and 3 if there was robust invasion.

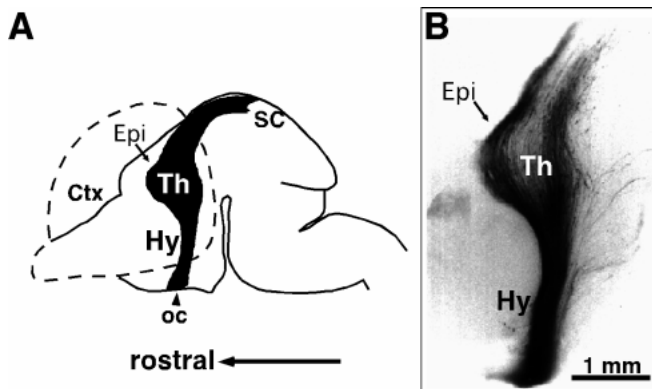
In floor plate cocultures, as well as the retinodiencephalic cocultures presented in Fig. 9, axon growth was examined and photographed using phase-contrast optics; cultures were photographed during the culture period as well as after fixation. Axon growth was assigned to one of three possible categories: explants that had more axon growth either (1) 'towards' or (2) 'away' from the floor plate or diencephalon, or (3) explants that had similar amounts of axon growth on both of these sides and were therefore scored as 'same.' These data were statistically analyzed using the chi-square test.

## RESULTS

### Retinal axon growth patterns in the diencephalon

To determine the distribution of retinal axons over the surface of the diencephalon, DiI was injected into one of the eyes of E15-19 rats. The resulting labeled retinal axon projection from the chiasm to the superior colliculus was examined in whole mounts (Fig. 1). For this paper, the diencephalon is subdivided into three regions: in a ventral-to-dorsal order these are the hypothalamus, ventral and dorsal thalamus, and epithalamus (Fig. 2); the most caudal part of the dorsal diencephalon, the pretectum, is only rarely referred to in this paper. In this paper, what is referred to as the dorsal-ventral axis is, at earlier embryonic stages, referred to as the posterior-anterior axis in certain studies (e.g. Puelles and Rubenstein, 1993).

Retinal axons overlying the lateral surface of the hypothalamus were in a compact bundle. At the border between the hypothalamus and thalamus, the retinal axon bundle splayed out dramatically over the thalamic surface (Fig. 1). At all ages examined, it was apparent in both whole mounts and coronal sections that retinal axons remained at the surface of the hypothalamus and did not penetrate the underlying hypothalamic tissue. However, while axons and growth cones are visible at the thalamic surface, many penetrate the thalamic tissue at the hypothalamic/thalamic border and course parallel to and slightly beneath the surface (data not shown). More dorsocaudally, the pathway constricted as retinal axons swerved caudally avoiding the epithalamus and forming a band of axons extending towards the pretectum and superior colliculus. This pathway is illustrated for an embryo injected in utero on E17 and fixed 16 hours later (Fig. 1). At E15 and E19, the retinal axon pattern was similar,



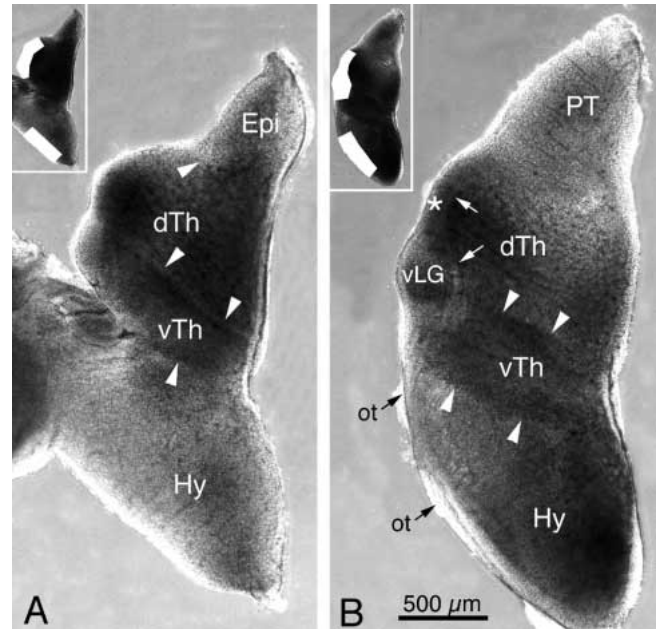
**Fig. 1.** The pattern of retinal axon growth is dramatically different in dorsal and ventral diencephalon. (A and B) Schematic and actual images, respectively, of retinal axon patterning in E17 rat diencephalon. The cortex (Ctx; dashed outline in A) was removed to expose the underlying diencephalon. (A) The solid black area represents the portion of the retinal pathway from the optic chiasm (oc) to the rostral superior colliculus (SC). Retinal axons are bundled as they course dorsally over the hypothalamus (Hy) but splay out dramatically over the surface of the thalamus (Th). The axons swerve caudally towards the superior colliculus; those that are rostral-most in the thalamus turn at the border between thalamus and epithalamus (arrows in A and B). (B) Retinal axons labeled by DiI injected into the retina in utero are shown extending over the diencephalon (this is a negative image of a fluorescence photo). Due to the difficulty of labeling all retinal axons by in utero injections, the central part of the pathway overlying the thalamus is lightly labeled.

although at E15, there were many fewer labeled retinal axons in the thalamus. Within the thalamus, labeled growth cones were seen at all ages examined; they were most numerous at E17, but were also observed at E15 and E19 (data not shown), reflecting the broad temporal span of retinal ganglion cell neurogenesis (Morest, 1970; Prada et al., 1991; Snow and Robson, 1994). These in vivo observations suggest that the hypothalamus and epithalamus are inhibitory or repulsive for retinal axon growth, and that the thalamus is stimulatory.

### Hypothalamic and epithalamic repulsion of retinal axons by substrate-bound cues

To test whether molecular differences along the dorsal-ventral axis of the diencephalon might dictate the pattern of retinal axon growth, explants of E15 rat retina were cocultured at a distance from coronal sections of E15-19 rat diencephalon in collagen gels; sections that together covered the full rostral-caudal extent of the diencephalon. One retinal explant was positioned ventrally near the lateral surface of the hypothalamus, and a second dorsally near the lateral surface of the thalamus or epithalamus. After roughly 3 days, the cultures were fixed and the retinal explants filled with DiI to reveal retinal axons both in the collagen and in the diencephalic sections; by comparing fluorescence and phase contrast images, it appeared that most, if not all, retinal axons were DiI-labeled.

Retinal explants placed dorsally extended axons that often invaded the thalamus (Figs 3, 4). Interestingly, retinal axons in E19 thalamus tended to be longer and more numerous than those in E17 thalamus (Fig. 3; compare B with A). Retinal axons generally did not invade the hypothalamus and



**Fig. 2.** Diencephalic regions are apparent in unstained vibratome sections of live brain as processed for coculture experiments. (A) A 'rostral' section from an E17 rat that includes epithalamus (Epi) and hypothalamus (Hy) rostral to the optic tract; the lateral geniculate (LG) is caudal to this section. (B) A 'caudal' section from an E17 rat that includes optic tract (ot), as well as the ventral LG (vLG) and dorsal LG (marked by \*). The thalamic radiation, which forms the medial border of the LG, is indicated with arrows. Boundaries between the epithalamus/dorsal thalamus (dTh), dorsal thalamus/ventral thalamus (vTh), and ventral thalamus/hypothalamus are indicated with arrowheads. Solid white areas shown in the insets approximate the locations of some of the diencephalic explants used for the experiments summarized in Fig. 7. PT, pretegmentum.

epithalamus (Figs 3, 4), but instead turned away from these regions, or contacted them and either formed blunt terminations or turned and grew along their surface (Figs 3B, 4A). The hypothalamic inhibition was apparent whether the axons approached the lateral, medial or dorsal surfaces of the hypothalamus (Fig. 4A,A'). These data are summarized in Fig. 5. Nearly all of the sections that exhibited greater retinal axon invasion of the thalamus had no retinal axons in the hypothalamus or epithalamus. The paucity of retinal axons within the hypothalamus or epithalamus was not due to an overall decrease in retinal axon outgrowth since axon growth into the surrounding collagen gel was exuberant. A small proportion of sections exhibited either greater retinal axon growth into hypothalamus or equivalent growth in hypothalamus and thalamus; the vast majority of these sections were from caudal diencephalon. Retinal axons also generally failed to penetrate the ventral aspect of E15 diencephalic sections (data not shown), indicating that the hypothalamic inhibitory activity is present as early as E15. Since mutant mice may be useful tools to further study the mechanisms of axonal patterning in the diencephalon, these coculture experiments were repeated using E14 rat retinal explants paired with E17 mouse diencephalic tissue (developmentally equivalent to E18 rat diencephalon); the results were the same as those obtained with E17-18 rat diencephalon (Fig. 5).

These data suggest that retinal axons are inhibited or repulsed by substrate-bound activities in the embryonic rodent hypothalamus and epithalamus. The data also suggest that the embryonic thalamus is stimulatory for retinal axon growth, and that there is a maturation-dependent increase in this stimulatory activity. A similar phenomenon has been described in the developing cortex, where it has been shown that cortical and thalamic axons respond to a maturation-dependent upregulation of axon growth-promoting molecules in the developing cortical plate (Tuttle et al., 1995).

### Retinal axons selectively invade target nuclei in vitro

The data above suggest that retinal axons respond to molecular differences along the dorsal-ventral axis of the embryonic diencephalon. Cocultures were used to determine whether retinal axons might also be sensitive to molecules that are differentially distributed along the rostral-caudal axis of the thalamus. In these experiments, retinal explants were paired in collagen gels with explants from the lateral aspect of E17 or E19 thalamus or hypothalamus (Fig. 2, insets). The thalamic explants contain nuclei that underlie the pathway of retinal axons; one of these nuclei, the lateral geniculate nucleus (LG), is a target of retinal axons, while others, for example the lateral dorsal, lateral posterior, reticular thalamic and medial geniculate nuclei receive sparse or no retinal input in vivo. One thalamic explant was dissected from each of 3 to 4 serial, coronal sections. Cultures were fixed after 3 days, the retinal explants injected with DiI, and the amount of retinal axon invasion into each cocultured thalamic explant assessed.

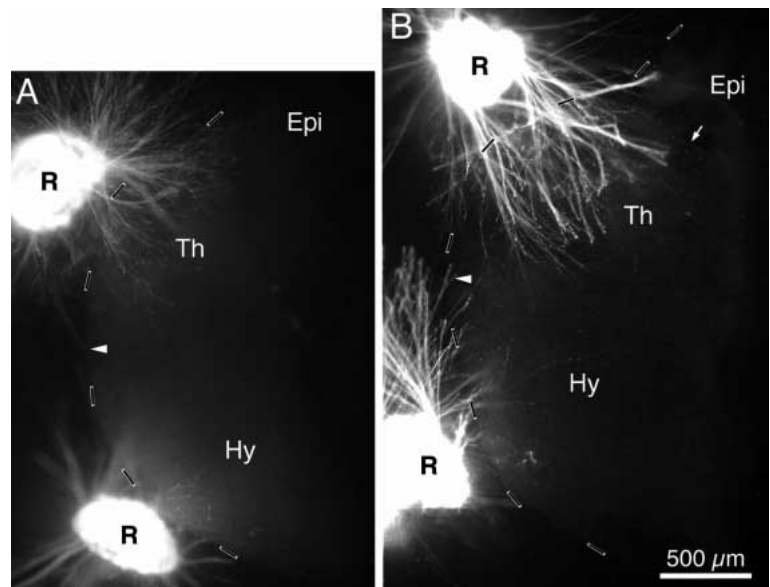
Fig. 6 illustrates a coculture series for thalamic explants from a single E19 brain. Explants that contained predominantly nontarget thalamic nuclei were sparsely invaded by retinal axons (Fig. 6A,B). In contrast, retinal axon invasion was robust in the explant that contained predominantly LG (Fig. 6C). Upon contacting the LG explant, retinal axons defasciculated and extended throughout the explant, where they typically remained rather than reentering the collagen. Retinal axons were often branched within the LG explants (data not shown). These data are summarized in Fig. 7. The ability of retinal axons to discriminate target thalamic nuclei from nontarget nuclei was pronounced in cocultures in which the lateral thalamic explants were dissected from E19 brains; the effect was less strong but still statistically significant with explants from E17 brains. While the majority of thalamic explants exhibited moderate to robust invasion by retinal axons, most hypothalamic explants were either devoid of retinal axons or contained only a few retinal axons.

These data demonstrate that retinal axons can distinguish between target and non-target regions along both the rostral-caudal and dorsal-ventral axes of the diencephalon, even when presented as isolated explants. Further, they confirm the distinctions between target and non-target regions defined in the preceding experiment using whole sections of diencephalon, and suggest that these distinctions are mediated by growth-promoting molecules upregulated in the LG when retinal axons begin to extend branches into it and by inhibitory molecules present in the hypothalamus.

### Neurocan and tenascin are enriched in repulsive regions of the diencephalon

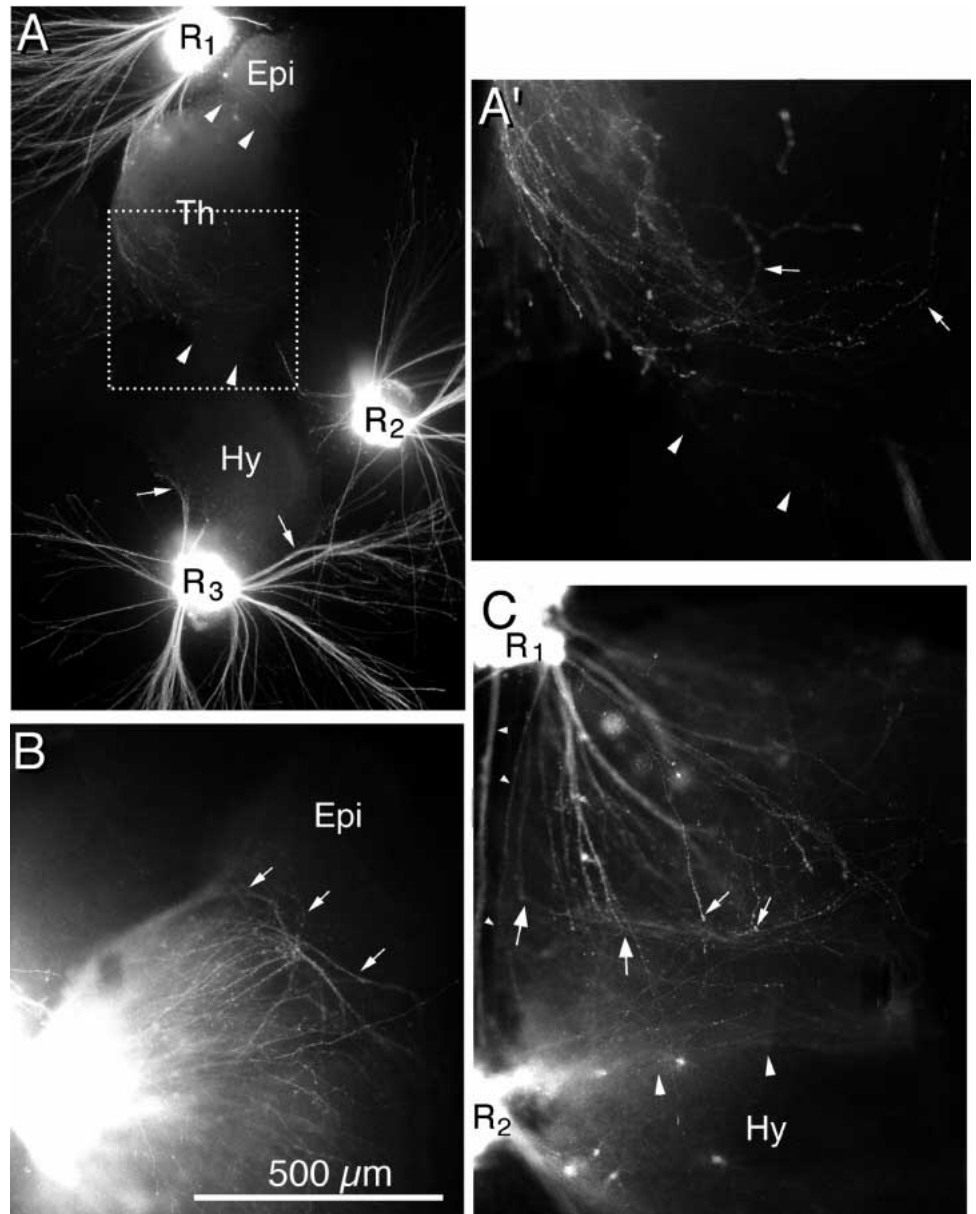
The *in vivo* and *in vitro* data presented above suggest that activities that repulse retinal axons are present in the embryonic hypothalamus and epithalamus, but not in the thalamus. We therefore examined the distribution of the extracellular matrix (ECM) molecules, neurocan (a chondroitin sulfate proteoglycan), tenascin and chondroitin sulfate (CS), which have been shown to be inhibitory for axon growth, including retinal axons, *in vitro* (Lochter et al., 1991; Snow et al., 1991; Snow and Letourneau, 1992; Perez and Halfter, 1993; Friedlander et al., 1994). Sections of E17 diencephalon were immunostained with polyclonal antibodies to neurocan and tenascin, and with a monoclonal antibody to CS (Avnur and Geiger, 1984).

Immunostaining for neurocan and tenascin was higher in the hypothalamus and epithalamus than in the thalamus, with the staining intensity changing abruptly at the thalamic/hypothalamic and thalamic/epithalamic borders (Fig. 8). While most of the hypothalamus was intensely stained, reduced levels of staining were seen in locations corresponding to the preoptic and suprachiasmatic nuclei (data not shown), both of which become innervated by retinal axons (Johnson et al., 1988; Levine et al., 1991, 1994). The immunostaining pattern for CS was similar (data not shown). The neuromeric border between dorsal and ventral thalamus (Papalopulu, 1995) also stained intensely for tenascin (Fig. 8B), as well as neurocan and CS (data not shown). Interestingly, this is the



**Fig. 3.** Retinal axon growth is inhibited by hypothalamus and stimulated by thalamus. Explants of E15 rat retina (R) were placed at a distance from the lateral surface of caudal sections (see Fig. 2B) of E17 (A) or E19 (B) rat diencephalon and cocultured for 3 days in collagen gels. After fixation, the retina were injected with DiI. In this, and subsequent figures, the DiI filled the retinal explants and extended slightly beyond their borders. The retinal axons rarely invaded the hypothalamus, but often invaded the thalamus. The approximate border between the thalamus and hypothalamus is indicated with an arrowhead. Retinal axon invasion of thalamus was more robust at E19. Retinal axons tended not to invade the epithalamus (Epi). The arrow in B marks the thalamic/epithalamic border. For the diencephalic sections, dorsal is at the top of the photos; the lateral surface is outlined with dashes, and the medial surface (midline) is at the right edge of each photo.

**Fig. 4.** Repulsive activities in the diencephalon are broadly distributed in hypothalamus and epithalamus. E15 rat retinal explants were cocultured with sections of E17 rat diencephalon; after 3 days the cultures were fixed and the retinas injected with DiI. Three different cultures are shown (A,B,C). (A) Retinal explants positioned lateral to the epithalamus (Epi, R<sub>1</sub>), or medial (R<sub>2</sub>) and lateral (R<sub>3</sub>) to the hypothalamus (Hy) of a rostral section (see Fig. 2A). No retinal axons extended into the hypothalamus or epithalamus. Instead axons often turned and, as they do in vivo, grew along the surface of the hypothalamus (arrows in A). In contrast, numerous axons from R<sub>1</sub> extended into the thalamus; however, none of these ventrally extending axons entered the hypothalamus, but instead turned, many reversing their direction to extend dorsally and remain in the thalamus. Arrowheads point to the epithalamic/thalamic and thalamic/hypothalamic borders. The area delineated by dotted lines is illustrated at higher magnification in A'. (A') Some of the axons that have turned away from the hypothalamus are indicated with arrows. (B) A retinal explant placed at a distance from the lateral edge of the thalamus (the retina is out of the field of view to the left; the fluorescent glare at the left is due to intense axonal labeling). The retinal axons extending dorsally made right angle turns at the thalamic/epithalamic border (arrows). No retinal axons were in the epithalamus. (C) Retinal explants placed at a distance lateral to the dorsal thalamus (R<sub>1</sub>) or at the hypothalamic/ventral thalamic border (R<sub>2</sub>) in a caudal section (see Fig. 2B). Axons from R<sub>1</sub> invaded the dorsal thalamus and extended ventrally. Many of these axons made right angle turns as they approached the dorsal thalamic/ventral thalamic border (large arrows); others appear to have stopped near the border (small arrows). Nearly all axons that extended ventrally beyond this border remained in the ventral thalamus; many turned at the ventral thalamic/hypothalamic border (large arrowheads). Two axon bundles from R<sub>1</sub> extended within the collagen along the lateral edge of the diencephalon (small arrowheads at left margin of photo); unlike the axons that extended within the section, these axons maintained a straight course. Axons from R<sub>2</sub> did not enter the hypothalamus but did extend into the ventral thalamus, although they had access to both regions.



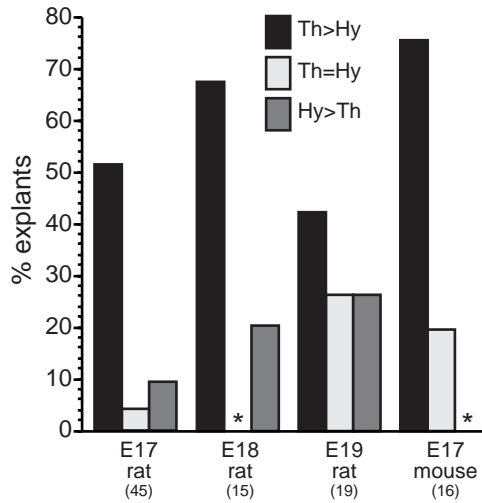
border at which retinal axons sometimes stopped or turned (Fig. 4C). These findings indicate that neurocan, tenascin and CS are enriched in regions of the E17 rat diencephalon that inhibit or repulse retinal axons in vitro.

#### Retinal target nuclei stimulate, and non-target regions repulse retinal axons at a distance

The data presented in the preceding sections suggest that retinal axon growth is affected by repulsive and stimulatory activities associated with distinct regions of the diencephalon. To determine whether these activities can also affect retinal axon growth at a distance, coculture experiments were carried

out that differ from those shown in Figs 3 and 4 in that axon growth was analyzed at 1.5 rather than 3 days in vitro, and by phase contrast rather than fluorescence microscopy. In these experiments, retinal explants were cocultured with coronal sections of either rostral (Fig. 2A) or caudal (Fig. 2B) diencephalon. Retinal explants were positioned at a distance from the lateral surface of the epithalamus, thalamus or hypothalamus.

In the first series of experiments, explants of E15 rat retina were paired with sections of E17 rat diencephalon. Axons extending from retinal explants placed at a distance from rostral hypothalamus or epithalamus were repulsed (Figs 9A,B, 10A).



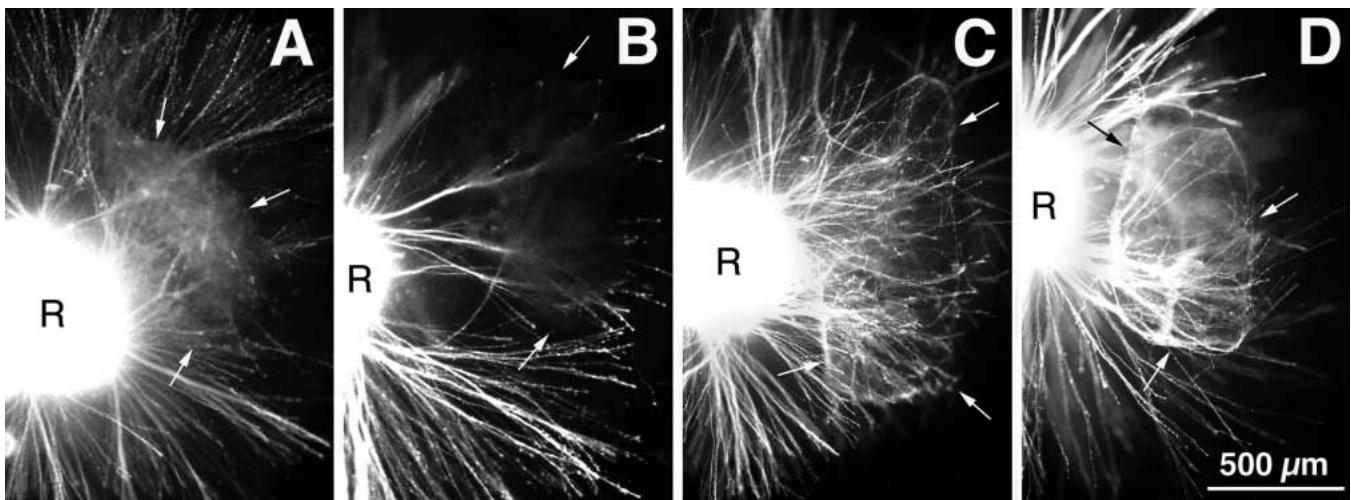
**Fig. 5.** Quantitation of differences in retinal axon invasion of thalamus and hypothalamus. E14–16 rat retinal explants were cocultured for 3 days in collagen gels at a distance from the hypothalamus and thalamus in living, coronal sections of either E17, E18, E19 rat, or E17 mouse diencephalon. For each age, the percentage of sections that had greater axon ingrowth in thalamus (Th>Hy), hypothalamus (Hy>Th), or equivalent amounts of growth (Th=Hy), is presented. 90% of the rat sections and 67% of the mouse sections that exhibited greater retinal axon invasion of the thalamus, had no axons in the hypothalamus. In addition, 86% of the 21 total sections that exhibited either greater growth in hypothalamus or equivalent growth in hypothalamus and thalamus were from caudal diencephalon. By chi-square analysis, the four sets of data were not significantly different, except for E17 and E19 rat diencephalon cocultures ( $P=0.04$ ). Summed percentages do not equal 100% since cases that had no axon growth into thalamus or hypothalamus are included in the calculations but not presented.  $n$  values are in parentheses. Asterisks indicate zero percentage values. The data are from five experiments.

In contrast, retinal axon growth from explants placed at a distance from either caudal hypothalamus or LG was not repulsed (Figs 9C, 10A). Repulsion by caudal versus rostral epithalamus was not significantly different; these data were, therefore, combined. Thus, a soluble activity that repulses retinal axons is released in vitro by epithalamus and rostral hypothalamus from E17 rats.

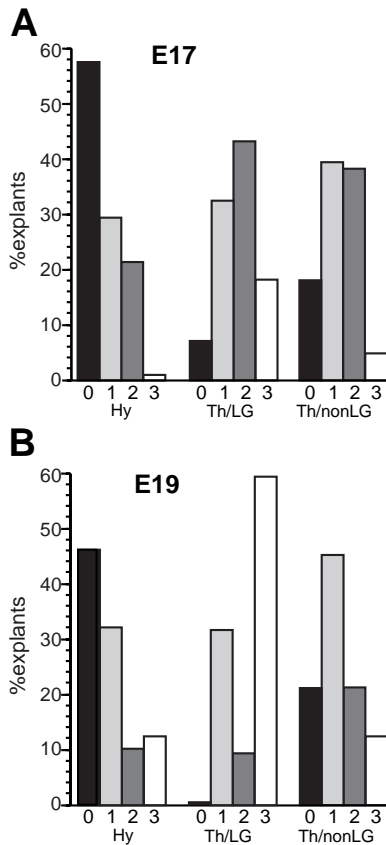
Similar experiments were carried out using explants of E14–15 mouse retina cultured in collagen gels at a distance from rostral or caudal sections of E14–15 mouse diencephalon (Fig. 10B; E14 mouse is developmentally similar to E15 rat). Both rostral and caudal hypothalamus were repulsive for retinal axons. In contrast, retinal axon growth was specifically stimulated by LG; axons were repulsed if retinal explants were positioned at a distance from thalamus in rostral sections that do not contain LG. Thus, a soluble repulsive activity is released by E14–15 mouse hypothalamus, as well as non-target regions of thalamus. In addition, a soluble stimulatory activity is released from thalamus containing LG.

### Floor plate is repulsive for rat and chick retinal axons

A ventral midline CNS structure, the floor plate, releases a soluble activity shown to repel certain populations of spinal cord and hindbrain axons (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Tamada et al., 1995; Shirasaki et al., 1996), as well as mouse retinal axons (Wang et al., 1996). We therefore tested whether the soluble retinal axon repulsive activity released by the hypothalamus could be mimicked by a floor plate-derived activity. Floor plate from E13 rat spinal cord was cocultured at a distance in collagen gels with explants of E15 rat or E6 chick retina, or as a control, E15 rat neocortex. E6 chick retina (developmentally similar to E15 rat retina) was used since, when cultured alone, the axon outgrowth tends to be more radially symmetric than outgrowth from rat retinal explants. The cultures were analyzed at 1.5 days in vitro.

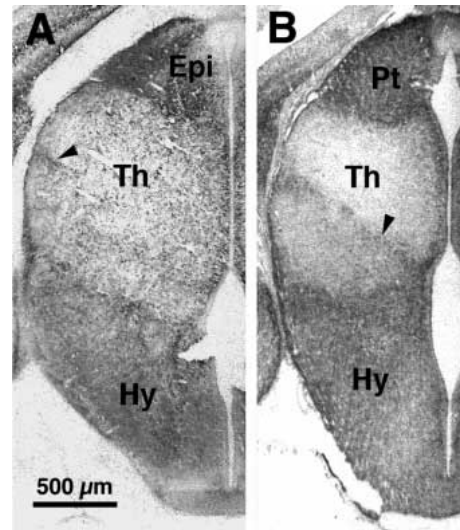


**Fig. 6.** Retinal axons can discriminate their target thalamic nucleus. E15 retinal explants (R) were cocultured for 3 days with explants of lateral thalamic nuclei dissected from serial coronal sections of E19 rat diencephalon. (A–D) Fluorescence images of DiI-injected retinal explants (to the left). The thalamic explants in A–D are presented in a rostral to caudal progression, and mainly contain the following nuclei: (A) the lateral dorsal thalamic nucleus; (B) the reticular, lateral posterior and lateral dorsal thalamic nuclei; (C) the dorsal and ventral LG nuclei; (D) the dorsal LG and medial geniculate nuclei. The retinal axons robustly invaded the LG (C), and to a lesser degree the caudal-most explant (D). Few axons invaded the more rostral thalamic nuclei (A and B), but instead extended around the thalamic explants (those that extended over are out of focus). Arrows approximate the borders of the thalamic explants.



**Fig. 7.** Selective invasion of the LG by retinal axons becomes more pronounced at later embryonic ages. The histograms summarize data obtained from cocultures of E15 retinal explants and explants of hypothalamus or thalamus dissected from the lateral surface of coronal sections through either E17 (A) or E19 (B) diencephalon (as illustrated in Fig. 2). Thalamic explants were either of the LG (Th/LG), or non-target nuclei (Th/nonLG). Retinal axon growth in the diencephalic explants was scored on a scale of 0 to 3, with 0 representing no axon invasion and 3, robust invasion. The y-axis is the percentage of diencephalic explants that exhibited these different levels of retinal axon invasion. At both ages, retinal axon invasion of LG was significantly greater than invasion of the hypothalamic explants. However, the percentage of LG explants with robust levels of retinal axon invasion is greater at the older ages. The data are from six experiments (3 at each age) totaling 135 E17 and 96 E19 cocultures. The differences between seven pairings of the data in A and B were analyzed by chi-square analysis, and found to be significant:  $P < 10^{-10}$ : E17 Hy and Th/LG, E17 LG and E19 LG, E19 Th/LG and Th/nonLG, E19 Hy and Th/LG;  $P < 0.004$ : E17 Hy and Th/nonLG, E17 Th/LG and Th/nonLG, E19 Hy and Th/nonLG.

Axons extending from either E15 rat or E6 chick retina were repulsed by floor plate (Figs 9D,E, 10C). The dramatic asymmetry in retinal axon growth was apparent throughout the culture period. On the side of the retinal explant facing the floor plate, retinal axons tended to be reduced in number, short, fasciculated and often directed away from the floor plate. Since repulsion of temporal and nasal retinal axons was not significantly different, these data were combined. In contrast, explants of E15 rat neocortex typically had greater axon outgrowth from the side of the explant facing the floor plate (Figs 9F, 10C; also see Richards et al., 1997), demonstrating



**Fig. 8.** Distribution of immunostaining for the ECM molecules, neurocan and tenascin, correlates with the localization of inhibitory activities in diencephalon. Neurocan (A) and tenascin (B) immunolabeling in coronal sections of E17 rat diencephalon. Dark reaction product indicates immunopositive regions. Immunostaining for both molecules is high in hypothalamus (Hy) and epithalamus (Epi), and low in the thalamus (Th). The staining intensity changes abruptly at the hypothalamic/thalamic and the thalamic/epithalamic borders. The border between the dorsal and ventral thalamus stains intensely for both molecules (arrowhead in B), although only the intergeniculate leaflet is apparent in the section shown in A (arrowhead). Pt, pretectum.

that the floor plate explants were not releasing a nonspecific toxin that repulses retinal axons. These data demonstrate that retinal axons are repelled *in vitro* by a soluble floor plate activity that mimics the activities released by the hypothalamus, epithalamus and non-target regions of rostral thalamus.

## DISCUSSION

### Region-specific repulsive and stimulatory activities

*In vivo*, retinal axons extend over the caudal hypothalamus in a large fascicle; only a fraction invade the underlying tissue to form the retinohypothalamic projection (Johnson et al., 1988; Levine et al., 1991, 1994). At the border between the hypothalamus and thalamus, the axons defasciculate and spread over the surface of the ventral and dorsal thalamus, avoiding the epithalamus as they turn caudally toward the SC. This pattern of growth suggests that the hypothalamus and epithalamus are repulsive, and that the thalamus is stimulatory for retinal axons; our *in vitro* data support this hypothesis. We demonstrate a target-specific activity in thalamus that stimulates retinal axon growth, and appears to act in both soluble and substrate-bound forms. In addition, we show the existence of both substrate-bound and soluble repulsive activities in the hypothalamus and epithalamus. Our data also reveal a soluble repulsive activity associated with non-target regions of thalamus.

These activities may be substrate-bound *in vivo* since they appear to be regionally restricted, and because many molecules that diffuse *in vitro*, appear not to do so *in vivo* (Marti et al.,

1995; Reilly and Melton, 1996). In addition, retinal axon-repulsive activities described previously at the optic chiasm and in the SC/tectum are substrate-bound (Walter et al., 1987; Godement and Bonhoeffer, 1989; Simon and O'Leary, 1992; Wizenmann et al., 1993; Sretavan et al., 1994; Wang et al., 1995). Experimental evidence also indicates that substrate-bound cues in *Xenopus* diencephalon guide retinal axons (Harris, 1989; Walz et al., 1997).

The *in vivo* observation that the majority of retinal axons extend over but do not invade hypothalamus, correlates with the *in vitro* observation that retinal axons can extend over an inhibitory substrate but do so in a highly fasciculated fashion (Bray et al., 1980). In rats, temporal retinal axons extend through caudal colliculus, even though, *in vitro*, this region is repulsive for these axons (Simon and O'Leary, 1992). Thus, while much of tissue that retinal axons grow over or through *in vivo* is repulsive *in vitro*, this repulsion may dictate the degree of retinal axon fasciculation and branching rather than preventing retinal axon growth altogether. In addition, we demonstrate *in vitro* the existence of stimulatory activities in LG and repulsive activities in adjacent, nontarget regions of thalamus. Thus, both stimulatory and repulsive activities may play a role in establishing target-specific innervation by retinal axons *in vivo*.

#### Cells that might mediate the hypothalamic repulsive activity

A population of neurons in the ventral-most aspect of the hypothalamus express a transmembrane molecule, CD44, that inhibits retinal axon growth *in vitro* (Sretavan et al., 1994). The CD44-positive neurons, which are coincident with and caudal to the optic chiasm, extend axons dorsally along the lateral wall of the diencephalon (Sretavan et al., 1994), forming the tract of the postoptic commissure (tpoc) (Easter et al., 1993). The optic tract parallels the rostral border of the tpoc (Burrill and Easter, 1995). The hypothalamic repulsive activity described here cannot be attributed entirely to the tpoc neurons or their axons since the localization of the activity includes areas rostral and medial to these structures.

We demonstrate that floor plate repulses both E6 chick and E15 rat retinal axons. Floor plate also repulses mouse retinal axons (Wang et al., 1996). The floor plate/ventral midline clearly plays a role in forebrain patterning (Hatta et al., 1991, 1994; Barth and Wilson, 1995; Ericson et al., 1995). As defined by morphological criteria, floor plate extends as far rostrally as the mid-diencephalon in zebrafish (Macdonald et al., 1994). As defined by the expression of floor plate markers, floor plate extends even further rostrally in zebrafish (Macdonald et al., 1994; Barth and Wilson, 1995), chick (Ericson et al., 1995) and mouse (Marti et al., 1995; Shimamura et al., 1995). Our data also suggest that retinal axons are repulsed by

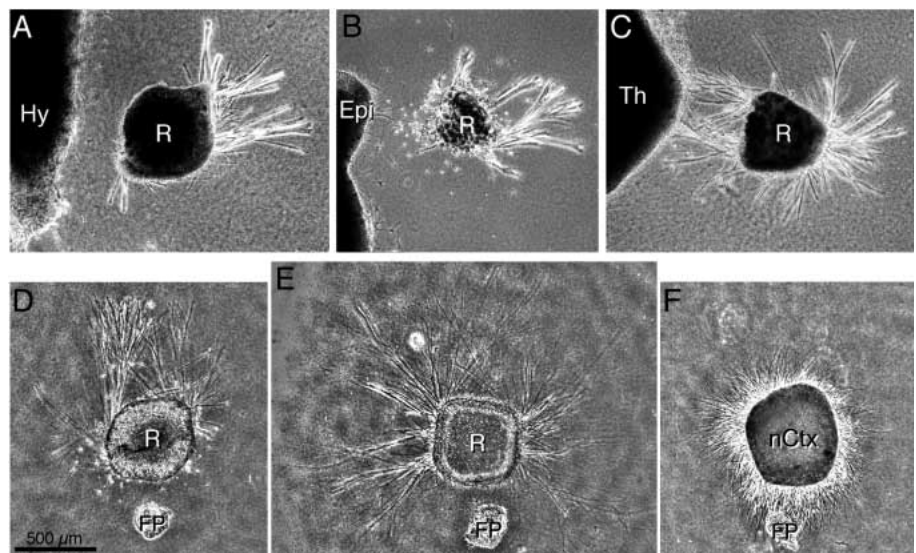
the neuromeric border between dorsal and ventral thalamus, the zona limitans intrathalamica, which also expresses floor plate markers (Macdonald et al., 1994; Barth and Wilson, 1995; Shimamura et al., 1995).

Using tissue from E17 rats, we show that rostral, but not caudal, hypothalamus repulses retinal axons. This result is consistent with the finding that floor plate and rostral, but not caudal, midline diencephalic tissue induce the expression of forebrain-specific markers (Ericson et al., 1995), a function attributed to the morphogen, sonic hedgehog (SHH), which is expressed at the midline in rostral, but not caudal diencephalon (Ericson et al., 1995). It is possible, therefore, that SHH-expressing cells in diencephalon affect the patterning of molecules that directly influence retinal axon pathfinding.

#### Molecules that might mediate the hypothalamic and epithalamic repulsive activities

Using immunohistochemical techniques we show that neurocan and tenascin are enriched in regions of E17 rat diencephalon that retinal axons avoid. While both of these molecules are localized in the ECM, neurocan (Oohira et al., 1988, 1994; Rauch et al., 1991) and tenascin (Grumet et al., 1985) are enriched in soluble brain fractions and culture supernatants. Therefore, these ECM molecules could contribute to either the soluble or substrate-bound repulsion observed *in vitro*.

Other molecules shown to repulse retinal axons may mediate the diencephalic repulsive activities. These include the Eph receptor tyrosine kinase ligands, ephrin-A5 (AL-1/RAG) (Drescher et al., 1995; Winslow et al., 1995) and ephrin-A2



**Fig. 9.** Rostral hypothalamus, epithalamus and floor plate release soluble activities that repulse retinal axons. (A-C) Collagen cocultures of E15 rat retina placed at a distance from the lateral surface of coronal sections of E17 rat diencephalon. Retinal axons were repelled by rostral hypothalamus (Hy, A) and epithalamus (Epi, B), but not by thalamus that contained LG (Th, C). In A, the few retinal axons emerging from the side of the explant facing the hypothalamus are very short and directed away from the hypothalamus. (D-F) Collagen cocultures of E15 rat retina (D), E6 chick retina (E) or E15 rat neocortex (F) placed at a distance from explants of E13 rat floor plate. (D) Rat retinal axon growth is directed away from the floor plate. (E) Chick retinal axon outgrowth is robust everywhere except from the side facing the floor plate. (F) Neocortex (nCtx) exhibited greater axon growth on the side facing the floor plate. All photos were taken at 1.5-2 days *in vitro*. R, retina.



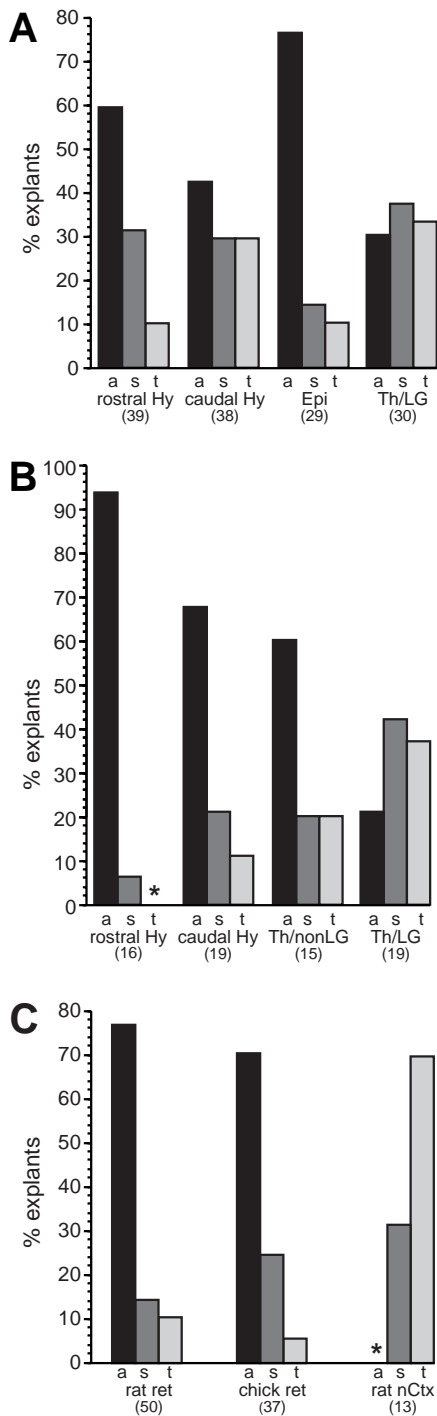
(ELF-1) (Nakamoto et al., 1996), as well as a 33 kDa protein isolated from caudal chick tectum (Stahl et al., 1990), all of which are anchored to the cell membrane via a glycosylphosphatidylinositol linkage. An EphA3(Mek4)-AP receptor affinity probe labels ventral hypothalamus in E15-E17 rat brains, indicating the presence of ephrin-A ligands; however, these ligands alone cannot account for the hypothalamic repulsive activity since their distribution is more restricted than the activity (R. Tuttle, T. McLaughlin and D. O'Leary, unpublished observations), and they do not activate their receptors when presented in a soluble form (Davis et al., 1994).

The secreted growth cone collapsing molecule, collapsin-1/semaphorin III/D, has been shown by in situ hybridization to be expressed at the ventral, diencephalic midline in E2 and E4.5 chick (Shepherd et al., 1996), and in the ventral diencephalon of E15 mouse (Puschel et al., 1996). While retinal growth cones have been reported to be insensitive to collapsin-1 (Luo et al., 1993), retinal ganglion cells express transcripts for the putative collapsin receptors, neuropilin-1 (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) and neuropilin-2 (Chen et al., 1997), and retinal axons immunostain for neuropilin-1 (Kawakami et al., 1996). Therefore, collapsin-1 may mediate a component of the diencephalic repulsive activity.

The floor plate secretes at least two repulsive activities (Serafini et al., 1996; Wang et al., 1996), one of which is mimicked by Netrin-1 (Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1996; Varela-Echavarría et al., 1997). While *Netrin-1* is expressed in E4 chick ventral diencephalon (Kennedy et al., 1994), its expression in rodent hypothalamus is much more restricted than the hypothalamic repulsive activity shown here (H. Simon and D. O'Leary, unpublished observations). In addition, Netrin-1 may stimulate rather than repulse retinal axons (Deiner et al., 1997). Therefore, it is likely that a molecule distinct from Netrin-1 is mediating the hypothalamic repulsion.

### Regionalization of the retinal axon repulsive activities within the diencephalon

Early neuronal populations and their axonal projections in zebrafish (Krauss et al., 1991; Macdonald et al., 1994) and mouse (Shimamura et al., 1995) forebrain often correlate with



**Fig. 10.** Summary of retinal axon response to diencephalon and floor plate in 1.5-day collagen gel cocultures. (A) E15 rat retinal explants were cocultured with living coronal sections through either rostral or caudal E17 rat diencephalon; explants were positioned at a distance from the epithalamus (Epi), LG (Th/LG), or hypothalamus (Hy). (B) E14-15 mouse retinal explants were cocultured with living coronal sections through either rostral or caudal E14-15 mouse diencephalon; explants were positioned at a distance from LG (Th/LG), thalamus that does not contain LG (Th/nonLG), or hypothalamus (Hy). (C) Explants of E15 rat retina (rat ret) or neocortex (rat nCtx), or E6 chick retina (chick ret) were cocultured at a distance from floor plate explants. Retinal axon growth was categorized as being towards (t) or away (a) from the diencephalon or floor plate, or symmetric (s) (see Materials and methods). Data in A, B and C are from three sets of experiments, except for the cocultures with epithalamus, which are from two experiments; in B, two of the three sets of experiments used E14 mouse tissue. *n* values are in parentheses. Asterisks indicate zero percentage values. In A, the data for cocultures of retina with LG versus rostral hypothalamus were found to be significantly different ( $P=0.02$ ). On the other hand, data for retina cocultured with LG versus caudal hypothalamus were not significantly different. In B, the data for the cocultures of retina with LG versus either rostral or caudal hypothalamus were found to be significantly different ( $P=9 \times 10^{-5}$  or 0.01, respectively). However, the difference between retina cocultured with thalamus that does not contain LG (Th/nonLG) versus hypothalamus (either rostral or caudal) was not significantly different. In C, the repulsion of retinal axons by floor plate was not significantly different than that by either epithalamus or rostral hypothalamus. However, the effect of floor plate versus caudal rat hypothalamus on retinal axon growth was significantly different ( $P=0.005$ ).

borders of gene expression. Our data suggest that retinal axon patterning in rodent diencephalon, and the distribution of molecules that influence retinal axon growth, correlate with domains delineated on the basis of gene expression and other criteria (Figdor and Stern, 1993; Puelles and Rubenstein, 1993).

The diencephalon has been hypothesized to consist of neuromeres, which are presumptive forebrain analogs of hindbrain rhombomeres (reviewed in Papalopulu, 1995; Lumsden and Krumlauf, 1996). Figdor and Stern showed in chick that early axonal projections correlate with diencephalic neuromeres (D), which they defined by morphological features, lineage restriction analysis, peanut agglutinin-binding and acetylcholinesterase activity (Figdor and Stern, 1993). Puelles and Rubenstein (1993) divide the forebrain, or prosencephalon, into 'prosomeres' (P) on the basis of morphological features and gene expression patterns. By these criteria, the rostral diencephalon (i.e. excluding the pretectum) is hypothesized to be composed of the following neuromeres: the epithalamus and dorsal thalamus are assigned to D2 (or P2), whereas the ventral thalamus and hypothalamus are assigned to D1 by Figdor and Stern, but to separate prosomeres by Puelles and Rubenstein.

The retinal axon repulsive activities that we describe are localized to the hypothalamus and epithalamus, while the retinal axon stimulatory activity is localized within the dorsal and ventral thalamus. Our in vitro data suggest therefore that the epithalamus and dorsal thalamus are distinct domains within the D2/P2 neuromere. The data further suggest that the ventral thalamus and hypothalamus are distinct domains within the D1 neuromere.

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