Axon Guidance in the Mouse Optic Chiasm: Retinal Neurite Inhibition by Ephrin "A"-Expressing Hypothalamic Cells in Vitro

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In the mammalian visual system, retinal axons undergo temporal and spatial rearrangements as they project bilaterally to targets on the brain. Retinal axons cross the neuraxis to form the optic chiasm on the hypothalamus in a position defined by overlapping domains of regulatory gene expression. However, the downstream molecules that direct these processes remain largely unknown. Here we use a novel in vitro paradigm to study possible roles of the Eph family of receptor tyrosine kinases in chiasm formation. In vivo, Eph receptors and their ligands distribute in complex patterns in the retina and hypothalamus. In vitro, retinal axons are inhibited by reaggregates of isolated hypothalamic, but not dorsal diencephalic or cerebellar cells. Furthermore, temporal retinal neurites are more inhibited than nasal neurites by hypothalamic cells. Addition of soluble EphA5-Fc to block Eph "A" subclass interactions decreases both the inhibition and the differential response of retinal neurites by hypothalamic reaggregates. These data show that isolated hypothalamic cells elicit specific, position-dependent inhibitory responses from retinal neurites in culture. Moreover, these responses are mediated, in part, by Eph interactions. Together with the in vivo distributions, these data suggest possible roles for Eph family members in directing retinal axon growth and/or reorganization during optic chiasm formation. © 2000 Academic Press

Key Words: Ephs; ephrins; retinal axon guidance; optic chiasm; hypothalamus; nasal retina; temporal retina.

INTRODUCTION

During development of the visual pathways, the optic chiasm represents an important intermediate target where retinal axons undergo several temporal and spatial rearrangements (Guillery et al., 1995; Colello and Coleman, 1997; Chan and Chung, 1999). In addition, in mammals, retinal axons from each eye diverge in the optic chiasm to project to targets on both sides of the brain. The molecular and cellular cues that both position the optic chiasm on the hypothalamus and direct retinal axons have been areas of intense investigation. The chiasm forms on the hypothalamus in a position defined by overlapping zones of regulatory protein expression domains (Marcus et al., 1999). The region of the developing chiasm is further defined by a zone occupied by a palisade of radial glia and an early differentiating population of neurons (CD44/SSEA neurons; reviewed in Mason and Sretavan, 1997). We hypothesize that these cells mediate general retinal axon patterning and, by selective inhibition, axon divergence (Marcus and Mason, 1995; Mason et al., 1996).

In vitro studies have implicated both contact-mediated (Sretavan and Reichardt, 1993; Wang et al., 1995; Wizenmann et al., 1993; Godement et al., 1994) and diffusible (Wang et al., 1996) inhibitory cues in directing retinal axon growth and divergence in the chiasm. Furthermore, the coincidence between commissure formation and regulatory protein expression domains has been suggested to form inhibitory zones that discourage growth cone exploration into inappropriate regions (Wilson et al., 1997). However, the identity of the downstream effector molecules responsible for guiding commissure formation, including the optic chiasm, remains largely unknown.

Recently, a number of different molecular families have been implicated in presenting inhibitory cues to growing axons. These include chondroitin sulfate proteoglycans,
netrins, collapsin/semaphorins, Robo-related immunoglobulin proteins and their slit ligands, and the Eph family of receptor tyrosine kinases (reviewed in Tessier-Lavigne and Goodman, 1996; Varela-Echaverria and Guthrie, 1997; Stoeckli and Landmesser, 1998; Guthrie, 1999). To date, at least 14 distinct Eph family members have been identified, and these receptors are divided into two subfamilies based on their ability to bind either glycosyl phosphatidylinositol (GPI)-anchored (“A” subfamily) or transmembrane (“B” subfamily) ligands (Davis et al., 1994; Gale et al., 1996; Eph Nomenclature Committee, 1997). Repulsive interactions between Eph-related receptors and their ligands, the ephrins, have been implicated in several aspects of neural development, including the development of topographic maps, axonal pathway selection, targeted cell migration, and the establishment of regional pattern (reviewed in Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999; O’Leary and Wilkinson, 1999).

In order to identify molecules involved in directing retinal axon growth through the developing optic chiasm, we developed an in vitro paradigm to test for inhibitory interactions between retinal axons and cells isolated from the hypothalamus upon which the chiasm forms. This assay revealed that retinal neurites are specifically inhibited by hypothalamic cells, and that neurites from temporal retina are more inhibited than neurites from nasal retina. We next determined that members of both the “A” and the “B” subfamilies are present along the retinofugal pathway in patterns that suggest a role in its development. Members of the “A” subfamily of receptors and ligands occupy adjacent domains in the developing hypothalamus, and we further studied possible roles of this subfamily by analyzing the effects of blocking endogenous receptor–ligand interactions in vitro. These experiments demonstrate that endogenous “A” subclass ephrins present on hypothalamic cells contribute to both the inhibition and the differential response of retinal neurites by chiasmatic reaggregates, suggesting a role for local Eph receptor–ligand interactions in directing retinal axon growth during optic chiasm formation. Moreover, our results, in conjunction with previous reports on possible roles of Eph family members in the developing visual system, suggest that specific receptor–ligand interactions underlie distinct aspects of visual system pathway development and organization.

**MATERIALS AND METHODS**

Experiments were performed on C57Bl/6J mice and Sprague-Dawley rats obtained from timed-pregnancy breeding colonies. Time of conception was considered midnight before the day a plug was found and noon the following day 0.5 (0.5). Pregnant mice containing E14 embryos were anesthetized with a mixture of ketamine and xylazine, and the embryos were removed one at a time by cesarean section. Pregnant rats containing E16 embryos were sacrificed by CO2 asphyxiation and the embryos removed and kept on ice until use. P0 and P5 rat pups were sacrificed by decapitation.

**Antibody Fusion Protein Binding**

The distributions of the ephrin-A and ephrin-B subclasses were visualized by receptor–antibody fusion protein binding on 15-μm-thick unfixed frozen sections and cellular reaggregates, as described previously (Gale et al., 1996; Marcus et al., 1996a). Receptor–antibody fusion proteins consisting of the extracellular portions of the Eph receptors EphA2 (Eck), EphA5 (Ehk1, Bsk), EphB1 (Eik, Cek6), and EphB2 (Cek5, Nuk) and the ephrins ephrin-A1 (B61) and ephrin-B1 (Eik-L) fused to the human IgG1 Fc domain were used (Davis et al., 1994; Gale et al., 1996). Briefly, 15-μm-thick sections, collected on subbed slides, were blocked for 30 min in a solution containing 10% normal goat serum, 2% BSA, 0.02% Na azide (block). Slides were then incubated in 2 μg/ml of receptor or ligand fusion proteins in 0.5× block for 1 h. The tissue was then fixed in fresh 4% paraformaldehyde, washed in PBS, heat treated at 70°C for 1 h to destroy endogenous phosphatase activity, and incubated for 1 h in goat anti-human IgG alkaline phosphatase-conjugated secondary antibody (1:1000 in 0.5× block; Promega). Sections were color developed in a solution of NBT and BCIP, followed by fixation in 4% paraformaldehyde, and mounted.

Living cultures were incubated either overnight or for 30 min in 50 μg/ml of EphA2-Fc or EphA5-Fc, or 12 μg/ml of an Fc control. The concentration of the receptor–antibody fusion proteins used was that previously determined to be effective in binding available ligand (Gale et al., unpublished results). The Fc control concentration was matched to the protein concentration of the Fc tag used in the receptor–antibody fusion proteins. Cultures were washed with calcium- and magnesium-free PBS, fixed in 4% paraformaldehyde, and processed as for the tissue sections outlined above.

**In Situ Hybridization**

Fifteen micrometer cryostat sections prepared for in situ hybridization using probes for ephrin-A3, -A4, -A5, and -B2 for the analysis in Marcus et al. (1996a), were examined for expression in the hypothalamus.

**Culture Methods**

**Retinal explants.** Retinal explants from E14 mouse embryos were collected in ice-cold DMEM/F12 medium with 15 mM Hepes buffer (Gibco). For orientation, a small cut was made prior to dissection, at the ventral point of the diamond-shaped opening of the eye cup formed by the pigmented epithelium. Retiniae were dissected free from the associated pigmented epithelium, lens, vitreous, and vasculature, and a 250-μm-wide strip of retina incorporating the periphery of either temporal (DT and VT) or nasal retina (DN and VN) was cut. Retiniae were plated ganglion cell side down on glass coverslips prepared with polylysine (100 μg/ml, Specialty Media) and laminin (20 μg/ml, Sigma) and covered with 80–100 μl of serum-free medium (SFM: DMEM/F12 supplemented with 1% BSA, 20 units/ml penicillin/streptomycin, and Sigma I-1884 (5 mg/ml insulin, 5 mg/ml transferrin, 5 μg/ml sodium selenite) containing 0.4% methyl cellulose (Sigma), as described previously (Marcus et al., 1996b; Wang et al., 1995). Treated cultures had 50 μg/ml of either EphA2-Fc or EphA5-Fc or 12 μg/ml of the Fc control diluted directly in SFM-containing methyl cellulose.

**Cellular reaggregates.** While initial experiments were performed on retiniae and cells isolated from mice, it was difficult to distinguish retinal neurites from neurites emanating from the test cellular reaggregates. The data reported here derive from cocultures...
of mouse retina with rat cellular reaggregates; both combinations gave qualitatively similar results. Four sources of rat cells were used: late E16 chiasm (including surrounding hypothalamic cells) and dorsal diencephalon, and P4–P5 whole cerebella and isolated cerebellar granule cells. E16 brains were dissected free from the skull and the region of the developing optic chiasm and hypothalamus isolated, as described previously (Wang et al., 1996; Marcus et al., 1996b). To obtain dorsal diencephalic cells, the cortex was removed and an approximately 400-μm-diameter piece of diencephalon just lateral to the dorsal midline was isolated. Tissue chunks were collected in DMEM/F12 medium and dissociated using a modification of previously published methods (Alder et al., 1996; Wang et al., 1995). Briefly, tissue was sequentially incubated in medium containing 0.08% trypsin, 0.25% trypsin plus 0.1% collagenase, and 0.05 mg/ml DNase containing 1 ml of 0.05 mg/ml trypsin inhibitor, for 15 min each at 35.5°C. The tissue was dissociated using fire-polished pipets and passed over a 33-μm Nytex filter. Dissociated whole cerebella and cerebellar granule cells were obtained according to previously published methods (Gao et al., 1991; Hatten et al., 1998).

Dissociated cells were resuspended at 2–3 × 10^6 cells/ml in medium containing 10% horse serum. Three-hundred to 500 μl of the suspended cells was added to uncoated Nunc Lab-Tek (Naperville, IL) 7-mm culture wells and incubated overnight at 35.5°C. The next day, cellular reaggregates were resuspended in a large volume of SFM and maintained at 35.5°C for at least 3 h prior to use. Cellular reaggregates (approximately 50–80 μm in diameter) were transferred in less than 5 μl of SFM to coverslips containing retinal explants and positioned using fine forceps (see Gao et al., 1995). Briefly, tissue was sequentially incubated in medium containing 0.08% trypsin, 0.25% trypsin plus 0.1% collagenase, and 0.05 mg/ml DNase containing 1 ml of 0.05 mg/ml trypsin inhibitor, for 15 min each at 35.5°C. The tissue was dissociated using fire-polished pipets and passed over a 33-μm Nytex filter. Dissociated whole cerebella and cerebellar granule cells were obtained according to previously published methods (Gao et al., 1991; Hatten et al., 1998).

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To unambiguously identify neurites from mouse retinal explants from cellular processes arising from rat cellular reaggregates, mouse retinal neurites were labeled with a mouse-specific antibody, M6, according to previously published methods (Baird et al., 1992; Lagenaur et al., 1992). Labeled cultures were coverslipped with Gelmount (Biomeda Corp.) and photographed on a Zeiss Axiohot microscope.

### Analysis

Possible effects of the receptor-antibody fusion proteins on retinal neurite outgrowth were evaluated by comparing retinal neurite lengths from explants cultured for 24 h alone or in the presence of 50 μg/ml EphA2-Fc or EphA5-Fc or 12 μg/ml of the Fc control. In our analysis “retinal neurites” include processes emanating singly or as bundles from the retinal explants.

For coculture analysis, cultures were blinded to both treatment condition and retinal origin (i.e., nasal or temporal retinal strip from either the right or left eye), and the behaviors of retinal neurites encountering cellular reaggregates were scored by two independent observers (Fig. 1). Positive behaviors included those in which a majority of the retinal neurites appeared to grow over the reaggregates and their neurites (Fig. 1.3) or those in which retinal axons appeared to freely grow onto the cellular portion of the reaggregates (Fig. 1.4). Negative behaviors included an avoidance category in which retinal neurites stopped short or clearly turned away from the reaggregates (Fig. 1.1) and a mixed category in which less than 50% of the retinal neurites grew freely over the reaggregates (Fig. 1.2). An ambiguous category included encounters which did not clearly fall in any of the categories or those in which the two independent observers did not agree (Tables 1 and 2).

Possible effects of the different treatment conditions on neurites from the four retinal quadrants were analyzed by dividing each retinal explant in thirds and only including those encounters with retinal neurites arising from the end thirds of each retinal explant. Only the retinal neurites in the outer thirds were analyzed because the middle third was thought to contain a mixture of the two populations.

A total of 580 encounters between retinal neurites and cellular reaggregates, from 161 retinal explants, were included in the analysis. Statistical significance was evaluated using the χ² test. Figures were assembled with Adobe Photoshop. For some images, brightness or contrast levels were globally altered in order to enhance clarity.

### RESULTS

#### Cellular Reaggregate Cultures

As a first step in elucidating the molecules which direct axon growth through the hypothalamus, we developed an in vitro paradigm to readily assay interactions between retinal axons and molecules expressed by endogenous hypo-

#### TABLE 1

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<td></td>
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<td>Nt</td>
<td></td>
<td>Nt</td>
<td></td>
<td>Nt</td>
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<tr>
<td>Avoidance</td>
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<td>(37)</td>
<td>(17)</td>
<td>(28)</td>
<td>58.6 (85)</td>
<td>10.0 (5)</td>
<td>10.0 (9)</td>
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<td>28.3 (41)</td>
<td>18.0 (9)</td>
<td>5.6 (5)</td>
<td>5.6 (1)</td>
<td>33.0 (31)</td>
<td>16.0 (20)</td>
<td>42.5 (17)</td>
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<td>86.9 (126)</td>
<td>28.0 (14)</td>
<td>15.6 (14)</td>
<td>11.1 (2)</td>
<td>72.3 (68)</td>
<td>44.8 (56)</td>
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<td>72.0 (36)</td>
<td>84.4 (76)</td>
<td>88.9 (16)</td>
<td>27.7 (26)</td>
<td>55.2 (69)</td>
<td>32.5 (13)</td>
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<tr>
<td>Ambiguous</td>
<td>(3)</td>
<td>(6)</td>
<td>(0)</td>
<td>(1)</td>
<td>(3)</td>
<td>(5)</td>
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<tr>
<td>Total n</td>
<td>(148)</td>
<td>(56)</td>
<td>(90)</td>
<td>(19)</td>
<td>(97)</td>
<td>(130)</td>
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Note. Data represent the percentage of the total number of encounters (minus those scored as ambiguous) for each culture condition. The raw numbers are given in parentheses. Total inhib, avoidance = mixed inhib categories; chiasm., chiasmatic reaggregates; d. dien., dorsal diencephalic reaggregates; mixed cb, mixed cerebellar reaggregates; granule c., purified cerebellar granule cell reaggregates.
lamic cells (see Marcus et al., 1999, for a detailed explanation of the anatomical descriptors used in this paper).

**Retinal Axons Are Specifically Inhibited by Cellular Reaggregates from the Chiasmatic Region of the Hypothalamus**

Previous studies from our lab revealed that all retinal neurites are inhibited by dissociated cells from the chiasmatic region (Wang et al., 1995; Marcus et al., 1996b). Furthermore, contralaterally ("crossed") and ipsilaterally ("uncrossed") projecting retinal axons responded differentially to dissociated chiasm cells. Specifically, in cocultures of retinal explants with cells dissociated from the region of the developing chiasm, uncrossed axons, which primarily arise from the ventrotemporal (VT) retina, grew fewer and shorter neurites than crossed axons from the rest of the retina (dorsoventral (DV), dorsonasal (DN), and ventronasal (VN) retina). Moreover, uncrossed axons avoided groupings of glia and neurons, while crossed axons extended on them (Wang et al., 1995).

While these studies uncovered several interesting interactions between retinal axons and dissociated hypothalamic cells, encounters of retinal axons with groupings of cells were rare and highly variable. In order to more rigorously evaluate inhibitory interactions between retinal neurites and clusters of cells from the chiasmatic region, dissociated hypothalamic cells were reaggregated overnight in serum-containing medium, and then individual reaggregates of uniform size were plated at a distance from the retinal explants (Fig. 1). Four different sources of cells were used to make cellular reaggregates: dissociated cells isolated from the chiasmatic region ("chiasmatic reaggregates")—which include developing hypothalamic

| TABLE 2 |
|------------------|---|---|---|---|---|---|
|                | DT  | VT  | DN  | VN  | Temporal | Nasal |
| Untreated      |     |     |     |     |          |       |
| Avoidance      | 62.5 (25) | 64.5 (20) | 36.4 (8) | 40.0 (6) | 62.2 (56) | 40.8 (20) |
| Mixed inhibb   | 32.5 (13) | 29.0 (9) | 40.9 (9) | 20.0 (3) | 32.2 (29) | 30.6 (15) |
| Total inhibc   | 95.0 (38) | 93.5 (29) | 77.3 (17) | 60.0 (9) | 94.4 (85) | 71.4 (35) |
| Permissived    | 5.0 (2) | 6.5 (2) | 22.7 (5) | 40.0 (6) | 5.6 (5) | 28.6 (14) |
| Ambiguous      | (0) | (1) | (0) | (0) | (1) | (0) |
| Total n        | (40) | (32) | (22) | (15) | (90) | (49) |
| Fc control     |     |     |     |     |          |       |
| Avoidance      | 64.3 (9) | 50.0 (7) | 31.6 (6) | 27.3 (3) | 63.2 (24) | 25.5 (13) |
| Mixed inhibb   | 21.4 (3) | 35.7 (5) | 36.8 (7) | 36.4 (4) | 26.3 (10) | 35.3 (18) |
| Total inhibc   | 85.7 (12) | 85.7 (12) | 68.4 (13) | 63.6 (7) | 89.5 (34) | 60.8 (31) |
| Permissived    | 14.3 (2) | 14.3 (2) | 31.6 (6) | 36.4 (4) | 10.5 (4) | 39.2 (20) |
| Ambiguous      | (0) | (0) | (1) | (1) | (1) | (2) |
| Total n        | (14) | (14) | (20) | (12) | (38) | (51) |
| EphA5-Fc       |     |     |     |     |          |       |
| Avoidance      | 36.5 (8) | 35.3 (6) | 5.6 (1) | 46.7 (7) | 32.3 (20) | 18.8 (13) |
| Mixed inhibb   | 22.7 (5) | 17.6 (3) | 33.3 (6) | 6.7 (1) | 19.4 (12) | 16.1 (10) |
| Total inhibc   | 59.1 (13) | 52.9 (9) | 38.9 (7) | 53.4 (8) | 51.6 (32) | 37.1 (23) |
| Permissived    | 40.9 (9) | 47.1 (8) | 61.1 (11) | 46.7 (7) | 48.4 (30) | 62.9 (39) |
| Ambiguous      | (2) | (0) | (2) | (0) | (3) | (1) |
| Total n        | (24) | (17) | (20) | (15) | (62) | (62) |
| Dorsal di      |     |     |     |     |          |       |
| Avoidance      | 0.0 (0) | 28.6 (2) | 14.3 (1) | 20.0 (1) | 13.0 (3) | 8.7 (2) |
| Mixed inhibb   | 25.0 (2) | 0.0 (0) | 28.6 (2) | 0.0 (0) | 17.4 (4) | 17.4 (4) |
| Total inhibc   | 25.0 (2) | 28.6 (2) | 42.9 (3) | 20.0 (1) | 30.4 (7) | 26.1 (6) |
| Permissived    | 75.0 (6) | 71.4 (5) | 57.1 (4) | 80.0 (4) | 69.6 (16) | 73.9 (17) |
| Ambiguous      | (2) | (1) | (0) | (1) | (4) | (1) |
| Total n        | (10) | (8) | (7) | (6) | (23) | (23) |

Note. Data represent the percentage of the total number of encounters (minus those scored as ambiguous). In parentheses are the raw numbers for each condition. DT (dorso temporal), VT (ventro temporal), DN (dorso nas al), and VN (ventro nas al) are data from the end one-thirds of temporal and nasal explants, respectively (see Materials and Methods for details). Uncrossed axons arise almost exclusively from VT retina.

a "Temporal" and "nasal" represent data from entire temporal and nasal retinal explants.

b Mixed inhib (mixed inhibitory), ≤50% of retinal neurites grew over reaggregates.

c Total inhib (total inhibitory) = avoidance + mixed inhib.

d Permissive, retinal neurites freely crossed or grew onto reaggregates.

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neurons and glia), dorsal diencephalon, whole cerebellum, or purified cerebellar granule cells. Retinal neurites were unambiguously identified from processes arising from the cellular reaggregates by coculturing mouse retinal explants with reaggregates of rat cells and visualizing the retinal neurites with the mouse neuron-specific antibody, M6, at the conclusion of the culture period.

Numerous retinal neurites extend radially out from the cut edge of retinal explants grown on a planar substrate coated with polylysine and laminin alone (e.g., see Fig. 2 in Wang et al., 1995). Neurites emerged from retinal explants singly and as bundles, and both were analyzed. Retinal neurites encountering reaggregates of cells from the four cellular sources exhibited a range of behaviors. In some cases, retinal neurites continued along their original trajectories when encountering cellular reaggregates (Figs. 1.3 and 2D). In other cases, retinal growth cones grew directly onto the cell bodies within the reaggregates (Figs. 1.4 and 2F). Reaggregates which retinal neurites grew directly onto, or which the majority of retinal axons grew over, were considered to present either positive or neutral cues to retinal axons.

Over 85% of encounters between retinal neurites (from both nasal and temporal strips) and chiasmatic reaggregates were considered inhibitory (Table 1; Fig. 3). Of these, the majority (>67%) were classified as demonstrating avoidance vs a mixed response. The inhibition of retinal neurites by chiasmatic reaggregates was significantly greater than that observed with reaggregates of dorsal diencephalic (28%), mixed cerebellum (16%), or purified granule cells (11%) (P < 0.001, Table 1). The majority of retinal neurites grew freely over reaggregates of dorsal diencephalic cells (Figs. 2C, 2D and 3), whereas retinal growth cones were frequently found on top of cellular reaggregates composed of mixed cerebellar or purified cerebellar granule cells (Figs. 2E, 2F and 3). Thus, retinal axons can detect and respond differentially to cues presented by cellular reaggregates from different sources. Moreover, retinal neurites are specifically inhibited by reaggregates of cells from the hypothalamus on which the chiasm forms.

**FIG. 1.** Schematic diagram of reaggregate cultures. Cellular reaggregates from four different cellular sources were cocultured for 24–28 h with a strip of nasal or temporal retina. Following fixation, cultures were blinded, and the behaviors of retinal neurites to individual reaggregates classified by two independent observers according to the following criteria: 1(-), "avoidance," retinal neurites clearly avoid cellular reaggregates; 2(-), "mixed inhibitory," retinal neurites demonstrate a mixed response of growth over and growth around the cellular reaggregates; 3(+), "growth over," retinal neurites continue along their original trajectories when encountering cellular reaggregates; 4(+), "growth on," retinal neurites grow onto cell bodies of cellular reaggregates. Reaggregates from categories 1 and 2 were considered to present negative or inhibitory cues for retinal axon growth, whereas those from categories 3 and 4 were considered to present positive or neutral cues to retinal axons.

**Temporal and Nasal Retinal Axons Are Differentially Affected by Chiasmatic Reaggregates**

Both inhibitory and permissive cues associated with a zone centered around the chiasmatic midline are implicated in directing retinal axon divergence (Godement et al., 1994; Sretavan et al., 1994; Marcus and Mason, 1995; Wang et al., 1995). In addition, a diffusible signal(s) from the chiasmatic region suppresses the growth of all optic axons, regardless of their retinal origin (Wang et al., 1996). To determine whether crossed and uncrossed axons were differentially or equally affected by the inhibition uncovered in the in vitro assay described above, we compared the
behaviors of uncrossed retinal neurites arising from VT retina with crossed neurites arising from DT, DN, and VN retina (see Materials and Methods).

Comparison of the behaviors of retinal neurites from the four retinal quadrants revealed that the degree of retinal neurite inhibition was not equal across the retina (Table 2; Fig. 4). These differences did not correspond to an uncrossed (ventrotemporal) vs a crossed retinal origin as in our previous in vitro model (Wang et al., 1995), but rather reflected a temporal vs nasal distribution. Over 90% of encounters between chiasmatic reaggregates and temporal (both dorsal and ventral) retinal neurites were inhibitory, compared to about 70% of encounters with nasal retinal neurites (P < 0.001). Several explanations can account for the difference between these results and our former study. First, in our former study, retinal neurites growing among dissociated chiasm cells may be primed to respond differentially to larger clusters of chiasmatic cells. Alternatively, in the
The present study, inclusion of serum in the medium (necessary for the formation of cellular reaggregates) may have altered the expression of cell surface molecules required for eliciting differential behaviors of crossed and uncrossed retinal neurites. Nevertheless, the present assay provides a highly reproducible and easily quantifiable method for measuring position-dependent retinal neurite responses to chiasmatic cells. Because the cultures are grown on a planar substrate in a large volume of medium, these responses most likely represent contact-mediated, or short-range, diffusible cues.

**Eph Receptors and Their Ligands Are Distributed along the Retinofugal Pathway**

Recently, a number of molecular families with inhibitory properties have been uncovered. We investigated whether one of these, the Eph family of receptor tyrosine kinases and their ligands, the ephrins, might contribute to the inhibitory response of retinal axons to chiasmatic reaggregates, by determining the distributions of Eph family members in the mouse during the period of optic chiasm formation.

In a previous study, we used receptor- and ligand-antibody fusion proteins to localize their corresponding ligands and receptors in the developing mouse retina (Marcus et al., 1995a). These studies demonstrated that receptors and ligands from either the "A" and the "B" specificity subclasses distribute in opposing gradients along the temporonasal or dorsoventral axes by E10.5–E11. This region overlaps with previously described territories of gene expression and an early differentiating population of neurons implicated in guiding retinal axon growth through the hypothalamus (Fig. 5a, e). Thus, the complementary distributions of the "A" subclass receptors and ligands were already present at E11.5–E12.5, prior to the first retinal axon ingrowth into the hypothalamus (Fig. 5a, B and C), indicating binding sites independent of those present on the retinal axons themselves. Little or no binding was detectable in the optic stalks. The localization of members of ephrin-A ligands in the region of the developing hypothalamus is in agreement with previous reports in both mouse (Donoghue et al., 1996; Zhang et al., 1996) and zebrafish (Brennan et al., 1997; Macdonald et al., 1997).

Ephrin-B1-Fc bound two domains in the developing hypothalamus. The data suggest that EphA receptors within the region of the optic chiasm, in a pattern reminiscent of a previously described radial glial palisade that straddles the chiasmatic midline (Fig. 5a, B, and H; and Fig. 5b, e and f; see also Fig. 2 in Marcus et al., 1995). The complementary distributions of the "A" subclass receptors and ligands were already present at E11.5–E12.5, prior to the first retinal axon ingrowth into the hypothalamus (Fig. 5a, B and C), indicating binding sites independent of those present on the retinal axons themselves. Little or no binding was detectable in the optic stalks. The localization of members of ephrin-A ligands in the region of the developing hypothalamus is in agreement with previous reports in both mouse (Donoghue et al., 1996; Zhang et al., 1996) and zebrafish (Brennan et al., 1997; Macdonald et al., 1997).

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The first domain overlapped with the distribution of the ephrin-A1-Fc binding straddling the chiasmatic midline, suggesting that receptors of both the "A" and "B" specificity subclasses colocalize with the chiasmatic glial palisade (Fig. 5a, G and H; Fig. 5b, f). The second domain (Fig. 5a, A and D) overlapped in part with the region labeled...
by the EphA5-Fc antibody fusion protein and contains the early born population of CD44/SSEA neurons (Fig. 5b, e') (Marcus et al., 1999; Mason and Sretavan, 1997). In contrast, no labeling was observed with either an EphB1- or EphB2-antibody fusion protein.

Previously we used probes specific for ephrin-A3, -A4, -A5, and -B2 to determine the specific identities of retinal ligands revealed by receptor-antibody fusion binding (Marcus et al., 1996a). Inspection of those data revealed high levels of ephrin-A5 in the hypothalamus (Fig. 5b, a' and b'). Interestingly, in contrast to the lack of binding with either an EphB1-Fc or EphB2-Fc receptor antibody-fusion protein, hybridization with an ephrin-B2 probe revealed expression flanking the midline of the developing chiasm (Fig. 5b, c', d', and f'; Nakagawa et al., 2000). Ephrin-B2 labeling is concentrated in the region overlapping the radial glial palisade, coincident with the expression of EphA and EphB receptors revealed by antibody fusion protein binding. The overlapping expression of “B” subclass receptors and ligands may explain the discrepancy between the ephrin-B expression revealed by binding studies and in situ hybridization, as ligands may be masked by endogenous receptors in sites where overlaps occur (Sobieszczuk and Wilkinson, 1999). Further in situ hybridization studies will be necessary to precisely determine the distributions of individual receptors and ligands present in the hypothalamus.

“A” Subclass Ephrins Contribute to Retinal Neurite Inhibition by Chiasmatic Reaggregates

Several studies suggest that growth cone extension is regulated by domains of regulatory genes expressed in the early neuroepithelium (see Wilson et al., 1997; Marcus et al., 1999). In particular, many commissures appear to form in locations predicted by different expression domains. However, it remains unclear which, if any, of the guidance molecules lying upstream of these regulatory proteins influence growth cone extension. “A” subclass ephrins are expressed along the ventral aspect of the developing chiasm. This region coincides with previously described territories of regulatory gene expression (Marcus et al., 1999), prompting us to investigate whether endogenous hypothalamic ephrins can affect retinal neurite extension.

First we determined whether “A” subclass ephrins contributed to be expressed in the cultures. Both EphA2-Fc and EphA5-Fc bound to chiasmatic reaggregates in culture (Figs. 6E–6H). Little or no binding was detected on the retinal neurites (data not shown). In vivo, “A” subclass receptors and ligands demonstrate complementary temporal–nasal distributions (Marcus et al., 1996a). The apparent lack of retinal binding could reflect either a low number of ligands on the retinal axons or that existing ligands are masked by binding to endogenous receptors on the same or adjacent retinal axons. Next we added either EphA2- or EphA5-receptor-antibody fusion proteins to disrupt potential receptor-ligand interactions in cocultures of retinal explants with chiasmatic reaggregates (Figs. 6C and 6D). To control for possible inhibitory-releasing effects of the Fc tag, cocultures were incubated in the presence of soluble Fc protein added in a concentration that matched that of the Fc tag present on the antibody fusion proteins (Figs. 6A and 6B).

Addition of EphA5-Fc, EphA2-Fc, or the Fc control did not noticeably affect the numbers or lengths of retinal neurites grown on polylysine/laminin alone (neurite lengths: untreated, 1013 ± 196 μm, n = 12; EphA5-Fc treated, 1013 ± 186 μm, n = 10; EphA2-Fc treated, 955 ± 182 μm, n = 6). In contrast, addition of EphA5-Fc, but not EphA2-Fc, significantly decreased the frequency of inhibitory responses of neurites from all poles of the retina to chiasmatic reaggregates (P < 0.001, Table 1). Fc addition to the cocultures resulted in a small, but significant, decrease in the number of inhibitory responses of retinal neurites (P < 0.001 vs Fc control). Thus, signaling by ephrins contributes to the inhibition of retinal neurites by chiasmatic reaggregates.

We next investigated whether “A” subclass ephrins contributed to the differential response of temporal vs nasal neurites to untreated chiasmatic reaggregates by determining the frequency of inhibitory responses from entire temporal or nasal retinal strips in the presence of EphA5-Fc (see Materials and Methods). Addition of EphA5-Fc decreased the inhibition of temporal and nasal neurites by 42.8 and 34.3%, respectively, when compared to untreated controls (Table 2, Figs. 4 and 7). If signaling via Eph receptors is responsible for the greater inhibition of temporal vs nasal neurites by chiasmatic reaggregates, blocking this signal would cause nasal and temporal neurites to respond equally to the cellular reaggregates. In support of this hypothesis, when the data from Fig. 7 are expressed as the difference in the percentage of inhibition of temporal vs nasal retinal neurites, the disparity in the amount of inhibition between temporal and nasal neurites (23%) was reduced in the presence of EphA5-Fc (14.5%) (Fig. 8). Although addition of EphA5-Fc reduced the inhibition seen by all four retinal quadrants, it did not differentially affect uncrossed retinal axons from VT retina vs crossed axons arising from DT retina (Table 2). Furthermore, comparison of EphA5-Fc-treated cultures with control cultures containing dorsal diencephalic reaggregates indicates that the addition of EphA5-Fc does not totally block the inhibitory effects of chiasmatic reaggregates on temporal or nasal neurites (Fig. 7). Several factors could account for why this difference was not totally abolished. First, the addition of exogenous EphA5-Fc may have incompletely blocked available ligand on the hypothalamic cells or the retinal axons themselves. Second, despite demonstrations of promiscuous binding between receptors and ligands of a given subclass in vitro, it is unclear whether such promiscuous binding occurs in vivo. Consistent with this idea, we found that EphA2-Fc was much less effective than EphA5-Fc in blocking inhibitory interactions between retinal neurites.
and chiasmatic reaggregates, even though both EphA2-Fc and EphA5-Fc were equally effective in localizing ephrin distributions in the hypothalamus (see also Krull et al., 1997). Third, other spatially restricted molecules could also contribute to the different behaviors of temporal and nasal neurites in response to chiasmatic reaggregates. A growing list of molecules with distinct temporal–nasal and dorsal–ventral distributions have been isolated (reviewed in Kaprielian and Patterson, 1994). Molecules with distinct temporal–nasal distributions include Eph-related receptors and ephrins (Cheng et al., 1995; Marcus et al., 1996a), the winged helix transcription factors BF-1 and BF-2 (Hatini et al., 1994; Huh et al., 1999), the homeobox containing gene SOHO-1 (Deltchev et al., 1994), and the cell surface protein TOPa (Savitt et al., 1995). Nevertheless, the reduction in the number of inhibitory responses of retinal neurites to chiasmatic reaggregates is consistent with the addition of soluble EphA5-Fc in blocking inhibitory (or repulsive) receptor–ligand interactions between endogenous retinal axons and hypothalamic cells.

**DISCUSSION**

Previous studies have implicated inhibitory signals in directing retinal axon growth and divergence through the hypothalamus during optic chiasm formation. Here we developed a novel in vitro assay to show that retinal
neurites are specifically inhibited by signals associated with reaggregates of chiasmatic cells and that temporal retinal neurites are more inhibited than nasal ones. Using a combination of receptor–antibody fusion protein binding and in situ hybridization we demonstrate that both “A” and “B” subclass receptors and ligands occupy the hypothalamus in patterns that implicate them in directing retinal axon growth. “A” subclass ligands are expressed ventral to the developing chiasm, and we demonstrate that disrupting signaling between “A” subclass receptors and ligands contributes both to the inhibition and the differential response of retinal neurites to chiasmatic cells. Eph family interactions have been implicated in several developmental processes including the establishment of regional pattern, axon guidance, and the encoding of positional labels. Below we discuss how our results implicate this family in similar roles during optic chiasm development.

Eph Family Members and the Establishment of Regional Pattern

Using antibody fusion proteins we demonstrate that “A” subclass receptors and ligands occupy adjacent, complementary domains in the hypothalamus. Eph-related receptors and their ligands are expressed in adjacent domains throughout the mouse embryo (Flenniken et al., 1996; Gale et al., 1996), suggesting that signaling at the interface between adjacent domains contributes to the segmental organization of the developing embryo (Holder and Klein, 1999). Consistent with such a role, expression of a truncated Sek1 receptor in zebrafish and Xenopus embryos results in abnormal hindbrain segmentation, possibly through restricting the movement of cells between adjacent rhombomeres (Xu et al., 1995; Mellitzer et al., 1999). Likewise, repulsive receptor–ligand interactions appear to be necessary for restricting neural crest cell migration in the trunk and branchial arches (Krull et al., 1997; Smith et al., 1997). The recent identification of Krox-20 as a direct transcriptional activator of EphA4 suggests one mechanism by which the identity and movement of cells are coupled to produce sharply restricted segmental domains (Theil et al., 1998).

Studies of regulatory gene expression patterns suggest that the forebrain, like the hindbrain and spinal cord, consists of a series of transverse and longitudinally organized domains (Figgiori and Stern, 1993; Puelles and Rubenstein, 1993). Furthermore, commissures frequently form at locations predicted by domains of regulatory gene expression (see Wilson et al., 1997). Interestingly, the complementary domains of “A” subclass receptors and ligands we observed in the hypothalamus correspond to regulatory gene expression domains implicated in patterning retinal axon growth through this region (Marcus et al., 1999) and, as such, might be downstream targets of these genes. Related to these findings, expression of a truncated Sek1 receptor in the zebrafish embryo results in the mis specification of forebrain structures in the misspecification of forebrain structures, including the eyes and the ventral forebrain (Xu et al., 1996). Together, these results suggest that Eph family members may play an early developmental role in patterning the hypothalamus on which the optic chiasm forms.

In addition to the complementary patterns of “A” subclass neurites are specifically inhibited by signals associated with reaggregates of chiasmatic cells and that temporal retinal neurites are more inhibited than nasal ones. Using a combination of receptor–antibody fusion protein binding and in situ hybridization we demonstrate that both “A” and “B” subclass receptors and ligands occupy the hypothalamus in patterns that implicate them in directing retinal axon growth. “A” subclass ligands are expressed ventral to the developing chiasm, and we demonstrate that disrupting signaling between “A” subclass receptors and ligands contributes both to the inhibition and the differential response of retinal neurites to chiasmatic cells. Eph family interactions have been implicated in several developmental processes including the establishment of regional pattern, axon guidance, and the encoding of positional labels. Below we discuss how our results implicate this family in similar roles during optic chiasm development.

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class receptors and ligands in the hypothalamus, we detected a subregion of EphB expression within the ephrin-A-expressing domain and a region of overlapping expression of “A” and “B” receptors with ephrin-B2 centered on the chiasmatic midline. Thus, receptors and ligands are not restricted to mutually exclusive domains. Sites of overlapping expression have also been detected by in situ hybridization in somites and the branchial arches (Wang and Anderson, 1997). The visualization of ephrin-B2 by in situ hybridization, but not by receptor-antibody fusion protein binding, is consistent with masking of ligand by endogenous receptors in sites where overlaps occur (see also Sefton et al., 1997; Sobieszczuk and Wilkinson, 1999).

**Eph Family Interactions and Axon Guidance**

Eph-related receptors and their ligands have also been implicated as axon guidance molecules in several systems including the topographic mapping of retinotectal (Cheng et al., 1995; Drescher et al., 1995), retinogeniculate (Feldheim et al., 1998), and hippocampeoseptal (Gao et al., 1996; Zhang et al., 1996) projections; axon fasciculation (Winslow et al., 1995); the development of cortical (Castellani et al., 1998) and hippocampal (Stein et al., 1999) circuits; guidance of sensory and motor axons (Gao et al., 1998; Ohta et al., 1997; Wang and Anderson, 1997); and the establishment of tracts across the midline (Henkemeyer et al., 1996; Orioli et al., 1996; Park et al., 1997). Here we demonstrate that Eph-related receptors and ligands are present both in the retina and in the hypothalamus upon which the chiasm forms and that “A” subclass interactions contribute to the inhibition of retinal neurite growth by hypothalamic cells in vitro. Addition of EphA5-Fc to our cultures could, therefore, exert its effects at the hypothalamic or retinal levels.

In vivo, “A” subclass ephrins occupy a domain just ventral to the optic chiasm, where they may restrict retinal axons from growing into inappropriate regions of the hypothalamus. Consistent with such a role, ephrin-A5, which is known to be expressed in the hypothalamus (Fig. 5b and see Donoghue et al., 1996; Zhang et al., 1996), is implicated in inhibiting retinal axon growth into the inferior colliculus in both zebrafish (Brennan et al., 1997) and mice (Frisen et al., 1998) and to exclude limbic thalamic afferents from innervating sensorimotor cortex (Gao et al., 1998). Ligands are also present in a high nasal to low temporal gradient in the retina, and recent experiments in chick demonstrate that altering the retinal expression of “A” subclass ephrins modulates retinal ganglion cell responses to ephrins expressed as a growth substrate or in the tectum (Hornberger et al., 1999). In these experiments, retinal axons were more responsive to the repellent action of ephrin-A5 when “A” subclass ephrins were removed from the retinal axons. Addition of EphA5-Fc in our cultures would likewise be expected to lower the amount of ligand available and increase the pool of free retinal receptors and sensitivity to external cues. However, we observed that the inhibition of retinal axons in our cultures was lessened, not enhanced.

Thus the addition of exogenous EphA5-Fc clearly bound to ligand on the chiasmatic reaggregates, although we cannot rule out additional effects of the EphA5-Fc treatment on the retinal axons themselves.

In agreement with the present findings, analyses of axonal defects in EphA8 mutant mice suggest how ephrin-rich territories may exclude axons, thereby forcing their growth across the midline (Frisen and Barbacid, 1997; Park et al., 1997). In normal mice, EphA8-expressing neurons in the superior colliculus send axons toward the inferior colliculus that expresses high levels of ephrin-A2 and ephrin-A5. At the border between the superior and inferior colliculi, EphA8-expressing axons turn and cross the midline. In mutant animals, neurons that normally express EphA8 receptors cannot respond to ephrins present in the inferior colliculus and instead project their axons ipsilaterally toward the spinal cord.

Targeted disruption of EphB2 also leads to defects in commissure formation (Henkemeyer et al., 1996). In mutant mice, axons forming the posterior part of the anterior commissure do not cross the midline, but rather penetrate into the ventral forebrain. Interestingly, the misdirected axons express ephrin-B ligands, suggesting that ephrin-B ligands on axons in the anterior commissure transduce an inhibitory signal in response to receptors along their pathway. The colocalization of Eph receptors with the region of the radial glial palisade in the hypothalamus suggests that similar interactions may act to guide ephrin-expressing retinal axons.

Addition of soluble EphA5-Fc did not reduce the inhibition of retinal neurites by chiasmatic reaggregates to control levels, suggesting that other inhibitory cues are still present in the cocultures. One candidate is CD44, a cell surface molecule present in the hematopoietic system and on a population of early differentiating hypothalamic neurons (reviewed in Mason and Sretavan, 1997). CD44 appears to inhibit retinal axon growth in vitro (Sretavan et al., 1994). In addition, the hypothalamus secretes an inhibitory diffusible cue (Wang et al., 1996; Tuttle et al., 1998) that we propose may function as a general guidance mechanism in intermediate targets to prime growth cones to perceive other, more specific cues (Wang et al., 1996). This diffusible cue is unlikely to involve signaling via Eph-related receptors since receptors are not activated by ephrins presented in soluble form (Davis et al., 1994).

**The Eph Family and the Encoding of Positional Labels**

In addition to roles in patterning and axon guidance, Eph-related receptors are implicated in encoding positional information, including the proper mapping of Eph-expressing retinal axons in their targets (reviewed in Flanagan and Vanderhaegen, 1998; O’Leary and Wilkinson, 1999). Prior to reaching their targets, however, retinal axons undergo several spatial and temporal rearrangements in the region of the optic chiasm (reviewed in Guillery et al.,

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Thus, proper position-dependent sorting of retinal axons at the chiasm is essential for the formation of topographically precise projections.

In the mammalian optic chiasm, retinal axons sort out in a position-dependent manner to project to targets on both the ipsilateral and the contralateral sides of the brain (Chan and Chung, 1999). Previously we hypothesized that the decision to cross or not cross the midline may be mediated by a threshold response of uncrossed axons from ventrotemporal retina to inhibitory cues associated with the chiasmatic midline (Mason et al., 1996). Eph family receptors and their ligands from each of the two specificity subclasses distribute in opposing gradients in the developing mouse retina (Marcus et al., 1996a). The orientation of these gradients is such that the highest concentration of receptors from both the “A” and “B” subclasses occupies the ventrotemporal portion of the retina (see also Connor et al., 1998), raising the question of whether signaling through these receptors might contribute to the sorting of retinal axons at the optic chiasm. In our experiments, crossed and uncrossed axons from DT and VT retina were equally affected by addition of EphA5-Fc (Table 2), suggesting that Eph “A” family interactions alone are insufficient to direct the divergence of ipsilaterally and contralaterally projecting retinal axons in the chiasm. In contrast, ectopic expression of ephrin “A” ligands leads to an increased ipsilateral projection in chick (Dutting et al., 1999). The reason for this increase is unclear. As shown previously, retinal axons avoid ectopically expressed ephrins in the tectum (Nakagawa et al., 1996). Retinal axons may likewise avoid ectopic patches of ephrins in the chiasm, resulting in the growth of retinal axons toward the ipsilateral optic tract. Alternatively, the modulation of retinal receptors by ectopically expressed ephrins in the retina may lead to errors in the position-dependent responses of retinal axons to chiasmatic cues (see below). Ephrin-B2 colocalizes with the radial glial palisade within which the chiasm forms, suggesting a possible role for the Eph “B” subfamily in retinal axon sorting at the chiasm (this study and Nakagawa et al., 2000). Further study will be necessary to test this hypothesis.

Rather than ventrotemporal, or uncrossed, axons displaying selective inhibition by the chiasmatic cells, temporal and nasal axons were differentially inhibited by chiasmatic reaggregates in culture. This differential response was partially blocked by the addition of EphA5-Fc, consistent with a role for “A” family interactions in these position-dependent responses. Recent analyses using double fluorescent dye labeling indicate retinal axons sort out in a position-dependent manner as they approach, confront the midline, and enter the optic tracts en route to their bilateral targets (Chan and Chung, 1999). These findings suggest that local cues in the hypothalamus contribute to retinal axon reorganization. Here we demonstrate that Eph family interactions contribute to the differential responses of temporal and nasal neurites to chiasmatic reaggregates, thereby implicating these interactions in the position-dependent sorting of retinal axons along their path.

SUMMARY

Recently isolated mutations in zebrafish indicate that axon patterning in the visual system is regulated by genes that act in different parts of the optic pathway (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). Eph-related receptors and ephrins are distributed throughout the retinofugal pathway, making them prime candidates for regulating the topographic organization of the visual system. Eph family members are also implicated in the mapping of retinal axons on the tectum (reviewed in Drescher et al., 1997; Flanagan and Vanderhaeghen, 1998) and the lateral geniculate nucleus (Feldheim et al., 1996), in intraretinal (Marcus et al., 1996a; Braisted et al., 1997; Holash et al., 1997; Setton et al., 1997; Hornberger et al., 1999) and intratectal (Braisted et al., 1997; Connor et al., 1998) organization, and in the development of thalamocortical projections (Gao et al., 1998). Here we suggest that Eph family interactions help define a developmental domain that directs retinal axon growth during chiasm formation by restricting axons from entering inappropriate regions of the brain and/or contributing to position-related rerouting of optic fibers. These findings suggest that specific combinations of Eph-related receptors and ephrins may act at multiple steps along a given pathway.

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