Domains of Regulatory Gene Expression and the Developing Optic Chiasm: Correspondence With Retinal Axon Paths and Candidate Signaling Cells

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ABSTRACT

In mammals, some axons from each retina cross at the optic chiasm, whereas others do not. Although several loci have been identified within the chiasmatic region that appear to provide guidance cues to the retinal axons, the underlying molecular mechanisms that regulate this process are poorly understood. Here we investigate whether the earliest retinal axon trajectories and a cellular population (CD44 and stage-specific embryonic antigen 1 [SSEA] neurons), previously implicated in directing axon growth in the developing chiasm (reviewed in Mason and Sretavan [1997] Curr. Op. Neurobiol. 7:647–653), correlate with the expression patterns of several regulatory genes (BF-1, BF-2, Dlx-2, Nkx-2.1, Nkx-2.2, and Shh). These studies demonstrate that gene expression patterns in the chiasmatic region reflect the longitudinal subdivisions of the forebrain in that axon tracts in this region generally are aligned parallel to these subdivisions. Moreover, zones defined by overlapping domains of regulatory gene expression coincide with sites implicated in providing guidance information for retinal axon growth in the developing optic chiasm. Together, these data support the hypothesis that molecularly distinct, longitudinally aligned domains in the forebrain regulate the pattern of retinal axon projections in the developing hypothalamus. J. Comp. Neurol. 1999:346–358, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: axon guidance; midline; forebrain; BF-1; BF-2; Dlx-2; Nkx-2.1; Nkx-2.2; Shh

In mammals, retinal axons from a given eye project to targets on both sides of the brain. The site of this divergence is the optic chiasm, and the successful sorting of retinal axons at this site is essential for the formation of binocular vision. The adult pattern of retinal projection arises during development (Guillery et al., 1995). Static and real time studies of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled retinal axons during both the initial (E12.5–E13.5, where E is embryonic day) and major (E15–E17) periods of retinal axon growth in the mouse have identified several loci in which guidance cues may direct retinal axon trajectories (Colello and Guillery, 1990; Godement et al., 1990; Sretavan, 1990; Sretavan and Reichardt, 1993; Godement et al., 1994;...
and Mason, 1995), we have chosen to follow the terminology of the chiasm has been described as the ventral diencephalon (e.g., Marcus respectively. Although in the past the region containing the optic section that are parallel or perpendicular to the longitudinal axis, of this paper, we define "horizontal" and "coronal" to indicate planes of this region of the alar plate at the rostral end of the brain. For the purposes of this paper, we define "horizontal" and "coronal" to indicate planes of section that are parallel or perpendicular to the longitudinal axis, respectively. Although in the past the region containing the optic chiasm has been described as the ventral diencephalon (e.g., Marcus and Mason, 1995), we have chosen to follow the terminology of the Prosomeric model because it offers a useful framework for integrating the topology of the developing optic pathway within the rest of the forebrain.

According to the Prosomeric model, the primary longitudinal subdivisions of the neuroaxis (roof, alar, basal, and floor plates), define the dorsoventral axis and extend to the front of the forebrain. The chiasmatic plate (indicated by the asterisk in A–C) forms in a midline region of the alar plate at the rostral end of the brain. For the purposes of this paper, we define "horizontal" and "coronal" to indicate planes of section that are parallel or perpendicular to the longitudinal axis, respectively. Although in the past the region containing the optic chiasm has been described as the ventral diencephalon (e.g., Marcus and Mason, 1995), we have chosen to follow the terminology of the Prosomeric model because it offers a useful framework for integrating the topology of the developing optic pathway within the rest of the forebrain.

In addition, the developing hypothalamus secretes a diffusible factor that suppresses the growth of all retinal axons (Wang et al., 1996). With the anatomical data, these results suggest that multiple cues direct retinal axon divergence (ipsi-contra pathfinding) within the developing hypothalamus.

Much less is known about the events that specify the position of the optic chiasm within the developing hypothalamus. According to the Prosomeric model of forebrain organization, the optic chiasm forms on the hypothalamus (Fig. 1), where longitudinally aligned domains of gene expression cross the anterior midline of the neuroaxis (Shimamura et al., 1995). Axon pathways are frequently located along boundaries or interfaces between domains of regulatory gene expression (Krauss et al., 1991; Figdor and Stern, 1993; Macdonald et al., 1994; Che´dotal et al., 1995; Shimamura et al., 1995; Gavalas et al., 1997; Mastick et al., 1997) leading to the suggestion that boundaries of gene expression influence axonal growth by regulating the expression of guidance molecules or patterning the local environment (reviewed in Wilson et al., 1993, 1997). Furthermore, loss of function studies have implicated several regulatory genes in patterning, neuron specification, and axon guidance in the forebrain (reviewed in Rubenstein and Shimamura, 1997).

Here we expand our previous studies on retinal axon growth and patterning in the developing mouse optic chiasm by examining patterns of regulatory gene expression in relation to both nascent retinal pathways and CD44/SSEA neurons in the developing hypothalamus. We find that CD44/SSEA neurons, whose axons contribute to the tract of the postoptic commissure (TPOC), are located within the ventral part of a longitudinal domain that

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**Fig. 1.** Schematic diagrams depicting the morphological relationships of the optic stalks and chiasmatoid plate with respect to major landmarks in the mid-gestation (E12.5) mouse brain. A: Dashed lines indicate the approximate boundaries between the major transverse subdivisions of the central nervous system, and the thick black line marks the location of the longitudinal boundary that separates the alar and basal plates as hypothesized by the Prosomeric model. According to the Prosomeric model, the primary longitudinal subdivisions of the neuroaxis (roof, alar, basal, and floor plates), define the dorsoventral axis and extend to the front of the forebrain. The chiasmatic plate (indicated by the asterisk in A–C) forms in a midline region of the alar plate at the rostral end of the brain. For the purposes of this paper, we define "horizontal" and "coronal" to indicate planes of section that are parallel or perpendicular to the longitudinal axis, respectively. Although in the past the region containing the optic chiasm has been described as the ventral diencephalon (e.g., Marcus and Mason, 1995), we have chosen to follow the terminology of the Prosomeric model because it offers a useful framework for integrating the topology of the developing optic pathway within the rest of the forebrain. B: Frontal view indicating the topological terminology and planes of section presented in this paper. Previous descriptions of this view as "horizontal" (Sretavan et al., 1994; Marcus and Mason, 1995; Marcus et al., 1995), while preserving the relationship of this region to head structures such as the eyes, do not take into account the curvature of the longitudinal axis created by the cephalic flexure. The location of the optic stalks and the CD44/SSEA neurons are indicated, and the dashed line depicts the location of the third ventricle as viewed through whole mount preparations (see Marcus and Mason, 1995). C: Mid-sagittal view. Relationship of the three different neuroepithelial layers (ventricular zone [VZ], subventricular zone [SVZ], mantle layer) is shown (left). Same orientations are maintained in Figs. 2–6. tel, telencephalon; dl, diencephalon; mes, mesencephalon; rhomb, rhombencephalon; poa, preoptic area; RP, Rathke's pouch; abn, antero-basal nucleus; MGE, medial ganglionic eminence; hyp, hypothalamus.
expresses Nkx-2.2 and partially overlaps with a domain of Shh expression. In contrast, lateral to the midline, retinal axons course along the dorsal part of the domain of Nkx-2.2 expression, where it appears to overlap with a domain of BF-2 and Dlx-2 expression. Retinal axons cross the neuraxis within a midline zone of overlapping Nkx-2.2 and BF-1 expression. Our results, in agreement with previous studies, demonstrate that axon tracts are generally aligned along the orthogonal subdivisions of the developing forebrain. Furthermore, the association of these tracts with regions of overlapping gene expression suggest that "overlap zones" between adjacent domains of gene expression may be important for determining the position of axon pathways. Thus, molecularly distinct, longitudinally aligned domains in the forebrain appear to be important for regulating retinal axon projections in the developing hypothalamus.

**MATERIALS AND METHODS**

Experiments were performed on C57Bl/6j mice obtained from timed-pregnancy breeding colonies. Time of conception was considered midnight before the day on which a plug was found, and noon the following day was considered 0.5 (E0.5). Pregnant mothers containing E11–E13 embryos were anesthetized with a mixture of ketamine and xylazine, and the embryos were removed one at a time by cesarean section. Embryos were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, and stored in phosphate buffer containing sodium azide.

**Dil labeling of retinal axons in fixed tissue**

Dil (Molecular Probes, Eugene, OR) labeling of retinal axons was performed as described previously (Marcus and Mason, 1995) except that solutions used for tissue preparation for in situ hybridizations contained 5 mg/ml of heparin (H-3393; Sigma Chemical Co., St. Louis, MO) to prevent RNA degradation. Whole-mount preparations that included optic stalks, chiasmatric plate, and associated hypothalamic structures were isolated (Fig. 1, Marcus and Mason, 1995), and Dil-labeled axons were photo-oxidized in the presence of diaminobenzidine to produce a permanent brown reaction product. After photoconversion, labeled axons were drawn with the aid of a camera lucida attachment. Tissue used for in situ hybridization was subsequently dehydrated through an ascending series of methanol.

**Immunocytochemistry**

Two antibodies that label a population of early differentiating neurons in the developing hypothalamus were used (Mason and Sretavan, 1997): monoclonal antibody (Mab) 480–1.1 (mouse immunoglobulin M [IgM]), from the Developmental Studies Hybridoma Bank (Solter and Knowles, 1978), which recognizes SSEA-1; and Mab KM201 (gift from E. Pure, Wistar Institute), which recognizes the cell surface molecule CD44 (Miyake et al., 1990). Immunostaining was performed on whole mounts both with and without Dil-photoconverted axons as described previously (Sretavan et al., 1994; Marcus and Mason, 1995). Labeling was revealed by incubating the preparations in a solution containing 0.5 mg/ml diaminobenzidine with or without 0.08% NiCl₂. Addition of NiCl₂ resulted in formation of a bluish black reaction product.

**In situ hybridization**

In situ hybridization was performed according to previously published methods (Shimamura et al., 1994). Two-color staining was obtained by using purple alkaline phosphatase and INT/BCIP (Boehringer-Mannheim, Indianapolis, IN) as the chromogenic substrates for the alkaline phosphatase.

**LacZ detection**

The distribution of BF-1 and BF-2 transcripts were analyzed in heterozygous mice containing one copy of a β-gal reporter gene, which replaces either the BF-1 or BF-2 coding sequence (Xuan et al., 1995; Hatini et al., 1996). Tissue used for LacZ detection was first washed in phosphate buffer containing 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% IGEPAL-CA-630 (Sigma) and then incubated overnight in 1 mg/ml X-gal (Molecular Probes, Eugene OR) diluted in 30 mM K₃Fe(CN)₆, 30 mM K₄Fe(CN)₆, and 2 mM MgCl₂.

**Tissue sectioning and analysis**

Labeled whole mounts were mounted between two cover-slips by using two or three smaller cover-slips as spacers, then viewed and photographed on a Zeiss Axiphot microscope. Preparations were subsequently embedded in agar and cut in a frontal, horizontal or sagittal plane at 30–50 μm on a vibratome. Sections were mounted on slides, coverslipped with Gel Mount (Biomedica Corp., Foster City, CA), photographed, and drawn with the aid of a camera lucida attachment. We analyzed more than 100 preparations in which various combinations of regulatory genes, Dil-labeled retinal axons, and CD44/SSEA-1 cells were visualized. Figures were prepared by using Adobe Photoshop. In some cases, the brightness or contrast of individual figures were globally altered to increase clarity.

**RESULTS**

We examined mouse embryos between E11 and E13 that had been treated to reveal retinal axons, SSEA-1-positive cells, and the expression of Dlx-2, Nkx-2.2, Nkx-2.1, BF-1, BF-2, and Shh regulatory genes. Preparations were viewed as whole mounts and then subsequently sectioned into frontal, sagittal, and horizontal planes to obtain a three-dimensional view of the relationship among retinal axons, hypothalamic cells, and regulatory genes in the region of the developing optic chiasm. The diagrams in Figure 1 show the morphological relationships of the optic stalks and chiasmatric plate (that region of the hypothalamus where the chiasm will form) with respect to major landmarks in the mid-gestation mouse forebrain. In this paper, we use the topological terminology defined in Shimamura et al. (1995) to describe the relationships of gene expression patterns, axon tracts, and morphological subdivisions. We describe how these terms relate to the nomenclature that is frequently used to describe the developing optic pathway in Figure 1.

Figure 1A is a schematic diagram of a lateral view of an embryonic mouse brain showing the approximate locations of the major brain subdivisions at E12.5 (see also Puelles and Rubenstein, 1993). The trajectory of the longitudinal axis, as predicted by the Prosomeric model, is also depicted. According to this model, the anterior-most
extensions of the primary dorsoventral subdivisions of the neuraxis (roof, alar, basal, and floor plates) extend into the forebrain. The eye fields and associated structures are contained within the alar plate, just dorsal to the alar-basal boundary. During early embryonic development, the optic vesicles evaginate from the lateral walls of the hypothalamus near the rostral end of the neural tube, where they are connected to the brain via the optic stalks (Puelles et al., 1987; Couly and Le Douarin, 1988; Eagle-son and Harris, 1990). The chiasmatic plate is the region of the hypothalamus between the optic stalks, and frontal and midsagittal views through this region are shown in Figure 1 (B and C, respectively). It is important to note that in mice at the ages studied in this paper, the alar and basal plates in the hypothalamus are oriented approximately 180° to those in the hindbrain and spinal cord. This difference is due to the curvature of the longitudinal axis caused by the bending of the brain at the cephalic flexure.

**Domains of gene expression in the rostral hypothalamus before retinal axon ingrowth**

Retinal axons growing along the optic stalks enter the region of the chiasmatic plate around E12.5 in mice (reviewed in Mason and Sretavan, 1997). As a first step toward identifying the building blocks (i.e., domains) that pattern the region of the hypothalamus on which the optic chiasm will form, we began our analysis at E11–E11.5, one day before the initial retinal axon ingrowth. We chose to study the expression of BF-1, BF-2, Dlx-2, Nkx-2.1, Nkx-2.2, and Shh, because these genes were previously shown to be expressed in this region (Price et al., 1991; Price et al., 1992; Bulfone et al., 1993; Hatini et al., 1994; Shimamura et al., 1995). In agreement with our previous studies (Bulfone et al., 1993; Shimamura et al., 1995), these genes define molecularly distinct domains that roughly correspond to the alar, basal, and intermediate longitudinal zones (see below and Fig. 7).

BF-1 is expressed in a large continuous domain within the alar plate that includes most of the telencephalon, preoptic area, and nasal part of the optic stalk (Fig. 2J-L) and retina (Hatini et al., 1994; Shimamura et al., 1995). BF-2 is also expressed in the alar plate, where its expression is complementary to BF-1. BF-2 expression is ventral to BF-1; it includes the temporal optic stalk and retina, and extends ventrally towards the postoptic area. Unlike BF-1, BF-2 does not extend across the rostral midline.

Dlx-2 is expressed in two major domains in the forebrain (Bulfone et al., 1993; Porteus et al., 1994). The telencephalic domain (domain 2) includes the septum, lateral, and medial ganglionic eminences, and the preoptic area. A more ventral longitudinal domain (domain 1) extends from the zona limitans to the postoptic area to either side of the midline. This ventral domain overlaps with the BF-2 domain described above and largely corresponds to the trajectory of the retinal axons that pioneer the optic chiasm. In addition, although Dlx-2 is primarily expressed in the alar plate, there is a small domain of expression in the basal plate in the tuberal hypothalamus, which was described previously (Puelles and Rubenstein, 1993) (Fig. 3A).

Nkx-2.1 and Shh also are expressed in two domains. Nkx-2.1 transcripts are found in a telencephalic domain, which includes the septum, the medial ganglionic eminence, and the preoptic area, whereas the second domain includes only hypothalamic regions located within the basal plate (mammillary, tuberal, and retrochiasmatic domains) (Shimamura et al., 1995). Shh expression in the telencephalon and hypothalamus is nested within the Nkx-2.1 domains (Ericson et al., 1995; Shimamura et al., 1995). In the telencephalon, Shh is expressed in the preoptic area and the medial ganglionic eminence. In the hypothalamus, Shh is expressed in the mammillary, tuberal, and retrochiasmatic areas, where it defines the anterior-most extension of the basal plate (Shimamura et al., 1995).

Nkx-2.2 is expressed in a longitudinal stripe along the entire neuraxis (Price et al., 1992; Saha et al., 1993; Barth and Wilson, 1995; Shimamura et al., 1995). Within the forebrain, Nkx-2.2 occupies a zone located between the ventral Nkx-2.1 and Shh and the dorsal BF-1 and Dlx-2 hypothalamic domains. In the forebrain and midbrain, Nkx-2.2 expression has been interpreted to correspond to the alar-basal boundary (Shimamura et al., 1995). One of the earliest axon tracts in the forebrain, the diencephalic tract of the posterior commissure (DPC), is largely co-linear with the stripe of Nkx-2.2 expression (Barth and Wilson, 1995; Shimamura et al., 1995).

As described above, all of these genes are expressed at the rostral end of the brain—although at differing dorsoventral positions relative to the longitudinal axis defined by the Prosomeric model—in the region of the chiasmatic plate and the optic stalks. Examination of frontal views of embryos stained by the use of two-color in situ RNA hybridization clearly shows the relationship of these genes to the optic stalk and chiasmatic plate. E11.5 embryos hybridized with Dlx-2 and Shh reveal their separate telencephalic and hypothalamic domains (Fig. 2A-C). In the hypothalamus, Dlx-2 and Shh are separated by a thin wedge of cells (arrow in Fig. 2B), demonstrating their distinct, non-overlapping hypothalamic domains. A near-midsagittal section shows that in this region, Shh expression is confined to the ventricular zone (VZ) (Fig. 2C). Dlx-2 is expressed in the subventricular zone (SVZ) within the hypothalamus and in both the VZ and SVZ in the lamina terminalis and preoptic area.

A frontal view of an E11–E11.5 brain stained for Shh and Nkx-2.2 shows Nkx-2.2 expression along the dorsal aspect of the Shh hypothalamic domain (Fig. 2D). Nkx-2.2 expression at the front of the brain forms a shallow, inverted “V” shape ventral to the optic stalks (Fig. 2H). This location corresponds approximately to the unlabeled region located between the Dlx-2 and Shh transcripts described above. A near-midsagittal section shows that whereas both Shh and Nkx-2.2 are expressed in the VZ, Nkx-2.2 is also expressed in the SVZ and postmitotic (i.e., mantle) layers of the neuroepithelium (Fig. 2F). In frontal views, Nkx-2.2 appeared to be expressed adjacent to the hypothalamic Shh domain, similar to observations at earlier ages (Shimamura et al., 1995). In sagittal sections, however, there appeared to be overlap in the Nkx-2.2 and Shh domains in the VZ, reminiscent of that described for Shh and nk2.2 (the zebrafish homologue of Nkx-2.2) in zebrafish (Barth and Wilson, 1995).

A frontal view of an E11–E11.5 brain stained for Nkx-2.1 and Nkx-2.2 demonstrates that Nkx-2.1 occupies separate telencephalic and hypothalamic domains. Nkx-2.2 is expressed along the dorsal aspect of the Nkx-2.1 hypothalamic expression domain (Fig. 2G-H). A near-midsagittal section reveals that both genes are expressed in the VZ,
SVZ, and mantle layers of the neuroepithelium. Moreover, Nkx-2.1 and Nkx-2.2 appear to occupy adjacent, non-overlapping domains (Fig. 2I).

A frontal view of an E11–E11.5 brain stained for BF-1 shows its expression in the telencephalon and nasal half of the optic stalk (Fig. 2J,K). In sagittal sections, BF-1 is
found in both the VZ and SVZ (Fig. 2L). BF-2 is expressed in the hypothalamus and temporal half of the optic stalk, adjacent to the domain of BF-1 expression (Hatini et al., 1994; data not shown).

Thus, several domains of regulatory gene expression are present in the region of the chiasmatic plate and future optic tracts. Next, we determined the relationship of these regulatory gene expression patterns to the pathways taken by pioneering retinal axons.

**Retinal axon trajectories and domains of regulatory gene expression**

Previous analyses of retinal axon growth in the hypothalamus identified several sites where retinal axons may be responding to guidance information (Sretavan et al., 1994; Marcus and Mason, 1995). In particular, retinal axons display distinct behaviors at four sites (Fig. 7a; Fig. 5 in Marcus and Mason, 1995). First, at the junction of the optic stalk with the hypothalamus, pioneering retinal axons enter at E12.5–E12.75 and turn ventrally along the pial surface of the brain (Fig. 7a, 1). Not highlighted by us previously, at this location we frequently observed a small number of axons course dorsally and medially before taking a more ventral trajectory to join the major cohort of pioneering retinal axons (Fig. 7a, asterisk). Second, after initially growing ventrally in lateral portions of the hypothalamus, contralaterally projecting axons turn toward the midline (Fig. 7a, 2). Third, in contrast to their initial ventral-directed growth, contralaterally projecting retinal axons grow laterally or slightly dorsally as they approach the midline (Fig. 7a, 3). Fourth, early ipsilaterally projecting axons grow ventrally in lateral portions of the hypothalamus where, along with contralaterally projecting axons from the other eye, they form the ipsilateral optic tracts (Fig. 7a, 4). This is in contrast to later ipsilaterally projecting axons, which first grow toward the midline and then make sharp 90° turns to join the ipsilateral optic tract (Godement et al., 1990; Sretavan, 1990). Here, we extend these results by examining the earliest retinal axon trajectories with respect to patterns of gene expression between E12.5 and E13.5, paying particular attention to those regions previously implicated in directing retinal axon growth in the chiasmatic plate and adjacent hypothalamic territories.

Examination of preparations containing Dil-labeled, photoconverted axons and double-labeled to indicate different domains of regulatory gene expression reveals that Dil-x-2, BF-2, and Nkx-2.2 are expressed in the chiasmatic plate (Figs. 3, 4, 7). Retinal axons forming the optic chiasm do not grow freely throughout any of these expression domains, but rather occupy a limited region that includes the ventral part of the Dil-x-2 and BF-2 expression domains and the dorsal part of the Nkx-2.2 expression domain. Interestingly, there is a correspondence between the lateral, slightly dorsal, curved trajectory taken by contralaterally projecting axons and the contours of the expression domains of these genes (arrowheads, Fig. 3A,B). Sagittal sections reveal retinal axons growing on the external (i.e., pial) surface of the brain, in close proximity to Dil-x-2 and BF-2-expressing cells in the SVZ and mantle layers (Fig. 3C–E). Although retinal axons are located predominantly within the ventral part of the Dil-x-2 and BF-2-positive domain, it is important to note that some axons extend beyond its ventral border (Fig. 3E). Furthermore, near the midline, contralaterally projecting axons cross through a region containing little or no BF-2 and Dil-x-2 transcripts.

**Fig. 3.** Retinal axons grow in a domain of Dil-x-2 and BF-2 expression. A,C,D,F: Coronal (A), sagittal (C,D) and horizontal (F) sections through the hypothalamus of E13 preparations containing Dil-photoconverted retinal axons (brown) and hybridized with a Dil-x-2 probe (purple). Dashed lines in A indicate the planes of section shown in C, D, and F. Arrow indicates an unlabeled, inverted V-shaped domain located between the alar and basal Dil-x-2-positive hypothalamic domains. B,E,G,H: Whole mount (B), and sagittal (E,H) and coronal (G) sections through the hypothalamus of preparations containing Dil-photoconverted retinal axons and treated with X-gal to visualize BF-2-positive cells (bluish green). Dashed lines in B indicate the planes of section shown in E, G, and H. In H, the preparation was also immunolabeled to reveal stage-specific embryonic antigen (SEEA)-1-positive cells (brownish black). Arrows in F and G indicate the midline, and arrowhead in F points to a growth cone growing in the optic tract. Small brown cells are non-specifically labeled blood cells. Retinal axons grow in the ventral part of the alar Dil-x-2 and BF-2 domain (arrowheads in A and B), except near the midline, where contralaterally projecting axons cross through a region with little or no BF-2 and Dil-x-2 transcripts. Scale bars = 90 µm (A,B), 50 µm (C–H).
Fig. 4. Nkx-2.2 and SSEA-1 are expressed in an inverted V-shaped pattern in the rostral forebrain. Frontal (A,B), sagittal (C,D,E), and horizontal (F,G) views of E13 preparations containing DiI-photoconverted retinal axons (brown) and hybridized with a Nkx-2.2 probe (purple) (C,D,F) or immunolabeled for stage-specific embryonic antigen (SSEA)-1 (brown-black) (B,E). The dashed lines in A and B indicate the planes of section in C, D, and E. Inset in F is a higher-power view of growth cones positioned within the Nkx-2.2-positive domain. G: Coronal section double-labeled for Nkx-2.2 (purple) and SSEA-1 (brown). Small brown cells are non-specifically labeled blood cells. In A, retinal axons are just entering the optic stalks (asterisk). In parasagittal sections (C), retinal axons are found within the dorsal part of the Nkx-2.2 expression domain. Near the midline (D), retinal axons occupy a more ventral position within the Nkx-2.2 expression domain. SSEA-1-positive neurons are located within the ventral part of the Nkx-2.2 expression domain, just ventral to retinal axons in lateral portions of the developing optic chiasm. Scale bars = 150 µm (A,B), 100 µm (C–E), 30 µm (F,G).

Fig. 5. BF-1 and stage-specific embryonic antigen (SSEA)-1 overlap in the chiasmatic midline zone. Frontal (A,B,F–H), sagittal (C,D), and horizontal (E) views of preparations treated with X-gal to visualize BF-1–positive cells (blue) and cells containing DiI-photoconverted retinal axons (brown) (A–E,H) or SSEA-1-immunolabeled cells (F,G). In A, the retinal axons are just beginning to cross the chiasmatic midline (arrow), whereas in B, retinal axons have already reached the contralateral optic tract. Dashed lines in B indicate the planes of section in C, D, and E. G,H: High-power views of the midline from frontal sections through the preparations in F and A, respectively. Retinal axons cross the midline in a zone of overlapping BF-1 and SSEA-1 expression. Small brown cells are non-specifically labeled blood cells. See Results for details. Scale bars = 90 µm (A,B,F), 50 µm (C–E), 15 µm (G,H).
Adjacent and just ventral to the domain occupied by Nkx-2.2, where it occupies a longitudinal domain that is an inverted V-shaped stripe located ventral to the optic stalks and BF-2-expressing cells (arrowhead, Fig. 3F). Nkx-2.2 expression of the domain of hypothalamic expression domains (see Discussion).

Between E12.5 and E13.5, BF-1 and BF-2 continue to define non-overlapping adjacent domains within the optic stalk and chiasmatic regions. BF-1 is dorsal to BF-2, and retinal axons pioneering the optic chiasm generally extend in BF-1-negative areas (Fig. 5). However, retinal axons encounter areas of BF-1 expression in two regions. The first is near the junction of the optic stalks with the brain, where a small number of retinal axons take a slightly dorsomedial course before turning ventrally to join the major cohort of pioneering retinal axons (Fig. 5A; Fig. 7a, asterisk). The second is near the midline. In this region, retinal axons cross through a ventrally oriented V-shaped extension of BF-1-expressing cells that occupies the midline zone of low Dlx-2 and BF-2 expression (Fig. 5; Fig. 7a, 3).

Retinal axons remain dorsal to the domain of Shh expression in the hypothalamus (Fig. 6). This domain defines the anterior-most extension of the basal plate, suggesting that the optic pathway is an alar tract (see also Shimamura et al., 1995; Mastick and Easter, 1996).

Fig. 6. The optic chiasm forms dorsal to a basal Shh domain. Frontal (A) and sagittal (B) views of E13 preparations containing Dil-photoconverted retinal axons and hybridized with an Shh probe. A: Composite of five camera lucida drawings from frontal sections through a preparation labeled as a whole mount. Dashed line indicates the location of the third ventricle. Retinal axons, located along the pial surface of the brain, grow dorsal to a basal domain of Shh. Shh transcripts are confined to the ventricular zone. Scale bars = 100 µm (A), 40 µm (B).

Nkx-2.2 expression and CD44/SSEA neurons

Previously, we and others identified an early differentiating population of neurons located in a region ventral to the optic stalks and dorsal to the postoptic recess (reviewed in Mason and Sretavan, 1997). This neuronal population, which expresses antigens including SSEA-1, CD44, β-tubulin, MAP-2, and L1 is arrayed similarly to—and located in the region of—a small group of cells identified as the anterobasal nucleus in the rat (Altman and Bayer, 1986; Easter et al., 1993). CD44/SSEA neurons send axons laterally and posteriorly and thus contribute axons to the TPOC. Retinal axons pioneering the optic chiasm follow a course that closely mirrors the contours of these cells. The correspondence of pioneering retinal axon trajectories with the topological organization of the CD44/SSEA neurons suggested that these cells provide guidance information to growing retinal axons (Sretavan et al., 1994; Marcus and Mason, 1995). Interactions of retinal axons with these cells are complex, however. In culture, neurons isolated from the developing hypothalamic region can inhibit retinal axon growth (Wang et al., 1995). In contrast, in vivo ablation of these cells results in failure of optic axons to exit the optic stalks, suggesting that the CD44/SSEA neurons may also promote axon growth into the brain (Sretavan et al., 1995).

During the initial period of retinal axon growth into the hypothalamus, the CD44/SSEA neurons are arrayed in an inverted V-shaped pattern reminiscent of the pattern of Nkx-2.2 expression (cf. Fig. 4A, B). We further examined the similarity of these expression patterns in preparations double-labeled with Nkx-2.2 and an antibody recognizing SSEA-1. In whole mounts, the SSEA-1-positive cells and the domain of Nkx-2.2 expression appeared to partially overlap but be displaced from one another within the neuroepithelium (data not shown). This view was verified in sections. Nkx-2.2 transcripts were located in the VZ and SVZ, where they formed a canopy over the SSEA-1-positive neurons located in the SVZ and the differentiating cellular layers (Fig. 4G). Compared with the SSEA-1-negative neurons located in the SVZ, the SSEA-1-positive neurons were arrayed in a fashion consistent with the Nkx-2.2 expression pattern. This view was further supported by the observation that Nkx-2.2 expression was not detectable in the VZ. In addition, the CD44/SSEA neurons were expressed in a pattern consistent with the Nkx-2.2 expression pattern, suggesting that the two populations of neurons may play a role in the development of the optic chiasm.
positive neurons, the domain of Nkx-2.2 expression extended farther laterally and dorsally, such that the SSEA-1-positive neurons were positioned within the ventral part of the Nkx-2.2 expression domain. Tissue double-labeled for Nkx-2.2 expression and an antibody recognizing CD44 gave similar results (data not shown).

**Distinct domains of regulatory gene expression occupy the midline zone**

Throughout the nervous system of most species, the midline of the neuraxis is a specialized region involved in dorsal-ventral patterning, cell determination, and axon guidance (reviewed in Tessier-Lavigne and Goodman, 1996). In the developing visual system, the optic chiasm is an important rostral midline locus where retinal axons from a given eye diverge to project to targets on both the ipsilateral and contralateral sides of the brain. A combination of immunocytochemical, axon-tracing, and in vitro studies has clearly identified the midline region to be unique, both anatomically and functionally (reviewed in Guilley et al., 1995; Mason and Sretavan, 1997). This view is further strengthened by our analysis of domains of regulatory gene expression in the region of the chiasmatic plate. Lateral to the midline, retinal axons grow in a region of overlapping expression of BF-2, Dlx-2, and Nkx-2.2. This region is located ventral to the telencephalic domain of BF-1 expression (Fig. 5D) and just dorsal to the location of the CD44/SSEA neurons that occupy the ventral part of the domain of Nkx-2.2 expression (Fig. 3H). In contrast, BF-2 and Dlx-2 are diminished or absent from the midline. Instead, in this region, a ventrally directed V-shaped extension from the domain of BF-1 expression converges with the tip of the inverted V-shaped stripe of Nkx-2.2 expression. These genes occupy the midline zone normally traversed by contralaterally projecting retinal axons (Fig. 5). Furthermore, SSEA-1-positive cells overlap with BF-1-expressing cells in this region (Fig. 5F,G), indicating that BF-1 and Nkx-2.2 overlap at the midline. Together, these results demonstrate that retinal axons cross the neuraxis in a rostral midline zone that is defined, in part, by the overlapping expression of distinct domains of regulatory gene expression.

**DISCUSSION**

In this study we describe the spatial relationships of the earliest retinal axons and hypothalamic cells with respect to domains of regulatory gene expression in E11–E13 mice. The relative patterns of BF-1, BF-2, Dlx-2, Nkx-2.1, Nkx-2.2, and Shh expression during this developmental period are not markedly different from their earlier established distributions, despite extensive changes in brain morphology. Moreover, the expression patterns of several of these genes correlate with the locations of hypothesized sites of axon guidance in the developing chiasm (reviewed in Mason and Sretavan, 1997; Fig. 7). Here we discuss the present data with respect to current hypotheses on whether molecularly distinct, longitudinally aligned domains regulate axonal growth in the forebrain.

**Patterns of gene expression in the hypothalamus reflect the longitudinal organization of the anterior brain**

Regionally restricted patterns of regulatory gene expression have led to a resurgence of studies focusing on forebrain morphogenesis and have prompted reevaluation of several models of forebrain organization (reviewed in Puelles and Rubenstein, 1993; Shimamura et al., 1995). In the Prosomeric model, the forebrain is divided into a series of transverse and longitudinal domains that are defined, in part, by regionally restricted patterns of regulatory gene expression (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Rubenstein and Shimmura, 1997; Shimamura et al., 1997). Analysis of several regulatory genes that are expressed in restricted longitudinal domains demonstrates that they cross the rostral midline of the neuraxis in the region of the chiasmatic plate (Barth and Wilson, 1995; Shimamura et al., 1995). Based on these previous studies and our present work, we adopt the terminology presented in the legend to Figure 1 in our description of the developing chiasm. Although this terminology is at odds with nomenclature defined relative to head structures such as the eyes, the terminology presented here is useful when considering the anterior-posterior and dorsal-ventral mechanisms that function to pattern the developing hypothalamus.

**The visual pathway is an alar tract**

The TPOC is one of the earliest longitudinal tracts in the brain and is collinear with cells that express the Nkx-2.2 homeobox gene (Barth and Wilson, 1995; Shimamura et al., 1995). Here we demonstrate that CD44- and SSEA-expressing neurons, which we believe are the mouse equivalent of the rat anterobasal nucleus (abn; Altman and Bayer, 1986; see also Marcus and Mason, 1995), are located within the Nkx-2.2 expression domain in the hypothalamus. The abn neurons are among the first cells to contribute to the TPOC (Easter et al., 1993). Furthermore, axons of the TPOC and the mesencephalic tract of the trigeminal nerve combine to form the dorsal longitudinal pathway that appears to mark the boundary between the alar and basal plates (Mastick and Easter, 1996). We find that axons in the optic chiasm and tracts are dorsal to the CD44/SSEA neurons and the TPOC; this implies that the visual pathway should be considered an alar tract.

**Retinal axon trajectories and CD44/SSEA neurons occupy distinct longitudinal domains in the developing hypothalamus**

Several regulatory genes are expressed in the hypothalamic region, through which retinal axons grow. In addition to the genes investigated in the present study, Pax-2 is expressed in the developing hypothalamus (Nornes et al., 1990). In mice deficient for Pax-2, the normal brain subdivisions are disrupted, as demonstrated by changes in Shh expression (Torres et al., 1996). Concomitant with these changes is a failure of optic chiasm formation. In agreement with these results, we find several correlations between axon pathways in the hypothalamus with gene-expression domains that reflect the orthogonal subdivisions of the brain. For example, CD44/SSEA neurons, whose axons contribute to the TPOC, are located within the ventral part of a longitudinal domain that both expresses Nkx-2.2 and partially overlaps with the dorsal-most expression of a basal Shh domain (present study and Barth and Wilson, 1995). Lateral to the midline, retinal axons course along the dorsal part of the Nkx-2.2 domain, where it overlaps with a domain of BF-2 and Dlx-2 expression. Nearer the midline, retinal axons traverse a zone of overlapping Nkx-2.2 and BF-1 expression.
The correspondence between axon trajectories and regions of overlapping domains of gene expression is in contrast to previous suggestions that axon pathways are located along boundaries or interfaces between domains of regulatory gene expression in the nervous system (Wilson et al., 1993, 1997). This discrepancy probably results from our simultaneous analysis of axons and multiple regulatory genes that thereby yielded a more complete view of the relationship of regulatory gene boundaries and axon tracts. However, it is possible that there are differences between axon pathfinding of longitudinal and optic chiasm tracts in the forebrain and transverse tracts in the hindbrain, whose trajectories are correlated with rhombomeric boundaries (Guthrie and Lumsden, 1991).

**Domains of regulatory gene expression and axon guidance**

How regulatory genes may direct axonal growth is not known. One possibility is that they regulate the expression of molecules that pattern adjacent tissues. In support of this, Shh has been shown to regulate expression of nk2.2 in the zebrafish (Barth and Wilson, 1995) and to induce cells expressing ventral forebrain markers in chicks, mice, and rats (Ericson et al., 1995; Chiang et al., 1996; Dale et al., 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997). In addition, axial, the zebrafish homologue of mouse HNF-3β (Strähle et al., 1993), is implicated in the spatial regulation of nk2.2 in the zebrafish (Barth and Wilson, 1995). BF-1, BF-2, and HNF-3β are all members of the winged-helix family of transcription factors (Lai et al., 1993; Hatini et al., 1994), and mice deficient for BF-1 display severe patterning defects in the forebrain (Xuan et al., 1995; and Huh et al., unpublished observations). These results suggest that domains of regulatory gene expression may indirectly influence the location of axonal pathways by regulating the release of a morphogen or triggering a cascade of local inductive interactions that pattern the environment adjacent to growing axons (see Wilson et al., 1993).

Several studies provide evidence that transcription factors directly regulate the expression of molecules involved in axon guidance (Gould and White, 1992; J ones et al., 1992). With regard to the present study, two examples are noteworthy. Eph family receptor tyrosine kinases and their ligands, the ephrins, are a large family of molecules implicated in both directing axonal growth and patterning in the nervous system (reviewed in Selton and Nieto, 1997; Varela-Echavarria and Guthrie, 1997; Holder et al., 1998). A number of Eph receptors and their ligands are expressed in the developing forebrain. In the zebrafish and *Xenopus,*

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**Fig. 7.** Schematic diagrams depicting retinal axon paths in relation to patterns of regulatory gene expression and hypothalamic cells in the anterior forebrain. a: Paths taken by retinal axons pioneering the optic chiasm. Sites where retinal axons exhibit distinct behaviors are marked with 1–4 and an asterisk (for details, see Results and Marcus and Mason, 1995). b–h: Retinal axon paths, depicted in relation to the patterns of regulatory gene expression and CD44/SSEA neurons. Lateral to the midline, retinal axons grow along the dorsal part of the Nkx-2.2 expression domain that appears to overlap with the ventral part of a domain that expresses BF-2 and Dlx-2. Retinal axons traverse a midline zone of overlapping Nkx-2.2 and BF-1 expression. CD44/SSEA neurons are positioned within the ventral portion of the Nkx-2.2 expression domain where it overlaps with the basal Shh domain. Taken together, these results suggest that “overlap zones” between adjacent domains of regulatory gene expression may be important for determining the position of axon pathways in the developing hypothalamus. Furthermore, the coincidence of unique combinations of regulatory genes with sites where retinal axons exhibit distinct behaviors supports the hypothesis that molecularly distinct, longitudinally aligned domains in the forebrain, regulate the pattern of retinal axon projections in the hypothalamus, poa, preoptic area; RP, Rathke’s pouch.
expression of a truncated Eph-related receptor results in a loss of hypothalamic structures, indicating a role for this family in patterning of the developing forebrain (Xu et al., 1996). In the mouse, Ephrin-A5 is expressed in the hypothalamus (Donoghue et al., 1996; Zhang et al., 1996) in a region that contains the CD44/SSEA neurons. Furthermore, hypothalamic cells that occupy the basal domain are inhibitory to retinal axons (Marcus et al., unpublished), consistent with a role for Eph family members in specifying a region inhibitory to retinal axon growth (see also Brennan et al., 1997). These results are consistent with a role for Eph family members in specifying a region inhibitory to retinal axon growth (see also Brennan et al., 1997).

Although the molecular pathways that regulate Eph family expression in the hypothalamus are unknown, the transcription factors Engrailed and BF-1 have been implicated in regulating ephrin expression in the tectum and retina, respectively (Logan et al., 1996; and Huh et al., unpublished observations).

The FORSE-1 antibody was isolated in a screen designed to identify cell surface antigens that are upregulated in response to Hox gene activation (Tode et al., 1995). In the forebrain, FORSE-1-immunopositive cells correlate with the expression of Dlx-2 and BF-1. FORSE-1 recognizes LeX, an antigen implicated in cell adhesion (Allendoerfer et al., 1995; Götz et al., 1996, and references therein). Interestingly, LeX is also recognized by a number of MAb's, including the SSEA-1 antibody used here to label the CD44/SSEA hypothalamic neurons. Consistent with the presence of LeX on the CD44/SSEA neurons, FORSE-1 labeling is identical to that of SSEA-1 labeling in the mouse hypothalamus (data not shown). The LeX epitope is also present on phosphacan, a chondroitin sulfate proteoglycan that binds different neural cell adhesion molecules (Grumet et al., 1994; Milev et al., 1994), further implicating this antigen in adhesive interactions important for proliferation, cell migration or axon guidance (Allendoerfer et al., 1995).

Correlations among selective adhesion, segmentation, and patterns of regulatory gene expression have been observed in the hindbrain (reviewed in Lumsden and Krumlauf, 1996) and the forebrain (Figer and Stern, 1993; Götz et al., 1996; Stoykova et al., 1997). For example, several members of the cadherin family of calcium-dependent adhesion molecules are distributed in patterns that correlate with domains of regulatory gene expression (reviewed in Redies and Takeichi, 1996), and in the forebrain, region-specific cell adhesion appears to be mediated in part by Pax-6 regulation of R-cadherin expression (Stoykova et al., 1997). In vitro, growth cones turn and grow along the boundary between two different adhesive substrates (Gomez and Letourneau, 1994; Burden-Gulley et al., 1995). A similar mechanism has been proposed to direct retinal axon growth in vivo (Godemont et al., 1990). Domains of regulatory gene expression, therefore, may indirectly influence the location of axonal pathways by regulating the distribution of different adhesive substrates in the nervous system (Wilson et al., 1993, 1997).

Summary

In this study we examined patterns of expression of regulatory genes (BF-1, BF-2, Dlx-2, Nkx-2.1, Nkx-2.2, and Shh) in relation to retinal axon pathways and cells in the hypothalamus adjacent to the region of the developing optic chiasm. Our results demonstrate that retinal and CD44/SSEA axons in the developing hypothalamus are oriented along longitudinally organized domains of regulatory genes. In addition, we observed several correlations between overlapping patterns of gene expression with sites previously implicated in providing guidance information for growing retinal axons (Fig. 7). Together, these results support the hypothesis that different combinations of regulatory genes act to direct the final pattern of retinal axon growth and divergence in the mouse optic chiasm. Future studies will be needed to decipher how the relationships between processes such as patterning, cellular differentiation, and the production of cell surface and secreted molecules combine to direct axonal growth in the brain. In the zebrafish, several mutations have been isolated that affect retinal axon guidance at multiple steps along the developing visual pathway (Karlstrom et al., 1997). Moreover, analyses of these mutants are beginning to unravel the molecular pathways involved in retinal axon guidance (Macdonald et al., 1997). In addition, mice deficient for all of the genes described in this paper (Kimura et al., 1995; Xuan et al., 1995; Chiang et al., 1996; Hatini et al., 1996; Anderson et al., 1997; Sussel et al., 1998) as well as others implicated in visual pathway development (Walter and Gruss, 1991; Torres et al., 1996) already exist, paving the way for uncovering the regulatory networks that underlie these processes.

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LITERATURE CITED


