Retroviral Misexpression of *engrailed* Genes in the Chick Optic Tectum Perturbs the Topographic Targeting of Retinal Axons

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We have investigated the role of the homeodomain transcription factor genes *En-1* and *En-2*, homologs of the *Drosophila* segment polarity gene *engrailed*, in regulating the development of the retinotopic map in the chick optic tectum. The En proteins are distributed in a gradient along the rostral–caudal axis of the developing tectum, with highest amounts found caudally. Previous evidence suggests that *En-1* and *En-2* may regulate the polarity of the rostral–caudal axis of the tectum and the subsequent topographic mapping of retinal axons. We have tested this hypothesis by using a recombinant replication-competent retrovirus to overexpress the *En-1* or *En-2* genes in the developing tectum. Anterograde labeling with the axon tracer Dil was used to analyze the topographic mapping of retinal axons after the time that the retinotectal projection is normally topographically organized. Overexpression of either *En-1* or *En-2* perturbed the topographic targeting of retinal axons. In *En*-infected tecta, nasal retinal axons form an abnor-

mally diffuse projection with numerous aberrant axons, branches, and arbors found at topographically incorrect locations, colocalized with domains of viral infection. In contrast, temporal axons did not form a diffuse projection or discrete aberrant arbors; however, many temporal axons were stunted and ended aberrantly rostral to their appropriate TZ, or in other cases either did not enter the tectum or formed a dense termination at its extreme rostral edge. These findings indicate that *En-1* and *En-2* are involved in regulating the development of the retinotopic map in the tectum. Furthermore, they support the hypothesis that *En* genes regulate the polarity of the rostral–caudal axis of the tectum, most likely by controlling the expression of retinal axon guidance molecules.

Key words: axonal guidance; gene transfer; homeobox; neural maps; recombinant retrovirus; retinotectal; transcription factors; visual system development

The projection of retinal ganglion cells to the optic tectum in nonmammalian vertebrates, or to its homolog, the superior colliculus in mammals, has served as a model system for studies of topographic map development. The targeting of retinal axons to their topographically correct termination sites in the tectum is believed to be controlled by guidance molecules distributed in a graded or restricted manner across the tectum (Bonhoeffer and Gierer, 1984; Gierer, 1987; Holt and Harris, 1993; Kaprielian and Patterson, 1994; Roskies et al., 1995). The differential expression of retinal guidance molecules must be regulated by transcription factors (Sanes, 1993).

Several lines of evidence suggest that *En-1* and *En-2*, two closely related homeodomain transcription factor genes, which are vertebrate homologs of the *Drosophila* segment polarity gene *engrailed*, may regulate the development of the retinotopic map in the tectum, and in particular the polarity of the rostral–caudal tectal axis. First, *En-1* and *En-2* are expressed in a caudal-to-rostral gradient in the tectum well before the arrival of retinal axons (Gardner et al., 1988; Martinez and Avarado-Mallart, 1990; Martinez et al., 1991). Levels of *En-1* and *En-2* mRNA and protein are highest in caudal tectum,

which is innervated by nasal retinal axons, and lowest in rostral tectum, which is innervated by temporal retinal axons (Gardner et al., 1988; Martinez et al., 1991). Second, the En proteins are the earliest identified molecules to be distributed in a gradient along the rostral–caudal axis of the tectum (Davis and Joyner, 1988; Davis et al., 1988; Gardner et al., 1988). Third, in ectopic tecta formed by mesencephalic transplants in which En is distributed in a normal or inverted gradient, the terminations of retinal axons along the rostral–caudal tectal axis correlate with the relative level of En protein: nasal axons terminate in regions of high En, whereas temporal axons either terminate in regions of low En or are not found in the ectopic tecta (Itasaki et al., 1991; Itasaki and Nakamura, 1992). Although these findings suggest that En may regulate tectal polarity, the correlation between the level of En and rostral–caudal polarity of the retinotopic map in the tectum may be coincidental, and the retinotectal map and the expression of *En-1* and *En-2* may be regulated independently by other transcription factors.

One approach to study more directly the role of En in retinotopic map formation would be to analyze the projections of retinocollicular axons in the *En-1* and *En-2* null-mutant mice, which have been produced by homologous recombination (Joyner et al., 1991; Wurst et al., 1994). Several points, however, would confound such an analysis. *En-1* homozygous mutant mice die around birth, days before topographic order develops in the retinocollicular projection. Furthermore, the deletion of *En-1* results in a reduced superior colliculus (Wurst et al., 1994), which would complicate interpretations of any aberrancies in early retinal axonal targeting. An additional complication is that *En-1* and *En-2* seem to be functionally redundant and can compensate for

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the loss of each other (Joyner et al., 1991; Millen et al., 1994; Hanks et al., 1995).

Therefore, to assess the role of *En* in the development of the retinotectal map, we have chosen the approach of using a replication-competent recombinant retrovirus to misexpress *En-1* or *En-2* in the developing chick tectum, combined with axonal labeling techniques to assay the effect of misexpression on the topographic mapping of retinal axons. The retroviral construct that we have used is based on the RCASBP vector (Hughes et al., 1987), which infects only replicating cells and has been used previously to express the alkaline phosphatase gene in chick tectum (Fekete and Cepko, 1993) and *Hox 4.6* in the chick limb bud (Morgan et al., 1992). We find that misexpression of *En-1* or *En-2* results in topographically aberrant terminations of nasal and temporal retinal axons. These results indicate that *En-1* and *En-2* are involved in the regulation of the retinotopic map in the tectum.

This work was presented previously as an abstract at the Annual Society for Neuroscience meeting (Friedman and O'Leary, 1995).

MATERIALS AND METHODS

Viral construction, production, and infection

The mouse *En-1* and *En-2* cDNAs, with the ATG start sites modified to an *NcoI* restriction site and subcloned into pClaNco12 shuttle vector, were gifts from A. Joyner and C. Logan. These cDNAs were subcloned into the *NcoI* and *HindIII* site of the SLAX shuttle vector (Morgan and Fekete, 1996). A partial *ClaI* fragment was then cloned into the *ClaI* site of the RCASBP A or B retroviral vector (Hughes et al., 1987; Homburger and Fekete, 1996). The completed expression construct is diagrammed in Figure 1. Subtype 0 chick embryo fibroblasts (CEFs) were transfected with the retroviral constructs by the CaPO_4 precipitation and glycerol shock. The transfected cells were propagated and expanded over 10 d in CEF growth medium [DMEM, 10% fetal bovine serum (FBS), 2% chick serum (CS), penicillin/streptomycin (P/S), and glutamine], by which time 100% of the cells were infected. The viral particles were collected in DMEM, 1% FBS, 0.2% CS, P/S, and glutamine over two 24 hr time periods. The viral-conditioned medium was centrifuged for 3 hr in a Beckman SW28 rotor at 21,000 rpm, the viral pellet was resuspended in 0.1 of the original volume, and the viral titer was determined (see Results).

Fertile White Leghorn eggs, subtype 0 (SPAFAS), were incubated at 37.5°C in a 100%-humidified incubator. After 36 hr (approximately stage 10–12), the eggs were windowed, and ~80 nl of viral concentrate containing 0.025% fast green and 80 $\mu\text{g}/\text{ml}$ polybrene (for B envelope virus) was injected into the embryonic mesencephalic vesicle with a forced air pipspritzer attached to a micromanipulator. An additional injection was made 12–20 hr later (approximately stage 14). The eggs were sealed with tape and returned to the incubator.

Immunohistochemistry

Cells and cryostat sections were fixed in 4% paraformaldehyde (PF) in PMF buffer (0.2 M PIPES, pH 6.95, 2 mM MgSO_4 , 4 mM EGTA) for 15 min, washed 3 $\times$ 5 min in PBS, incubated in 1 M glycine, pH 7.5, followed by 3% H_2O_2 for 2 min, and washed in PBS. DiI-injected animals were perfused, postfixed overnight in 4% PF, and sectioned on a vibratome. Sections were counterstained with neutral red, dehydrated, and mounted in DPX.

Gag detection. Cells and sections were blocked in 10% FBS in PBT (PBS, 0.1% Triton X-100) for 30 min and incubated for 2 hr at room temperature for cells and overnight at 4°C for sections, with the AMV 3C2 antibody (which recognizes the gag matrix protein P19) (Potts et al., 1987). Cells and sections were washed in PBT and incubated with goat anti-mouse peroxidase-conjugated or fluorescein-conjugated antibodies (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hr at room temperature and then washed 4 $\times$ 10 min in PBT. The peroxidase antibody was detected by reacting in 0.03% DAB, 0.03% H_2O_2 in PBT.

En detection. Cells and sections were blocked in PBSMT (PBS, 2% dry milk, 0.1% Triton X-100) for 30 min and incubated with a 1:1000 dilution $\alpha\text{En}1$ antibody (Davis et al., 1991) (a gift from A. Joyner), which recognizes the homeodomain of *En-1* and *En-2*, in PBSMT overnight at

4°C. The sections were washed 4 $\times$ 10 min in PBSMT, incubated 2 hr at room temperature with a goat anti-rabbit antibody (Jackson ImmunoResearch Labs), washed 2 $\times$ 10 min in PBSMT and 2 $\times$ 10 min in PBT, and reacted in DAB as described above.

In situ hybridization

cRNA probes were synthesized from *NcoI*-linearized *En-1* or *En-2* SLAX constructs. One microgram of linearized plasmid was mixed with T7 RNA polymerase (10 U) (New England Biolabs, Beverly, MA) in 1 $\times$ transcription buffer (40 mM Tris HCl, pH 8.25, 6 mM MgCl_2 , 2 mM spermidine, 10 mM dithiothreitol), ribonuclease inhibitor (Promega, Madison, WI), and nucleotides [1 mM each of GTP, ATP, and CTP, 0.65 mM UTP, and 0.35 mM digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN)]. The transcription reaction was incubated at 37°C for 2 hr. The RNA transcript was purified by incubation with 2 μl DNaseI (RNase-free, Promega) at 37°C for 15 min, followed by precipitation with 0.4 M LiCl in 70% EtOH at –20°C for 1 hr. The precipitate was resuspended in 5 mM Tris HCl, pH 8.0, 0.5 mM EDTA, and 50% formamide.

Twenty micrometer cryostat sections were thaw-mounted and air-dried on 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO) subbed slides, fixed for 10 min in 4% PF in PBS, washed 3 $\times$ 3 min in PBS, acetylated in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, permeabilized in 1% Triton X-100/PBS, and washed 3 $\times$ 5 min in PBS. The sections were then blocked for 6 hr in hybridization buffer [50% formamide, 4 $\times$ SSC (0.6 M NaCl, 60 mM sodium citrate, pH 7.0, 4 mM EDTA) and 1 $\times$ Denhardt's solution [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA (Fraction V), 20% dextran sulfate, 500 $\mu\text{g}/\text{ml}$ tRNA]]. The sections were hybridized with 1 mg/ml digoxigenin-labeled cRNA in hybridization buffer for 16 hr at 65°C. The sections were then washed in 0.2 $\times$ SSC for 1 hr at 72°C, washed 2 $\times$ 10 min in PBS, blocked with 10% heat-inactivated lamb serum in PBT, and then incubated with a 1:2000 dilution of Dig-specific antibody (Boehringer Mannheim) for 2 hr. The sections were then washed 3 $\times$ 10 min in PBS, equilibrated in AP buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 0.05 M MgCl_2 , 0.1% Tween 20, 2 mM Levamisole) for 5 min, and then reacted with 0.45 mg/ml 4-Nitroblue tetrazolium chloride (Boehringer Mannheim), 0.175 mg/ml 5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate, Boehringer Mannheim) in AP buffer for 16 hr at room temperature.

Axonal labeling and analysis

DiI was used as an anterograde axon tracer (Honig and Hume, 1986, 1989) to label retinal axons projecting to the tectum. Injections and analysis of labeling were carried out as described in detail by Nakamura and O'Leary (1989). Briefly, focal injections of a 10% solution of DiI (Molecular Probes, Eugene, OR) in dimethylformamide (Sigma) were made into the peripheral nasal or temporal retina on the 15th day of incubation. The embryos were incubated for an additional 2–3 d, perfused with 4% PF in 0.1 M phosphate buffer, and staged according to the criteria of Hamburger and Hamilton (1951). Whole mounts of the retina and the contralateral optic tectum were examined and photographed on a fluorescence microscope under rhodamine illumination to document the location of the DiI injection site and the distribution of DiI-labeled retinal axons. In addition, the location of the retinal injection sites was mapped onto drawings of the retina, and the trajectories and arborizations of labeled retinal axons in the tectum were plotted on tracings of the tecta. The tecta were then processed for the expression of the virally expressed genes in one of two ways. Whole mounts either were directly immunostained with the AMV 3C2 antibody for gag protein or were embedded in agar, vibratome-sectioned parallel to the rostral-caudal tectal axis, and then immunostained for gag protein. In some cases, sections through the tectal whole mounts were photographed under rhodamine fluorescence before immunostaining to document the distribution of DiI-labeled axons and arbors. Aberrant mapping of retinal axons in tectal whole mounts and sections was correlated with distribution of gag protein and the retinal location of the DiI injection site. Photomontages and figures were constructed with Adobe Photoshop.

RESULTS

Expression of virally introduced genes in vitro and in vivo

Figure 1 shows schematics of the replication-competent recombinant retroviruses used and their putative transcripts. Viral titer was determined by immunocytochemistry with either AMV 3C2

Figure 1. Recombinant retrovirus construct and putative transcripts. The *En-1* and *En-2* recombinant replication-competent retroviruses were made by cloning the murine *En-1* or *En-2* cDNAs into the *Cla*I site of the RCASBP construct. Alternate splicing results in the three putative transcripts shown: the first two transcribe viral genes, including the *gag* gene, and the third transcribes the *En-1* or *En-2* cDNA. SA, Splice acceptor; SD, splice donor; LTR, long terminal repeat. (Adapted from Fekete and Cepko, 1993.)

(Potts et al., 1987), which recognizes a viral *gag* protein, or α *Enhb1* (Davis et al., 1991), which recognizes the homeodomain of the *En-1* and *En-2* protein, or by *in situ* hybridization with *En-1* or *En-2* digoxigenin-labeled cRNAs. Essentially identical titers were detected by immunohistochemistry and *in situ* hybridization for the recombinant retroviruses used. For the *En-1* RCASBP envB construct, a titer of 2.7×10^8 IU/ml was detected with both the *gag* and *En* antibodies, and 2.5×10^8 IU/ml with cRNA probes. For the *En-2* RCASBP envA construct, a titer of 7.7×10^7 IU/ml was detected with both antibodies, and 5.0×10^7 IU/ml with cRNA probes. In addition, as a control for the effect of viral infection, we used both an RCASBP envB construct and an RCASBP envA construct, each of which contained the human placental alkaline phosphatase (*hPLAP*) cDNA in place of an *En* cDNA. The *PLAP* RCASBP envB and envA viruses had titers of 1×10^8 IU/ml and 1×10^9 IU/ml, respectively, as detected by alkaline phosphatase histochemistry. These findings demonstrate the effectiveness of retroviral infection and indicate that the retrovirally introduced genes are coexpressed in cells.

En is first evident in the tectum on E2 (Gardner et al., 1988; Gardner and Barald, 1991), and tectal neurogenesis occurs from E4 to E9 (La Vail and Cowan, 1971). Therefore, for the *in vivo* analysis of the influence of *En* on retinotectal mapping, embryos were infected at E2 (stage 10–12) with recombinant retrovirus containing *En-1* or *En-2* cDNA. To enhance the level of infection, about half of these embryos were reinfected at E3 (stage 14). In an initial set of *in vivo* experiments, we assessed the coexpression of the *En-1* ($n = 4$) and *En-2* ($n = 4$) genes and the *gag* gene at E4/E5 (stages 24–27), ~2 d before retinal axons first enter the tectum (Crossland et al., 1975; McLoon, 1985). At E4, the caudal to rostral gradient of endogenous *En* protein is readily demonstrable (Fig. 2A,B). Coexpression was determined by detecting *En* mRNA by *in situ* hybridization, and the *En* protein and a *gag* protein were determined by immunohistochemistry. Retrovirally infected cells and their progeny form dense columns of cells spanning from the ventricular to pial surfaces of the tectum, with a scattering of infected cells outside of these columns (Figs. 2C–E; also see Fig. 5). These columnar patterns were anticipated, be-

cause the replication-competent retrovirus infects only proliferating cells and the retrovirally introduced genes are passed on to their progeny. This labeling pattern is similar to that reported by others using replication-incompetent, recombinant retroviruses to mark tectal progenitor cells and their progeny (Gray et al., 1988; Galileo et al., 1990; Gray and Sanes, 1991). We find that *En* mRNA, *En* protein, and *gag* protein are coexpressed at the same infection site. An example of coexpression in rostral tectum is shown in Figure 2C–E for an embryo infected with a retrovirus containing *En-1* cDNA. Similar findings were obtained for *En-2* (data not shown). Thus, infected tectal cells express exogenous *En* mRNA and its protein product as well as the viral *gag* protein.

The coexpression of *En* and *gag* retrovirally introduced genes allowed us to use immunohistochemical localization of *gag* protein as a marker for *En* overexpression in subsequent experiments. One advantage of using *gag* as a marker for infection is that it is present only in infected cells, whereas both *En-1* and *En-2* endogenous proteins normally present in the chick tectum are recognized by the α *Enhb1* antibody (Fig. 2). Although staining for the endogenous *En* proteins is low in rostral tectum, caudally the staining is heavy enough to obscure detection of the retrovirally introduced *En*.

Effect of overexpression of *En* genes on the retinotectal map

In additional sets of embryos, we assessed the effects of misexpression of *En-1* (using an RCASBP envB retroviral construct; $n = 8$) and *En-2* (using an RCASBP envA retroviral construct; $n = 21$) on the topographic organization of the retinotectal projection. About half of these embryos were infected at E2 (stage 10–12), and the remaining embryos were infected at both E2 and E3 (stage 14 or 15), with recombinant retrovirus containing *En-1* or *En-2* cDNA or with virus containing the *hPLAP* cDNA in place of an *En* cDNA to control for nonspecific influences of retroviral infection. On the 14th or 15th day of incubation, by which time the retinotectal projection has taken on its mature-like topographical organization (Crossland et al., 1975; Thanos and Bonhoeffer, 1983, 1987; Nakamura and O'Leary, 1989), small injections of DiI were made into peripheral nasal or temporal retina to label retinal axons projecting to the contralateral tectum. On the 17th or 18th day of incubation, the embryos were fixed and staged, and the retinotectal projection and viral infection patterns were analyzed. In addition, the size and location of DiI injections in the retinas, and the projections of labeled retinal axons to the optic fissure, were analyzed. The projection of retinal axons within the retina appeared normal in all cases. Cases were not included if the injection site was not well localized, failed to label axons, or was located at a position not consistent with those in other cases.

To control for the effect of retroviral infection per se on the targeting of retinal axons, embryos were infected with a replication-competent retrovirus (RCASBP envA or envB) containing the *hPLAP* cDNA, and either nasal retina ($n = 4$ for envA subtype; $n = 4$ for envB subtype) or temporal retina ($n = 4$ for envA subtype) was injected with DiI. In each case, the pattern of retinal axonal terminations is indistinguishable from that seen in normal, aged-matched embryos labeled in the same manner (nasal, $n = 3$; temporal, $n = 2$). Examples of control-infected nasal and temporal cases are shown in Figure 3A,B. Labeled axons arising from either nasal or temporal retina extend rostrocaudally across the tectum, and all terminate in a small, well localized zone of dense arborizations in either caudal or rostral tectum, respectively. Only rarely were arbors located aberrantly outside of the

Figure 2. Overexpression of *En* in the tectum correlates with viral infection. *A, B*, Caudal (*C*) to rostral (*R*) graded distribution of *En* protein in normal E4 tectum. *A*, Sagittal section counterstained with neutral red and immunostained with the α *Enhb1* antibody, which detects both *En-1* and *En-2* protein. *B*, The same section as in *A*, but not counterstained to better illustrate the *En* gradient revealed with α *Enhb1* immunostaining. *C–E*, Adjacent sections through a tectum infected with the RCASBP envB replication-competent recombinant retrovirus containing *En-1* cDNA. Arrows mark coincident ectopic domains of *En-1* mRNA (*C*), *En* protein (*D*), and the viral gag protein (*E*) detected with *in situ* hybridization using digoxigenin-labeled *En-1* cRNA probe (*C*), or immunohistochemistry using the α *Enhb1* antibody for *En* protein (*D*) or the 3C2 antibody for a gag protein (*E*). The retrovirus was injected into the mesencephalic vesicle at E2 (stage 10) and E3 (stage 14). At E4 (stage 24), 48 hr postinfection, the embryo was fixed, and the expression patterns were analyzed. Rostral is to the left. Scale bars: *A, B*, 200 μ m; *C, D*, 50 μ m.

appropriate termination zone (TZ). Histochemical treatment of tissue sections verified that clusters of cells in the infected tecta contain enzymatically functional, exogenous alkaline phosphatase (Fig. 3*C,D*). These findings indicate that infection with the replication-competent retroviruses does not alter the targeting of retinal axons.

In contrast, the misexpression of *En-1* or *En-2* perturbs the targeting of nasal retinal axons. In these cases, we are able to define a zone of dense arborizations, which likely corresponds to the normal TZ of the labeled axons based on the location of the DiI injection site in the retina. This TZ, however, often appears to be less focused than that observed in normal chicks, with many labeled axons, branches, and arbors found around it or radiating from it (Fig. 4*A,E*). What is most striking, though, are the numerous mistargeted axons and aberrant arbors. The degree of aberrant targeting and arborization varies among cases. For example, in the *En-2*-infected tectum shown in Figure 4*A*, several well defined arbors are present at a distance from the dense TZ (Fig. 4*B–D*), whereas in the *En-2*-infected tectum shown in Figure 4*E*, a substantially greater number of aberrant arbors are present, giving the impression of a much more diffuse projection. Occa-

sionally, we observe small, dense ectopic TZs outside of the normal TZ, as illustrated for the *En-2*-infected tectum in Figure 4*E*. Immunostaining for gag protein, which correlates with domains of exogenous *En* protein, confirms the effective viral infection in these cases (Fig. 4*F,G*). A topographically disorganized projection was found in 8 out of 8 *En-1*-infected tecta and in 7 out of 11 *En-2*-infected tecta. Variations in the extent of infection, and the relationship between the domains of *En* overexpression and the trajectories and terminations of the labeled retinal axons, likely account for the variations in the degree of aberrancy in the retinotopic map observed between cases. These findings indicate that overexpression of *En* leads to an aberrant topographic mapping of nasal retinal axons.

In a subset of cases, we correlated aberrant arbors of nasal axons with domains of *En-1* ($n = 4$) or *En-2* ($n = 4$) retroviral infection. This was carried out in one of two ways: one, by photographing the distribution of labeled retinal axons and arbors in tectal whole mounts and then dissecting small regions of the whole mounts containing aberrant projections and processing them for gag immunostaining; or two, by photographing the distribution of labeled retinal axons and arbors in sections of

Figure 3. Retroviral infection per se does not alter retinal axon targeting. Tectal whole mounts showing the distribution of nasal (*A*) or temporal (*B*) retinal projections in control-infected embryos. These projections are indistinguishable from those observed in normal age-matched, uninfected embryos. *A*, An E16 (stage 42) embryo infected with an RCASBP envB retrovirus containing the *hPLAP* gene but not an *En* gene. Retrovirus was injected into the mesencephalic vesicle at E2 and E3 (stages 10 and 14). Nasal retinal axons were labeled with DiI on the 14th day of incubation, and the embryo was fixed on the 16th day of incubation. All of the labeled nasal retinal axons end in a well defined, topographically appropriate termination zone (TZ) consisting of dense arborizations. Branches and arborizations are rarely found outside of the TZ. *B*, An E16 (stage 42) embryo infected with an RCASBP envA retrovirus containing the *PLAP* gene but not an *En* gene. Retrovirus was injected into the mesencephalic vesicle at E2 (stage 11). Temporal retinal axons were labeled with DiI on the 16th day of incubation, and the embryo was fixed on the 18th day of incubation. All of the labeled temporal axons end in a TZ of dense arborizations at the topographically appropriate location; no aberrant branches or arbors are observed outside of the TZ. *C*, *D*, Alkaline phosphatase histochemistry of sagittal sections through the same infected tecta shown in *A* (*C*) and *B* (*D*). Columns of reaction product stretching to the pial surface indicate domains of infected tectal cells. Infection and expression of PLAP is extensive throughout the tectum (a few of the columns are marked with arrows in *C*). In *A* and *B*, rostral is to the bottom; in *C* and *D*, rostral is to the left. Scale bars: *A*, *B*, 200 μ m; *C*, *D*, 1 mm.

tectum and then processing the sections for gag immunostaining. Figure 5 shows an example of a case processed as a whole mount, and Figure 6 shows a case processed as sections. In the whole mount of an *En-1*-infected tectum illustrated in Figure 5, nasal axons form a dense TZ but also show a very diffuse projection with

many aberrant arbors present at ectopic locations (Fig. 5*A*). A collection of aberrant arbors coalescing at a topographically incorrect site (Fig. 5*B*) is coincident with a cluster of gag-positive cells indicative of a domain of ectopic *En* expression (Fig. 5*C*). In the sections of an *En-1*-infected tectum illustrated in Figure 6, the

Figure 4. Topographic mapping of nasal retinal axons is disrupted in *En*-2 infected tecta. Shown are whole mounts of two E15 (stage 41) tecta infected with *En*-2 (RCASBP envA) replication-competent recombinant retrovirus. In each case, the retrovirus was injected into the mesencephalic vesicle on E2 (stage 12), a focal injection of Dil was made into nasal retina on the 15th day of incubation, and the embryos were fixed and staged on the 17th day of incubation. *A*, A seemingly appropriately located termination zone (TZ) of dense arborizations is observed, but overall the mapping of nasal axons is aberrant. A diffuse network of labeled axons and branches surrounds the TZ, and numerous aberrant arbors and branches are found outside of it. Some of the aberrant arbors located at a sizable distance from the TZ are marked with arrows and shown at higher magnification in *B–D*. *E*, The mapping of nasal retinal axons is more diffuse than in the case shown in *A*. The TZ is poorly focused compared with control embryos of this age, and a large number of aberrantly targeted axons, branches, and arbors are evident. The arrow marks several arbors formed by multiple axons that have coalesced at an aberrant site rostral to the TZ. *F*, *G*, Gag immunostaining of sagittal sections through the same infected tecta shown in *A* (*F*) and *E* (*G*). Immunostaining for the viral gag protein, which correlates with domains of exogenous *En* protein, reveals extensive infection throughout the tecta. Many dense columns of infected cells (a few columns are indicated by arrows in *F*) span the tectal layers to the pial surface. At higher magnification, infected cells are found scattered outside of the heavily infected domains (not shown). Within a tectum, there is variability in the size and distribution of the infected columns of cells, and across cases there is variability in the number and distribution of the infected columns of cells. Scale bars: *A*, *E*, 200 μm; *B–D*, 50 μm; *F*, *G*, 0.5 mm.

dense primary TZ is nestled in a site of low gag immunostaining (Fig. 6*A–C*), whereas isolated topographically aberrant arbors of nasal axons colocalize with domains of dense gag immunostaining (Fig. 6*D–G*). In contrast to the aberrant topographic distribution of the nasal axons and arbors, their laminar distribution appears normal. These colocalizations of gag protein and aberrant arbors are evidence of a direct correlation between a site of infection and the presence of aberrant arborizations.

In another set of experiments, we analyzed the effect of *En*-2 overexpression on the targeting of temporal retinal axons. Normally, by E15 temporal axons project to a well defined TZ in the

rostral tectum (Nakamura and O'Leary, 1989). In contrast, the projection of temporal retinal axons is aberrant in 7 of the 10 *En*-2-infected cases examined at E15 or later. In two of the *En*-2-infected tecta, we observed labeled temporal axons that are stunted and end at various positions rostral to their appropriate TZ (Fig. 7*A*). Such stunted temporal axons are not seen in normal tecta at this age. In five of the seven abnormal cases, labeled temporal axons extend through the optic tract and arborize in pretectal nuclei, and they either form terminations that are restricted to the extreme rostral edge of the tectum abutting the optic tract (Fig. 7*B*) or the axons do not extend into the tectum

Figure 5. Zones of dense aberrant arborizations formed by nasal retinal axons correspond to sites of *En* retroviral infection. **A**, whole mount of an E16 tectum infected with the *En-1* replication-competent recombinant retrovirus (RCASBP envB) injected into the mesencephalic vesicle at E2 and E3 (stages 11 and 14). A focal injection of Dil was made into the nasal retina on the 15th day of incubation, and the embryo was fixed and staged on the 17th day of incubation. The topographically diffuse nasal projection resembles that observed in the *En-2*-infected tecta illustrated in Figure 4. Many labeled axons, branches, and arbors are distributed at aberrant sites. A site where multiple arbors coalesce at an aberrant site is marked by a box in **A** and shown at a higher magnification in **B**. This tissue was then processed for gag immunoreactivity. A cluster of FITC-labeled gag-positive tectal cells, indicative of a domain of exogenous *En* protein, is coincident with the aberrant arborization zone (**C**). The arrows in **B** and **C** mark the same point before and after gag immunohistochemistry. Scale bars: **A**, 200 μ m; **B**, **C**, 100 μ m.

(Fig. 7C). Again, immunostaining for the expression of the *gag* gene confirms effective retroviral infection (Fig. 7D–F) and, in particular, dense domains of infection in rostral tectum. The intercase differences in the targeting and terminations of the labeled temporal axons may be attributable to observed variations in infection between cases and to differences between cases in the probability that labeled axons will encounter ectopic domains of *En* expression. These findings indicate that *En* overexpression disrupts the projection of temporal retinal axons, but in a manner distinct from nasal retinal axons.

DISCUSSION

We have used recombinant replication-competent retroviruses to overexpress the *En-1* and *En-2* homeobox transcription factors in the developing chick tectum to assess their role in regulating the development of the retinotopic map. Overexpression of either *En-1* or *En-2* perturbed the topographic targeting of nasal and temporal retinal axons. The disruption of nasal retinal axons was evident by a diffuse TZ and numerous axons, branches, and arbors at aberrant topographic locations. The disruption of temporal axon targeting was evident by the presence of stunted axons that failed to reach their appropriate TZ, ending rostral to it, or by the finding in other cases that temporal axons either did not enter the tectum or formed a TZ at its extreme rostral margin. These findings indicate that *En-1* and *En-2* are involved in regulating the development of the retinotopic map in the tectum. Because *En-1* and *En-2* are transcription factors, they must act indirectly on axon mapping, most likely by controlling, either directly or through intermediaries, genes that encode retinal axon guidance molecules.

In the developing chick, most retinal axons initially grow past the appropriate rostral–caudal position of their TZ. The majority of arbors are formed by collateral branches that extend from the

primary retinal axons (Nakamura and O'Leary, 1989; Yates et al., in press). During normal development, collateral branching and arborization occur at topographically appropriate and inappropriate sites but are densest near the appropriate location. *In vitro* experiments suggest that branching is controlled by molecules anchored to the membrane of tectal cells by a phosphatidylinositol (PI)-linkage and is distributed differentially along the rostral–caudal tectal axis (Roskies and O'Leary, 1994). The final stage in the development of the topographically ordered projection is the elimination of aberrant axons, branches, and arbors and the consolidation of arborizations at the appropriate TZ (Nakamura and O'Leary, 1989).

We have examined the topographic organization of retinal projections in the *En*-infected tecta at ages when the projection has taken on its mature topographic order. Before preceding, however, we should consider whether our findings of a diffuse nasal retinal projection in the *En*-infected tecta might be explained by a slowing of overall development and consequently a delay in the normal elimination of aberrantly located arbors. Several lines of evidence seem to rule this out. First, two controls were carried out to take into account a possible slowing of normal development in the viral-infected chicks. All of the cases were staged according to the Hamburger and Hamilton (1951) criteria, which controls for a slowing of overall development, and the projections in the *En*-infected cases were extremely diffuse compared with those in age-matched control cases infected with the same parental virus that contained the *PLAP* gene instead of an *En* gene. Second, the aberrant arbors formed by nasal axons colocalize with domains of viral infection, which are coincident with domains of *En* overexpression. And third, although both temporal and nasal axons normally form transient aberrant arbors

Figure 6. Topographically aberrant arbors of nasal retinal axons correspond to sites of retroviral infection. Sagittal sections parallel to the rostral–caudal axis of an E14 (stage 40) tectum infected with the *En-1* replication-competent recombinant retrovirus (RCASBP envB) injected into the mesencephalic vesicle at E2 and E3 (stages 11 and 15). A focal injection of DiI was made into the nasal retina on the 15th day of incubation, and the embryo was fixed and staged on the 17th day of incubation. In addition to a dense primary termination zone (marked TZ in A and C), many labeled axons, branches, and arbors located at topographically aberrant sites (D–G) are shown. A, D, F, DiI-labeled nasal axons and arbors photographed under rhodamine illumination. B, E, G, The same sections as in A, D, and F after processing for gag immunostaining. C, E, G, Composites in which the axonal labeling in A, D, and F was overlaid on the gag immunostaining in C, E, and G using Adobe Photoshop. In E and G, the axonal labeling shown in D and F is inverted from a positive to a negative image. The arrowheads in A–C mark the same reference point before and after gag immunohistochemistry. The arrows in B and C mark the same column of gag-immunostained cells. The topographically aberrant arbors in D and F colocalize with clusters of dense gag immunostaining, indicative of domains of exogenous En protein. The laminar distribution of arbors within the primary TZ, and at aberrant locations, appears normal. In each panel, the pial surface is to the top. SGFS, Stratum griseum et fibrosum superficiale; SO, stratum opticum. Scale bars: A–C, 100 μ m; D, E, 50 μ m; F, G, 100 μ m.

at an earlier stage of development, we find that temporal axons do not have aberrant arbors in the En-infected cases, whereas the nasal axons do. This distinct effect of En viral infection on temporal and nasal populations cannot be accounted for by a slowing of development.

The aberrant retinal projections in the *En-1*- and *En-2*-infected embryos may be attributable to a stabilization of normally transient aberrant connections or to the promotion of aberrant connections coupled with their stabilization or both. In any case, our findings suggest that En-1 and En-2 regulate the expression of axonal guidance molecules that identify positional addresses in the tectum, and they control the targeting, branching, and arborization of retinal axons.

In Figure 8, we illustrate two possible scenarios that could account for our findings. One scenario is that En-1 or En-2 regulates the expression of a molecule(s) that operates as an attractant, a growth promoter, or a trophic activity specific for nasal retinal axons. In this scenario, misexpression of *En-1* or *En-2* would result in ectopic domains of molecules that positively influence the growth of nasal retinal axons resulting in the maintenance of aberrant nasal projections. These aberrant projections could result from a failure of nasal axons to remodel and prune incorrectly positioned axons, branches, and arbors, which normally occurs through competitive interactions between correctly

and incorrectly targeted retinal axons (Kobayashi et al., 1990; Simon et al., 1992, 1994; Goodman and Shatz, 1993), or to the promotion and retention of additional aberrant connections. In this scenario, correctly targeted temporal axons may be excluded from domains of En overexpression by competitive interactions with aberrantly terminated nasal retinal axons. Thus, the stunted temporal axons observed in the En-infected tecta could be in the process of retraction and perhaps elimination. An alternative scenario is that En-1 and En-2 regulate the expression of molecules that specifically inhibit or repulse temporal retinal axons. Temporal axons encountering these negative domains would be inhibited or repulsed and fail to establish appropriate connections within them. This could also account for the aberrancies in the mapping of nasal axons. The exclusion of temporal axons would remove the competition within these domains and allow nasal axons to stabilize normally transient aberrant arbors that project to them. Of course, En-1 and En-2 may regulate both sets of putative molecules (one set positive for nasal axons and one set negative for temporal axons), and the two scenarios presented in Figure 8 may work in concert.

For clarity, these scenarios describe the behaviors of temporal and nasal retinal axons as two uniform populations interacting with each other as well as with guidance molecules; however, these scenarios are an oversimplification, because the temporal-

Figure 7. The topographic targeting of temporal retinal axons is disrupted in *En*-infected tecta. *En-2* replication-competent recombinant retrovirus (RCASBP envA) was injected into the mesencephalic vesicle on either (*A*) E2 (stage 11) or (*B, C*) E2 and E3 (stages 12 and 14), a focal injection of Dil was made into temporal retina on the 15th (*A*) or 16th (*B, C*) day of incubation, and the embryos were fixed and staged on the 17th (*A*) or 18th (*B, C*) day of incubation. *A*, In an E15 (stage 41) *En-2*-infected tectum, several labeled temporal axons (marked by arrows) end aberrantly rostral to their appropriate termination zone (TZ) as club-like structures without any sign of arborization. *B*, An E16 (stage 42) *En-2*-infected case in which labeled temporal axons end in a TZ of dense arborizations aberrantly located at the extreme rostral edge of the tectum (*Tec*) where it abuts the optic tract (*ot*). *C*, An E17 (stage 43) *En-2*-infected case in which labeled temporal axons course through the *ot* and arborize in pretectal nuclei but do not enter the *Tec*. In *B* and *C*, the interface between the tectum and the optic tract is marked with arrows. *D–F*, Gag immunostaining of sagittal sections of the same cases shown in *A* (*D*), *B* (*E*), and *C* (*F*). In *A–C*, rostral is to the bottom; in *D–F*, rostral is to the left. Scale bars: *A*, 100 μ m; *B, C*, 200 μ m; *D–F*, 0.5 mm.

Figure 8. Schematic summary of findings and interpretations. *Normal*, In a normal embryo, temporal (*T*) retinal axons map in an orderly fashion to rostral (*R*) tectum, and nasal (*N*) retinal axons map in an orderly fashion to caudal (*C*) tectum. En protein is distributed in a caudal-to-rostral gradient in the tectum, with highest amounts caudally. *En Overexpression*, Infection of E2/E3 chick embryos with replication-competent recombinant retrovirus containing either the *En-1* or *En-2* cDNA results in the ectopic overexpression of *En* mRNA and protein in dense domains (circles). When these infected tecta are examined at a stage when the retinotectal projection is normally topographically precise, the topographic mapping of nasal and temporal retinal axons is disrupted. Nasal axons project diffusely with numerous arbors at topographically aberrant sites. In contrast, temporal axons are stunted and end rostral to their appropriate TZ (as indicated on the drawing). In other cases (not schematized), temporal axons either do not extend into the tectum or are restricted to its extreme rostral border. These results suggest that overexpression of *En-1* or *En-2* induces the expression of retinal axon guidance molecules at inappropriate levels. Two potential effects of *En* overexpression are diagrammed. First, *En* overexpression results in the overexpression of a molecule(s) that operates as an attractant or trophic activity (+) specifically for nasal axons. Second, *En* overexpression results in the overexpression of a molecule(s) that specifically inhibits or repels (–) the growth of temporal axons. Each of these scenarios, or a combination of them, is compatible with our findings. For further elaboration, see Discussion.

nasal axis of the retina maps smoothly along the rostral–caudal axis of the tectum. It is likely that this mapping continuum is controlled by a graded distribution of guidance molecules—regulated by the graded distribution of *En*—and differential sensitivities of retinal axons to these molecules, which depends on the point of axon origin along the temporal–nasal axis of the retina. For example, the mapping of nasal retina onto caudal tectum is attributable to differential responses of nasal axons to guidance molecules, depending on the central-to-peripheral point of axon origin within nasal retina. In an *En*-infected tectum, variability in the levels of exogenous *En* would be expected from one overexpression domain to another. This, combined with the graded distribution of endogenous *En* protein, would likely result in variability in the overall levels of *En* protein, and in turn retinal guidance molecules, from one overexpression domain to another. This interpretation could account for the aberrant mapping of nasal axons within the caudal tectum of *En*-infected embryos.

Our results showing that the overexpression of *En-1* or *En-2* perturbs the mapping of retinal axons, and the interpretation that this perturbation is attributable to *En*-induced misexpression of guidance molecules, are consistent with studies in the quail/chick chimera system, which suggest that the *En* gradient regulates tectal polarity and subsequently retinal axon targeting. When a graft of mesencephalon/metencephalon from an E2 quail is rotated 180° around its rostral–caudal axis and transplanted homo-

topically into an E2 chick, the *En* gradient in the graft regulates to coincide with the host *En* gradient (Martinez and Alvarado-Mallart, 1990). Similarly, the rostral–caudal polarity of the retinotectal projection within such a graft inverts to parallel that of the host (Ichijo et al., 1990). In other studies, the transplantation of E2 or E3 quail mesencephalon into E2 or E3 chick diencephalon results in the formation of an ectopic tectum rostral to the diencephalon/mesencephalon border, which is innervated by retinal axons entering the graft from its original caudal pole (retinal axons normally enter the tectum from its rostral edge) (Itasaki et al., 1991; Itasaki and Nakamura, 1992). The rostral–caudal polarity of the retinotectal projection to the ectopic tectum correlates with its *En* gradient. Nasal axons project to the region expressing high levels of *En*. Temporal retinal axons fail to enter the ectopic tectum when the high levels of *En* are expressed in the caudal pole (the site of axonal entry in the ectopic tectum). These transplantation experiments indicate a correlation between the level of *En* and the topographic terminations of nasal and temporal retinal axons: nasal axons terminate in regions of high levels of *En*, and temporal axons terminate in regions of low levels of *En*.

The correlative evidence provided by the quail/chick chimera transplant experiments and the more direct evidence provided by our recombinant retroviral overexpression experiments lead to the conclusion that *En-1* and *En-2* regulate the topographic targeting of nasal and temporal retinal axons along the rostral–caudal tectal axis. Furthermore, taken together, these studies suggest that the graded levels of *En-1* and *En-2* regulate the graded expression of at least a subset of molecules that guide the topographic targeting of retinal axons. For example, an activity preferentially associated with caudal tectum seems to promote or attract the growth of nasal retinal axons *in vitro* as well as have a trophic effect specific for nasal neurites (von Boxberg et al., 1993). In particular, however, three PI-anchored molecules that are expressed in a caudal-to-rostral gradient in the developing tectum, with highest levels caudally, may have a role in the topographic mapping of retinal axons. One is a 33 kDa protein, termed Repulsive Guidance Molecule, which acts as a repellent to growing temporal retinal axons *in vitro* (Walter et al., 1987a,b, 1990; Stahl et al., 1990; Müller and Bonhoeffer, 1994; Müller et al., 1995). The other two, Repulsive Axon Guidance Signal (RAGS) (Drescher et al., 1995) and Elf1 (Cheng and Flanagan, 1994; Cheng et al., 1995), are 25 kDa ligands for receptor protein tyrosine kinases of the Eph subfamily. *In vitro*, the RAGS protein is equally repulsive for both temporal and nasal retinal axons (Drescher et al., 1995), whereas Elf1 is a repellent for temporal axons but not nasal axons (Friedman et al., in press). Interestingly, the overexpression of *En-1* in rostral tectum results in an increased expression of Elf1 and RAGS (Logan et al., in press). That *En* regulates the expression of these two molecules which repel temporal axons is consistent with our finding that temporal axons aberrantly end rostral to their normal TZ in *En*-infected tecta.

It is presently unclear whether the *En* proteins act directly to regulate the genes encoding retinal axon guidance molecules or indirectly through intermediaries; however, the expression of the *En* genes in the tectum seems to be regulated by two distinct domains, one caudally at the mesencephalic/metencephalic border and one rostrally at the diencephalic/mesencephalic border. The *Wnt-1* proto-oncogene (Wilkinson et al., 1987; McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Gardner and Barald, 1991; Molven et al., 1991; Bally-Cuif et al., 1992; McMahon et al., 1992; Parr et al., 1993; Bally-Cuif and Wassef, 1994) and the *Pax-2*

paired-box transcription factor (Krauss et al., 1992) expressed at the mesencephalic/metencephalic border seem to be positive regulators of *En* expression. In contrast, an unidentified molecule(s) present at the diencephalic/mesencephalic border has been implicated as a negative regulator of *En* (Itasaki and Nakamura, 1992). These regulatory molecules may act in concert to establish the gradient of *En* across the tectum.

The structure of *En* genes is conserved across evolutionarily distant species. *En-1* and *En-2* are homologs of the *Drosophila* segment polarity genes *engrailed* and *invected* (Nusslein-Volhard and Wieschaus, 1980; Poole et al., 1985). Homologs of *engrailed* have been cloned at least partially and are expressed in similar patterns in zebrafish (Pattel et al., 1989; Ekker et al., 1992; Fjose et al., 1992), *Xenopus* (Pattel et al., 1989; Davis et al., 1991; Hemmati-Brivanlou et al., 1991), chick (Gardner et al., 1988; Pattel et al., 1989; Davis et al., 1991; Logan et al., 1992), mouse (Joyner and Martin, 1987; Davis et al., 1991; Logan et al., 1992), and human (Logan et al., 1992). This structural conservation among *engrailed* genes is underscored by the finding that the phenotype of midbrain and hindbrain structural deficits seen in *En-1* knockout mice (Wurst et al., 1994) can be partially rescued by the *Drosophila engrailed* gene placed under the control of the mouse *En-1* promoter (M. Hanks and A. Joyner, unpublished observations).

In addition to conservation of structure, the function of *En* proteins in defining caudal (i.e., posterior) fate of certain tissues seems to be conserved across species as well as by different *En* proteins within the same species. For example, the caudal midbrain is lacking in mice in which the *En-1* gene is deleted by homologous recombination (Wurst et al., 1994). Together, the experimental evidence presented here and that derived from quail/chick transplant experiments (see above) indicate that *En* proteins regulate the polarity of the rostral–caudal (i.e., anterior–posterior) tectal axis and confer caudal (i.e., posterior) tectal fate. *Drosophila engrailed* and *invected* are expressed in the posterior part of each segment of the *Drosophila* embryo (Kornberg, 1981; Di Nardo et al., 1985). Although a function for *invected* has not been determined, *engrailed* is involved in conferring posterior identity to each segment of the *Drosophila* embryo (Morata and Lawrence, 1975; Nusslein-Volhard and Wieschaus, 1980; Kornberg, 1981; Lawrence and Struhl, 1982; Di Nardo et al., 1985; Brower, 1986). In addition, *En-1* and *En-2* seem to have a similar role in regulating the retinotopic map (present study). This functional similarity is predicted by gene “knock-in” experiments in mice. For example, the structural deletions in the colliculus and cerebellum in *En-1* knockout mice are rescued when an additional copy of the *En-2* gene is placed under the control of the *En-1* promoter (Hanks et al., 1995). This finding supports the suggestion that the lack of an obvious structural defect in the superior colliculus of *En-2* homozygous mutants is attributable to compensation by *En-1* (Joyner et al., 1991; Millen et al., 1994). In the homozygous mutants, *En-1* may be more effective at compensating for the loss of *En-2* because *En-1* expression begins earlier in development than *En-2* (Davis and Joyner, 1988; Davis et al., 1988; McMahon et al., 1992). Taken together, these findings suggest that *engrailed*-like proteins may be general posterior-defining molecules in the developing tectum of vertebrates and in the segments of *Drosophila*.

Note added in proof: After the submission of this manuscript to *The Journal of Neuroscience*, a similar study was published in *Neuron* by N. Itasaki and H. Nakamura (A role for gradient *En* expression in positional specification on the optic tectum. *Neuron* 16:55–62). These investigators also misexpressed *En-1* and *En-2*

in the developing chicken optic tectum using an RCASBP retrovirus. Although the details differ between our study and theirs, both groups have obtained comparable results.

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