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The Spatial and Temporal Expression Patterns of Netrin Receptors, DCC and Neogenin, in the Developing Mouse Retina

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Recently it has been demonstrated that the guidance of retinal ganglion cell (rgc) axons through the optic disc is dependent on the DCC/netrin-1 axonal guidance system. To gain further insight into the function of the netrin receptors, DCC and Neogenin, in retinal development we have studied the expression patterns of these receptors in the embryonic mouse retina. Neogenin mRNA was restricted to a single neural cell type, the rgc. However, strong Neogenin mRNA expression was observed in the extending fiber cells of the developing lens suggesting a role for Neogenin in the migration events shaping the early lens. Our studies demonstrated that DCC mRNA was expressed at high levels in chains of closely opposed neurons as they migrated towards the emerging mantle layer in the early retina (E12·5–E13·5) suggesting a role for DCC in the migration of neurons out of the ventricular zone. DCC protein expression was high on rgc axons as they actively navigated through the optic disc into the optic nerve. At birth, when the majority of rgc axons had projected through the optic disc, DCC protein was no longer detectable on the distal axonal segments within the optic nerve despite significant DCC protein expression on the proximal axonal membranes in the nerve fiber layer. These observations suggest that a localized down-regulation of DCC protein occurs on projecting axonal membranes once the DCC guidance function is no longer required. We also demonstrated that DCC mRNA and protein were expressed by amacrine cells and Müller glial cells while DCC mRNA was detected in horizontal cells. Taken together, these expression patterns suggest a role for DCC in axon outgrowth and/or pathfinding for a variety of retinal neurons and in the migration of newly born neurons within the developing retina.

Key words: axon guidance; neural migration; eye development; retina; DCC; Neogenin.

1. Introduction

The neural retina provides a relatively simple and accessible model for the study of the molecular mechanisms governing neuronal differentiation, migration, and axon guidance as it contains only five distinct neural cell types that reside in readily discernible layers. Establishment of connections between neurons within the retina have been of particular interest since it is apparent there is a high degree of predetermination and precision in the selection of pathways and targets by extending axons (Cepko et al., 1996). Neurogenesis is initiated in the central region of the early vertebrate retina with the field of proliferation and differentiation spreading towards the periphery over the following days of gestation (Sidman, 1961). In the mouse, axons arising from dorso centrally-located retinal ganglion cells (rgcs) begin to extend around embryonic day 11·5 (E11·5) (Cepko et al., 1996). The rgcs differentiating in the peripheral regions arise over the next few days of gestation and their axons must navigate over a greater distance before exiting at the optic disc. Many different molecular interactions appear to act in a coordinated fashion to initiate rgc axon outgrowth and to steer axons through the optic disc and subsequently toward the appropriate targets in distant regions of the brain. Recently it has been demonstrated that the guidance of rgc axons through the optic disc is dependent on the DCC/netrin-1 axonal guidance system (Deiner et al., 1997).

It is now clear that the DCC/netrin-1 guidance system is largely responsible for directing axonal projections toward the ventral midline throughout the invertebrate and vertebrate embryonic central nervous system (CNS). The absolute requirement for the DCC/netrin-1 guidance system in the establishment of some commissural tracts has been dramatically demonstrated by the generation of mouse mutants lacking functional DCC or netrin-1 (Serafini et al., 1996; Fazeli et al., 1997). Both sets of mice exhibit severe defects in commissural axon extension in the developing spinal cord and lack several major

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commissures within the forebrain, including the corpus callosum and the hippocampal commissure. Both these mutants also demonstrate optic nerve hypoplasia due to the failure of the rgc axons to enter the optic nerve (Deiner et al., 1997). In vitro experiments have further demonstrated that the DCC guidance receptor expressed on *Xenopus* rgc axons triggers the reorientation of rgc growth cones in response to a netrin-1 gradient (de la Torre et al., 1997; Höpker et al., 1999).

Another DCC-like receptor, Neogenin, has been cloned from the chicken, mouse and human (Vielmetter et al., 1994; Keeling, Gad and Cooper, 1997; Meyerhardt et al., 1997). It has also been reported that netrin-1 is a ligand for Neogenin (Keino-Masu et al., 1996), however a role for Neogenin in axon guidance has not yet been demonstrated. The observation that high levels of Neogenin protein on rgc axons are correlated with the onset of axonal extension (Vielmetter et al., 1994) suggests that Neogenin may also play a role in rgc axon guidance.

Here we describe the expression patterns of DCC and Neogenin in the developing eye. We demonstrate that DCC mRNA and protein are not restricted to a specific retinal cell type but are expressed by rgcs, amacrine cells, horizontal cells, and Müller glial cells. In addition, immunohistochemical analysis revealed that the rgc axons express high levels of DCC protein during the period of active navigation through the optic disc where netrin-1 expression is localized. The spatial and temporal pattern of DCC expression further suggests a role for DCC in the migration of newly born retinal cells through the onl (outer neuroblastic layer) into the emerging inl (inner neuroblastic layer). In contrast to DCC, Neogenin mRNA appears to be restricted to a single neural cell type, the rgc.

2. Materials and Methods

Staging of Mouse Embryos

Mouse embryos were obtained from timed matings of ICR mice housed in the Ludwig Institute Animal Facility. The first day of pregnancy was considered to be embryonic day 0·5 (E0·5). The subsequent developmental stages were determined according to Kaufman (1992). All animal use was approved by the Ludwig Institute Animal Care and Use Committee according to the Australian Nation Health and Medical Research Council guidelines.

Preparation of ^{33}P-labeled RNA Riboprobes

A 2·4 kb mouse DCC cDNA (MB18, Cooper et al., 1995) cloned in pBluescript KS(+) (Stratagene, CA, U.S.A.) encoding the second, third and fourth immunoglobulin domains and the first five fibronectin type III repeats was used as the template for both the sense and anti-sense DCC riboprobes. The Neogenin anti-sense and sense riboprobes were synthesized using mouse Neogenin cDNA clone, BR11, encoding the last four fibronectin type III repeats, the transmembrane domain, and the first 55 amino acids of the cytoplasmic domain, as the template (Keeling et al., 1997). One μg of template was used to transcribe the ^{33}P-labeled sense and anti-sense riboprobes employing the Bresatec Message Maker Kit according to the manufacturer's instructions (Bresatec, Adelaide, Australia). To assess the integrity of the riboprobes, 1 μl of each labeled probe was run on a 1 % formaldehyde/agarose gel and then transferred to GeneScreen Plus nylon membrane (DuPont, NEN, MA, U.S.A.) via capillary action. The ^{33}P-labeled riboprobes were visualized using the PhosphorImager (Molecular Dynamics, CA, U.S.A.). Before hybridization, the riboprobes were precipitated and subjected to mild alkaline hydrolysis to reduce the probe length to approximately 100 bp. Hydrolysis was carried out in 60 mM Na_2CO_3 40 mM^{-1} $NaHCO_3$ (pH 10) for a time determined by the formula: (initial probe length − 0·1 kb)/[hydrolysis rate constant (0·11) × initial probe length × 0·1 kb] at 60°C. Specificity of hybridization was assessed using a 2·8 kb mouse VEGF receptor-2 riboprobe (Olerichs et al., 1993) hydrolysed according to the above formula.

In situ Hybridization

E11·5 to E13·5 embryos were dissected from uteri and immediately fixed in 4 % paraformaldehyde in phosphate buffered saline pH 7·4 (PBS) for at least 12 hr at 4°C. E15·5–E18·5 embryos were collected after perfusion of the mother with 4 % paraformaldehyde in PBS by injection into the left ventricle of the heart (Bolam, 1992). The embryos were initially embedded in 1 % low melting agarose (FMC Bioproducts, U.S.A.) to allow proper orientation before embedding in paraffin. Parasagittal or tranverse serial sections (4 μm) were cut and mounted onto 3′-aminopropyl-triethoxysilane-coated glass slides (Sigma, MO, U.S.A.) and stored at 4°C. Every 20th section was stained with hematoxylin and eosin to allow for histological orientation. In situ hybridization was carried out as described by Gad et al. (1997).

5-Bromo-2′-deoxyridine (BrdU) Staining

Pregnant ICR mice were injected intraperitoneally with 50 μg of BrdU (Sigma) per gram of body weight (0·01 ml g^{-1} in isotonic saline). Embryos were collected at the indicated time points post-injection and immersion fixed in 4 % paraformaldehyde for at least 24 hr before sectioning as described above. Incorporation of BrdU into embryonic tissue was assessed using a mouse monoclonal antibody to BrdU (Becton Dickinson, CA, U.S.A.) according to the protocol described by Gad et al. (1997). Bound antibody was detected using an avidin-biotin-horseradish

peroxidase (AB-HRP) complex (Vectastain ABC Kit, Vector Laboratories, CA, U.S.A.), and the chromogen, diaminobenzidine (DAB; Sigma), which produces a brown precipitate upon reaction with the AB-HRP-antibody complex. Negative control embryos were collected in parallel from a second group of pregnant females that had not been injected with BrdU. No background staining was observed in these controls using the anti-BrdU antibody (data not shown).

Immunohistochemical Analysis

The rabbit polyclonal antiserum, 2744, was raised against a purified preparation of the mouse DCC extracellular domain (mDCC-Fn2) comprising the four immunoglobulin domains and the first two fibronectin type III repeats. This DCC subdomain also has a FLAG peptide sequence (International Biotechnologies, CT, U.S.A.) at the C-terminus. The cDNA encoding mDCC-Fn2 was cloned into the pEE6 mammalian expression vector (Bebbington and Hentschel, 1987; Cockett, Bebbington and Yarranton, 1990) and transfected into CHO-K1 cells using the calcium phosphate method (Kingston, 1996). Clones expressing high levels of the mDCC-Fn2 protein were obtained by selection in 100 mM methionine sulfoxide. The expressed mDCC-Fn2 protein was purified from the culture supernatant by affinity chromatography using anti-FLAG monoclonal antibody (M2)-affinity beads (International Biotechnologies). Contaminating co-eluting proteins were removed by cation exchange chromatography. Purified protein was run on a 4–12 % gradient PAGE gel (Novex, SD, U.S.A.) and then silver stained to assess homogeneity.

DCC immunohistochemistry was performed on E15·5–E18·5 embryos transcardially perfused with 4 % paraformaldehyde and 2·5 % acrolein (v/v; Polysciences Inc., PA, U.S.A.) and then post-fixed in 4 % paraformaldehyde for 2–3 days. Whole heads were then sunk in 30 % sucrose (w/v) in PBS and cryostat sectioned at 10 μm. Sections were adhered to glass microscope slides and neutralized in 0·05 % sodium borohydride (w/v/; Sigma) in PBS for 10 min. The sections were then washed three times in PBS and blocked in 2 % normal goat serum 0·2 % Triton-X100 in PBS for 2 hr. Incubation in the primary antibody (2744; 1 : 1000) in blocking solution was carried out overnight. The following day the sections were washed three times with PBS and then incubated in goat anti-rabbit secondary antibody (1 : 500; Vector Laboratories) for 1 hr. After washing three times in PBS the sections were finally incubated in avidin-biotin solution (2·5 % nickel sulphate/0·02 % 3,3″-diaminobenzidine in 0·175 M sodium acetate) activated with 0·01 % (v/v) H_2O_2 until a dark precipitate formed. Sections were then washed in PBS, dehydrated through increasing concentrations of ethanol, immersed in two changes of 100 % xylene and coverslipped in DePeX (Sigma) mounting medium.

3. Results

To gain insight into the role of the DCC and Neogenin guidance receptors in retinal development we carried out in situ hybridization on the developing mouse retina throughout mid to late gestation (E11·5–E18·5). Using ^{33}P-labeled anti-sense riboprobes specific for mouse DCC or mouse Neogenin, we analysed mRNA expression in serial sections of the developing eye cut in parasagittal or transverse planes. The specificity of the hybridization signals generated by the anti-sense DCC and Neogenin riboprobes have been previously demonstrated (Gad et al., 1997). No detectable hybridization signal was observed using the control DCC and Neogenin sense riboprobes when hybridized from E11·5 to E18·5 whole embryo sections (Gad et al., 1997). A riboprobe specific for the endothelial cell marker, VEGF receptor-2 [Fig. 1(E)], produced the expected endothelial-specific hybridization pattern for this gene product (Olerichs et al., 1993) which was distinct from that observed for the DCC and Neogenin anti-sense riboprobes. In all cases, the hybridization patterns generated by the control riboprobes were distinct from those seen with either the DCC or Neogenin anti-sense riboprobes demonstrating the stringency of the hybridization protocol employed.

In addition, we used a rabbit polyclonal antiserum, raised to a purified preparation of a soluble DCC extracellular subdomain comprising the four immunoglobulin domains and the first two fibronectin type III repeats, to assess DCC protein expression in the developing retina of E15·5–E18·5 embryos. This antiserum specifically recognizes the DCC receptor in immunoblots of COS and HEK 293T cells transfected with the mouse DCC cDNA and does not cross react with the closely related mouse Neogenin receptor under the same assay conditions (data not shown). This antiserum has previously been used to demonstrate the pattern of DCC protein expression in the developing mouse forebrain (Shu et al., 2000). Presently there are no antibodies available that detect Neogenin protein in mouse tissues.

Expression of DCC mRNA Correlates with the Emergence of the Earliest Retinal Neurons

Postmitotic retinal neurons first appear within the vz of the optic cup around E11 of gestation in the mouse (Sidman, 1961; Cepko et al., 1996). Neurogenesis is initiated in the central region of the early retina with the field of proliferation and differentiation spreading to the periphery over the following days of gestation (Sidman, 1961). In the early phase of retinal differentiation (E11–E15) approximately half of the total complement of amacrine cells, rgcs, and cone photoreceptor cells are formed as well as a minor population of rod photoreceptors. In addition, the majority of horizontal cells, which make up only a

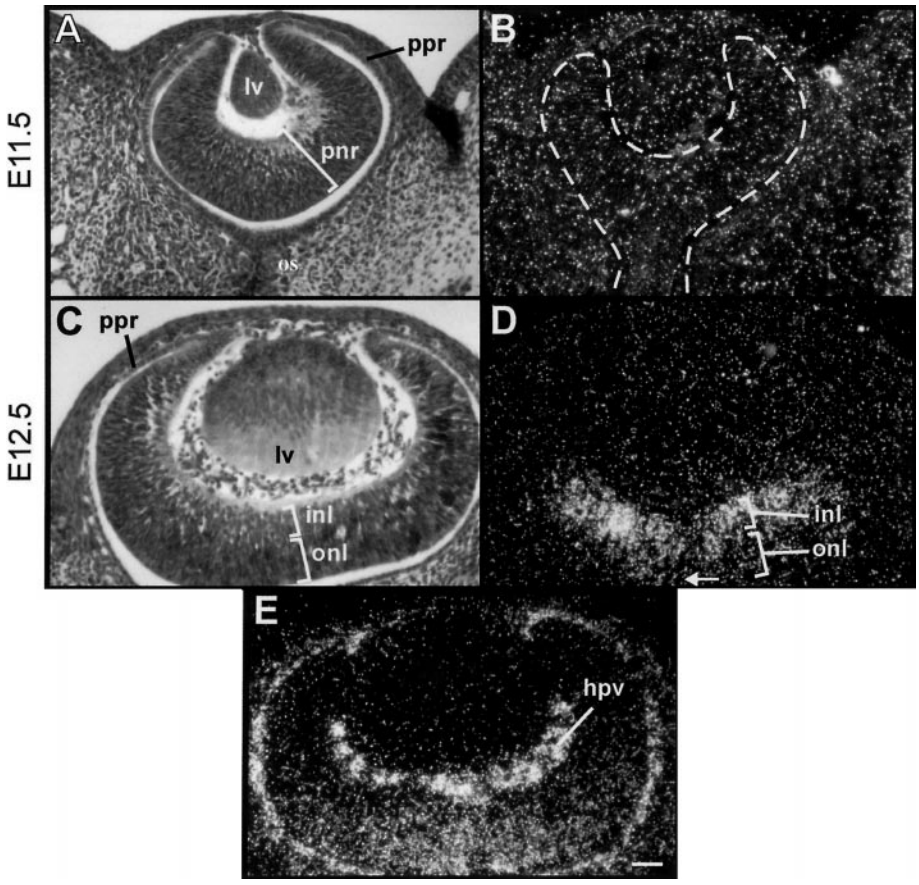

FIG. 1. DCC expression is initiated in the early neural retina between E11·5 and E12·5. DCC mRNA expression is not detected in transverse sections of the E11·5 eye (B). By E12·5, DCC mRNA is present in the central region of the neural retina (D). The arrow indicates expression at the outer edge of the onl. An adjacent transverse section of the E12·5 eye was hybridized with a control anti-sense riboprobe specific for the endothelial marker VEGFR-2 (E). Corresponding bright field images of adjacent hematoxylin and eosin stained transverse sections of the E11·5 (A) and E12·5 (C) eye. hpv, hyaloid plexus; inl; inner neuroblastic layer; lv, lens vesicle; onl; outer neuroblastic layer; os, optic stalk; ppr, presumptive pigment layer; pnr, presumptive neural layer of retina. Scale bar: 42 μm (A–E).

small fraction of all retinal neurons in the adult, are produced (Sidman, 1961; Cepko et al., 1996).

No DCC mRNA was detected in the E11·5 optic cup [Fig. 1(B)]. However, between E11·5 and E12·5 DCC mRNA expression was initiated. By E12·5, strong DCC mRNA expression was observed in the central region of the early retina [Fig. 1(D)]. Within the central retina, the most intense DCC expression was localized to the emerging inl where the first postmitotic neurons reside after migrating through the vz. In addition, weaker DCC mRNA expression was also seen in patches [arrow; Fig. 1(D)] at the outer edge of the vz where retinal progenitors are undergoing mitosis, and newly born neurons begin their migration toward the inl. Few DCC mRNA expressing cells occupied the inner regions of the vz in which S-phase cells reside. At this early stage of development the onl coincides with the vz. An adjacent hematoxylin and eosin stained serial section [Fig. 1(C)] shows that the peripheral edges of the optic cup are yet to develop the distinctive inner and outer neuroblastic layers. These peripheral regions comprise only the dividing neural progenitors. It is clear from Fig. 1(D) that no DCC mRNA expression was initiated in the peripheral regions at this stage.

By E13·5 the domain of high DCC mRNA expression in the inl above the vz had expanded towards the periphery, coincident with the broadening region of differentiation [Fig. 2(B)]. DCC mRNA expression was not detected in cells of the retina pigment layer or the developing lens, however some expression was noted in the developing eye lids. The proliferation of the neural progenitors at E13·5 was demonstrated by the extensive incorporation of the thymidine analogue, BrdU, within the vz [Fig. 2(A)]. The dark stained nuclei in Fig. 2(A) represent those neural progenitors progressing through S-phase during the 4 hr pulse period. The inl, containing only post-mitotic cells, is clearly demarcated with respect to the onl which contains the BrdU-positive proliferative population. DCC mRNA expression was most intense in the emerging inl of the E13·5 neural retina [Fig. 2(B)]. Towards the periphery of the retina the BrdU-positive cell population extended from the inner to the outer surface of the neural retina [vz; Fig. 2(A)]. In this region DCC mRNA was expressed in a decreasing

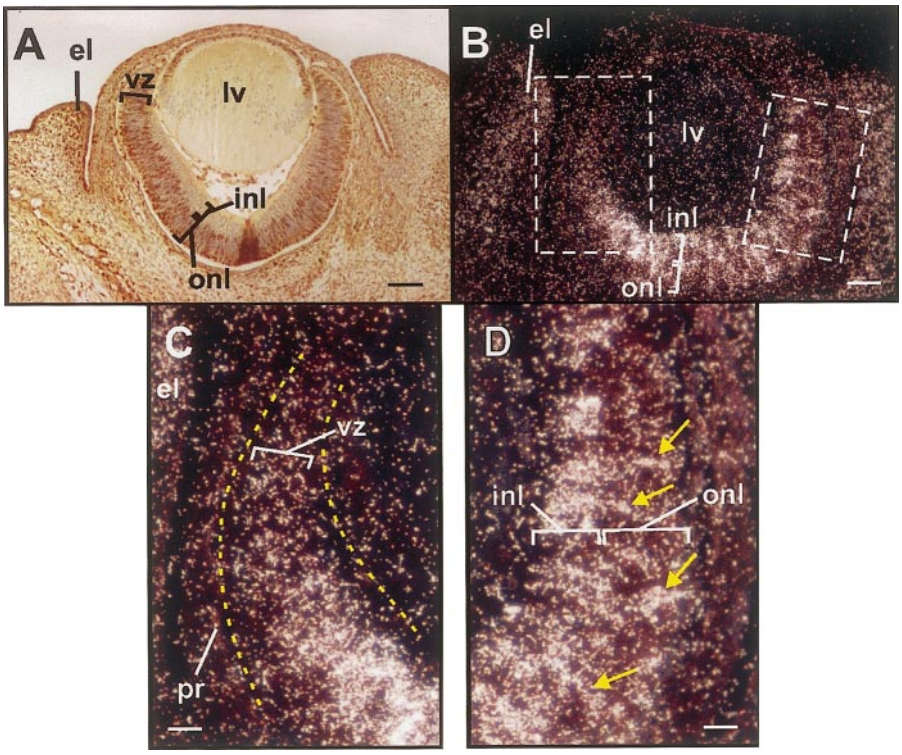

FIG. 2. By E13·5 the domain of DCC mRNA expression has expanded towards the periphery and is highest in the inl. BrdU incorporation at E13·5 (dark nuclei) (A) demonstrates that the inl is emerging above the vz. The vz coincides with the onl at this stage. Temporal is to the right and nasal to the left. Hybridization of an adjacent parasagittal section with the anti-sense DCC riboprobe demonstrates strong DCC mRNA expression in the inl (B). Higher magnification of the larger boxed region in B shows that DCC mRNA is absent from the most peripheral regions of the E13·5 retina (C). The dotted lines indicate the borders of the neural retina. Higher magnification of the smaller boxed region in (B) highlights the bright chains of DCC expressing cells (arrows) extending from the outer surface of the onl through to the inl (D). el, eye lid; inl, inner neuroblastic layer; lv, lens vesicle; onl, outer neuroblastic layer; pr, pigment layer; vz, ventricular zone. Scale bars: 90 μm (A), 77 μm (B), 190 μm (C), 220 μm (D).

gradient and had not yet been initiated at the extremities [Fig. 2(B) and (C)]. Interestingly, the region of DCC mRNA expression was more extensive in the temporal side of the developing retina when compared to the nasal region [Fig. 2(B)]. This may reflect a more advanced, differentiated state of the temporal vs nasal neural retina. A skewed differentiation gradient has also been noted in the developing *Xenopus* retina (Holt et al., 1988).

DCC is Expressed in Chains of Migrating Neurons

Bright chains of closely opposed DCC mRNA expressing cells were observed extending from the outer surface of the onl through to the expanding inl in the central region of the E13·5 retina [Fig. 2(B) and (D); arrows]. These chains were dispersed regularly throughout the region of the retina actively undergoing differentiation but were not observed in the undifferentiated peripheral regions [Fig. 2(C)]. This observation suggests that DCC mRNA expression was initiated in the earliest postmitotic neurons, immediately after they had undergone terminal differentiation, and was maintained as they migrated through the onl into the inl. Fig. 3 demonstrates that as the field of differentiation spread into the peripheral retina at E15·5 [Fig. 3(B)] and E16·5 [Fig. 3(D)] the intense domain of DCC mRNA expression above the onl also expanded towards the periphery. In the most peripheral regions, chains of cells strongly expressing DCC mRNA were again situated directly below the region of accumulating postmitotic neurons [E15·5; Fig. 3(B), E16·5; Fig. 3(H)]. In contrast, within the central E16·5 retina, where differentiation was more advanced, the DCC mRNA positive chains had disappeared [Fig. 3(F)]. These observations support the notion that DCC is expressed by newly-born retinal neurons as they migrate out of the vz.

DCC is Expressed in a Variety of Cell Types within the Retina

As retinal development proceeded to E15·5, DCC mRNA expression became restricted to two discrete domains that can be seen extending along the entire extent of the inner and outer edges of the retina [Fig. 3(B)]. High levels of DCC mRNA expression were observed throughout the inl. At this stage of development the majority of postmitotic neurons within this layer are amacrine cells and rgcs (Sidman, 1961). These cells are most likely the same DCC-expressing cells observed migrating in chains through the vz at

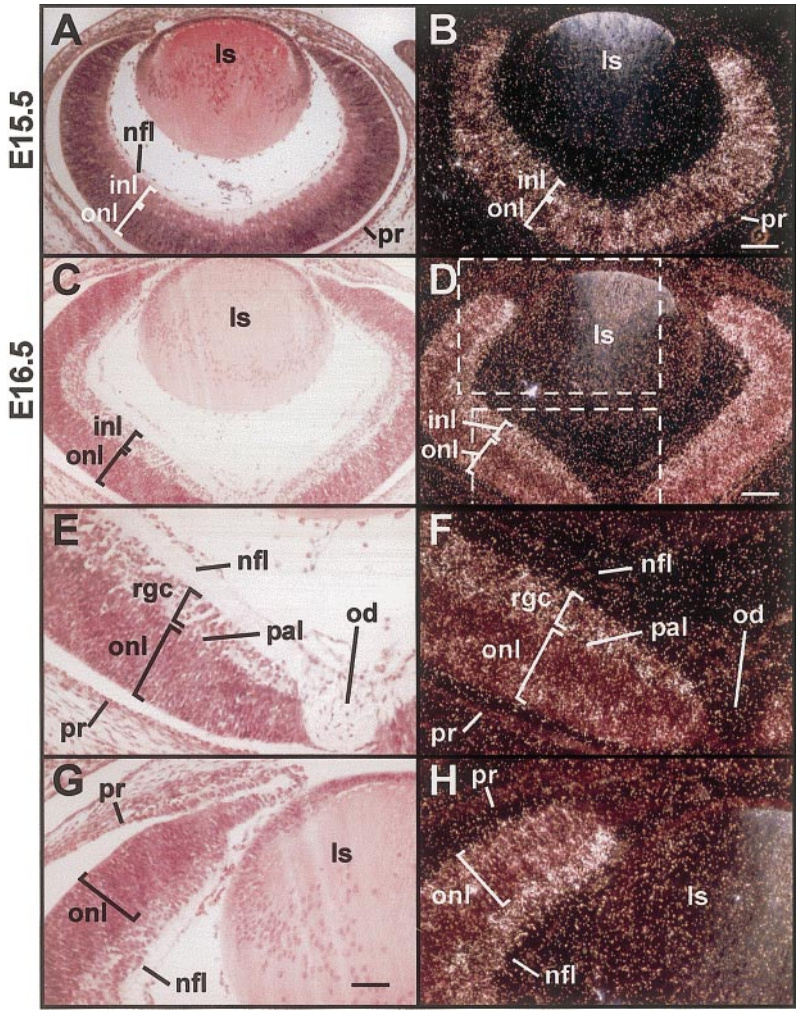

FIG. 3. Retinal ganglion cells and amacrine cells express DCC mRNA. At E15·5 (B) and E16·5 (D), high levels of DCC mRNA can be seen throughout the inl which contains newly differentiated RGCs and amacrine cells. Significant DCC mRNA expression was also detected in the outer region of the onl at this developmental stage. Higher magnification of DCC expressing cells (lower box in D) in the central retina at E16·5 (F). Higher magnification of the peripheral region of the E16·5 retina (upper box in D) shows DCC expressing cells continue to form distinct chains (H). Corresponding bright field images of adjacent hematoxylin and eosin stained transverse sections through the E15·5 eye (A) and parasagittal sections through the E16·5 eye (C, E, G). inl, inner neuroblastic layer; ls, lens; nfl, nerve fiber layer; od, optic disc; onl, outer neuroblastic layer; pal, presumptive amacrine layer; pr, pigment layer; rgc, retinal ganglion layer. Scale bars: 80 μm (A, B), 100 μm (C, D), 50 μm (E–H).

E12·5 and E13·5 (Figs 1 and 2). Also at this stage of development, DCC protein is present on the rgc axons within the nerve fiber layer [Fig. 5(A) and (B)] and on rgc axons passing through the optic disc [Fig. 6(A)].

Within the onl at E15·5, immunohistochemistry using the polyclonal antiserum, 2744, revealed elongated DCC-positive processes extending to the outer limiting membrane [Fig. 5(B); yellow arrows]. Such extended processes are characteristic of Müller glial cells indicating that DCC protein is produced by these glial cells. At the outer edge of the onl, cell bodies expressing significant levels of DCC protein were distributed sparsely [red arrows; Fig. 5(B)]. Overall, however, the low level of DCC protein detected in the onl at E15·5 is in contrast to the extensive expression of DCC mRNA seen throughout this region [Fig. 3(B)]. This disparity in the levels of DCC mRNA and protein expression may be due to the greater sensitivity of the in situ hybridization technique vs that of the immunohistochemical protocol employed. However, this disparity may also be due to regulation of DCC protein expression at the post-translational level since it has been demonstrated that DCC protein is a target for the ubiquitin degradation pathway (Hu et al., 1997).

By E16·5 of mouse development the neural retina shows increased evidence of stratification. Most of the ganglion, amacrine, cone and horizontal cells have been formed (Turner, Snyder and Cepko, 1990; Cepko et al., 1996) and the inl shows the first evidence of regionalization. Expression of DCC mRNA also becomes more regionalized at this stage [Fig. 3(D) and (F)]. Two distinct layers of DCC mRNA expressing cells were observed in the central E16·5 retina. The first of these DCC mRNA-positive layers coincided with the rgc layer and the presumptive amacrine cell layer located directly under the rgc layer [Fig. 3(F)]. The

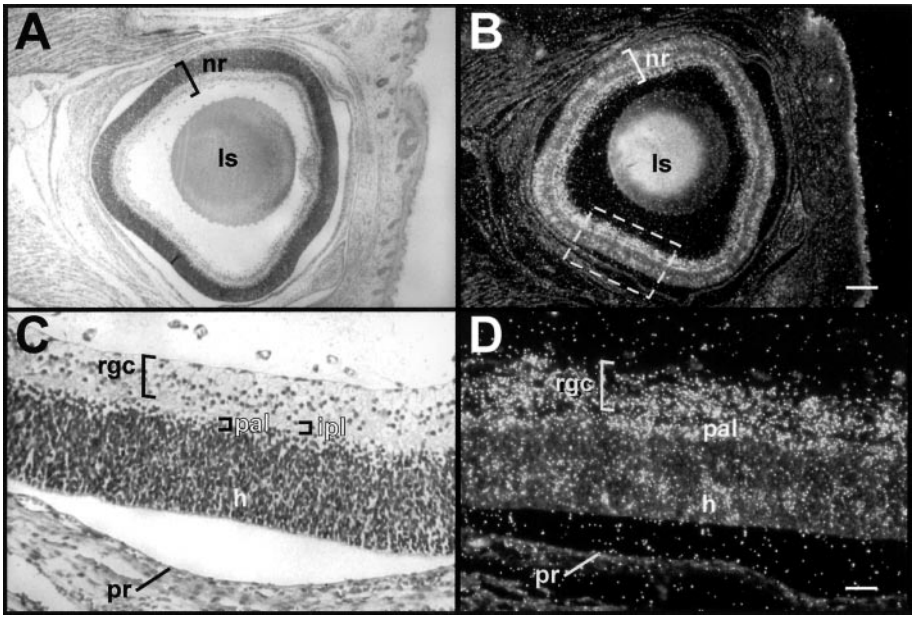

FIG. 4. DCC mRNA expression in the E18·5 retina. Bright field images of a hematoxylin and eosin stained parasagittal section through the E18·5 eye (A, C). An adjacent section hybridized with the anti-sense DCC riboprobe (B). Higher magnification of the E18·5 retina (box in B) showing DCC mRNA localization in the rgc, amacrine and horizontal cell layers (D). h, horizontal cell layer; ipl, inner plexiform layer, ls, lens; nr, neural retina; pal, presumptive amacrine layer; pr, pigment layer; rgc, retinal ganglion cell layer. Scale bars: 180 μm (A, B), 36 μm (C, D).

nerve fiber layer that overlays the inl has thickened by E16·5 indicating that an increased number of axons from the rgcs have extended into the optic disc [Fig. 3(C) and (E)]. DCC protein was still expressed on the axons within the fiber layer at this stage [Fig. 5(D); arrowheads] and on axons within the optic disc [Fig. 6(B)]. Amacrine cells bodies also express DCC protein at E16·5 [arrows; Fig. 5(C) and (D)].

The second DCC mRNA-expressing region in the E16·5 retina coincides with the outer region of the onl [Fig. 3(F)] which contains horizontal cells, cones and rods at this stage (Sidman, 1961). In contrast, no significant expression of DCC protein was detected within this region [Fig. 5(C)]. This may again be due to post-translation regulation of protein expression. DCC mRNA expression was almost completely absent from the intermediate region of the onl [Fig. 3(D) and (F)]. This area is predominantly occupied by proliferating cells and will eventually comprise of horizontal, bipolar, and Müller cells.

Differentiation of the neural retina continues until postnatal day 11. However by E18·5, all the horizontal cells, cones and most of the ganglion and amacrine cells have been born. A large percentage of rods have also been formed (Cepko et al., 1996). By E18·5 the rgc layer at the inner edge of the retina is well defined and retains strong expression of DCC mRNA [Fig. 4(B) and (D)]. At E18·5, the underlying acellular inner plexiform layer is now evident and the inner nuclear layer is forming which contains a large population of amacrine cells at its inner boundary (Sidman, 1961). Figs 4(D) and 5(F) demonstrate that the cells in this layer express high levels of DCC mRNA and protein. Finally, a band of DCC mRNA expressing cells was observed above the outer boundary of the neural retina [Fig. 4(B) and (D)]. This band of DCC expressing cells is well demarcated and aligns with the horizontal cell layer delineated by Sidman (1961). Again, we were not able to detect significant levels of DCC protein expression in this layer.

DCC Protein is Expressed on rgc Axons as they Navigate through the Optic Disc

Strong DCC protein expression was observed on the axons within the nerve fiber layer adjacent to the rgc layer at the inner edge of the inl from E15·5 through to E18·5 [Fig. 5(B), (D) and (F)]. At E15·5, all rgc axons within the optic disc also expressed high levels of DCC protein [Fig. 6(A)]. However, the intensity of DCC protein expression on those segments of the rgc axons residing in the optic nerve was greatly reduced by birth. Fig. 6(B) shows that DCC protein is present on only a subset of rgc fibers within the optic disc at E16·5. By E18·5, no DCC protein is detectable within the optic nerve [Fig. 6(C)] while protein is still present on the proximal rgc axons that lay within the nerve fiber layer overlying the rgc cells bodies [Fig. 5(F)]. These observations suggests that high levels of DCC protein are expressed on rgc axons only when axons are actively navigating through the optic disc.

Neogenin Expression in the Developing Eye

Very low levels of Neogenin mRNA were detected throughout the early neural retina at E11·5 (data not shown) and E12·5 [Fig. 7(B)] and therefore preceded the initiation of DCC mRNA expression by at least

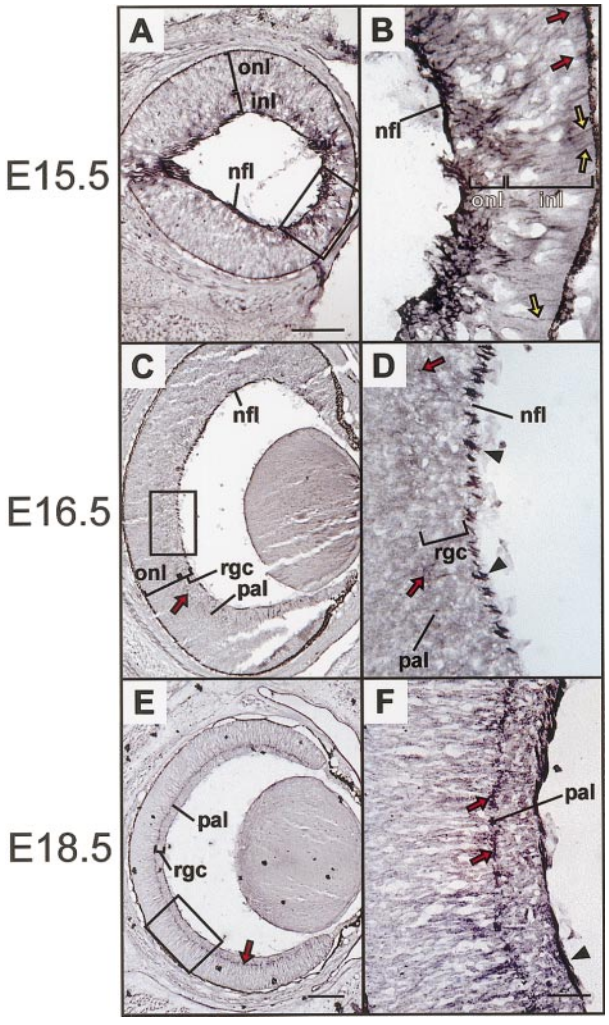

FIG. 5. Immunohistochemical analysis of DCC protein expression in parasagittal sections of E15·5, E16·5 and E18·5 retinae. At E15·5, DCC protein is seen in the rgc axons in the nerve fiber layer and in cell bodies throughout the inl (A, B). Red arrows in (B) indicate DCC expressing cells at the outer edge of the onl. Yellow arrows in (B) indicate Müller glial cell processes expressing DCC protein. DCC protein is expressed by amacrine cells (red arrows), rgcs and rgc axons (arrowheads) at E16·5 (C, D) and E18·5 (E, F). Control experiments in which the primary antibody was omitted produced no detectable staining (data not shown). inl, inner neuroblastic layer; nfl, nerve fiber layer; onl, outer neuroblastic layer; pal, presumptive amacrine layer; rgc, retinal ganglion cell layer. Scale bars: 200 μm (A, C, E), 50 μm (B, G, F).

24 hr. Neogenin mRNA expression had intensified by E15·5 [Fig. 7(D)] and become localized to the inl and the outer most region of the onl. No chains of Neogenin-expressing cells were observed in the vz as was seen with the DCC anti-sense riboprobe at these developmental stages.

Significant levels of Neogenin mRNA expression were also seen in the inner surface of the lens vesicle at E11·5 (data not shown) and E12·5 [Fig. 7(B)] where the forefront of fiber cells are extending towards the anterior region. At E15·5 [Fig. 7(D)], Neogenin expressing cells within the developing lens were situated in the equatorial region of the lens where the anterior capsular epithelial cells are gradually incorporated into the lens proper and develop into lens fiber.

By E16·5 Neogenin expression had localized to the emerging rgc layer [Fig. 8(B) and (C)] which also expresses DCC mRNA and protein at high levels. In contrast, Neogenin mRNA was no longer observed at the outer edge of the onl at this stage of gestation. At E17·5 significant levels of Neogenin mRNA were still seen in the rgcs [Fig. 8(E) and (F)]. Expression can also be seen in the pigment layer at E16·5 [Fig. 8(C)] and E17·5 [Fig. 8(F)]. Significant Neogenin mRNA expression was still present in the E16·5 lens [Fig. 8(B)]. At this stage of development the lens fibers have increased in length, with the most peripheral part of the lens being less differentiated. It is in this region of the lens that Neogenin expression is most intense. By E17·5, most, if not all, expression was lost from the lens [Fig. 8(E)]. Most lens cells have completed their elongation and migration to the anterior region of the lens at this stage of development.

4. Discussion

Within the CNS of the embryonic mouse, initiation of DCC mRNA expression is known to correlate with the onset of neurogenesis (Gad et al., 1997). Around the time of birth, DCC expression is down-regulated throughout the CNS except in structures such as the olfactory bulb, the hippocampus, and the cerebellum, which are known to sustain active neurogenesis well into postnatal life (Gad et al., 1997). Therefore, high levels of DCC mRNA expression are always associated with those regions of the developing CNS actively undergoing neurogenesis. We demonstrate here that strong DCC mRNA expression is also found in those regions of the neural retina where neural progenitors are actively differentiating (E12·5–E16·5). Within the E12·5 and E13·5 retina the most intense domains of DCC mRNA expression were found to coincide with the regions where the earliest formed retinal neurons were accumulating above the onl [Figs 1(D) and 2(B)]. These cells have been identified by Sidman (1961) as mostly differentiating rgcs, amacrine cells and some horizontal cells.

Significant DCC mRNA expression was also noted at the outer surface of the onl where mitosis occurs [Figs 1(D) and 2(D)]. We propose that these DCC expressing cells have just completed their final mitosis before migrating through the onl into the inl where the earliest rgcs and amacrine cells reside. The DCC mRNA-positive cells seen throughout the differentiating region of the neural retina [Fig. 2(D)] form an extensive network of chains. Since it is unlikely that DCC is expressed in cycling retinal progenitors (see below), the identity of the DCC expressing cells comprising these chains are likely to be newly born neurons migrating through the onl to take up their appropriate positions within the emerging inl.

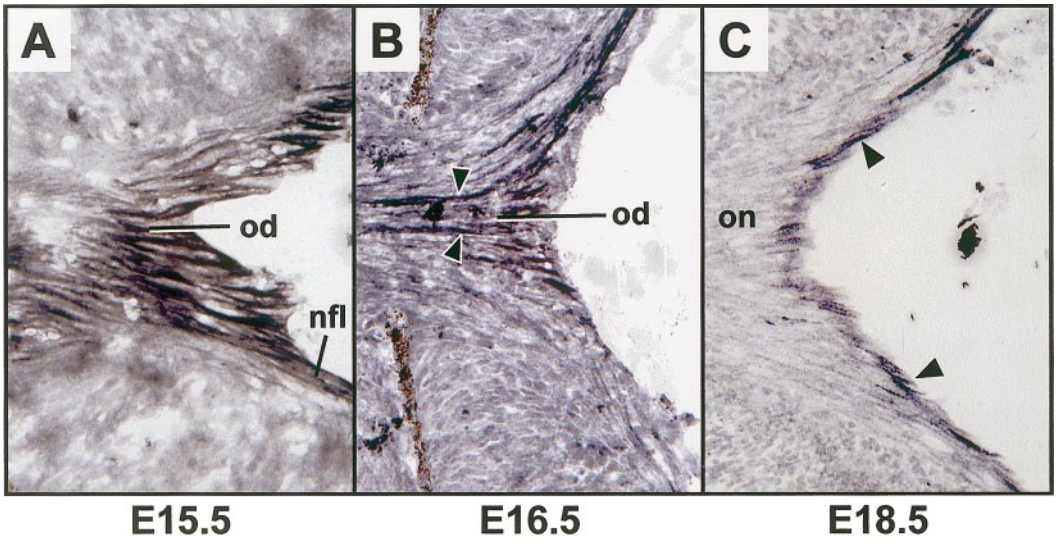

FIG. 6. Immunohistochemical analysis of DCC protein expression in parasagittal sections through the E15·5, E16·5 and E18·5 optic disc and optic nerve. Strong DCC protein expression is seen at E15·5 (A). At E16·5 (B) the number of fibers expressing DCC (arrowheads) is significantly reduced within the optic disc. No significant DCC protein is observed on RGC axons in the optic nerve by E18·5 (C) whereas DCC protein is present on proximal regions of these axons (arrowheads) within the nerve fiber layer. Control experiments in which the primary antibody was omitted produced no detectable staining (data not shown). nfl, nerve fiber layer; od, optic disc; on, optic nerve. Scale bars: 40 μm (A, C), 50 μm (B).

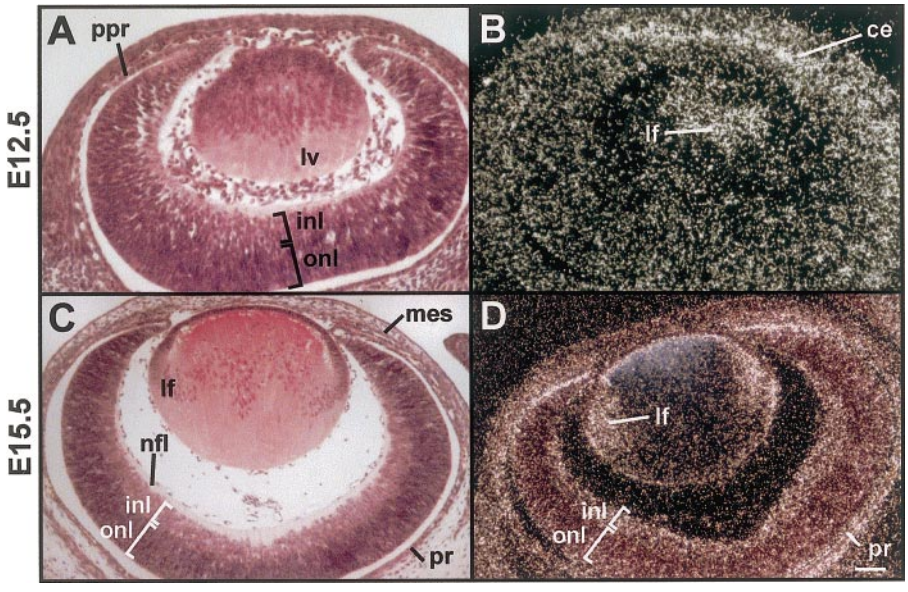

FIG. 7. Neogenin mRNA is found at low levels throughout the retina at E12·5 (B) and also in the lens vesicle. By E15·5, Neogenin expression is seen in the inl, the outer edge of the onl, the pigment layer, and in the region of dividing cells within the developing lens (D). Corresponding bright field images of adjacent hematoxylin and eosin stained transverse sections through the E12·5 (A) and E15·5 (C) eye. ce, corneal ectoderm; inl, inner neuroblastic layer; lf, lens fibers; lv, lens vesicle; mes, mesodermal condensation; nfl, nerve fiber layer; os, optic stalk; onl, outer neuroblastic layer; pnr, presumptive neural layer; ppr, presumptive pigment layer; pr, pigment layer. Scale bars: 42 μm (A, B), 80 μm (C, D).

That cycling neural progenitors do not express DCC mRNA is evidenced by the lack of hybridization signal in the more peripheral regions of the vz in the E12·5–E13·5 retina [Figs 1(D) and 2(C)]. In these regions, the progenitors are progressing through the cell cycle but little differentiation is occurring. The contention that DCC is not expressed in cycling neural progenitors is reinforced by our previous study of DCC expression using the thymidine analogue, BrdU, where it was clearly demonstrated that DCC mRNA expression colocalized with those postmitotic neurons that had recently migrated out of the vz throughout all regions of the embryonic mouse (Gad et al., 1997). DCC expression was never observed within the proliferative zones in our earlier study.

Previous studies have demonstrated the presence of DCC protein on rgcs and their axons within the nerve fiber layer, the optic disc, and within the optic nerve at E12 and E14 (Deiner et al., 1997) indicating that DCC is expressed on the earliest rgc fibers navigating through the optic disc. Our studies show that DCC protein expression remains high on following rgc

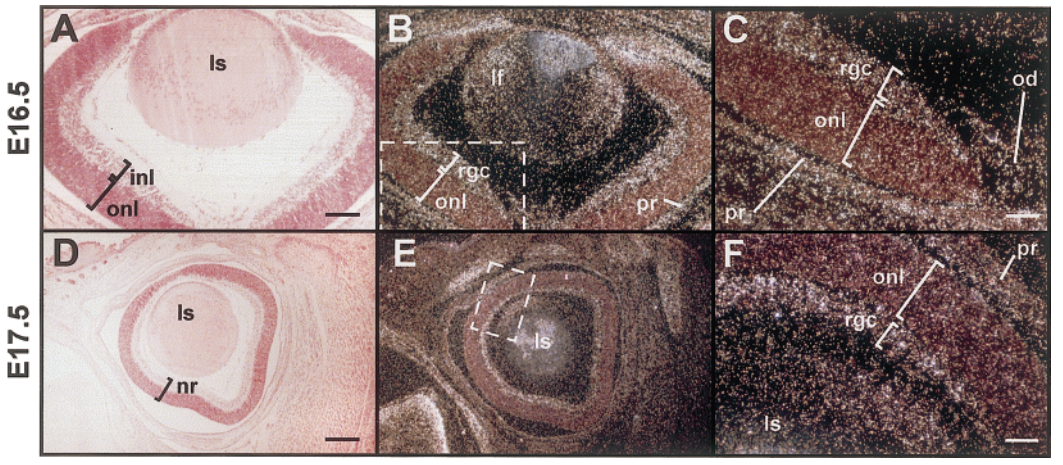

FIG. 8. At E16·5 (B, C) and E17·5 (E, F), Neogenin mRNA is expressed within the RGC layer and is also detected in the pigment layer and in the elongating cells of the lens. Higher magnification of the central retina (boxed region in B) at E16·5 (C). Higher magnification of the boxed region in (E) at E17·5 (F). Corresponding bright field images of adjacent hematoxylin and eosin stained parasagittal sections through the E16·5 (A), and E17·5 (D) eye. lf, lens fiber; nr, neural retina; od, optic disc; onl, outer neuroblastic layer; pr, pigment layer; rgc, retinal ganglion cell layer. Scale bars: 100 μm (A, B), 50 μm (C), 300 μm (D, E), 30 μm (F).

axons as they navigate through the disc at E15·5 and E16·5 (Fig. 6). Thus, DCC is present not only on the pioneering axons but is also present on the majority of axons subsequently projecting through the disc. This observation implicates DCC as an important guidance receptor for all axons navigating through the optic disc throughout retinal development.

We further observed that the intensity of DCC protein expression on distal segments of the rgc axons residing within the optic nerve was greatly reduced around birth. By E18·5, no DCC protein was detectable within the optic nerve while protein was still present on the proximal regions of the rgc axons that lay within the nerve fiber layer [Fig. 6(C)]. These observations suggest that high levels of DCC protein are expressed on rgc axons only when they are actively navigating through the optic disc. We have observed a similar phenomenon in a variety of projecting axons within the developing mouse forebrain (Shu et al., 2000). For example, high levels of DCC protein are present on cortical axons (originating from neurons in cortical layers 5 and 6) as they actively project through the internal capsule. However, expression is greatly reduced on the distal regions of these axons after the targeting phase has been completed whereas significant DCC expression is still apparent on the proximal axonal membranes of these neurons. Thus, localized down-regulation of DCC protein on projecting axonal membranes when the DCC-guidance function is no longer required may be a general mechanism for controlling axon navigation throughout the embryonic CNS. Such regulation of DCC protein expression at the post-translational level may occur via the ubiquitin degradation pathway (Hu et al., 1997).

DCC mRNA and protein expression were also detected in Müller glial cells, and cells within the amacrine cell layer. DCC mRNA was also expressed in the horizontal cell layer. Recently it has been demonstrated that the guidance of rgc axons through the optic disc is dependent on the DCC guidance receptor (Deiner et al., 1997). However, the axonal projections of the amacrine and horizontal cells have not yet been examined in these mice. The data presented here suggest that DCC may also be employed as an axon guidance receptor for these neural cell types. This notion is supported by the observation that DCC protein expression is highest in the amacrine cell layer during the period when axons are actively extending (Fig. 5). DCC protein has also been observed in both the amacrine and horizontal cells of the developing *Xenopus* retina (de la Torre et al., 1997).

Initiation of Neogenin mRNA expression preceded DCC mRNA expression by at least 24 hr with low levels of Neogenin detected throughout the early neural retina at E11·5–E12·5 (Fig. 7). In contrast to DCC, the expression of Neogenin mRNA in the late embryonic retina (E17·5; Fig. 8) is restricted to the mature rgc layer. The degree of optic nerve hypoplasia observed in mice lacking DCC was found to be highly variable with some mutant retinae displaying only a mild reduction in optic disc size (Deiner et al., 1997). In these instances Neogenin may be replacing DCC as the rgc axon guidance receptor steering rgc axons through the optic disc. Immunostaining using a monoclonal antibody that recognizes chicken Neogenin has also demonstrated Neogenin protein on the rgc axons in the developing chick retina (Vielmetter et al., 1994). In addition, this study reported antibody staining in both the inner and outer plexiform layers suggesting Neogenin expression in amacrine, bipolar and horizontal cells. In the mouse, amacrine and horizontal cells express DCC mRNA but we did not detect Neogenin mRNA in these cells. Since the extracellular domains of DCC and Neogenin are closely

related at the amino acid level (Keeling et al., 1997) it is possible that the monoclonal antibody used in this chick study is also able to detect DCC protein. Finally, strong Neogenin expression in the extending fiber cells of the developing lens suggests a role for Neogenin in the migrational events shaping the early lens.

The nature of the DCC and Neogenin ligands within the embryonic mouse retina is unclear. In the developing chick retina a relatively steep gradient of netrin-1 protein originating from the outer edge of the onl and extending to the inl has been reported (MacLennan et al., 1997). However, Deiner et al. (1997) were unable to detect any netrin-1 protein within these regions of the E11, E14 or E16 mouse retina. This lack of netrin-1 expression suggests that other DCC and Neogenin ligands are present. Recently, a second mouse netrin, netrin-3, has been identified (Püschel, 1999; Wang et al., 1999), however its expression pattern within the developing retina has not yet been determined.

Retinal neural progenitors are multipotential and are capable of giving rise to all cell types found within the adult neural retina (Cepko et al., 1996). In addition, it is believed that retinal cell fate is dictated by the local environment of the neuroblast (Cepko et al., 1996). The spatial and temporal pattern of DCC mRNA expression within the early retina indicates that DCC expression is initiated immediately after the neural progenitors have undergone their final division. This raises the intriguing notion that DCC may play a role in the cell fate determination of the rgc and amacrine cell populations. The continued high level of DCC expression observed in the young neurons as they migrate toward the emerging inl further suggests that DCC may play a role in the migration of retinal cells through the onl. DCC expression is not restricted to a specific mature retinal cell type but is expressed by Müller glial cells and rgcs, as well as in the amacrine and horizontal cell layers. Thus, the DCC guidance receptor is likely to be involved not only in axonal navigation of rgc axons through the optic disc but is also likely to be involved in establishing the intricate network of axonal and dendritic connections between the distinct neural populations observed in the adult retina.

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