Newborn horizontal cells migrate bi-directionally across the neuroepithelium during retinal development

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Summary

Cell migration plays an important role during the development of the retina. In this work we have studied the migration of newborn horizontal cells in avian embryonic retina. Using the pattern of the early expressed transcription factors Lim1 and Prox1 we have shown that horizontal cells migrate bi-directionally from their site of birth, close to the ventricular side, to the adjacent (vitreal) side of the neuroepithelium, where they align just next to the prospective ganglion cell layer before migrating back again to their final laminar position in the external part of the inner nuclear layer. The migration occurs between Hamburger and Hamilton stages 24 and 33, which is equivalent to embryonic day 4.5 and 8. Between stages 26 and 30 the horizontal cells reside close to the ganglion cell layer and intra ocular injections of a cytochalasin D, an actin polymerisation blocker that inhibit migration, at stage 29 interfered with the migration of the horizontal cells to their final destination. Furthermore, using biolistic gene transfer with a green fluorescence protein expression vector of retinal slices we were able to record ventricle-directed migration by time-lapse microscopy. Combining biolistics with immunohistochemistry we showed that transfected cells, which have also been translocated in a ventricular direction were positive for the horizontal cell markers Lim1 and Prox1. The alternative path of migration that is described in this work differs from the generally accepted one for horizontal cells and this knowledge will influence the view of how the molecular determination of horizontal cells is specified.

Key words: Biolistic gene transfer, Chicken, Cytochalasin D, GABA, Lamination, Lim1, Migration, Prox1, Retina

Introduction

A crucial element in the development of a mature and functional central nervous system (CNS) is the migration undertaken by newborn neurons in order to position themselves in appropriate layers. This is a complex process governed and guided by intrinsic and extrinsic factors and several distinct modes of migration have been identified in the developing cerebral cortex alone (Nadarajah and Parnavelas, 2002). Contrary to most other parts of the CNS, the retina is not believed to develop in an obvious inside-out or outside-in fashion. The retinal neurons are generated during a short period at early embryonic stages and the cells can in principle populate any layer in the retina (Adler and Belecky-Adams, 1999; Livesey and Cepko, 2001; Prada et al., 1991). Although cellular birth dates and factors influencing cell fate for the six classes of neurons in the retina are available (Altshuler et al., 1991; Jean et al., 1998) less is known about retinal cell migration during the lamination process.

Currently there are two modes of migration identified in the developing avian retina (Prada, 1983). Early in chick retinal development, before Hamburger and Hamilton stage (st) 22-25 (equivalent to embryonic day 4, E4), cycling progenitor cells are undergoing radial migration manifested by a nuclear translocation across the neuroepithelium, while the cellular end-feet remain connected to the ventricular zone. After cell division at the ventricular side, post-mitotic cells that are committed to a certain precursor lineage, are thought to detach their end-feet and migrate freely to a specific lamina where they will start the maturation process. The free migration is predominant in chicken retina from st24 to st33. Furthermore, after the initial period (from st25 and beyond) with uniform mitotic activity over the entire surface of the retina, the development spreads in a centrifugal gradient both in a dorsal to ventral and nasal to temporal direction. Thus, the central parts of the retina are always more mature relative the peripheral parts (Prada et al., 1991). The first cells to withdraw from the cell cycle and differentiate are the retinal ganglion cells, followed by the overlapping birth of amacrine cells, horizontal cells (HC), cone photoreceptors and Müller glia cells. The last cells to withdraw from the cell cycle are the bipolar cells and rod photoreceptors. The majority of the retinal ganglion cells, amacrine cells and HC withdraw from the cell cycle around st19-22 (E3) but the cellular birth of these classes are not fully completed until st25-28 (Altshuler et al., 1991; Kahn, 1974; La Vail et al., 1991; Young, 1985).

This work was initiated by an observation in our previous work (Karlsson et al., 2001), in which we found that cells thought to be HCs and that expressed nerve growth factor and its receptor TrkA, were translocated from the vitreal to the

ventricular side of the retina. In the present study we have investigated how prospective HC, defined here as cells positive for the homeobox-containing transcription factors Lim1 (Lhx1) and Prox1 (Adler and Belecky-Adams, 1999; Dyer et al., 2003; Liu et al., 2000; Tomarev et al., 1996), migrate during retinal development. The current view holds that HCs migrate only a short distance from their place of birth to their final position (Adler, 2000; Mey and Thanos, 2000; Prada, 1983; Stone, 1988). However, based on the distinct spatiotemporal changes of the Lim1/Prox1 labelling patterns, we propose that newborn Lim1-positive (+) HC precursors undergo a previously undocumented pattern of free migration before attaining their final position in the external part of the inner nuclear layer. The results do not exclude that other cells may take a similar migration route. In short, the observed migration starts at st24-26, when newborn horizontal cells first undergo vitreal-directed migration from their site of birth to the lining of the prospective ganglion cell layer where they reside from st27-30, before undergoing a phase of ventricle-directed migration to reach their definite position in the external part of the inner nuclear layer by st33-34. In order to further collect evidence for the proposed migration we inhibited cell motility using a cytoskeleton inhibitor and using biolistic gene transfer together with timelapse microscopy we could document ventricle-directed migration.

Materials and methods

Embryos and embryo culture

Fertilised White Leghorn eggs were incubated in a humidified incubator at 37°C. Prior to any manipulations or analysis, embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Alternatively, eggs were incubated for 72 hours before they were aseptically cracked open into sterile high wall Petri dishes (#1005, Falcon) and the embryo cultured therein at 37°C (Dunn, 1974). Manipulations of embryo cultures were subsequently carried out at the desired stages. The animal work follows the European community guidelines and the ARVO statements for use of animals in ophthalmic and vision research. Experiments were scrutinized by the local ethics committee for experimental animals.

Time-lapse and particle-mediated gene transfer

Particle-mediated gene transfer ('biolistics') using a Helios Gene gun (BioRad) was used to transfect retinal cells with a green fluorescent protein (GFP) expression vector (Lo et al., 1994; Yang et al., 1990). One micron gold particles (BioRad) were coated with the pEGFP-C3 plasmid. Cartridges for gene gun transfection were prepared according to the manufacturer's instructions. Retinas for transfection were dissected in temperature controlled Neurobasal medium (no. 21103, Invitrogen, supplemented with 0.05 M Hepes and 1% penicillin/streptomycin) and flat-mounted with the ganglion cell layer facing up on a nitrocellulose filter (13006-50-N; Sartorius). After flat-mounting, the remaining medium was carefully removed not to obstruct the gold particles. The tissue was then immediately bombarded at 160 psi using the Gene gun. The He gas pressure used to accelerate the gold particles was calibrated so that the particles would penetrate up to 25 μm into the retina. Thus, only cells with their soma located not more than a few cell diameters into the retina were hit by the particles. Retinas on filters were then incubated in medium at 37°C in a humidified atmosphere containing 5% CO_2 for various times. After bombardment, the retinas were incubated for 4-6 hours before being cut into 0.20 mm thin slices using a vibratome and several slices with retina were positioned on a glass coverslip in the bottom of a Syke's Moore chamber (no. 1943-11111, Bellco Glass Inc., USA). The slices (flipped 90 degrees relative to the cut) were fixed using Vaseline so that a cross section of the retina was visible. The chamber was then filled with medium, sealed and coupled to a temperature controller (FCS-100, Shinko Technos, Japan) keeping a constant temperature at 37°C. Regions with many successfully transfected cells were selected and the time-lapse specimens were studied using a Zeiss Axioplan2 microscope with Axiovision software (3.0.6.1, Carl Zeiss Vision GmbH). For combined GFP transfection and immunohistochemistry, retinas were transfected as above and kept flat-mounted under the same conditions for the desired time (ranging from 15 minutes to 24 hours). The flat-mounted retinas were subsequently fixed with 4% PFA at 4°C, washed in PBS, cryoprotected in 30% sucrose, embedded in OCT (Sakura, The Netherlands), frozen, cut in 10 μm thick sections using a cryostat and collected on SuperFrost Plus glasses (Menzel-Gläser, Germany). Immunohistochemistry was carried out as described below.

BrdU incorporation

For 5-bromo-2′-deoxyuridine (BrdU) incorporation, 40 μg of BrdU (Sigma, St Louis, MO) was injected into the yolk of embryo cultures 12-16 hours before the desired stage of analysis. The embryo was kept in culture until analysis and the eyes were prepared for immunohistochemistry as described below. Only the central part of the retina was used to count cells that had incorporated BrdU, in order to adhere to a specific embryonic stage.

Migration inhibition assay

To inhibit migration, 0.2-2 μl of 2 mM actin polymerisation inhibitor cytochalasin D (Bruijns and Bult, 2001) (Sigma-Aldrich) was injected into the vitreous body of st29 embryos using pulled capillaries and a SP100i digital infusion syringe pump (World Precision Instruments, Sarasota, FL). The cytochalasin D was dissolved in 62% DMSO and was supplemented with Fast Green (C8686, Kodak) for visualisation purposes. Injections were carried out four times at 12-hour intervals and the embryos were sacrificed upon reaching st33. We also carried out injections with the microtubule inhibitor Colcemid (Molecular Probes, Leiden, The Netherlands) at a concentration of 100 μM (containing 1% DMSO) with 24 hours intervals. Apart from the frequency of the injections, the procedure was otherwise similar to that for cytochalsin. Controls received vehicle solution also containing Fast Green using similar regimens.

Immunohistochemistry

After dissection, whole eyes were fixed in 4% PFA at 4°C, washed with PBS for 10-15 minutes, cryoprotected in 30% sucrose at 4°C, embedded in OCT freezing medium, frozen and cut in a cryostat. Horizontal sections, 10 μm thick (12 μm for st44 and st45 eyes) were taken from the centre of the specimen at the level of the lens and collected on SuperFrost Plus glasses. For immunohistochemistry, the sections were rehydrated in PBS for 15 minutes and then blocked in PBS containing 1% foetal calf serum and 0.1% Triton X-100 for 30 minutes. Primary and secondary antibodies were diluted in this solution. Primary antibodies were allowed to react with the samples for 2 hours at room temperature or overnight at 4°C, and secondary antibodies for 2 hours at room temperature. Primary antibodies used in this study were directed towards Lim1/2 (1:5-50, 4F2, Developmental Studies Hybridoma Bank DSHB), Ng-CAM [1:2000, (de la Rosa et al., 1990)], Prox1 (1:4000, gift from Dr M. Nakafuku), GABA (1:1000, A2052; 1:500, A0310, Sigma), Pax6 (1:200, PAX6, DSHB), Lim3 (1:200, 67.4E12, DSHB), Ap2α (1:200, 3B5, DSHB), Islet1 (1:200, 40.2D6, DSHB), Chx10 (1:4000, gift from Dr J. Ericson), calretinin (1:1000, 1741-1007, Anawa, Zürich), Brn3a (1:100, MAB1585, Chemicon), Brn3b (1:200, sc-6026, Santa Cruz) and BrdU (1:50, M20105S, Biosite). Secondary antibodies were obtained from Vector Laboratories (Burlingame, CA) and Molecular Probes (Leiden, The Netherlands) and diluted 1:200. Sections were studied using a Zeiss Axioplan2 microscope and Axiovision.

Graphical enhancing and preparation for publication were performed using Axiovision and Photoshop (v6.0.1, Adobe).

Results

Horizontal cell precursors undergo free bi-directional migration

We have used transcription factors in early HCs to study their development during phases of chick retinogenesis. Antibodies directed towards the transcription factor Lim1 reveal a distinct and gradual spatial-temporal change in labelling pattern during st24-35 (Fig. 1A-I). Lim1 immunoreactivity is present already at st21 (Fig. 2A) in the central area of the retina. At this stage and until st24, labelled cells are mostly scattered throughout the neuroepithelium (Fig. 1A). From st25 until st27, some Lim1+ cells are still scattered throughout the neuroepithelium although it is clear that, in the central part of the retina, Lim1+ cells are lining up on the vitreal side of the neuroepithelium directly below the prospective ganglion cell layer (Fig. 1B). During st28-29 the pattern observed at st27 has spread throughout the periphery and while the majority of Lim1+ cells in the central retina are present on the vitreal side, occasional cells can still be found in the middle of the neuroepithelium. At st29 the alignment of Lim1+ cells at the vitreal side is complete (Fig. 1C). By st30-32 the vitreal alignment of Lim1+ cells breaks up and a massive ventricle-directed migration is observed, particularly at st31 when the HCs again become largely scattered throughout the neuroepithelium (Fig. 1D-F). By st32 a second alignment of Lim1+ cells at the external part of the future inner nuclear layer become increasingly obvious and by st33 all Lim1+ cells in the central retina have reached their final destination in the inner nuclear layer (Fig. 1F-G). From st33 and beyond the Lim1 expression pattern remains stable throughout development (Fig. 1G-H). In the less mature peripheral areas though, the Lim1 pattern described above can still be observed for some time (Fig. 1I-J). At no time during development did Lim1+ cells co-label with the retinal ganglion cell marker, Ng-CAM (Fig. 1A-H) or any of the Brn3 transcription factors (results not shown).

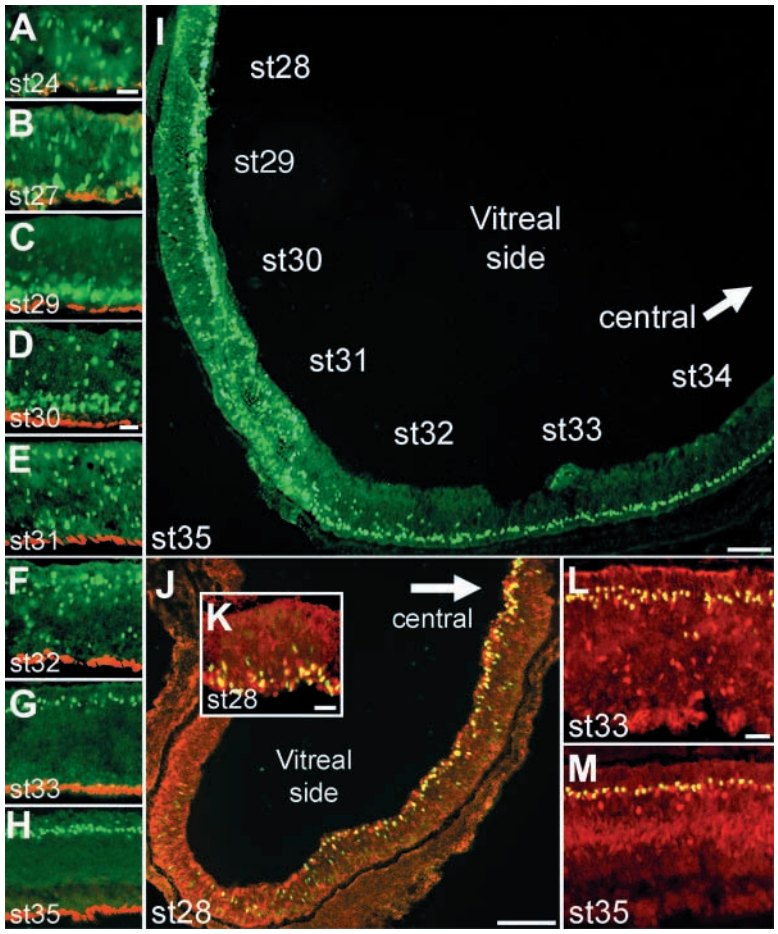


Fig. 1. Fluorescence immunohistochemistry for Lim1 and Prox1 in stage 24-35 developing avian retina. Micrographs showing immunohistochemistry for (A-H) Lim1 (green) in horizontal cells (HCs) and Ng-CAM (red) on ganglion cells in stage 24-35 retina. (I) Overview of Lim1 immunoreactivity in stage 35 retina. The gradual spatial maturation with corresponding developmental stages are indicated. (J-M) Prox1 (red) and Lim1 (green) immunoreactivity in (J, K) stage 28, (L) stage 33 and (M) stage 35 retinas. Arrows in I and J indicate the central region of the retina. st, stage. Scale bars: in A (for A-C), D (for D-H), K, L (for L-M) are 20 μm and I (for I-J) is 100 μm.

Lim1 and Prox1 patterns largely overlap in horizontal cell precursors

Another marker for early retinal development shown to be expressed in both putative HC, bipolar cells and some amacrine cells is the transcription factor Prox1 (Adler, 2000; Belecky-Adams et al., 1997; Dyer et al., 2003). We used antibodies directed toward Prox1 together with Lim1 antibodies to confirm our observations. Prox1 could not be detected in the retina before st24. The Prox1 expression at this stage was diffuse and labelled cells were found scattered throughout the NE. From st28 and beyond, an extensive overlap of the patterns was observed as most labelled cells started to co-express both markers while aligned next to the putative ganglion cell layer. Occasional cells that were only positive for one of the two markers were noticed (Fig. 1J-K). By st33, extensive co-expression between Prox1 and Lim1 was found in the future HC layer, and simultaneously, cells only positive for Prox1 were found scattered outside the HC layer at various positions in the inner nuclear layer (Fig. 1L). It is plausible that these Prox1+ cells are HCs that migrate in a ventricular direction. At st35 cells, which are probably bipolar cells, form a distinct layer in the outer part of the internal inner nuclear layer (Fig. 1M; Fig. 5B,E,H). In the HC layer all Lim1+ cells were also Prox1+, but not all Prox1+ cells were Lim1+.

Migrating Lim1+ cells are post-mitotic

To determine whether the migrating cells were post-mitotic and to exclude the possibility that the migrating Lim1+ cells were cycling progenitors that translocate their soma, we injected BrdU into the yolk of embryo cultures at different stages of development and analysed incorporation 12-16 hours later. When BrdU was injected at early stages and analysed at st21 and st23, as much as one third of the Lim1+ cells were also

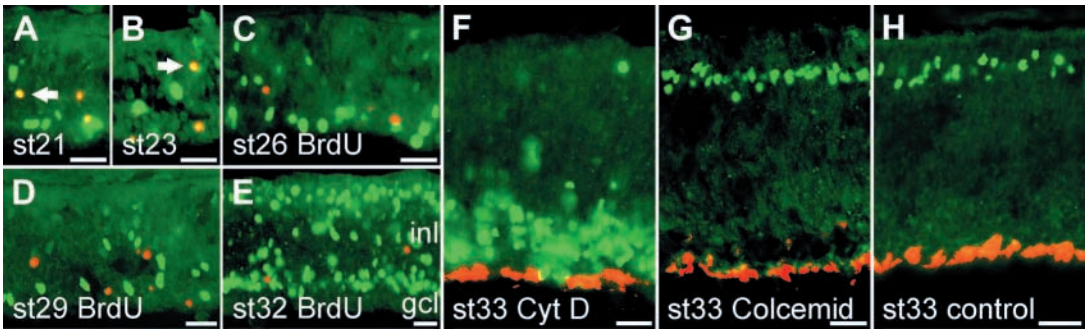


Fig. 2. Micrographs showing Lim1 immunohistochemistry in BrdU-, cytochalasin D- and Colcemid-treated retinas. Fluorescence micrographs showing (A-E) Lim1 (green) and BrdU (red) labelling in retinas at different developmental stages. See also Table 1. Arrows in A and B indicate double labelled cells. (F-H) Micrographs showing Lim1 (green) and Ng-CAM (red) in (F) cytochalasin D-treated, (G) Colcemid-treated and (H) control-treated retinas. Embryos were analysed at stage 33. gcl, ganglion cell layer (always oriented down); inl, inner nuclear layer; st, stage. Scale bars: 20 μm.

BrdU+, showing that these cells had undergone S-phase during the time between injection and analysis (Fig. 2A,B, Table 1). Embryos injected and analysed at later stages (st26, st29 and st32) showed few or no cells with co-labelling for Lim1 and BrdU when analysed (Fig. 2C-E, Table 1). Our result is in concordance with the reported birthdays for HCs according to some reports (Altshuler et al., 1991; Kahn, 1974) but in disagreement with some other reports (Adler and Belecky-Adams, 1999; Prada et al., 1991) that place the birthdays of HCs between E6-7 (st28-33). These results show that Lim1+ HCs are post-mitotic before undergoing bi-directional migration.

Cytochalasin D but not Colcemid inhibits ventricle-directed migration

In order to determine that the observed spatiotemporal Lim1 pattern did not originate from transient Lim1 gene expression in different layers of the retina we inhibited migration in the retina at the time before the ventricle oriented migration. We injected cytochalasin D, a blocker of actin polymerisation known to inhibit migration (Bruijns and Bult, 2001), into the eyes of st29 embryos. The inhibitor was administered at 12-hour intervals and the embryos were analysed upon reaching st33. In all treated eyes, an almost complete inhibition of migration was observed as the vast majority of Lim1+ cells could be found on the vitreal part of the retina by the time of analysis (Fig. 2F). In control embryos, only treated with vehicle, all Lim1+ cells had reached the external part of the inner nuclear layer by the time of analysis (Fig. 2H). When administering lower doses of cytochalasin D the migration was only partially obstructed as Lim1+ cells could be found scattered at all levels in the retina by the time of analysis (results not shown). When injecting 2 μl of the microtubule inhibitor Colcemid (Glasgow and Daniele, 1994), at a concentration of 100 μM at 24-hour intervals we saw no effect on the migration (Fig. 2G). Effects on cells in M-phase could however be seen. Higher concentrations of Colcemid or more frequent injections led to the death of embryos and could therefore not be assessed. These results indicate that the HC migration is an actin-dependent and post-mitotic process in accordance with the accepted migration mechanism.

Table 1. Number of Lim1-positive cells in embryonic retina with BrdU incorporation

Stage*	*n*	Lim1 cells†,‡	Lim1+BrdU+†,‡	%§
21 (E3.5)	10	12.4±3.0	2.0±1.3	16.1
23 (E4)	12	30.3±6.4	8.7±1.5	28.7
26 (E5)	9	32.6±7.7	2.6±2.0	7.9
29 (E6.5)	5	51.2±6.6	0.0±0.0	0
32 (E7.5)	5	125.2±9.0	0.0±0.0	0

The yolk sacs of in vitro cultured embryos were injected with BrdU 12-16 hours before analysis using immunohistochemistry for Lim1 and BrdU. The total number of Lim1+ and the number of Lim1+BrdU+ labelled cells were counted in the central region of the retina. See Fig. 2A-E. *n*=number of retinas analysed.

*Stages according to Hamburger and Hamilton (Hamburger and Hamilton, 1951)

†Number of Lim1 cells in representative part of central retina from embryo injected with BrdU 12-16 hours before the analysis.

‡±s.d.

§Percentage of Lim1+BrdU+ out of total number Lim1+ cells.

Time-lapse microscopy at st31 reveals ventricle-directed migration

We also used a direct approach to study if cells undergo ventricle-directed migration in the retina, which would be in agreement with the hypothesised HC migration. Dissected and flat-mounted st31 retinas were bombarded on the vitreal side (ganglion cell layer-face) by 1 μm gold particles coated with a GFP expression vector using a Gene gun. The acceleration of the gold particles was calibrated so that the particles would penetrate up to 20-25 μm into the retina. Thus, only cells with their soma located not more than a few cell diameters into the retina were hit (Fig. 3F). A fraction of those cells were transfected and later expressed the transgene. GFP expression could be detected after 30-60 minutes. For fluorescence time-lapse microscopy, 200 μm thick slices of the biolistically transfected retinas were cut and mounted in a microscope-fitted tissue chamber. The results showed that GFP-expressing cells could be found in the internal regions of the neuroepithelium (Fig. 3) and the time-lapse analysis showed that cells migrated away from the vitreal side of the neuroepithelium (Fig. 3A-C).

In order to determine if cells that were transfected by the GFP expression vector using the Gene gun method belong to the HC population, we bombarded retinas and analysed sections taken 18-24 hours after bombardment, using immunohistochemistry. Experiments showed that cells expressing GFP that were located in the internal parts of the retina could be labelled for Lim1 (Fig. 3D) and Prox1 (Fig. 3E) while those GFP-expressing cells that remained on the vitreal side were positive for the retinal ganglion cell markers Islet1

(Fig. 3G) and Ng-CAM (Fig. 3F,H). Some GFP-expressing cells in the internal part of the retina remained unlabelled after staining with Lim1 and could represent a small population of mitotic cells as shown in Fig. 2D,E. The results also showed that gold particles that did not lead to successful transfection were transported into the internal retina with time (Fig. 3F compare with E,G,H). These are particles that either have not successfully transfected the cells but have penetrated the cells or are particles that were not successfully loaded with expression vector. The vast majority of the delivered particles belong to these categories (Fig. 3D).

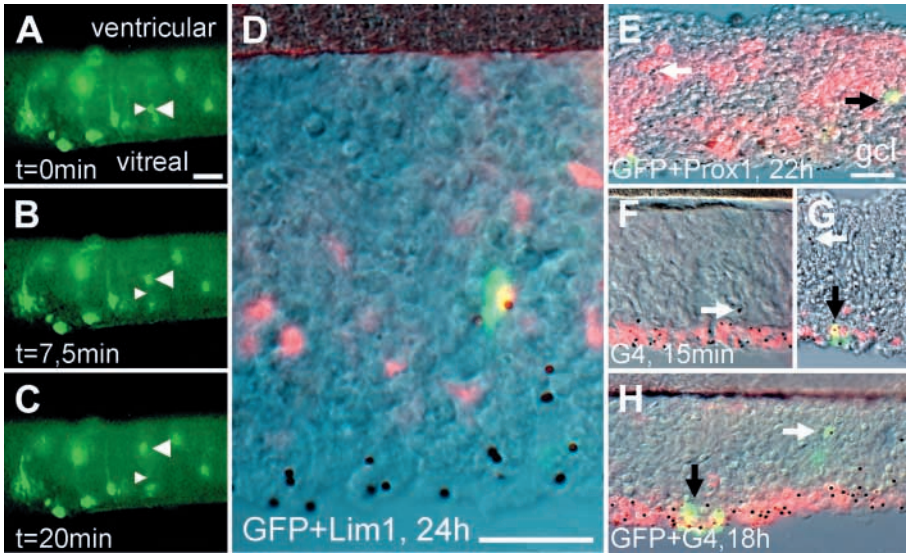


Fig. 3. GFP and Lim1 immunohistochemistry in biolistically transfected stage 31 retinas. (A-C) A time-lapse series of a retinal slice from a biolistically transfected stage 31 retina using a green fluorescent protein (GFP) expression vector. A cell indicated by a large arrowhead is migrating towards the ventricular side in relation to its starting point (small arrowhead). (D) Lim1 (red) and GFP (green) co-labelling in cells fixed and cryosectioned 24 hours after gene transfer. (E-H) GFP (green), and other markers (red) in fixed and cryosectioned retinas at different time points after transfection. White arrows indicate gold particles in the internal retina. Black arrows indicate GFP and antibody co-expression. (E) GFP and Prox1 22 hours after transfection. (F) Ganglion cell marker Ng-CAM (G4) expression and gold particle penetration 15 minutes after transfection. GFP is not yet expressed. (G) Ganglion cell marker Islet1 and GFP, 22 hours after transfection. (H) GFP and Ng-CAM (G4) in a retina 18 hours after transfection. Gold particles as well as cells expressing GFP are found on the ventricular side of the retina (white arrow). Scale bars: in A (for A-C), D,E (for E-H) 20 μm.

The Lim1-expressing cells are both GABA- and calretinin-positive horizontal cells

We wanted to confirm that Lim1 and Prox1 cells were indeed HCs and therefore co-labelled them with antibodies directed towards GABA and calretinin, two well-known markers for HCs (da Costa Calaza et al., 2000; Ellis et al., 1991; Yazulla, 1986). At st44, we could find a perfect overlap of Lim1 and GABA at the level of the HCs (Fig. 4A-D). Co-labelling of GABA and Prox1 in the HC layer was also found at this stage (results not shown). At st45 we found a perfect overlap between Lim1 and calretinin (Fig. 4E-H). These labelled cells have the typical shape of HCs as visualised by both GABA and calretinin immunoreactivity (Fig. 4A,E). Both GABA and calretinin were also found in those parts of the inner nuclear layer and ganglion cell layer where one can expect to find these markers (results not shown).

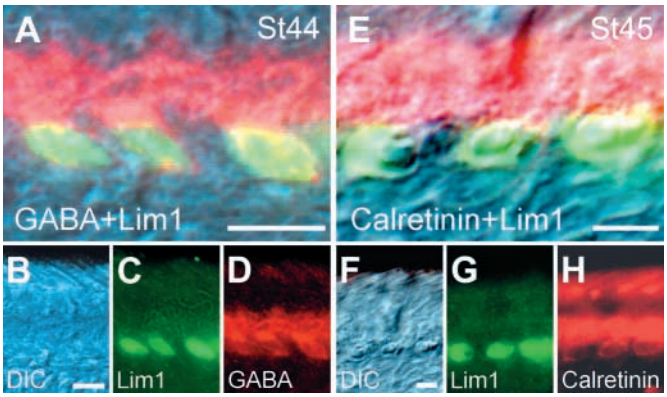
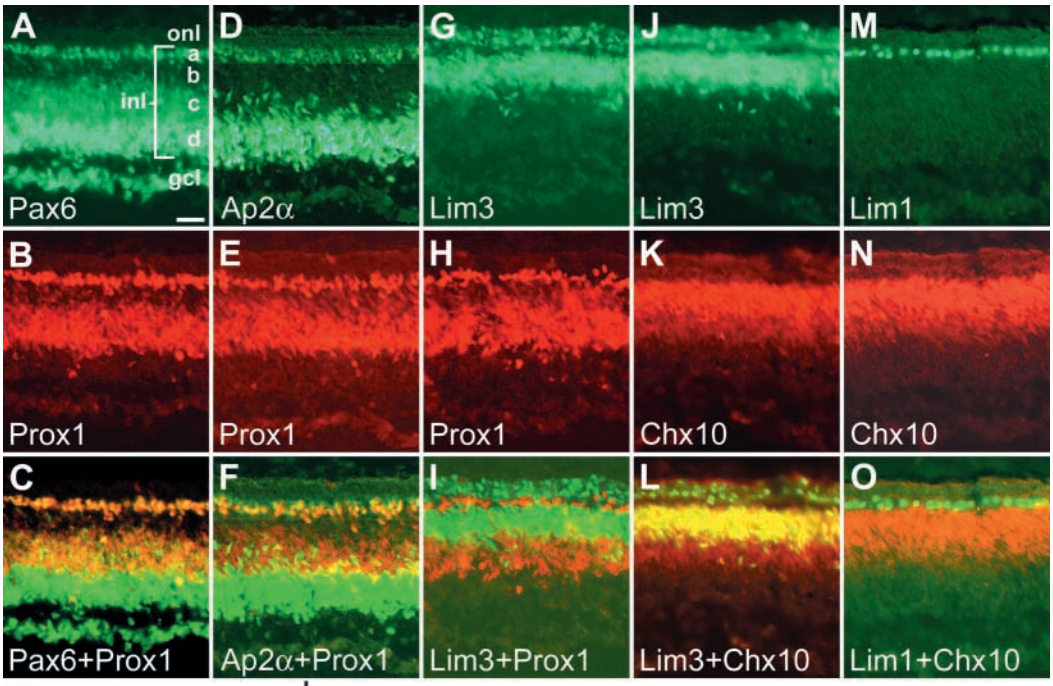


Fig. 4. Lim1+ cells are also positive for GABA and calretinin. Fluorescence and differential interference micrographs of (A-D) GABA (red) and Lim1 (green) immunoreactivity in HCs of a stage 44 retina. (A) Merged image of B, C and D. (E-H) Calretinin (red) and Lim1 (green) immunoreactivity in stage 45 retina. (E) Merged image of F, G and H. Scale bars: in A,B (for B-D), E,F (for F-H) 10 μm.

Retinal lamination as shown by transcription factor expression is in accordance with migration of HC to their mature position

Pax6, a fundamental transcription factor for eye development, can be found in the ganglion cell layer, the internal inner nuclear layer (INL, layers c and d in Fig. 5A) and in the outer external INL (layer a in Fig. 5A, the HC layer), while the ONL and inner part of the external INL (layer b in Fig. 5A) are unlabelled (Belecky-Adams et al., 1997; De Melo et al., 2003) (Fig. 5A-C). Double labelling with Pax6 and Prox1 reveals an almost complete co-expression at the level of the HCs, and to some degree co-expression in the outer internal INL (Fig. 5A-C). Ap2α, another transcription factor present in the developing retina (Hilger-Eversheim et al., 2000) is expressed by amacrine cells in the inner internal INL as well as in the HC layer (Fig. 5D). When co-labelling with Ap2α and Prox1, cells in the HC layer express both factors whereas in the rest of the INL, the co-localisation of the two factors is less obvious (Fig. 5D-F).

Unlike Pax6 and Ap2α, labelling for the transcription factor Lim3 produces a strong signal on the ventricular side of the developing retina (Fig. 5G,J) and double labelling with Lim3 and Prox1 at st35 shows no overlap between these transcription factors. Instead, the factors are expressed in distinct laminas (Fig. 5G-I). Among post-mitotic retinal cells,



P Layer	Pax6	Ap2α	Prox1	Lim3	Chx10	Lim1
ONL				+		
Outer external INL (a,HC)	+	+	++			++
Inner external INL (b)				++	++	
Outer internal INL (c)	+		++		+	
Inner internal INL (d)	++	++				
GCL	++					

Fig. 5. Lamination of the stage 35 retina as shown by transcription factor immunoreactivity. Representative fluorescence micrographs showing transcription factor immunoreactivity in the central region of stage 35 retina. (A) Pax6, (B) Prox1, (D) Ap2α, (E) Prox1, (G) Lim3, (H) Prox1, (J) Lim3, (K) Chx10, (M) Lim1 and (N) Chx10. (C,F,I,L,O) Merged image of the fluorescence micrographs presented in the top two rows. (P) Schematic summary of the immunoreactivity shown in A-O. (+) Moderate and (++) strong immunoreactivity. The results are representative of and were collected from more than three animals in each case. ONL/onl: outer nuclear layer, INL/inl: inner nuclear layer (a: outer external INL, b: inner external INL, c: outer internal INL, d: inner internal INL), GCL/glc: ganglion cell layer. Scale bar: A (for A-O) 20 μm.

the transcription factor Chx10 is exclusively expressed in bipolar cells (Chen and Cepko, 2000; Dyer, 2003) and labelling for Chx10 reveals a band of cells just below the HC layer; the inner external INL (Fig. 5J-L). Chx10 overlaps with Lim3 (Fig. 5J-L) but not with Lim1 (Fig. 5N-O) or Prox1 (Fig. 5B,E,H,K), except for a slight overlap between Prox1 and weak Chx10 expression in the central INL (compare Fig. 5B,E,H with K,N). These patterns distinguish the HCs from their nearest surrounding cells and are in clear agreement with the proposed HC migration into their mature positions next to the outer plexiform layer.

Discussion

We have presented evidence that horizontal cells migrate bi-directionally from their site of birth, close to the ventricular side, to the adjacent (vitreal) side of the neuroepithelium, where they align just next to the prospective ganglion cell layer, before migrating back again to their final laminar position in the outer part of the inner nuclear layer. The first cells start moving by st24 and migration is completed by st33. Both indirect and direct evidence for the cell migration are presented here and our results modulate a generally accepted view of how newborn horizontal cells migrate in the developing retina (Fig. 6) (Adler, 2000; Mey and Thanos, 2000; Prada, 1983; Stone, 1988).

Evidence that region- and cell type-specific transcription factors regulate morphogenesis and differentiation of the vertebrate nervous system comes from numerous studies. The studies of combinatorial expression of homeobox transcription factors including Lim1 that define subclasses of motor neurons in the spinal cord has been instrumental to the model where the combinatorial repertoires of transcription factors act to generate diverse cell types by regulating tissue-specific gene expression (Ericson et al., 1997). Targeted deletion of the Lim1 gene leads to loss of large parts of the head region including the retina (Shawlot and Behringer, 1995; Shawlot et al., 1999), thus it has not been possible to study the precise role of Lim1 in the retina. Our results together with others (Liu et al., 2000) show that retinal HCs express Lim1, in both birds and mammals, and that it is expressed throughout development. We show that Lim1 expression first appears in the avian retina concomitant with the postulated time of the birth of HCs. Lim1 expression may be initiated shortly before or after the last S-phase of the new HCs (Fig. 2, Table 1) and this is in agreement with its role as a cell-type-specifying transcription factor. Prox1 controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina by regulating the exit from the cell cycle (Dyer and Cepko, 2001; Dyer et al., 2003). The Prox1 expression in the chick retina is in agreement with the proposed function in the mammalian retina (Dyer et al., 2003). Similarly to Lim1, Prox1 is expressed throughout HC development suggesting roles also in the later developing retina, in regulating tissue-specific gene expression.

Although the bi-directional migration of HCs has not explicitly been demonstrated previously, ventricle-directed

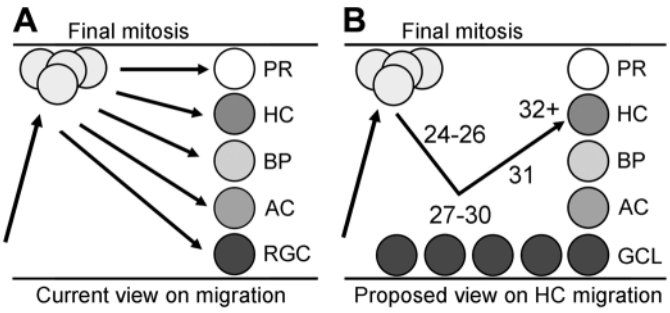


Fig. 6. Hypothetical models for cell differentiation and migration in chick embryo retina. (A) In the current model for retinal development cells migrate directly to their final lamina. (B) In the model suggested by this work, HCs migrate bi-directionally across the neuroepithelium before attaining their final position. Numbers denote developmental stages. PR: photoreceptors, HC, horizontal cells; BP, bipolar cells; AC, amacrine cells; RGC, retinal ganglion cells; GCL, ganglion cell layer.

migration of newborn retinal neurons finds indirect support in both previous studies and more recent ones. Based on serial sections Hinds and Hinds suggested as long ago as 1978 that cells in the mouse retina may take a route of migration that includes passing the vitreal side of the neuroepithelium before ending up in the inner nuclear layer (Hinds, 1979; Hinds and Hinds, 1978). It was suggested that this alternative route could explain the presence of displaced amacrine cells in the ganglion cell layer. However, this suggestion was rebutted by others (Prada et al., 1987; Prada, 1983). Although Prada and co-workers were opposed to the idea of ventricle-directed migration, they indicated the presence of HC-like cells in the vitreal side of the retina (Prada et al., 1984). Despite their migration dispute, both Hinds and Prada generally agreed that newborn HC only migrated from their place of birth directly to their final position close to the outer plexiform layer (Fig. 6A). Other researchers, however, disagreed with this view, resting their case on observations of "common neuroblasts of horizontal and amacrine cells", which at E7 (st30-31) were located close to the ganglion cell layer before some of these neuroblasts underwent ventricular-directed migration and ending up close to the outer plexiform layer by E8.5-9 (st35) (Gallego, 1986; Tarrés and Gallego, 1984). Moreover, in a report from 1987 Prada describes two populations of amacrine cell precursors based on their shape and migratory behaviour; the 'smooth' and 'multipodial' amacrine cell (Prada et al., 1987). The 'smooth' amacrine cells were radially disposed on the vitreal side at E5 (st26-28) but "diminished drastically" by E7 (st30-31) at the same time as free neuroblasts were found close to the ventricular side, indicating them to be HCs. We suggest that the 'smooth' amacrine cells, in fact, were the migrating HCs reported on herein.

More recent reports indicate the presence of retinal cells with immature HC markers located vitreally in the neuroepithelium of birds and mammals. Liu et al. found that in the E16.5-E18.5 mouse retina some Lim1+ cells could be found outside their proper lamina (Liu et al., 2000). In two reports on GABA expression during development in the chick and human embryo, 'ectopic' expression was found at time points that corresponds to our current observations (Hokoc et al., 1990; Nag and Wadhwa, 1997). Furthermore, a previous observation in our laboratory, which prompted us to undertake this study, showed how the pattern for nerve growth factor expression changed from being located vitreally at E5.5-E6 (st27-29) to be expressed in the external inner nuclear layer later (Karlsson et al., 2001). The cells were identified as HCs.

The detailed temporal analysis of the genes expressed by HCs (Fig. 1) gives strong support to the ventricle-directed migration. This is further strengthened by the fact that the translocation of Lim1 cells is inhibited by the cytochalasin D injection (Fig. 2F-H) in a dose-dependent manner showing that the changing patterns are not the result of transient expression in various layers of the retina. Another piece of evidence is the time-lapse recording of cells that move in the direction of the ventricle towards the prospective lamina of differentiated HCs (Fig. 3A-C). Similar cells can be identified as Lim1+ or Prox1+ cells (Fig. 3D-E). The time-lapse recording is a powerful technique in combination with particle-mediated gene transfer. The conditions for the preparation of biolistic particles and the bombardment were carefully controlled but there was a notable variation in the distribution of particles as well as transfection rate over the surface of the retina. The variation was partly batch related but was also seen between separate bombardments and this was most probably a result from the actual construction of the Gene gun. This variation made quantification difficult and is therefore not presented here.

The identification of Lim1+ cells as HCs was verified using GABA and calretinin and the conspicuous morphology of mature HCs next to the outer nuclear layer (Fig. 4A-H). On a related topic, recent work by Nadarajah et al. showed that certain GABAergic cortical interneurons display a ventricle-directed migration before attaining their mature positions in the cortex (Nadarajah et al., 2002) and these observations could suggest that these cells may have additional features in common.

Within the INL, and in the retina in general, the early born cells are in the inner part and the late born cells in the outer part, with the clear exception for HCs. The bi-directional migration of HCs, as shown in this work, gives an explanation to this order and organisation. The order is further supported by patterns of complementary expression of the homeobox transcription factors in layers and sub layers of the retina (Fig. 5). HCs express transcription factors that are also expressed by cells in the early formed inner retinal layers. In addition to the HC-specific Lim1 expression, Pax6 and Ap2α are expressed by HCs. Pax6 is also expressed by cells in the ganglion cell layer and internal INL and Ap2α is expressed by amacrine cells in the internal INL (Fig. 5A-F). In contrast, and complementary to this pattern, Lim3 and Chx10 (Fig. 5J-O) are expressed by cells in the external INL with the clear exception of HCs. This exception can be explained by the migration of HCs into this region.

Pax6, Lim3 and Chx10 are known to confer tissue-specific gene expression and their own expression has been shown to be regulated by morphogens such as Sonic hedgehog (Ericson et al., 1997). Retinal differentiation as well as lamination is thought to be modulated by external signals polarised from either the pigment epithelium (Raymond and Jackson, 1995) or from the retinal ganglion cells (Wang et al., 2002). HCs will be exposed 'en route' during migration to signals present in the inner part of the retina. These signals can provide the HCs with instructions necessary for their proper maturation.

Furthermore, HCs may 'escape' from signals present in the outer parts of the retina during a critical period. This implies that the fate and maturation of HC neurons are not necessarily specified by their final position in relation to the polarised signals in the retina as proposed previously (Adler, 2000; Adler and Belecky-Adams, 1999).

HCs are born early but establish synaptic contacts with bipolars and photoreceptors that are born late, and the function of the HCs is to modulate the visual information from photoreceptors to bipolars. This is reflected in the functional architecture of the retina where HCs are organised laterally. The proposed migration and the actual detour to the ganglion cell layer that leads to a delay of several days for the differentiating HCs, may constitute a mechanism by which the temporal sequence is achieved for the appropriate functional connections to be established within the outer plexiform layer.

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References

Adler, R. (2000). A model of retinal cell differentiation in the chick embryo. *Prog. Retin. Eye Res.* **19**, 529-557.

Adler, R. and Belecky-Adams, T. (1999). Cell fate determination in the chick embryo retina. In *Cell Fate and Cell Linage Determination* (ed. S. Moody), pp. 463-474. San Diego: Academic Press.

Altshuler, D. M., Turner, D. L. and Cepko, C. L. (1991). Specification of cell type in the vertebrate retina. In *Development of the Visual System* (ed. D. M. K. Lam and C. J. Shatz), pp. 37-58. Cambridge, USA: MIT Press.

Belecky-Adams, T., Tomarev, S., Li, H. S., Ploder, L., McInnes, R. R., Sundin, O. and Adler, R. (1997). Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest. Ophthalmol. Vis. Sci.* **38**, 1293-1303.

Bruijns, R. H. and Bult, H. (2001). Effects of local cytochalasin D delivery on smooth muscle cell migration and on collar-induced intimal hyperplasia in the rabbit carotid artery. *Br. J. Pharmacol.* **134**, 473-483.

Chen, C. M. and Cepko, C. L. (2000). Expression of Chx10 and Chx10-1 in the developing chicken retina. *Mech. Dev.* **90**, 293-297.

da Costa Calaza, K., Hokoc, J. N. and Gardino, P. F. (2000). Neurogenesis of GABAergic cells in the chick retina. *Int. J. Dev. Neurosci.* **18**, 721-726.

de la Rosa, E. J., Kayyem, J. F., Roman, J. M., Stierhof, Y. D., Dreyer, W. J. and Schwarz, U. (1990). Topologically restricted appearance in the developing chick retinotectal system of Bravo, a neural surface protein: experimental modulation by environmental cues. *J. Cell Biol.* **111**, 3087-3096.

De Melo, J., Qiu, X., Du, G., Cristante, L. and Eisenstat, D. D. (2003). Dlx1, Dlx2, Pax6, Brn3b, and Chx10 homeobox gene expression defines the retinal ganglion and inner nuclear layers of the developing and adult mouse retina. *J. Comp. Neurol.* **461**, 187-204.

Dunn, B. E. (1974). Technique of shell-less culture of the 72-hour avian embryo. *Poult. Sci.* **53**, 409-412.

Dyer, M. A. (2003). Regulation of proliferation, cell fate specification and differentiation by the homeodomain proteins prox1, six3, and chx10 in the developing retina. *Cell Cycle* **2**, 350-357.

Dyer, M. A. and Cepko, C. L. (2001). Regulating proliferation during retinal development. *Nat. Rev. Neurosci.* **2**, 333-342.

Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat. Genet.* **34**, 53-58.

Ellis, J. H., Richards, D. E. and Rogers, J. H. (1991). Calretinin and calbindin in the retina of the developing chick. *Cell Tissue Res.* **264**, 197-208.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Gallego, A. (1986). Comparative studies on horizontal cells and a note on microglial cells. *Prog. Retinal Res.* **5**, 165-206.

Glasgow, J. E. and Daniele, R. P. (1994). Role of microtubules in random cell migration: stabilization of cell polarity. *Cell Motil. Cytoskeleton* **27**, 88-96.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hilger-Eversheim, K., Moser, M., Schorle, H. and Buettner, R. (2000). Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. *Gene* **260**, 1-12.

Hinds, J. W. (1979). Early neuron differentiation in the CNS: a comparison of several regions studied with serial thin sections. In *Neural Growth and Differentation* (ed. E. Meisami and M. A. B. Brazier), pp. 133-151. New York: Raven Press.

Hinds, J. W. and Hinds, P. L. (1978). Early development of amacrine cells in the mouse retina: an electron microscopic, serial section analysis. *J. Comp. Neurol.* **179**, 277-300.

Hokoc, J. N., Ventura, A. L., Gardino, P. F. and De Mello, F. G. (1990). Developmental immunoreactivity for GABA and GAD in the avian retina: possible alternative pathway for GABA synthesis. *Brain Res.* **532**, 197-202.

Jean, D., Ewan, K. and Gruss, P. (1998). Molecular regulators involved in vertebrate eye development. *Mech. Dev.* **76**, 3-18.

Kahn, A. J. (1974). An autoradiographic analysis of the time of appearance of neurons in the developing chick neural retina. *Dev. Biol.* **38**, 30-40.

Karlsson, M., Mayordomo, R., Reichardt, L. F., Catsicas, S., Karten, H. and Hallbook, F. (2001). Nerve growth factor is expressed by postmitotic avian retinal horizontal cells and supports their survival during development in an autocrine mode of action. *Development* **128**, 471-479.

La Vail, M. M., Rapaport, D. H. and Rakic, P. (1991). Cytogenesis in the monkey retina. *J. Comp. Neurol.* **309**, 86-114.

Liu, W., Wang, J. H. and Xiang, M. (2000). Specific expression of the LIM/homeodomain protein Lim-1 in horizontal cells during retinogenesis. *Dev. Dyn.* **217**, 320-325.

Livesey, F. J. and Cepko, C. L. (2001). Vertebrate neural cell-fate determination: lessons from the retina. *Nat. Rev. Neurosci.* **2**, 109-118.

Lo, D. C., McAllister, A. K. and Katz, L. C. (1994). Neuronal transfection in brain slices using particle-mediated gene transfer. *Neuron* **13**, 1263-1268.

Mey, J. and Thanos, S. (2000). Development of the visual system of the chick. I. Cell differentiation and histogenesis. *Brain Res. Brain Res. Rev.* **32**, 343-379.

Nadarajah, B., Alifragis, P., Wong, R. O. and Parnavelas, J. G. (2002). Ventricle-directed migration in the developing cerebral cortex. *Nat. Neurosci.* **5**, 218-224.

Nadarajah, B. and Parnavelas, J. G. (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat. Rev. Neurosci.* **3**, 423-432.

Nag, T. C. and Wadhwa, S. (1997). Expression of GABA in the fetal, postnatal, and adult human retinas: an immunohistochemical study. *Vis. Neurosci.* **14**, 425-432.

Prada, C., Puelles, L., Genis-Galvez, J. M. and Ramirez, G. (1987). Two modes of free migration of amacrine cell neuroblasts in the chick retina. *Anat. Embryol.* **175**, 281-287.

Prada, C., Puga, J., Perez-Mendez, L., Lopez, R. and Ramirez, G. (1991). Spatial and temporal patterns of neurogenesis in the chick retina. *Eur. J. Neurosci.* **3**, 559-569.

Prada, C. and Ramirez, G. (1983). A Golgi study of the cell cycle and early neuron and glia differentiation in the chick retina. In *Ramon y Cajal's Contribution to the Neurosciences* (ed. S. Grisolia), pp. 117-123. Amsterdam, NY: Elsevier Science Publishers.

Prada, F. A., Armengol, J. A. and Genis-Galvez, J. M. (1984). Displaced horizontal cells in the chick retina. *J. Morphol.* **182**, 221-225.

Raymond, S. M. and Jackson, I. J. (1995). The retinal pigmented epithelium is required for development and maintenance of the mouse neural retina. *Curr. Biol.* **5**, 1286-1295.

Shawlot, W. and Behringer, R. R. (1995). Requirement for Lim1 in head-organizer function. *Nature* **374**, 425-430.

Shawlot, W., Wakamiya, M., Kwan, K. M., Kania, A., Jessell, T. M. and

Behringer, R. R. (1999). Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. *Development* **126**, 4925-4932.

Stone, J. (1988). The origins of the cells of vertebrate retina. *Prog. Retinal Res.* **7**, 1-19.

Tarrés, M. A. G. and Gallego, A. (1984). Embryonic development of HC and photoreceptors in the chick retina. *Proc. Int. Soc. Eye Res.* **3**, 22.

Tomarev, S. I., Sundin, O., Banerjee-Basu, S., Duncan, M. K., Yang, J. M. and Piatigorsky, J. (1996). Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. *Dev. Dyn.* **206**, 354-367.

Wang, Y. P., Dakubo, G., Howley, P., Campsall, K. D., Mazarolle, C. J., Shiga, S. A., Lewis, P. M., McMahon, A. P. and Wallace, V. A. (2002). Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. *Nat. Neurosci.* **5**, 831-832.

Yang, N. S., Burkholder, J., Roberts, B., Martinell, B. and McCabe, D. (1990). In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc. Natl. Acad. Sci. USA* **87**, 9568-9572.

Yazulla, S. (1986). GABAergic mechanisms in the retina. *Prog. Retinal Res.* **5**, 1-52.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. *Anat. Rec.* **212**, 199-205.