Dlx1 and *Dlx2* function is necessary for terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina

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Summary

Dlx homeobox genes, the vertebrate homologs of *Distal-less*, play important roles in the development of the vertebrate forebrain, craniofacial structures and limbs. Members of the Dlx gene family are also expressed in retinal ganglion cells (RGC), amacrine and horizontal cells of the developing and postnatal retina. Expression begins at embryonic day 12.5 and is maintained until late embryogenesis for *Dlx1*, while *Dlx2* expression extends to adulthood. We have assessed the retinal phenotype of the *Dlx1*/*Dlx2* double knockout mouse, which dies at birth. The *Dlx1/2* null retina displays a reduced ganglion cell layer (GCL), with loss of differentiated RGCs due to increased apoptosis, and corresponding thinning of the optic nerve. Ectopic expression of *Crx*, the cone and rod photoreceptor homeobox gene, in the GCL and neuroblastic layers of the mutants may signify altered cell fate of uncommitted RGC progenitors. However, amacrine and horizontal cell differentiation is relatively unaffected in the *Dlx1/2* null retina. Herein, we propose a model whereby early-born RGCs are *Dlx1* and *Dlx2* independent, but Dlx function is necessary for terminal differentiation of late-born RGC progenitors.

Key words: Apoptosis, *Brn3b*, BrdU birthdating, *Chx10*, *Crx*, *Dlx1*, *Dlx2*, Homeobox, Mouse, Ocular retardation, Retina

Introduction

During vertebrate retinogenesis a diverse and specialized array of neurons is generated from a relatively homogeneous population of retinal progenitors (Masland, 2001). Six classes of neuron and one class of glial cell are generated in a specified temporal order as the vertebrate retina differentiates (Sidman, 1961; Young, 1985). The birth order of neuronal classes is well conserved (La Vail et al., 1991; Steimke and Hollyfield, 1995). In the mouse, retinal ganglion cells (RGCs) are established first, followed by horizontal cells, cones, amacrine cells, rods, bipolar cells, and Müller glia (Cepko et al., 1996). Considerable overlap occurs and multiple cell types can be generated at any given stage during retinal development (Altshuler et al., 1991).

The transition from uncommitted multipotent to lineage-restricted progenitors may be regulated by basic helix-loop-helix (bHLH) transcription factors (Marquardt and Gruss, 2002). Expression of bHLH genes is controlled by lateral inhibition through Delta/Notch signaling pathways, resulting in mosaic-like expression patterns in the retina and other tissues (Artavanis-Tsakonas et al., 1999; Kuroda et al., 1999; Fode et al., 2000; Marquardt et al., 2001). These repressive interactions may result in a heterogeneous pool of progenitors with distinct retinogenic potentials (Marquardt and Gruss, 2002). Terminal differentiation of these progenitors to particular retinal neurons is accomplished, in part, through specific sets of transcription factors, particularly homeobox genes. In the mouse, null mutation of genes encoding homeodomain transcription factors such as *Chx10* and *Prox1* has resulted in abnormal retinal morphogenesis and the loss of specific cell types (Burmeister et al., 1996; Dyer et al., 2003).

The vertebrate *Distal-less* (Dlx) homeobox gene family consists of six known murine members (Panganiban and Rubenstein, 2002) organized in three bigenic gene clusters (McGuinness et al., 1996; Sumiyama et al., 2002; Ghanem et al., 2003). Four Dlx family members have been implicated in neurogenesis: *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* (Bulfone et al., 1993; Anderson et al., 1997a; Liu et al., 1997). *Dlx1* and *Dlx2* demonstrate similar expression in the forebrain, and subtle defects in forebrain differentiation of the *Dlx*2 single knockout suggest functional redundancy (Qiu et al., 1995; Eisenstat et al., 1999). Mice, in which both *Dlx1* and *Dlx2* have been knocked out, die at birth and display severe craniofacial (Qiu et al., 1997) and central nervous system defects (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000;

Anderson et al., 2001). Cells born after embryonic day (E) 12.5 in the striatum do not fully differentiate (Anderson et al., 1997a), resulting in a loss of migration of GABAergic interneurons to the neocortex and olfactory bulb (Anderson et al., 1997b; Bulfone et al., 1998). *Dlx1* and *Dlx2* are both expressed in the developing retinal neuroepithelium by E12.5 (Eisenstat et al., 1999). Expression of *Dlx1* is largely restricted to the ganglion cell layer (GCL); perinatally its expression is downregulated. Expression of *Dlx2* is maintained throughout the lifetime of the mouse with expression restricted to RGC, amacrine and horizontal cells (de Melo et al., 2003).

In this study we assess the retinal phenotype of the *Dlx1/Dlx2* null mouse. We demonstrate a loss of approximately one-third of RGCs in the mutant while other retinal neuronal classes appear unaffected. We further demonstrate that late-born RGCs are dependent on *Dlx1* and *Dlx2* function for their terminal differentiation, unlike the initial population of RGCs, which properly differentiate and migrate in the absence of *Dlx1* and *Dlx2*. The observed decrease in RGCs in *Dlx1/Dlx2* mutants is partly due to increased apoptosis among late-born RGCs.

Materials and methods

Animal and tissue preparation

Dlx1/Dlx2 knockout mice were generated as previously described (Qiu et al., 1997; Anderson et al., 1997a). Ocular retardation mutant mice and controls (129s/sv-Chx10$^{or\text{-}J/+}$) were purchased (Jackson Laboratory). Embryonic age was determined by the day of appearance of the vaginal plug (E0.5), confirmed by morphological criteria. Eyes were dissected from E16.5 and 18.5 embryos, while E13.5 eyes were left in situ. Tissues were processed as described (de Melo et al., 2003). All animal protocols were conducted in accordance with guidelines set by the Canadian Council on Animal Care and the University of Manitoba. *Dlx1/Dlx2* null mice and ocular retardation mice were genotyped as described (Qiu et al., 1995; Burmeister et al., 1996). For comparative studies all mutants were paired with wild-type littermate controls.

Retinal explant cultures

Eyes were dissected from embryos in sterile 1× PBS and transferred to dishes containing DMEM/F12 media (Gibco/Invitrogen). Retinas were dissected under sterile conditions from eyes with the lens and iris in situ and transferred onto Millicell-CM 0.4 μm filters (Millipore) with the lens facing away from the membrane. Filters were transferred to 6-well culture plates containing media enriched with 1× N2 supplement, 1× MEM sodium pyruvate, 2 mmol/l L-glutamine (all Gibco/Invitrogen), 5 μg/ml insulin (Sigma), and 1 U/ml penicillin/1 mg/ml streptomycin (Sigma). Explants were cultured at 37°C with 5% CO_2 in a humidified incubator for 7 days.

Histological staining, immunofluorescence and combined immunohistochemistry/in-situ hybridization

Tissues stained with Cresyl Violet dye were immersed for 2 minutes and then transferred through graded alcohol washes before mounting with Permount (Fisher Chemicals) and coverslips. Immunofluorescence was performed on cryosections as described (de Melo et al., 2003). Primary antibodies used were: mouse anti-BrdU (1:200, Chemicon), rabbit anti-BRN3a (1:100, courtesy of Dr E. Turner), rabbit anti-BRN3b (1:200, Babco), goat anti-BRN3b (1:200, Santa Cruz), rabbit anti-calretinin (1:3000, Chemicon), rabbit anti-caspase-3 (1:60, Cell Signalling Technologies), mouse anti-Chat (1:100, Chemicon), rabbit anti-CHX10 (1:700, courtesy of Dr T. Jessell), rabbit anti-CRALBP (1:250, courtesy of Dr J. C. Saari), mouse anti-cyclinD1 (1:100, Cell Signalling Technologies), rabbit anti-DLX1 (1:700), rabbit anti-DLX2 (1:250), rabbit anti-GFAP (1:3000, DAKO), mouse anti-islet-1 (ISL1) (1:600 Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-L1 (1:5000, courtesy of Dr C. F. Lagenaur), mouse anti-NF165 (1:50, Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-phosphohistone H3 (1:1000, Upstate), rabbit anti-PROX1 (1:500, courtesy of Dr M. Nakafuku), rabbit anti-PAX6 (1:800, Sigma), mouse anti-Rho4D2 (1:80, courtesy of Dr R. Molday), rabbit anti-SIX3 (1:500, courtesy of Dr G. Oliver), mouse anti-syntaxin (1:6000, Sigma), rabbit anti-VSX1 (1:10, courtesy of Drs R. L. Chow and R. R. McInnes). Peanut agglutinin (1:2000, Vector Laboratories) was also used. Secondary antibodies and fluorescent tertiary molecules used were FITC-conjugated goat anti-rabbit (1:100, Sigma), Biotin-SP-conjugated goat anti-rabbit (1:200), Biotin-SP-conjugated goat anti-mouse (1:200), Biotin-SP-conjugated rabbit anti-goat (1:200, all Jackson ImmunoResearch), Streptavidin conjugated Oregon Green-488 (1:200, Molecular Probes), and Streptavidin conjugated Texas Red (1:200, Vector Laboratories). Negative controls omitted the primary antibody. Non-radioactive in-situ hybridization was performed using digoxigenin-UTP labeled *Crx* riboprobes combined with immunohistochemistry utilizing CHX10 antibodies. The *Crx* cDNA was obtained from Dr C. Cepko. Single and combined in-situ hybridization and immunohistochemistry was performed with sense probe used as controls (Eisenstat et al., 1999). TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics) as per the manufacturer's instructions.

BrdU labeling and birthdating

Timed pregnant animals were injected with BrdU (5 mg/μl). For pulse labeling experiments, animals were sacrificed after 1 hour. For birthdating experiments, animals were sacrificed at E18.5. Sections were treated with 50% formamide/2× SSC for 2 hours at 65°C, 2× SSC for 5 minutes at 65°C, 2N HCl at 37°C for 30 minutes followed by 0.1 mol/l boric acid pH 8.5 at RT for 10 minutes.

Cell counting and statistical analysis

For cryosections, pooled counts from a series of matched sections of paired *Dlx1/Dlx2* mutant and wild-type retinas were taken at regularly spaced intervals to completely survey each retina. Six sets of eyes consisting of one *Dlx1/Dlx2* mutant and one wild-type eye from littermate pairs were used for quantification at E18.5 (five sets for ISL1, BrdU and phosphohistone H3 counts). Eyes were sectioned at 12 μm. Sections through the widest region of the optic nerve head were used as a centered start point and were matched histologically. The start section and sections 120 and 240 μm above and below were used for immunohistochemistry. Results from five sections were pooled to provide a count for each eye. BRN3b+ (Pou4f2 – Mouse Genome Informatics) cells located in the GCL were counted as RGCs; PAX6+ cells in the inner neuroblastic layer (NBL) but not the GCL or outer NBL, were counted as amacrine cells; and NF165+ cells located in the outer NBL were counted as horizontal cells. Comparisons between sets of count data were made using the paired t-test to determine statistical significance. For cell death and cell proliferation counts, sections were immunostained with antibodies to activated caspase-3. Sections from E13.5 and 16.5 embryos 60 and 120 μm above and below the start section were used due to smaller eye size. For BrdU birthdating studies, the proportion of BRN3b-expressing RGCs labeled with BrdU represents the number of RGCs born at the time of BrdU pulsing.

For retinal explants, sections from each mutant explant were histologically matched with those from a wild-type littermate and then immunoassayed with cell-type specific markers and with DAPI stain (Vector). Total cell numbers/section were determined by counting DAPI+ cells, then immunoreactive cells were counted and proportions were determined.

Morphometry

Six paired sets of littermate *Dlx1/Dlx2* mutant and wild-type eyes were processed. Sections were centered on the thickest region of the optic nerve head, which was taken as the midpoint. Sections 12, 24 and 36 μm above and below, including the middle section were immunostained with L1 N-CAM antibody to visualize the optic nerve. Thickness of the optic nerve head was measured in three regions (Fig. 3A) using Image-Pro Plus 4.5 software (Media Cybernetics), a mean thickness was determined and comparisons were made using the paired *t*-test.

Microscopy and imaging

Images were acquired using an Olympus IX70 inverted microscope with Fluoview 2.0 confocal laser scanning, an Olympus BX51 fluorescent microscope, or an Olympus SZX12 fluorescent stereomicroscope. These microscopes utilized a SPOT 1.3.0 digital camera (Diagnostic Instruments Inc.). Images were processed using Adobe Photoshop 5.5 (Adobe Systems) and were formatted, resized and rotated for the purposes of presentation.

Results

Dlx1/*Dlx2* knockout mice demonstrate a significant loss of RGCs by E18.5

Dlx1/Dlx2 mutant retinas at E13.5 and 16.5 were histologically indistinguishable from wild type (data not shown). Mutants at E18.5 demonstrated diminished cellularity in the GCL, which contains RGCs and displaced amacrine cells (Fig. 1A,B, arrows). Development of the lens, iris, cornea/sclera, pigment epithelium and outer neuroretina all appeared normal (Fig. 1A,B). *Dlx1/Dlx2* mutants die shortly after birth, so postnatal development could not be assessed. To determine if the differentiation of cells known to express *Dlx1* and/or *Dlx2* (de Melo et al., 2003) was affected in the mutants, we used cell-type-specific antibodies to RGCs and amacrine and horizontal cells. Antibodies to BRN3a (POU4F1 – Mouse Genome Informatics), BRN3b and ISL1 were used as markers specific to RGCs (Liu et al., 2000; Ma et al., 2004; Mu and Klein, 2004). There was a notable decrease in the number of cells expressing BRN3a, Bn3b and ISL1 in all mutants analyzed [Fig. 1C,D (BRN3a), E,F (BRN3b); Fig. 2A; see Fig. S2 in the supplementary material]. There were significant reductions of 33.76% for BRN3b ($t=5.81$, $P<0.05$, $n=5$) and 39% for ISL1 ($t=7.74$, $P<0.05$, $n=5$) in the mutants. Neither displaced nor ectopic expression of BRN3a, BRN3b or ISL1 was detected. Severely diminished numbers of BRN3b-expressing cells were observed as early as E16.5 in mutants, whereas no difference was observed at E13.5 (data not shown). SIX3 and PAX6 are expressed in the GCL in both RGCs and displaced amacrine cells at E18.5 (Belecky-Adams et al., 1997; Inoue et al., 2002). We also observed decreased mutant GCL cells expressing these markers [Fig. 1G,H (SIX3), I,J (PAX6)]. Differences were less marked compared with BRN3a or BRN3b, indicating that displaced amacrine cells in the GCL may not be affected.

Amacrine cells were quantified by counting PAX6 expressing cells located in the inner NBL but not in the GCL or outer NBL, in order to exclude potential RGC and horizontal cells. No significant difference was observed in mutant retinas ($t=1.76$, $P>0.05$, $n=5$) (Fig. 2B). Antibodies specific to the amacrine cell markers syntaxin and calretinin (Barnstable et al., 1985; Haverkamp and Wässle, 2000) were also analyzed. At E18.5, no difference could be determined in the NBL, where most amacrine cells are localized [Fig. 1K,L (syntaxin), M,N (calretinin)]. Also, calretinin-expressing cell numbers were unaffected in the mutant GCL, although total GCL numbers were reduced (Fig. 1M,N). This was also evident for choline acetyl-transferase (Chat) expressing amacrine cells (Haverkamp and Wässle, 2000) (data not shown). Horizontal cells identified by antibodies to NF165 (Haverkamp and Wässle, 2000), a neurofilament protein, and PROX1, a homeodomain transcription factor (Dyer et al., 2003), were also unaffected in the *Dlx1/Dlx2* mutant [Fig. 1O,P; Fig. 2A (NF165); Fig. 1Q,R (Prox1)]. These cells appeared with normal frequency and in the correct regions of the central/outer NBL [Fig. 1O,P (arrows), Q,R (asterisks)]. Horizontal cells quantified by NF165 expression in the outer NBL showed no significant differences compared with wild-type littermates ($t=1.79$, $P>0.05$, $n=5$) (Fig. 2A).

As *Dlx1/Dlx2* mutants die at P0, to determine whether late-born cell classes were affected, retinal explant cultures were collected at E18.5 and cultured for 7 days (Fig. 2B; see Fig. S1A-H in the supplementary material). Expression of peanut agglutinin, a marker for cone photoreceptors (Chen et al., 1994) (see Fig. S1A,B in the supplementary material), and Rho4D2, a marker for rod photoreceptors (Davidson et al., 1994) (see Fig. S1C,D in the supplementary material), could not discern any differences between mutant and wild-type tissues. Antibodies to the transcription factors CHX10 and VSX1 were used to identify rod and cone bipolar cells, respectively (Chow et al., 2001; Hatakeyama et al., 2001). No abnormalities in the number or histological placement of these cells could be detected in the *Dlx1/Dlx2* mutants (see Fig. S1E-H in the supplementary material). No amacrine or horizontal cell differences could be identified among mutant and wild-type explants consistent with our findings in the intact E18.5 retina (see Fig. S1I-P in the supplementary material). Hence, amacrine and horizontal interneurons, which normally express *Dlx1* and/or *Dlx2* in the developing retina, are unaffected by their absence. Finally, we used CRALBP and GFAP expression as markers for Müller glia (Bunt-Milam and Saari, 1983; Kuhrt et al., 2004). No difference was observed between wild-type and mutant retinas (data not shown). Moreover, there were no significant differences between mutant and wild-type explants in the proportion of late developing retinal cell classes: rods, cones, bipolar interneurons or Müller glia (Fig. 2B). These results support a specific loss of RGC in the *Dlx1/Dlx2* double mutant.

The optic nerve is reduced in *Dlx1*/*Dlx2* mutant mice corresponding to RGC loss

The optic nerve of *Dlx1/Dlx2* mutants displayed no gross anatomical abnormalities. To determine whether the *Dlx1/Dlx1* mutants displayed aberrant optic nerve morphology, we performed morphometric measurements. Measurements of the thickness of the optic nerve were made at the region where the nerve exited the retina (optic nerve head) in order to standardize the region of measurement (Fig. 3A, arrows). Antibodies to L1 were used to stain unmyelinated axons of the optic nerve (Bartsch et al., 1989) (Fig. 3A,B). Measurements revealed a mean optic nerve thickness of 318.10±59.76 μm in wild-type animals, while paired mutants had a mean thickness of 244.17±62.20 μm (Fig. 3C), a significant 23% decrease ($t=3.99$, $P<0.005$, $n=10$).

There is increased apoptosis in developing RGCs in *Dlx1/Dlx2* mutants

In order to explain the loss of RGCs, we assessed cellular proliferation and apoptosis in the retina. BrdU pulse labeling experiments demonstrated no significant differences between the populations of S-phase cells in mutants compared with wild-type retinas (see Fig. S3B in the supplementary material). In addition, studies using an antibody to cyclin D1 (data not shown), a general proliferation marker (Tong and Pollard, 2001) and an antibody to phosphohistone H3, an M-phase marker (Ajiro et al., 1996), were unable to identify any differences between mutant and wild-type retinal proliferation dynamics (see Fig. S3A in the supplementary material). Antibodies specific to activated caspase-3, an effector caspase, were then utilized to quantify apoptosis. Mutant retinas displayed significantly increased numbers of activated caspase-3 positive cells beginning at E13.5, 1 day after DLX1 and DLX2 expression is normally established in the retina (Eisenstat et al., 1999). Mutants at E13.5 displayed a significant 3-fold increase ($t=5.96$, $P<0.005$, $n=6$) in apoptotic cells (Fig. 4A, asterisk). Mutant retinas at E16.5 had a significant 66% increase ($t=6.04$, $P<0.005$, $n=6$) in activated caspase-3+ cells (Fig. 4A, cross). However, by E18.5 the number of apoptotic cells in *Dlx1/2* mutants returned to levels similar to those of wild-type littermates ($t=1.81$, $P>0.05$, $n=6$) (Fig. 4A). Virtually identical patterns of apoptosis were yielded by TUNEL assays, confirming results generated using activated caspase-3 expression (see Fig. S4A-I in the supplementary material).

To determine whether the apoptotic cells were RGCs, we performed co-expression experiments with antibodies to activated caspase-3 and BRN3b. At E13.5 and E16.5, we found that nearly all apoptotic cells in the mutant retina were also BRN3b immunoreactive (Fig. 4D,G, boxes, inset). Although apoptotic cells were distributed throughout the retinal neuroepithelium, at E16.5 more activated caspase-3 expression was localized to the inner retina. Therefore, we attribute the increase in apoptosis in the mutant retina to increased RGC death between E13.5 and E18.5. These results support a requirement for DLX1 and/or DLX2 in the survival and/or terminal differentiation of this subpopulation of RGCs.

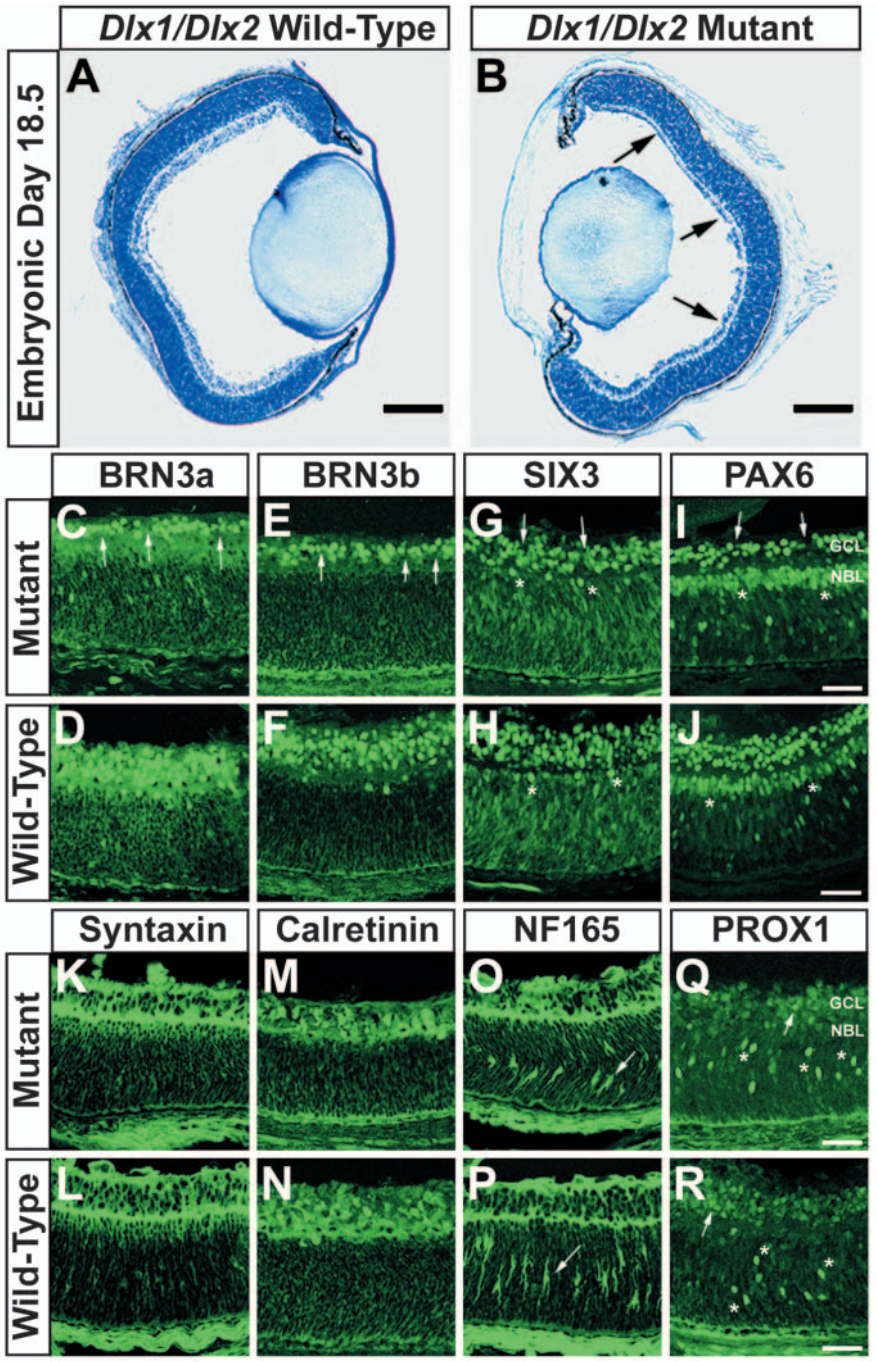

Fig. 1. Histological characterization of the *Dlx1/Dlx2* mutant retina. (A,B) Cresyl Violet staining of *Dlx1/Dlx2* mutant and wild-type retinas at E18.5. The mutant displays a reduced GCL (B, arrows) but the remainder of the retina appears spared. (C-F) Expression of BRN3a (C,D) and BRN3b (E,F) in mutant and wild-type retina. Decreased numbers of BRN3a and BRN3b immunoreactive cells (C,E, arrows) indicate that fewer RGCs are present in the mutants. (G-J) Expression of SIX3 and PAX6 in mutant and wild-type retina. SIX3 and PAX6 are expressed in fewer cells in the mutant GCL than in the wild type (G,I, arrows). Expression of SIX3 and PAX6 in the neuroblastic layer appears unaffected in mutants (G,H,I,J, asterisks). (K-N) Expression of amacrine cell markers in the mutant and wild-type retina. (K,L) Syntaxin expression appears unchanged. (M,N) Calretinin immunoreactive cells are expressed in the mutant retina in numbers similar to those of wild type. (O-R) Horizontal cell markers are expressed in normal number and position in the *Dlx1/Dlx2* mutants. Expression of NF165 (O,P, arrows) and PROX1 (Q,R, asterisks) are unaffected in the mutant. PROX1 expression in the GCL (Q,R, arrows) supports normal AII amacrine cell development. Scale bars: 250 μm in B; 50 μm in I,J,Q,R.

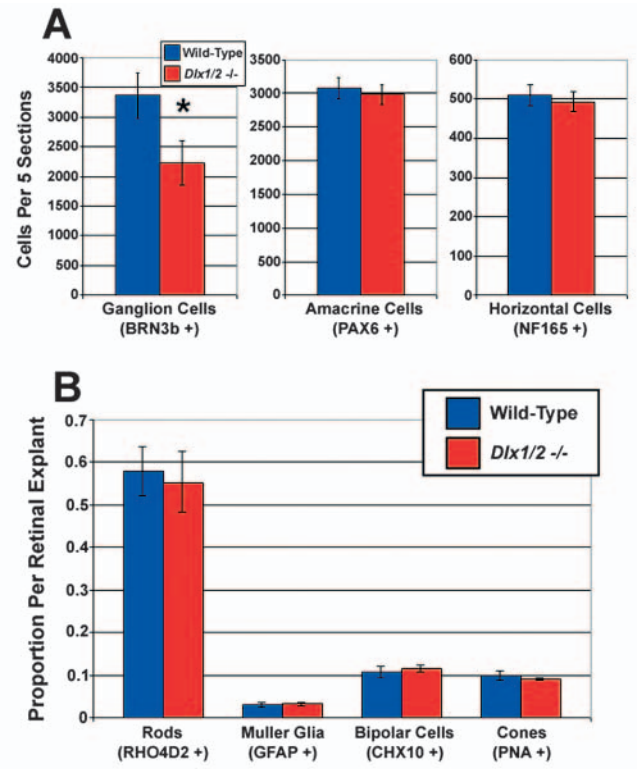

Fig. 2. Quantification of early-born cells in the E18.5 mutant and wild-type retinas and late-born cells in retinal explants cultured for 7 days. (A) Mutant retinas featured a significant 33.76% reduction in the number of BRN3b-expressing RGCs in the mutant retinas compared with wild-type retinas (asterisk). No significant differences in the number of PAX6-expressing amacrine or NF165-expressing horizontal cells were identified. (B) Late-born retinal cell classes not identifiable in E18.5 retinas were quantified in 7-day-old explant cultures of paired mutant and wild-type retinas collected at E18.5. No significant differences in the number of rods, cones, Müller glia or bipolar interneurons as identified by Rho4D2, peanut agglutinin, glial fibrillary acidic protein (GFAP), and CHX10 immunoreactivity, respectively, were found (rods t=1.03, $P>0.05$, $n=3$; cones t=1.26, $P>0.05$, $n=3$; Müller glia t=0.64, $P>0.05$, $n=3$; bipolar cells t=0.79, $P>0.51$, $n=3$). PNA, peanut agglutinin.

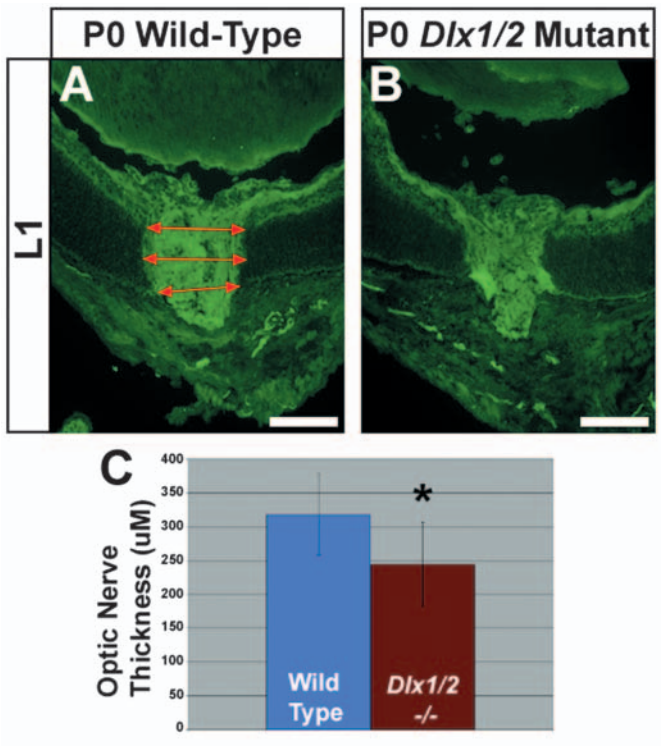

Fig. 3. Reduced optic nerve thickness in *Dlx1/Dlx2* mutant eyes. (A,B) Wild-type (A) and *Dlx1/Dlx2* mutant (B) optic nerves. Three measurements were made of the optic nerve as it exits the retina (optic nerve head) using the boundary between the GCL and NBL (A, top arrow), the outer retinal limit (A, bottom arrow) and at a point equidistant between these markers (A, middle arrow).There was a significant 23% decrease in the thickness of the mutant optic nerve (asterisk). Scale bars: 125 μm.

There is a complete loss of late-born RGCs in the *Dlx1*/*Dlx2* double mutant

As the earliest BRN3b-expressing RGCs are established before the onset of *Dlx1* and *Dlx2* expression in the developing retina, we hypothesized that *Dlx1*/*Dlx2* function is required for the terminal differentiation of a subclass of late-born RGCs. In retinal explants, there is loss of all pre-existing RGCs within 3 days of culture, due to RGC axon severance resulting from optic nerve transection during tissue preparation (Caffé et al., 1989; Tomita et al., 1996). However, the GCL remains with its displaced amacrine cells. Thus, any detected RGCs in explants cultured beyond 3 days are likely to have differentiated ex vivo. Retinas were collected at E18.5, as any subsequent terminally differentiated RGCs could be considered late-born relative to the total population. Explants were cultured for 7 days to ensure that all pre-existing RGCs were cleared. In wild-type explants, rare BRN3b expressing cells could be identified after 7 days of culture (Fig. 5A, box). These may represent newly specified RGCs. However, in *Dlx1*/*Dlx2* mutant explants, BRN3b-expressing cells could not be detected (Fig. 5B), suggesting that late-born RGCs are present only in wild-type explants. Subsequently, BrdU birthdating experiments were performed (Fig. 5C-T). A single BrdU pulse was delivered to timed-pregnant animals at E12.5, 13.5, 16.5 and 18.0 and embryos were collected at E18.5. BrdU expression marked cells born on the date of the BrdU pulse. Co-labeling with BRN3b and BrdU identified RGCs in mutant and wild-type retinas pulsed with BrdU at E12.5 (Fig. 5C-D,L-N), E13.5 (Fig. 5F-H,O-Q), and E16.5 (Fig. 5R-T,U). However, no co-labeling was evident in either mutant or wild-type retinas that were BrdU pulsed at E18.0. Proportions of RGCs born at the time of BrdU pulsing were determined. RGCs generated at E12.5 formed a significantly larger proportion of the population in mutant retinas (Fig. 5U, asterisk). However, for RGCs generated at E13.5 and 16.5, wild-type retinas displayed significantly larger proportions (Fig. 5U, cross, #). The difference was more pronounced at E16.5 than E13 (nearly 3-fold versus 60%). These results support a loss of late-born RGCs in the *Dlx1*/*Dlx2* double mutant, with early-born RGCs constituting a larger proportion of the total mutant RGC population compared with controls.

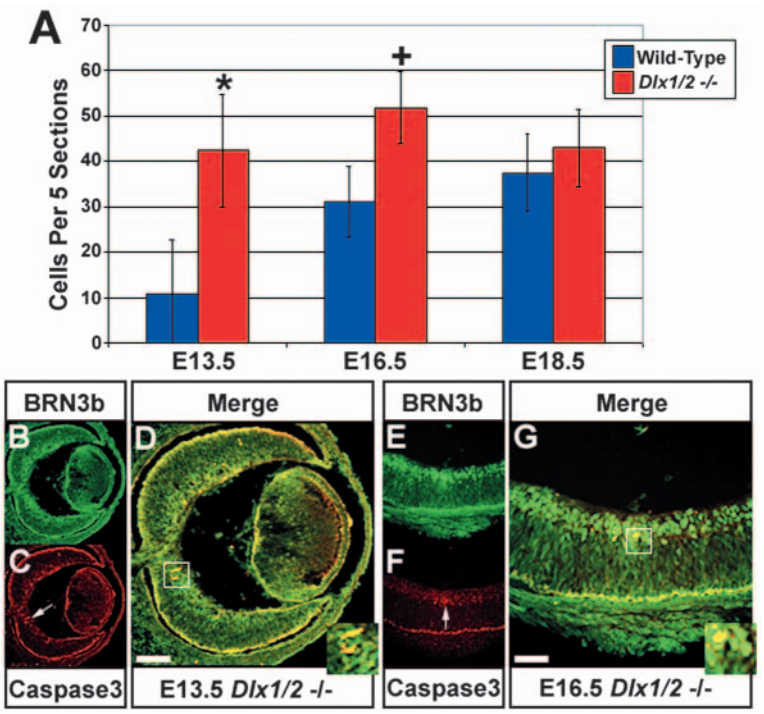

Fig. 4. (A) Quantification of cell death in the *Dlx1/Dlx2* mutant. At E13.5, there is a significant (asterisk) 3-fold increase in activated caspase-3-expressing cells in the mutant. This amount of apoptosis is less marked at E16.5 (cross), when there is a significant 66% increase in the number of apoptotic cells. There is no statistical difference in cell death by E18.5. (B-G) Co-localization of activated caspase-3 and BRN3b indicates that cell death is occurring among RGCs (D, box, insert) at E13.5 and at E16.5 (G, box, insert). Scale bars: 100 μm in D; 50 μm in G. Inserts in D and G represent 2-fold enlargements.

Analysis of ocular retardation mice support the role of *Dlx1* and *Dlx2* in RGC differentiation in the developing retina

Our retinal explant cultures and BrdU birthdating experiments support the requirement of *Dlx1* and *Dlx2* for differentiation of late-born RGCs. Ocular retardation (*Chx10* or^{J}/or^{J}) mutant mice demonstrate severe microphthalmia as well as aniridia, a poorly developed optic nerve, decreased RGCs and a complete loss of rod bipolar cells. *Chx10* is a paired domain homeobox transcription factor that patterns the outer retina but ultimately becomes restricted to rod bipolar cells in the mature retina (Burmeister et al., 1996). Chx10 mutants were studied to determine whether their RGC population, which is significantly reduced and very late in appearance, expresses *Dlx1* and/or *Dlx2*. The expression of *Dlx1* or *Dlx2* in the Chx10 mutant RGC population may suggest a role for Dlx genes in their differentiation. By E13.5, or^{J}/or^{J} mice completely lacked BRN3b-positive RGCs (Fig. 6A), present in the inner retina in wild-type controls (Fig. 6D). These mutants also lacked DLX1 and DLX2 retinal expression at this developmental stage (Fig. 6B,C). By E16.5, expression of DLX1 and DLX2 had become established in the mutants (Fig. 6H,I), as well as BRN3b (Fig. 6G). Patterns of DLX1, DLX2 and BRN3b expression in the or^{J}/or^{J} mutants appeared similar to wild-type controls, except for a marked decrease of immunoreactive cells (Fig. 6G-L). DLX1 appeared to be expressed in transitory cells, migrating to the inner retina, while DLX2 identified both migrating and nascent GCL cells. At E18.5, BRN3b expressing cells in the or^{J}/or^{J} mutants had localized to the inner retina, as in the wild type, but were severely diminished in number (Fig. 6M,P). At this stage DLX1 expression could not be detected in either wild-type or mutant retinas (Fig. 6N,Q). The downregulation of DLX1 expression occurred approximately 2 days earlier in the 129s/sv strain than in previously described CD-1 mice (de Melo et al., 2003). DLX2 remained robustly expressed in both mutant and wild type at this stage (Fig. 6O,R). At E16.5, we found co-expression between DLX2 and BRN3b, confirming that RGCs in the mutant retina expressed DLX2 (Fig. 7C). Co-localization between DLX2 and BRN3b was also observed throughout the retina at E18.5 (Fig. 7F). Whereas DLX2 single positive cells were identifiable, all observed BRN3b-expressing RGCs co-expressed DLX2. In ocular retardation retinas, RGCs differentiated after E13.5 and all BRN3b-expressing RGCs co-expressed *Dlx1* and/or *Dlx2*. Hence, Dlx genes may function in RGC differentiation in or^{J}/or^{J} mutants.

There is increased Crx homeobox gene expression in the *Dlx1*/*Dlx2* double mutant

Rod photoreceptors and bipolar interneurons are relatively late-born cells in the murine retina, most developing postnatally. We explored the possibility that there was a fate switch of retinal progenitors in the *Dlx1/Dlx2* mutant. *Crx* encodes a homeodomain protein expressed by photoreceptors in the developing outer retina (Furukawa et al., 1997). At E18.5, we observed regionalization of the outer retina (Fig. 8A). CHX10 protein is expressed in the outer NBL in wild-type embryos (Fig. 8A, brown nuclear staining). *Crx* RNA is also expressed in the outer neuroretina adjacent to the pigment epithelium (Fig. 8A, blue cytoplasmic staining). In the *Dlx1/Dlx2* mutant there is increased *Crx* RNA expression in the outer retina (Fig. 8B, blue stain) and ectopic *Crx* expression in the central retina and GCL (Fig. 8B, arrows, box). Ectopic *Crx* expression in the mutant, particularly in the GCL, is clearly demonstrated by single in-situ hybridization (Fig. 8D, box, inset) compared with wild-type littermates (Fig. 8C). CHX10 expression in the outer retina appears to decrease in the same region that there is increased *Crx* expression. This is consistent with the observation that as cells commit to photoreceptor cell fates, they downregulate *Chx10* (Green et al., 2003).

Discussion

Dlx1 and *Dlx2* are necessary for the terminal differentiation of RGCs

We have demonstrated a reduction of RGCs and an optic nerve of reduced thickness in the neonatal *Dlx1/Dlx2* mutant. The first RGCs are established by E11.5 (Wang et al., 2002), approximately 1 day before the onset of *Dlx1* and *Dlx2* expression in the outer retinal neuroepithelium (Eisenstat et al., 1999). Therefore, there is an established population of RGCs that have migrated to their correct locations independent of *Dlx1/Dlx2* function. However, throughout murine

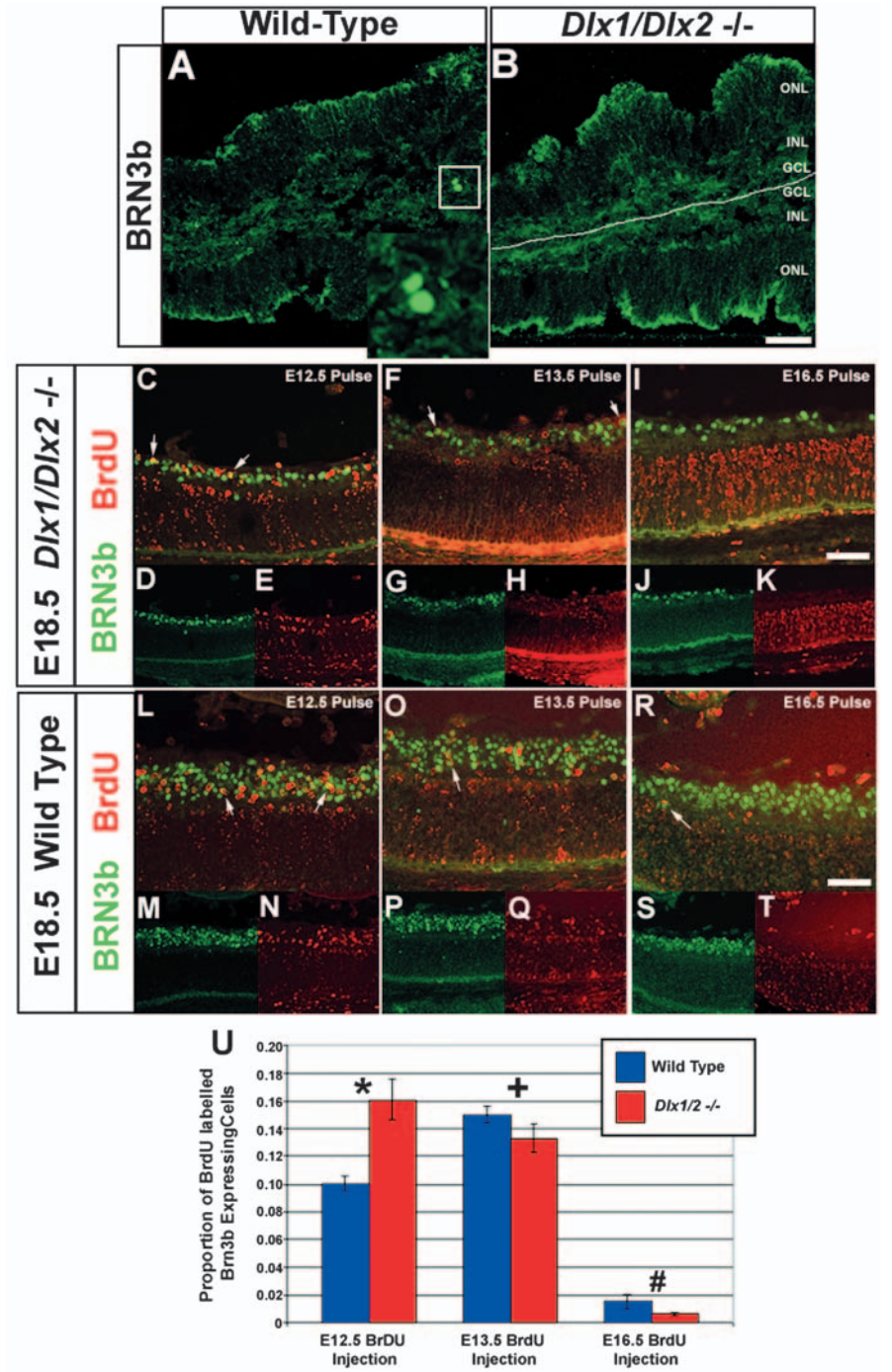

Fig. 5. Loss of function of *Dlx1/Dlx2* results in a loss of late-born RGCs in the developing retina. (A,B) Expression of BRN3b in 7-day-old explant cultures of wild-type and *Dlx1/Dlx2* mutant retinas collected at E18.5. BRN3b immunoreactivity is detected in wild type (A, box, insert) but not in mutant explant cultures. (C-T) BrdU birthdating assays identify retinal cells generated at the time of pulsing. (C-E,L-N) BrdU pulses at E12.5 label RGCs as identified by BRN3b protein expression in mutant (C, arrows) and wild-type (L, arrows) retinas collected at E18.5. (F-H,O-Q) E13.5 BrdU pulses also readily identify RGCs in both mutant (F, arrows) and wild-type (O, arrows) retinas. (I-K,R-T) BrdU pulses at E16.5 in mutant and wild-type retinas. Co-expression with BRN3b can be seen only in the wild-type retina (R, arrow). (U) Quantification of BRN3b-expressing RGCs born at E12.5, 13.5 and 16.5. RGCs born at E12.5 (asterisk) represent 10% of RGCs labeled in E18.5 wild-type retinas, but 16% of the RGCs in *Dlx1/Dlx2* mutants yielding a significantly larger proportion of the population at E18.5 (t=8.8, $P<0.05$, $n=5$). RGCs born at E13.5 (cross) form a significantly larger proportion of the population of RGCs in wild-type retinas compared with mutants (15 versus 13.3%; t=3.32, $P<0.05$, $n=5$). RGCs born at E16.5 (#) form a significantly larger proportion (~3 fold) of the population of wild-type retinas than mutant retinas (1.5 versus 0.6% of E18.5 population; t=4.85, $P<0.05$, $n=5$).

retinogenesis RGCs continue to be generated to the early postnatal period (Sidman, 1961; Young, 1985). *Dlx1* and *Dlx2* may specify a subset of RGCs that complete differentiation after the initial RGC population has been established. These Dlx-specified RGCs differentiate throughout mid- to late embryogenesis and represent a relatively late-born population of RGCs.

No other retinal cell classes appeared to be affected by the loss of *Dlx1* and *Dlx2*. Amacrine cells and horizontal cells both express *Dlx2* during development and maintain expression of *Dlx2* in the mature adult retina (de Melo et al., 2003). Most amacrine cell subclasses, including those identified by tyrosine hydroxylase, ChAT, GABA, PROX1 and calretinin immunoreactivity, and virtually all horizontal cells also express *Dlx2* (de Melo et al., 2003) (J.d.M. and D.D.E., unpublished). Therefore, we hypothesized that loss of *Dlx1/Dlx2* would play a significant role in the development and maintenance of these retinal cell types. The extensive role of both *Dlx1* and *Dlx2* in interneuron differentiation in the developing forebrain (Anderson et al., 1997a; Anderson et al., 1997b) further substantiated this hypothesis. However, the present study suggests that *Dlx1* and/or *Dlx2* are not required for either the generation or differentiation of amacrine or horizontal cells. This may be due, in part, to redundancy of function with other Dlx genes expressed in the developing retina, such as *Dlx5* (Zhou et al., 2004). The onset of *Dlx5* expression at approximately E16.5 is several days after the onset of expression of *Dlx1* and *Dlx2* (G.D. and D.D.E., unpublished). *Dlx5* RNA expression is unaffected in the *Dlx1/Dlx2* mutant retina (data not shown). Similar genetic redundancy is seen among members of the *Brn* POU domain homeobox gene family. *Brn3a*, *Brn3b* and *Brn3c* are expressed in RGCs. *Brn3b* knockouts display severe RGC loss (Gan et al., 1996; Erkman et al., 1996), while *Brn3c* knockout mice do not (Xiang et al., 1997). *Brn3b/Brn3c* double knockout mice display a more severe RGC phenotype, suggesting that *Brn3c*

and *Brn3b* are partially redundant. *Brn3c* is sufficient to initiate RGC development even though it is not required for proper RGC genesis (Wang et al., 2002). Analysis of the retinal phenotype of *Dlx5* knockout (Levi et al., 2003; Long et al., 2003) and *Dlx5/Dlx6* double knockout mice (Merlo et al., 2002; Robledo et al., 2002) may further illuminate the role of Dlx homeobox genes in amacrine and horizontal cell development.

Dlx1 and *Dlx2* function is necessary for the differentiation of late-born RGCs

As *Dlx1/Dlx2* mutants die at birth, we established explant cultures to study postnatal retinal differentiation. All RGCs generated prior to establishment of the cultures were lost due to transection of the optic nerve. Newly specified RGCs, while not abundant, were detected in explants from wild-type retinas but not in mutant explants. We suspected that the inability to detect RGCs in the *Dlx1/Dlx2* mutants was due to a failure of RGC terminal differentiation and/or survival during tissue culture, suggesting that late-born RGCs require *Dlx1* and *Dlx2*. Since cultures were established from E18.5 retinas, all RGCs generated could be considered late-born relative to the total birthdate distribution of RGCs. BrdU birthdating experiments at select stages of embryogenesis labeled neurons undergoing their final S-phase and exit from the cell cycle. Co-labeling with BRN3b, a specific marker for RGCs, allowed us to identify RGCs born on the day of the BrdU pulse. *Dlx1/Dlx2* mutants contained a greater proportion of RGCs born before

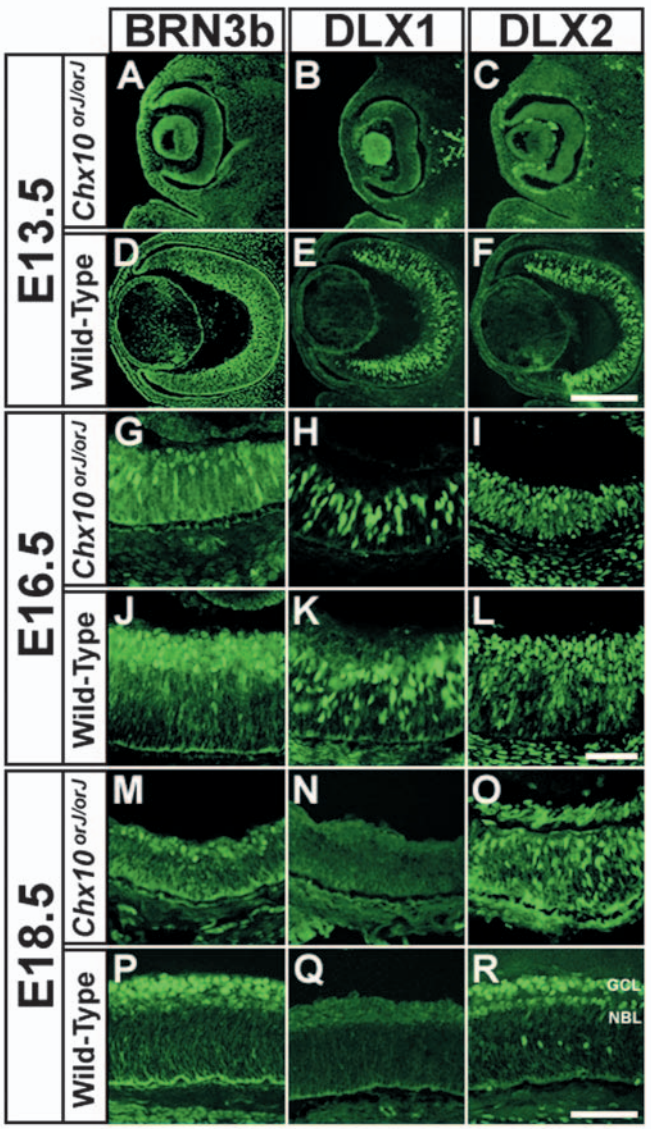

Fig. 6. Expression of DLX1, DLX2 and BRN3b in the ocular retardation (or^J/or^J) mouse. (A-C) Or^J/or^J mutants (*Chx10* null) lack expression of BRN3b (A), DLX1 (B) and DLX2 (C) at E13.5, indicating absent RGCs. (D-F)Wild-type controls exhibit normal patterns of expression of these markers. By E16.5, there are BRN3b-expressing cells in or^J/or^J mutants (G). BRN3b expression coincides with the onset of DLX1 and DLX2 expression (H, DLX1; I, DLX2). (J-L) Expression in controls (J, BRN3b; K, DLX1; L, DLX2). By E18.5, BRN3b expression is localized in the central inner retina in both or^J/or^J mutants and controls (M, mutant; P, wild type). DLX1 expression is not detected (N, mutant; Q, wild type) at E18.5. DLX2 expression is found in the inner and outer retina in both mutants and wild types (O, mutant; R, wild-type). The expression pattern of DLX2 is considerably disorganized in the or^J/or^J mutant compared with wild-type controls at this stage (O,R). Scale bars: 100 μm in F; 50 μm in L; 50 μm in R.

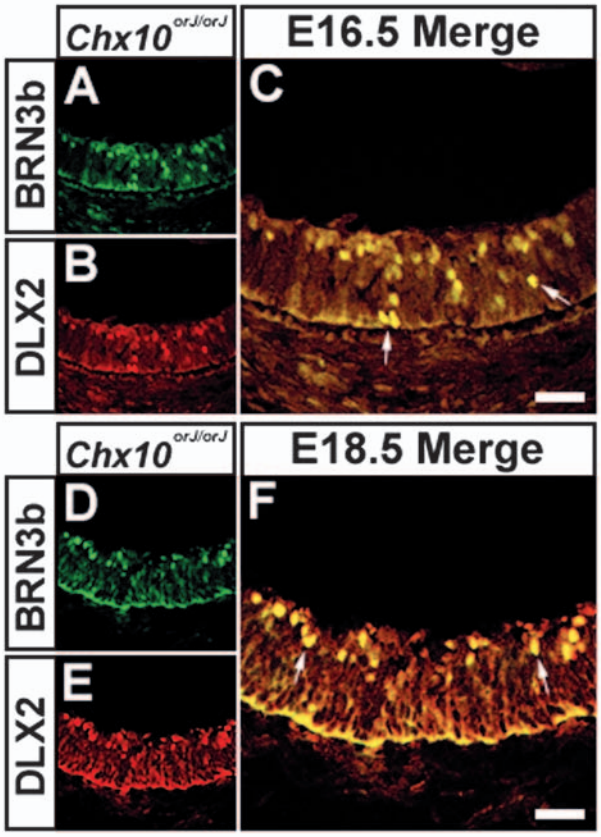

Fig. 7. Co-localization of DLX2 with BRN3b in the ocular retardation (or^J/or^J) mutant. (A-C) Expression of BRN3b (A) and DLX2 (B) in the E16.5 retina with merged image (C). Co-expression is extensive between DLX2 and BRN3b at this time point (C, arrows), without BRN3b single-positive cells detected at this stage. (D-F) Expression of BRN3b (D) and DLX2 (E) in the E18.5 retina with merged image (F). Similar to E16.5, extensive co-localization is evident (F, arrows) and BRN3b single-positive cells are not detected. Scale bars: 33 μm.

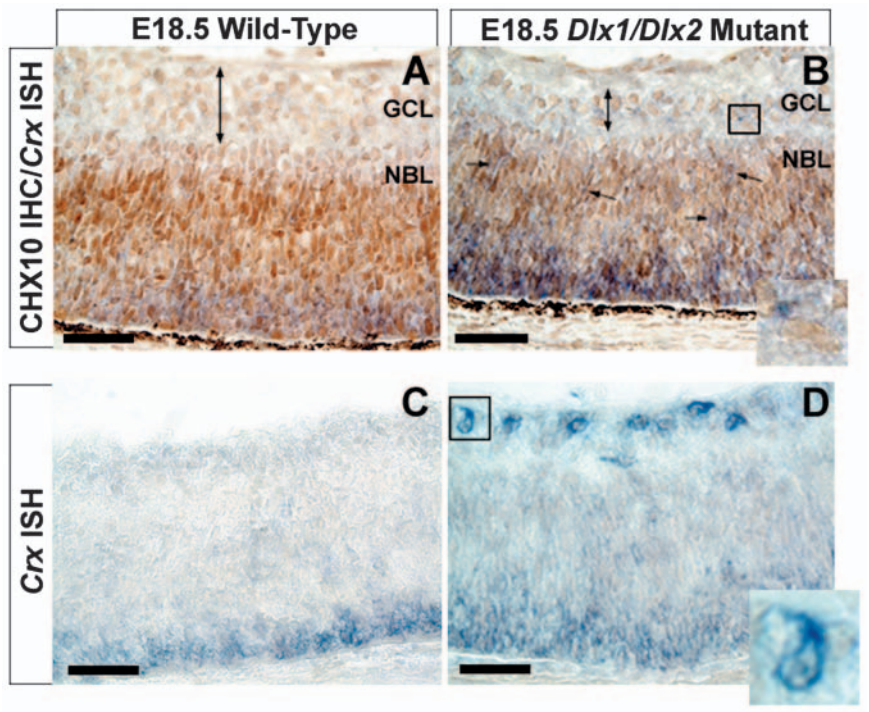

Fig. 8. Altered *Chx10* and *Crx* homeobox gene expression in the E18.5 *Dlx1/Dlx2* mutant retina. (A) Combined CHX10 immunohistochemistry with *Crx* digoxigenin in-situ hybridization in wild-type retina. CHX10 (brown stain) is localized primarily in the outer retina throughout the NBL. *Crx* RNA expression (blue stain) can be found in the extreme outer retina. (B) In the *Dlx1/Dlx2* mutant there is increased *Crx* expression in the outer retina as well as ectopic expression in the central retina (arrows) and GCL (box, insert). Note the reduced GCL (double-headed arrows). (C) *Crx* in-situ hybridization of E18.5 wild-type retina. (D) *Crx* in-situ hybridization of E18.5 *Dlx1/Dlx2* null retina. Ectopic *Crx* expression is clearly identified in the GCL of the mutant (D, box). Scale bars: 40 μm. Inserts in B,D represent a 3-fold enlargement.

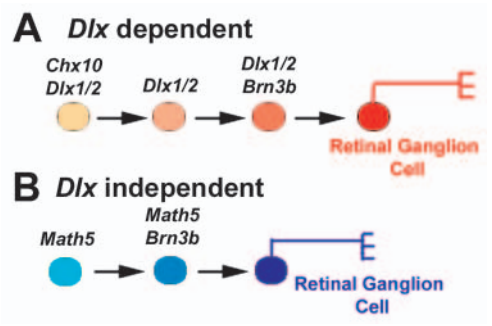

Fig. 9. Model for the generation of RGCs through a *Dlx1/Dlx2* dependent pathway (A). *Dlx1/Dlx2*-expressing cells downregulate *Chx10*. These cells may then establish *Brn3b* expression and terminally differentiate into RGCs. The majority of RGCs are derived from *Math5/Brn3b* co-expressing populations of retinal progenitors (B). We suggest that *Dlx1/Dlx2*-specified RGCs might represent a distinct late-born RGC population in the murine retina.

E13.5, whereas late-born RGCs were more prevalent in wild-type retinas. Hence, late-born RGCs may fail to terminally differentiate due to a requirement for *Dlx1* and/or *Dlx2*. These cells are lost by apoptosis, as we have shown using activated caspase-3 and TUNEL assays. This loss of late-born RGCs results in early-born RGCs comprising a greater proportion of the RGC population in *Dlx1/Dlx2* mutants.

Brn3b expression is established well before the onset of *Dlx1* and *Dlx2* in the retinal neuroepithelium. However, at birth, all BRN3b-expressing cells co-express DLX2 (de Melo et al., 2003). We suggest that the lost late-born RGCs in the *Dlx1/Dlx2* mutants are Dlx-dependent and may not require *Brn3b,* although expression of BRN3b protein is established later as these neurons develop (de Melo et al., 2003). Of significance, *Brn3b* knockout mice lose approximately 70-80% of RGCs (Erkman et al., 1996; Gan et al., 1996; Lin et al., 2004), leaving 20-30% of RGCs specified through alternative mechanisms. Of interest, there is increased *Dlx1* and *Dlx2* expression in *Brn3b* null mice (Mu et al., 2004). This compensatory increase in Dlx gene expression may be required for differentiation of the remaining RGC pool in the BRN3b mutant. In the *Dlx1/Dlx2* mutants, one-third of RGCs are lost, indicating that nearly 70% of RGCs develop unhindered by the loss of *Dlx1/Dlx2*. We suggest that the surviving RGC population in *Brn3b* knockout mice may comprise late-born RGCs with terminal differentiation that requires Dlx gene expression, while surviving RGCs in the *Dlx1/Dlx2* knockouts are Dlx-independent (refer to model, Fig. 9). Dlx-dependent RGCs may derive from distinct retinal progenitor pools as specified by bHLH genes. The bHLH transcription factor *Math5* (*Atoh7* – Mouse Genome Informatics) identifies a subpopulation of retinal progenitors, in which *Brn3b* expression commits cells to an RGC fate (Liu et al., 2001; Wang et al., 2001; Yang et al., 2003). By contrast, *Dlx1/Dlx2*-expressing lineages originate from progenitor pools defined by expression of the bHLH gene *Mash1* in the developing central nervous system (Cassarosa et al., 1999; Andrews et al., 2002; Letinic et al., 2002; Yun et al., 2002). However, the genetic interaction between Dlx genes and *Mash1* remains to be defined in the developing retina.

In addition to BrdU birthdating experiments and analysis of retinal explants, analysis of the ocular retardation mouse (or^J/or^J), in which *Chx10* function is lost and there is a severe loss of RGCs, provides support for a Dlx-dependent RGC population. In the orJ/orJ mouse only a limited number of late-born RGCs are present and differentiation of these RGCs is coincident with Dlx expression. We have shown that all RGCs that develop in the or^J/or^J mutant mouse express DLX2 and also express DLX1 before it is down-regulated at E18.5. Normally, expression of CHX10 leads to downregulation of the cyclin dependant kinase inhibitor $p27^{Kip1}$. Loss of cellularity in the or^J/or^J mutant may result from diminished cellular proliferation due to unchecked $p27^{Kip1}$ (Polyak et al., 1994; Toyoshima and Hunter, 1994; Green et al., 2003). We suggest that the proliferation of the *Math5* expressing progenitor pool is regulated by this mechanism. In the or^J/or^J mutant, aberrant cell cycle regulation results in the absence of *Math5* specified

BRN3b-expressing RGCs in the or^J/or^J retina by E13.5. However, in MASH1 expressing retinal progenitors, expression of Dlx genes may restore repression of $p27^{Kip1}$ by direct or indirect means. We have previously shown that expression of *Dlx1* and *Dlx2* originates in cells expressing *Chx10*, and that *Dlx1/Dlx2* and *Chx10* expression soon segregates into distinct retinal neuronal populations (de Melo et al., 2003). *Chx10* may work with *Mash1* to promote bipolar cell genesis (Hatakeyama et al., 2001; Marquardt and Gruss, 2002) while *Dlx1/Dlx2* may promote RGC development from *Mash1* progenitor pools. In the or^J/or^J mutant only *Dlx1/Dlx2* expressing RGCs undergo proper proliferation and differentiation of RGCs begins after E13.5. *Dlx1/Dlx2* derived lineages may potentially define a unique functional subclass of RGCs.

RGCs undergo increased apoptosis in the *Dlx1/Dlx2* mutant retina

Increased and ectopic *Crx* expression in the *Dlx1/Dlx2* mutant suggests that upon loss of *Dlx1* and/or *Dlx2*, some retinal progenitors may commit to photoreceptor differentiation pathways as an alternative to cell death. *Math5* mutant mice feature an absence of RGCs and an increase in the number of cone photoreceptors, possibly due to a binary fate switch (Brown et al., 2001). A similar cell fate switch may partly explain the decrease in RGCs in the *Dlx1/Dlx2* mutant retina. Unlike *Math5* mutants, the *Dlx1/Dlx2* mutant is not viable beyond birth. As a consequence, we cannot characterize photoreceptors in a mature retina. However, in explant cultures no significant differences were determined when quantifying rods and cones. Hence, aberrant *Crx* expression in the *Dlx1/Dlx2* mutant may be transient or may result indirectly from a loss of *Dlx1* and/or *Dlx2* function.

In the *Dlx1/Dlx2* mutants there were increased apoptotic cells. We attribute this increase in cell death to a loss of late-born RGCs that require *Dlx1/Dlx2* expression for their terminal differentiation. The utilization of caspase-mediated apoptotic pathways in the modulation of RGC number has been demonstrated in chick (Mayordomo et al., 2003). Our results suggest that similar mechanisms are involved in the clearance of RGCs with incomplete differentiation. Failure of RGC development in the *Dlx1/Dlx2* mutants may be due to several mechanisms. Interestingly, these cells express BRN3b protein at the time of death, suggesting that lethality results from a failure of later developmental processes. The direct transcriptional downstream targets of *Dlx1* and *Dlx2* remain largely undefined except for the *Dlx5/Dlx6* intergenic enhancer (Zerucha et al., 2000; Zhou et al., 2004). Regulation of survival factors and/or apoptosis may be mechanisms by which specific retinal neuronal classes are maintained. Characterization of the genetic networks regulated by *Dlx1* and *Dlx2* presents a challenging direction to further define the role of Dlx genes in the developing retina.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/2/311/DC1

References

Ajiro, K., Yoda, K., Utsumi, K. and Nishikawa, Y. (1996). Alteration of cell cycle-dependent histone phosphorylations by okadaic acid. Induction of mitosis-specific H3 phosphorylation and chromatin condensation in mammalian interphase cells. *J. Biol. Chem.* **271**, 13197-13201.

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. 38-58. Cambridge: MIT Press.

Anderson, S. A., Qiu, M., Bulfone, A., Eisenstat, D. D., Meneses, J., Pederson, R. and Rubenstein, J. L. R. (1997a). Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* **19**, 27-37.

Anderson, S. A., Eisenstat, D. D., Shi, L. and Rubenstein, J. L. R. (1997b). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* **278**, 474-476.

Anderson, S. A., Marin, O., Horn, C., Jennings, K. and Rubenstein, J. L. R. (2001). Origins and migratory pathways of cerebral cortical interneurons derived from the basal telencephalon. *Development* **128**, 353-363.

Andrews, G. L., Yun, K., Rubenstein, J. L. and Mastick, G. S. (2002). Dlx transcription factors regulate differentiation of dopaminergic neurons of the ventral thalamus. *Mol. Cell. Neurosci.* **23**, 107-120.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch Signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Barnstable, C. J., Hofstein, R. and Akagawa, K. (1985). A marker of amacrine cell development in rat retina. *Brain Res.* **352**, 286-290.

Bartsch, U., Kirchhoff, F. and Schachner, M. (1989). Immunohistological localization of the adhesion molecules L1, N-CAM, and MAG in the developing and adult optic nerve of mice. *J. Comp. Neurol.* **284**, 451-462.

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.

Brown, N. L., Patel, S., Brzezinski, J. and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. *Development* **128**, 2497-2508.

Bulfone, A., Kim, H. J., Puelles, L., Porteus, M. H., Grippo, J. F. and Rubenstein, J. L. R. (1993). The mouse Dlx-2 (Tes-1) gene is expressed in spatially restricted domains of the forebrain, face, and limbs in midgestation mouse embryos. *Mech. Dev.* **40**, 129-140.

Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pederson, R., Axel, R. and Rubenstein, J. L. R. (1998). An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. *Neuron* **21**, 1273-1282.

Bunt-Milam, A. H. and Saari, J. C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J. Cell Biol.* **97**, 703-712.

Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldman, D., Kalnins, V. I. et al. (1996). Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat. Genet.* **12**, 376-383.

Caffé, A. R., Visser, H., Jansen, H. G. and Sanyal, S. (1989). Histotypic differentiation of neonatal mouse retina in organ culture. *Curr. Eye Res.* **10**, 1083-1092.

Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. *Development* **126**, 525-534.

Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M. and Ezzidine, D. (1996). Cell fate determination in the vertebrate retina. *Proc. Natl Acad. Sci. USA* **93**, 589-595.

Chen, J., Tucker, C. L., Woodford, B., Szel, A., Lem, J., Gianella-Borradori, A., Simon, M. I. and Bogenmann, E. (1994). The human blue

opsin promoter directs transgene expression in short-wave cones and bipolar cells in the retina. *Proc. Natl. Acad. Sci. USA* **91**, 2611-2615.

Chow, R. L., Snow, B., Novak, J., Looser, J., Freund, C., Vidgen, D., Ploder, L. and McInnes, R. R. (2001). Vsx1, a rapidly evolving paired-like homeobox gene expressed in cone bipolar cells. *Mech. Dev.* **109**, 315-322.

Davidson, F. F., Loewen, P. C. and Khorana, H. G. (1994). Structure and function in rhodopsin: replacement by alanine of cysteine residues 110 and 187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin II state. *Proc. Natl. Acad. Sci. USA* **91**, 4029-4033.

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.

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

Eisenstat, D. D., Liu, J. K., Mione, M., Zhong, W., Yu, G., Anderson, S. A., Ghattas, I., Puelles, L. and Rubenstein, J. L. R. (1999). DLX-1, DLX-2, and DLX-5 expression define distinct stages of basal forebrain differentiation. *J. Comp. Neurol.* **414**, 217-237.

Erkman, L., McEvilly, R. J., Luo, L., Ryan, A. K., Hooshmand, F., O'Connell, S. M., Keithley, E. M., Rapaport, D. H., Ryan, A. F. and Rosenfeld, M. G. (1996). Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. *Nature* **381**, 603-606.

Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. L. and Guillemot, F. (2000). A role for neural determination genes In specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* **14**, 67-80.

Furukawa, T., Morrow, E. M. and Cepko, C. L. (1997). Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 531-541.

Gan, L., Xiang, M., Zhou, L., Wagner, D. S., Klein, W. H. and Nathans, J. (1996). POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells. *Proc. Natl. Acad. Sci. USA* **93**, 3920-3925.

Ghanem, N., Jarinova, O., Amores, A., Long, Q., Hatch, G., Park, B. K., Rubenstein, J. L. and Ekker, M. (2003). Regulatory roles of conserved intergenic domains in vertebrate Dlx bigene clusters. *Genome Res.* **13**, 533-543.

Green, E. S., Stubbs, J. L. and Levine, E. M. (2003). Genetic rescue of cell number in a mouse model of microphthalmia: interactions between Chx10 and G1-phase cell cycle regulators. *Development* **130**, 539-552.

Hatakeyama, J., Tomita, K., Inoue, T. and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. *Development* **128**, 1313-1322.

Haverkamp, S. and Wässle, H. (2000). Immunocytochemical analysis of the mouse retina. *J. Comp. Neurol.* **424**, 1-23.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J. E. and Kageyama, R. (2002). Math3 and NeuroD regulate amacrine cell fate specification in the retina. *Development* **129**, 831-842.

Kuhrt, H., Walski, M., Reichenbach, A. and Albrecht, J. (2004). Rabbit retinal organ culture as an in vitro model of hepatic retinopathy. *Graefes Arch. Clin. Exp. Ophthalmol.* **242**, 512-522.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H. and Honjo, T. (1999). Delta-induced notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J. Biol. Chem.* **274**, 7238-7244.

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

Letinic, K., Zoncu, R. and Rakic, P. (2002). Origin of GABAergic neurons in the human neocortex. *Nature* **417**, 645-649.

Levi, G., Puche, A. C., Mantero, S., Barbieri, O., Trombino, S., Paleari, L., Egeo, A. and Merlo, G. R. (2003). The Dlx5 homeodomain gene is essential for olfactory development and connectivity in the mouse. *Mol. Cell. Neurosci.* **22**, 530-543.

Lin, B., Wang, S. W. and Masland, R. H. (2004). Retinal ganglion cell type, size, and spacing can be specified independent of homotypic dendritic contacts. *Neuron* **43**, 475-485.

Liu, J. K., Ghattas, I., Liu, S., Chen, S. and Rubenstein, J. L. R. (1997). *Dlx* genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. *Dev. Dyn.* **210**, 498-512.

Liu, W., Khare, S. L., Liang, X., Peters, M. A., Liu, X., Cepko, C. and Xiang, M. (2000). All Brn3 genes can promote retinal ganglion cell differentiation in the chick. *Development* **127**, 3237-3247.

Liu, W., Mo, Z. and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. *Proc. Natl. Acad. Sci. USA* **98**, 1649-1654.

Long, J. E., Garel, S., Depew, M. J., Tobet, S. and Rubenstein, J. L. (2003). DLX5 regulates development of peripheral and central components of the olfactory system. *J. Neurosci.* **23**, 568-578.

Ma, W., Yan, R. T., Xie, W. and Wang, S. Z. (2004). bHLH genes cath5 and cNSCL1 promote bFGF-stimulated RPE cells to transdifferentiate toward retinal ganglion cells. *Dev. Biol.* **265**, 320-328.

Marin, O., Anderson, S. A. and Rubenstein, J. L. R. (2000). Origin and molecular specification of striatal interneurons. *J. Neurosci.* **20**, 6063-6076.

Marquardt, T. and Gruss, P. (2002). Generating neuronal diversity in the retina: one for nearly all. *Trends Neurosci.* **25**, 32-38.

Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001). Pax6 is required for the multipotent state of progenitor cells. *Cell* **105**, 43-55.

Masland, R. H. (2001). The fundamental plan of the retina**.** *Nat. Neurosci.* **4**, 877-886.

Mayordomo, R., Valenciano, A. I., de la Rosa, E. J. and Hallböök, F. (2003). Generation of retinal ganglion cells is modulated by caspase-dependent programmed cell death. *Eur. J. Neurosci.* **18**, 1744-1750.

McGuinness, T., Porteus, M. H., Smiga, S., Bulfone, A., Kingsley, C., Qiu, M., Liu, J. K., Long, J. E., Xu, D. and Rubenstein, J. L. R. (1996). Sequence, organization, and transcription of the Dlx-1 and Dlx-2 locus. *Genomics* **35**, 473-485.

Merlo, G. R., Paleari, L., Mantero, S., Genova, F., Beverdam, A., Palmisano, G. L., Barbieri, O. and Levi, G. (2002). Mouse model of split hand/foot malformation type I. *Genesis* **33**, 97-101.

Mu, X. and Klein, W. H. (2004). A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. *Semin. Cell Dev. Biol.* **15**, 115-123.

Mu, X., Beremand, P. D., Zhao, S., Pershad, R., Sun, H., Scarpa, A., Liang, S., Thomas, T. L. and Klein, W. H. (2004). Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. *Development* **131**, 1197-1210.

Panganiban, G. and Rubenstein, J. L. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. *Development* **129**, 4371-4386.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M. and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* **8**, 9-22.

Qiu, M., Bulfone, A., Martinez, S., Meneses, J. J., Shimamura, K., Pederson, R. A. and Rubenstein, J. L. R. (1995). Null mutation of Dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. *Genes Dev.* **9**, 2523-2538.

Qiu, M., Bulfone, A., Ghattas, I., Meneses, J. J., Christenses, L., Sharpe, P. T., Presley, R., Pedersen, R. A. and Rubenstein, J. L. R. (1997). Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx1, Dlx2, and Dlx1 and Dlx2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. *Dev. Biol.* **185**, 165-184.

Robledo, R. F., Rajan, L., Li, X. and Lufkin, T. (2002). The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev.* **16**, 1089-1101.

Sidman, R. L. (1961). Histogenesis of mouse retina studied with thymidine-H3. In *Structure of the Eye* (ed. G. K. Smelser), pp. 487-506. New York: Academic Press.

Steimke, M. M. and Hollyfield, J. G. (1995). Cell birthdays in Xenopus laevis retina. *Differentiation* **58**, 189-193.

Sumiyama, K., Irvine, S. Q., Stock, D. W., Weiss, K. M., Kawasaki, K., Shimizu, N., Shashikant, C. S., Miller, W. and Ruddle, F. H. (2002). Genomic structure and functional control of the Dlx3-7 bigene cluster. *Proc. Natl. Acad. Sci. USA* **99**, 780-785.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L. Nakanishi, S., Guillemot, F. and Kagayama, R. (1996). Mammalian hairy and enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. *Neuron* **16**, 723-734.

Tong, W. and Pollard, J. W. (2001). Genetic evidence for the interactions of cyclin D1 and p27(Kip1) in mice. *Mol. Cell. Biol.* **21**, 1319-1328.

Toyoshima, H. and Hunter, T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* **15**, 24-29.

Wang, S. W., Mu, X., Bowers, W. J., Kim, D. S., Plas, D. J., Crair, M. C., Federoff, H. J., Gan, L. and Klein, W. H. (2002). Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth. *Development* **129**, 467-477.

Xiang, M., Gan, L., Li, D., Chen, Z. Y., Zhou, L., O'Malley, B. W., Jr, Klein, W. and Nathans, J. (1997). Essential role of POU-domain factor Brn-3c in auditory and vestibular hair cell development. *Proc. Natl. Acad. Sci. USA* **94**, 9445-9450.

Yang, Z., Ding, K., Pan, L., Deng, M. and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. *Dev. Biol.* **264**, 240-254.

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

Yun, K., Fischman, S., Johnson, J., Hrabe de Angelis, M., Weinmaster, G. and Rubenstein, J. L. (2002). Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. *Development* **129**, 5029-5040.

Zerucha, T., Stuhmer, T., Hatch, G., Park, B. K., Long, Q., Yu, G., Gambarotta, A., Schultz, J. R., Rubenstein, J. L. and Ekker, M. (2000). A highly conserved enhancer in the Dlx5/Dlx6 intergenic region is the site of cross-regulatory interactions between Dlx genes in the embryonic forebrain. *J. Neurosci.* **20**, 709-721.

Zhou, Q. P., le, T. N., Qiu, X., Spencer, V., de Melo, J., Du, G., Plews, M., Fonseca, M., Sun, J. M., Davie, J. R. and Eisenstat, D. D. (2004). Identification of a direct Dlx homeodomain target in the developing mouse forebrain and retina by optimization of chromatin immunoprecipitation. *Nucleic Acids Res.* **32**, 884-892.