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

Jimmy de Melo¹, Guoyan Du²,³, Mario Fonseca²,³ Leigh-Anne Gillespie³, William J. Turk³, John L. R. Rubenstein⁴ and David D. Eisenstat¹,²,³,⁵,∗

¹Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg, Manitoba, R3E 3J7, Canada
²Department of Pediatrics and Child Health, University of Manitoba, Winnipeg, Manitoba, R3A 1S1, Canada
³Manitoba Institute of Cell Biology, Cancer Care Manitoba, Winnipeg, Manitoba, R3E 0V9, Canada
⁴Department of Psychiatry, University of California, San Francisco, CA 94143, USA
⁵Department of Ophthalmology, University of Manitoba, Winnipeg, Manitoba, R3E 0V9, Canada

*Author for correspondence (e-mail: eisensta@cc.umanitoba.ca)

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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; Steinke 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 Dlx2 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; Bullone 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 (Qi et al., 1997; Anderson et al., 1997a). Ocular retardation mutant mice and controls (129sv-C57Bl6-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 (Qi 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 to dishes containing DMEM/F12 media (Gibco/Invitrogen). Retinal explant cultures controls. Comparative studies all mutants were paired with wild-type littermate genotyped as described (Qiu et al., 1995; Burmeister et al., 1996). For retinal explants, sections from each mutant explant were used as a centered start point and were matched histologically. 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 spaced intervals to completely survey each retina. Six sets of eyes 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 immunostained 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), IJ (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 cholera 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 318.1±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 littersmates ($t=1.81$, $P>0.05$, $n=6$) (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, 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.
There is a complete loss of late-born RGCs in the \( \text{Dlx1/Dlx2} \) double mutant

As the earliest BRN3b-expressing RGCs are established before the onset of \( \text{Dlx1} \) and \( \text{Dlx2} \) expression in the developing retina, we hypothesized that \( \text{Dlx1/Dlx2} \) 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 \( \text{Dlx1/Dlx2} \) 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 \( \text{Dlx1/Dlx2} \) 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 or1/or1) 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, or1/or1 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 or1/or1 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 or1/or1 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 129sv/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 or1/or1 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 (I, 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 the 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 homebox 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
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 (or1/or1), in which Chx10 function is lost and there is a severe loss of RGCs, provides support for a Dlx-dependent RGC population. In the or1/or1 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 or1/or1 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 dependent kinase inhibitor p27Kip1. Loss of cellularity in the or1/or1 mutant may result from diminished cellular proliferation due to unchecked p27Kip1 (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 or1/or1 mutant, aberrant cell cycle regulation results in the absence of Math5 specified...
BRN3b-expressing RGCs in the orJ/orJ retina by E13.5. However, in MASH1 expressing retinal progenitors, expression of Dlx genes may restore repression of p27Kip1 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 orJ/orJ 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


