Chapter 87
Roles of Homeobox Genes in Retinal Ganglion Cell Differentiation and Axonal Guidance

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87.1 Introduction

Among the six major classes of retinal neurons, retinal ganglion cells (RGC) are the only projection neurons. RGCs differentiate in the mouse retina around E11.5 followed by horizontal, cone, and amacrine cells. The early generated RGC are located in the central inner retina, and the axons of these RGC commence their journey to central nervous system (CNS) targets immediately after terminal differentiation

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(Marquardt and Gruss 2002). Combinations of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors (TF) play important roles in RGC cell fate specification and influence RGC axonal pathfinding choices *en route*. In this review, we will focus on the roles of HD transcription factors in these processes.

87.2 *Brn-3* Genes

The POU-domain is a bipartite DNA-binding protein domain, containing a POU-specific region and a POU-homeodomain region. The class IV POU-domain proteins, BRN3a, BRN3b, and BRN3c (*POU4f1*, *POU4f2,* and *POU4f3*, respectively) are the homologs of *Unc-86* in *C. elegans*. *Brn-3* genes are expressed in the embryonic and adult CNS, and are required for sensorineural development and survival.

All three *Brn-3* POU-homeodomain genes are expressed in the developing retina, specifically in postmitotic RGC. *Brn3b* expression is first detected in the earliest RGC at E11.5 in the central inner mouse retina, followed by *Brn3a* and *Brn3c* expression 2 days later (Xiang et al. 1995; Pan et al. 2005). Through E15.5 to the adult retina, *Brn3a* and *Brn3b* expression overlaps in 80% of RGC. However, *Brn3a* is the predominant gene expressed in P5 retinas, with few RGC expressing *Brn3b* (Quina et al. 2005). Only ~15% of RGC express *Brn3c* (Xiang et al. 1995).

The overlapping expression pattern and a similar specific DNA-binding site [(A/G)CTCATTAA(T/C)] of these three BRN-3 proteins suggest their functional redundancy in retinogenesis. However, targeted mutations of *Brn-3* genes in mice show distinct defects, but only the *Brn3b*$^{-/-}$ mouse shows an obvious retinal phenotype. In *Brn3b* null mice there is loss of 60–80% RGC in adult retinas, depending on the background genetic strain (Erkman et al. 1996; Gan et al. 1996). This RGC loss is due to enhanced apoptosis after E15.5, but not to defects of initial cell fate specification or migration. *Brn3b* is also required for RGC axon pathfinding and fasciculation (Erkman et al. 2000). *Brn3a* null mutants die at birth, with loss of dorsal root ganglion and trigeminal neurons (Erkman et al. 1996; Xiang et al. 1996). *Brn3c*$^{-/-}$ mice display deficits in balance and complete deafness, attributed to loss of vestibular and auditory hair cells (Erkman et al. 1996; Xiang et al. 1997). Neither *Brn3a* nor *Brn3c* mutants show obvious defects in retinal development.

Brn3a and *Brn3c* are identified as downstream of *Brn3b* in retinogenesis, and there is reduced *Brn3a* expression in *Brn3b* mutants (Erkman et al. 1996). Despite the dominant roles of *Brn3b* in retinal development, several independent groups have reported that all three *Brn-3* genes are functionally equivalent in retinogenesis. Overexpression of *Brn3a*, *Brn3b,* or *Brn3c* in chick retinal progenitors exerts a similar effect in promoting RGC differentiation (Liu et al. 2000). Knocking-in the *Brn3a* coding sequence into a *Brn3b* null background mouse rescues RGC from apoptosis and restores RGC axonal pathfinding (Pan et al. 2005).

A recent study has shown that conditional deletion of *Brn3a* alters RGC dendritic stratification without influencing RGC axon central projections. However, the

conditional *Brn3b* knockout mice show reduced RGC numbers, loss of axonal projections to medial (MTN) and lateral terminal nuclei (LTN) and corresponding visual sensory defects (Badea et al. 2009).

87.3 *Dlx* Genes

Dlx genes are the vertebrate orthologs of *distal-less* (*Dll*). There are six *Dlx* genes identified in mice, which are arranged into three bigene clusters (*Dlx1/Dlx2*, *Dlx5/Dlx6*, and *Dlx3/Dlx7*), and are localized on mouse chromosomes 2, 6, and 11, respectively (Ghanem et al. 2003). Within the intergenic regions of *Dlx1/2* and *Dlx5/6*, several *cis*-acting regulators have been characterized, including *I12a* and *I12b* between the *Dlx1* and *Dlx2* genes, and *I56i* and *I56ii* separating *Dlx5* and *Dlx6* (Poitras et al. 2007). Two conserved enhancer elements, URE1 and URE2, have also been found in the 5′ flanking region of *Dlx1* (Hamilton et al. 2005) (Du and Eisenstat, unpublished). These *cis*-acting elements are important for cross-regulatory interactions between the *Dlx* genes. One example is that DLX1 and DLX2 regulate *Dlx5/Dlx6* expression by acting on *I56i* (Zhou et al. 2004).

Dlx1 and *Dlx2* were first detected in the retinal neuroepithelium on E12.5 including mitotic cells adjacent to the ophthalmic ventricle (Eisenstat et al. 1999). Our recent study reported DLX2 immunostaining in E11.5 retina, with DLX2 expressed in a dorsal (high) to ventral (low) gradient (de Melo et al. 2008). At E13.5, both DLX1 and DLX2 are expressed throughout the retina, with boundaries in peripheral and central inner retina. Interestingly, some other "retinal" homeobox genes are expressed in a nearly complementary manner to DLX2 at this stage. At E13.5, the highest level of PAX6 expression is observed in the most peripheral retina and BRN3b is expressed in the inner central retina, where DLX2 is absent. By E18.5, DLX1 and DLX2 expressions are highly restricted to the ganglion cell layer (GCL) and inner part of the neuroblastic layer (NBL), where they are co-expressed with markers for RGC, amacrine, and horizontal cells. DLX1 expression resembles DLX2 in embryonic retina, but decreases dramatically after birth and cannot be detected in adult retina. However, DLX2 is robustly expressed in the GCL and inner nuclear layer (INL) throughout adulthood (de Melo et al. 2003).

Although the role of *Dlx* genes in forebrain development is well reported, very few studies have described *Dlx* gene function in retina development. Homozygous deletion of *Dlx1* and *Dlx2* is perinatally lethal, and leads to a 33% reduction of RGC number due to enhanced apoptosis of late-born RGCs (de Melo et al. 2005). *TrkB*, a receptor for brain derived neurotrophic factor (BDNF) mediated signalling, was identified as a DLX2 downstream target during mouse retinal development and may contribute to RGC survival (de Melo et al. 2008).

The role of *Dlx5* and *Dlx6* genes in retinogenesis is still not clear. *In situ* hybridization revealed *Dlx5* mRNA expression in retina by E16.5. In P0 and adult retina, *Dlx5* mRNA is co-expressed with DLX2 in the GCL and INL. The *Dlx5/Dlx6* intergenic

enhancer (*I56i*) is co-expressed with DLX5, DLX1, and DLX2 in RGC, amacrine, and horizontal cells (Zhou et al. 2004). There is no published report regarding the retinal phenotype of *Dlx5/Dlx6* knockout mice.

87.4 *Vax* Genes

Vax (Ventral anterior homeobox-containing) genes are a homeodomain gene subfamily, closely related to *Emx* genes, sharing sequence homology, similar chromosomal location, and expression patterns (Hallonet et al. 1998). In the mouse, *Vax1* mRNA is first detected at E8, in the anterior neural ridge and adjacent ectoderm. During embryogenesis, *Vax1* expression is restricted to the derivatives of these regions, including basal forebrain, ventral optic vesicle, optic disk, stalk, and chiasm (Hallonet et al. 1998). The targeted deletion of *Vax1* shows defects in RGC axonogenesis and axonal-glial associations, without influencing expression of *Pax2* and *BF1*. In addition, *Vax1*$^{-/-}$ axons fail to fasciculate and do not extend toward the hypothalamic midline, leading to an absence of optic chiasm development. These RGC axon pathfinding defects are partially due to the loss of some important axon guidance cues, including *Netrin-1* and *EphB3*, but not *Slit1*(Bertuzzi et al. 1999). Another obvious phenotype of *Vax1* mutants is the failure of choroid fissure closure, known as coloboma. *Pax6* and *Rx* are ectopically expressed in the *Vax1* mutant optic nerve. However, *Pax2* expression remains unaffected in the mutants.

VAX2 shares an identical homeodomain with VAX1, and the *Vax2* gene is tightly linked with *Emx1* in mouse and human. By E9, *Vax2* transcripts are detected in the ventral optic vesicle, with lower expression in the optic nerve and stalk. *Vax1* and *Vax2* then share overlapping expression patterns in ventral retina and optic stalk. By E12, *Vax2* expression is restricted to the ventral neural retina in the whole retinal population. However, at later embryonic stages, *Vax2* is only detected in ventral RGC. *Vax2* is not expressed in the adult retina (Bertuzzi et al. 1999; Mui et al. 2002).

Consistent with its predominant ventral retinal expression pattern, *Vax2* plays a major role in ventralizing embryonic retina. Misexpression of *Vax2* in the dorsal retina is able to alter the expression of the putative dorsal-ventral marker genes, including upregulation of ventral retinal markers *EphB2/EphB3*, *Pax2,* and *Vax2* itself, and downregulation of the dorsally restricted TF, *Tbx5* (Barbieri et al. 1999). In addition, ectopic *Vax2* expression in dorsal retina is sufficient to induce profound axon pathfinding defects of dorsal RGC (Schulte et al. 1999). In agreement with these *Vax2* gain-of-function studies, the ventral RGC from *Vax2* null mice show complete dorsalization. The RGC axons from *Vax2*$^{-/-}$ ventral retina aberrantly project to the lateral rostral edge of the superior colliculus (SC) together with all the dorsal RGC axons, instead of medial rostral SC, the destination of all the wild-type ventral RGC axons. The expression of *EphB2*/*EphB3* is absent in *Vax2*$^{-/-}$ ventral retina.

87.5 Islet Genes

ISL1 and ISL2 are a subfamily of LIM homeodomain TF, characterized by two zinc-finger motifs (LIM domain) and a homeodomain. Both ISL1 and ISL2 play important roles in determining motor neuron subtype identity, axonal projections and peripheral innervations (Shirasaki and Pfaff 2002). Most of the work has been done in the spinal cord of vertebrate and invertebrate models. *Isl1* and *Isl2* are also expressed in the embryonic and postnatal retina.

Isl2 expression is first detected in the E13 retina, and by E17, almost all the *Isl2* positive cells are RGC (Pak et al. 2004). *Isl2* is expressed at high levels in the dorsal retina, with weak expression in the ventral-temporal region. By repressing *Zic2* and *EphB1*, *Isl2* specifies the contralateral projection of RGC axons. In comparison to the specific RGC expression of *Isl2*, *Isl1* expression shows different patterns from embryonic to postnatal retina. Prior to E15.5, *Isl1* is predominantly expressed in RGC. However, from E15.5 to the adulthood, *Isl1* expression is detected in RGC, amacrine cells, and bipolar cells (Elshatory et al. 2007). Recent work has shown that under the regulation of ATOH7 (formerly MATH5), *Isl1* defines a distinct but overlapping subpopulation of RGC with *Brn3b* (Mu et al. 2008; Pan et al. 2008)

87.6 Summary

In this review, we have described four major families of homeobox genes which play important roles in RGC differentiation as well as axonal pathfinding. The mechanism underlying how these HD TFs affect axonal pathfinding is not entirely known. One possibility is that the downstream targets directly regulated by these HD TF are responsible for axonal guidance. Examples of this are the repression of *EphB1* by *Isl2*, and *Vax1/Vax2* regulation of *EphB2/EphB3* expression. The roles of *Dlx* homeobox genes in RGC axonal guidance have not yet been reported. However, in the mouse telencephalon, *Dlx1* and *Dlx2* promote the tangential migration of GABAergic interneurons by repressing axonal growth (Cobos et al. 2007) and inhibiting Neuropilin-2 expression(Le et al. 2007). It is possible that the genetic program defining RGC identity also encodes a unique "sensory" network for their axons, determining how and where RGC axons respond to guidance cues *en route* to CNS targets.

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