Functional equivalence of Brn3 POU-domain transcription factors in mouse retinal neurogenesis

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Summary

POU-domain transcription factors play essential roles in cell proliferation and differentiation. Previous studies have shown that targeted deletion of each of the three POU-domain Brn3 factors in mice leads to the developmental failure and apoptosis of a unique set of sensory neurons in retina, dorsal root ganglia, trigeminal ganglia and inner ear. The specific defects associated with the removal of each Brn3 gene closely reflect their characteristic spatiotemporal expression patterns. Nevertheless, it remains elusive whether Brn3 factors are functionally equal and that their distinct Brn3 spatiotemporal expression patterns. Nevertheless, it remains elusive whether Brn3 factors are functionally equal and that their unique roles in neurogenesis are determined by the Brn3b locus, we showed here that Brn3a is expressed in a spatiotemporal manner identical to that of endogenous Brn3b. In addition, Brn3a functionally restored the normal development and survival of retinal ganglion cells (RGCs) in the absence of Brn3b and fully reinstated the early developmental expression profiles of Brn3b downstream target genes in retina. These results indicate that Brn3 factors are functionally equal and that their unique roles in neurogenesis are determined by the distinctive Brn3 spatiotemporal expression patterns.

Key words: Brn3, POU-domain, Retina, Neurogenesis, Retinal ganglion cells, Transcription factors, Pou4f

Introduction

A central issue in the development of the vertebrate nervous system is how its vast number of distinct neurons are generated. POU-domain transcription factors play pivotal roles in cell differentiation processes (Wegner et al., 1993). The Class IV POU-domain Brn3 factors Brn3a, Brn3b and Brn3c (Pou4f1, Pou4f2 and Pou4f3, respectively – Mouse Genome Informatics) share a highly conserved DNA-binding POU-domain with ~95% amino acid sequence identity and an ~70% sequence identity in regions outside the POU-domains (Xiang et al., 1995). In vitro DNA-binding assays have shown that all Brn3 proteins bind to the same specific consensus DNA sequences (Gruber et al., 1997; Xiang et al., 1995). Expression studies have revealed that Brn3 genes are expressed in distinct but often overlapping patterns in developing dorsal root and trigeminal ganglia, RGCs, auditory and vestibular neurons, and in selected midbrain nuclei associated with motor and sensory controls (Fedtsouva and Turner, 1995; Gerrero et al., 1993; Ninkina et al., 1993; Turner et al., 1994; Xiang, 1998; Xiang et al., 1997a; Xiang et al., 1995; Xiang et al., 1993). It is postulated that Brn3 genes are required for the cell differentiation of these sensory neurons (Turner et al., 1994; Xiang et al., 1993).

To investigate the roles of Brn3 genes, we and others have created targeted mutations in Brn3 genes and have shown that individual null mutations lead to distinct defects in the development of sensory neurons (Xiang et al., 1997a). Mice carrying Brn3a-null mutation suffer from a selective loss of neurons in somatosensory ganglia and in selective brainstem nuclei, display uncoordinated limb movement and impaired sucking, and die shortly after birth (McEvilly et al., 1996; Xiang et al., 1996). A detailed examination of the role of Brn3a in neurogenesis has demonstrated the axonal growth and pathfinding defects in trigeminal and dorsal root neurons prior to their programmed cell death (Eng et al., 2001). Last, Brn3a is required for axon pathfinding and target field innervation of spiral and vestibular ganglion neurons in the inner ear (Huang et al., 2001).

In contrast to the studies using Brn3a mutants, Brn3b-null mutants display a loss of ~70% of RGCs in adult retinal ganglion cell layer (GCL) (Erkman et al., 1996; Gan et al., 1996). Further studies by using Brn3b-lacZ and Brn3b-AP knock-in mice have shown that Brn3b is not required for cell fate specification and migration of RGCs. Rather, Brn3b expression is essential for axon growth, pathfinding, fasciculation and survival of RGCs (Gan et al., 1999; Wang et al., 2000). Analysis of RGC axons in postnatal Brn3b-null mice also reveals that Brn3b is involved in the pathfinding of RGC axons at multiple points along their pathways and in the establishment of topographic order in the superior colliculus (Erkman et al., 2000).

In the third group of Brn3 mutants, Brn3c-null mice lack...
vestibular and auditory hair cells in the inner ear, are deaf and have impaired balancing abilities (Erkman et al., 1996; Xiang et al., 1997a). Recently, Brn3c was shown to be essential for the differentiation of a small number of RGCs (Wang et al., 2002). This finding is exacerbated in Brn3b and Brn3c double knockout mice, where significantly more RGCs are lost than in either Brn3b or Brn3c single mutants (Wang et al., 2002).

The unique phenotypes associated with Brn3 knockouts illustrate that each Brn3 gene has a great degree of functional specificity. One explanation of their functional uniqueness is that the unique protein sequences of Brn3 factors could render each characteristic biochemical properties and thus, distinctive transcriptional activities. Conversely, based on their highly conserved POU-domains and identical DNA-binding properties, all Brn3 factors could also be functionally equivalent in transcriptional activities. The distinctive neuronal defects related to the loss of each Brn3 gene could simply reflect its characteristic spatiotemporal expression patterns. To test these hypotheses, we perform in vivo gene-replacement experiments to investigate whether knocking-in one Brn3 gene to replace the other can functionally rescue the neuronal defects associated with the loss of the other Brn3 gene. We demonstrate that targeted replacement of Brn3b with Brn3a corrects the retinal defects identified in Brn3b-null mice. The RGCs expressing Brn3a<sub>Δt</sub> in the absence of Brn3b are able to form fasciculated axons, to generate proper axon projection, and to avoid the fate of programmed cell death. Furthermore, Brn3a<sub>Δt</sub> expression from the Brn3b locus restores the early developmental expression profiles of Brn3b downstream target genes. Our results strongly argue for the functional equivalence of Brn3 transcription factors and imply a shared Brn3 regulatory pathway in the development of various sensory neurons.

Materials and methods

Animals

Brn3b<sub>lacZ</sub> and Brn3b<sub>APΔt</sub> knock-in mice were generated by targeted gene disruption as previously described (Gan et al., 1999). A Brn3a<sub>Δt</sub> knock-in construct was generated with the same 5′ and 3′ Brn3b genomic DNA fragments as those in a Brn3b<sub>lacZ</sub> knock-in construct for homologous recombination in embryonic stem (ES) cells (Gan et al., 1999). The DNA fragment containing Brn3b-coding regions in two exons and a single intron was replaced with Brn3a cDNA sequences containing only the entire open read frame (ORF). In addition, a DNA fragment containing the lacZ sequence tag was inserted 3′ to Brn3a translation terminal codon and was used as a specific in situ hybridization probe to monitor the expression of Brn3a<sub>Δt</sub>. This construct placed the Brn3a ORF under the control of Brn3b transcriptional regulation. To generate Brn3b<sub>Δt</sub> knock-in mice, a NotI-linearized Brn3b<sub>Δt</sub> targeting construct was electroporated into ABI (129S6) embryonic stem (ES) cells (a gift from A. Bradley, Baylor College of Medicine). Nine targeted clones were obtained from a total of 96 G418- and FIAU-resistant ES clones. Targeted clones were confirmed by Southern blotting genotyping. Two targeted ES cell clones were injected into C57BL/6J blastocysts to generate mouse chimeras and heterozygous Brn3b<sub>Δt</sub> mutant mice were generated in 129S6 and C57BL/6J mixed background as previously described (Gan et al., 1999; Gan et al., 1996). Embryos were identified as E0.5 at noon on the day at which vaginal plugs were first observed.

Histochemistry

To compare retinas and optic nerves in Brn3b<sub>lacZ</sub>, Brn3b<sub>APΔt</sub> and Brn3b<sub>APΔt</sub> mice, eye cups and brain tissues containing optic nerves, optic chiasms and optic tracks from weight- and gender-matched mice at 6 weeks age were paraffin-embedded and sectioned at 10 µm. Optic nerves were cut at ~1 mm anterior to the optic chiasms. Sections were de-waxed and stained with Hematoxylin and Eosin. Areas of the optic nerves were photographed and the sizes of optic nerves were computed and compared by Scion Image.

Immunohistochemistry, in situ hybridization, BrdU labeling and X-gal staining

Staged embryo and tissue samples were dissected and immediately fixed in 4% paraformaldehyde. The samples were then embedded and frozen in OCT medium (Tissue-Tek). For immunolabeling, cryosections were cut at 15 µm. The working dilutions and sources of antibodies used in this study were: mouse anti-bromodeoxyuridine (BrdU) (1:200, Becton Dickson), mouse anti-Brn3a (1:100, Santa Cruz), goat anti-Brn3b (1:2,000, Santa Cruz), rabbit anti-phospho-histone 3 (1:400, Santa Cruz) and anti-nonphosphorylated neurofilament H (SMI-32) (1:2,000, Sternberger Monoclonals). Alexa-conjugated secondary antibodies (Molecular Probes) were used at a concentration of 1:400. For in situ hybridization experiments, 20 µm cryosections were used as previously described (Li and Joyner, 2001).

Detection of β-galactosidase activity was determined by X-gal staining (Gan et al., 1999). Briefly, retinal tissues were fixed in 4% paraformaldehyde in PBS at 4°C for 30 minutes. Whole retinas or 16 µm frozen sections were stained overnight at room temperature with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl<sub>2</sub> in PBS. For bromodeoxyuridine (BrdU) (Sigma) pulse-labeling experiments, pregnant females were injected intraperitoneally with 100 µg BrdU/gram body weight 1 hour before they were sacrificed. Embryo processing and BrdU labeling were performed as previously described (Mishina et al., 1995).

Lipophilic dye tracing

For optic nerve labeling, E17.5 embryonic mouse heads were fixed overnight in 4% paraformaldehyde in PBS. After the right eyes were enucleated, crystals of DiI (Molecular Probes) were implanted unilaterally into the optic discs. After incubation at 37°C in PBS containing 0.1% sodium azide for 2 weeks, the brains were dissected. The labeled optic nerves were exposed and visualized with a Nikon fluorescence dissecting microscope.

Results

Expression of Brn3a and Brn3b in developing retina

To better elucidate the roles of Brn3a and Brn3b in retinal development, we compared their spatiotemporal expression patterns in embryonic retinas. Immunostaining experiments with anti-Brn3b first detected Brn3b expression in the central retina at E11.5 (Fig. 1A). As retinal development progressed, Brn3b expression expanded circumferentially towards peripheral retinal regions, peaking at E12.5 to E15.5 when the majority of RGCs were generated (Fig. 1D,G,J). The retinal expression of Brn3b was clearly detected in the migrating RGCs in the neuroblast layer (NBL) and in the post-migration RGCs in the newly formed GCL at the inner surface of the developing retina. Analysis of Brn3a expression by anti-Brn3a immunolabeling illustrated that Brn3a expression was not detectable at E11.5 (Fig. 1B) and that the onset of Brn3a expression started 1 day later than that of Brn3b at E12.5 (Fig. 1E). Similar to Brn3b, Brn3a expression was first observed in the central retina and expanded toward the peripheral retina (Fig. 1E,H,K). However, Brn3a expression was limited to the
post-migration RGCs in the GCL where its expression overwhelmingly overlapped with that of Brn3b (inserts in Fig. 1F,I,L). At E15.5, both Brn3a and Brn3b were strongly expressed in the GCL and their prominent expression in RGCs continued in adult retina (Fig. 1L; data not shown).

In mice, most RGC precursors become postmitotic, differentiate and migrate to the GCL at E11 to E18 with a peak at E13 to E15 (Hinds and Hinds, 1974; Young, 1985). The early onset of Brn3b expression in the NBL suggests that Brn3b could play a role in regulating the proliferation of retinal progenitors. To address this possibility and to detect whether Brn3b is expressed in the proliferating retinal progenitors, we analyzed Brn3b expression with respect to cell cycles by using specific cell cycle markers at E12.5 when both Brn3b-positive and proliferating progenitor cells were readily detectable. Retinal sections from BrdU-treated wild-type embryos were double-immunolabeled with anti-Brn3b and anti-BrdU as the S-phase or anti-phospho-histone 3 Ser10 (PH3) as the M-phase markers for proliferating cells. As shown in Fig. 1N, immunolabeling with anti-BrdU revealed the proliferating progenitors at S phase throughout E12.5 retina and the adjacent tissues. Anti-Brn3b showed labeled cells in both the NBL and the GCL (Fig. 1M). Double-immunolabeling images illustrated that Brn3b-positive cells did not colocalize with BrdU-positive cells (Fig. 1O). Similarly, double-immunolabeling with anti-Brn3b and anti-PH3 revealed no Brn3b expression in cells at M phase (Fig. 1P-R). Thus, the above results indicate that Brn3b is expressed exclusively in postmitotic retinal cells and are in agreement with previous expression studies of Brn3b in E15.5 retina (Trieu et al., 1999). We have previously shown that the loss of Brn3b does not affect the initial differentiation or the migration of RGCs to the GCL (Gan et al., 1999). The postmitotic expression of Brn3b indicates that Brn3b is unlikely to play a direct role in regulating the proliferation of retinal progenitors and further confirms our earlier findings that Brn3b executes its late role in regulating the terminal differentiation events of RGCs.

Previously, studies of adult retinas have shown that the Brn3-b-null mutation leads to the reduced retinal expression of Brn3a (Gan et al., 1996). To test if the loss of Brn3a expression is caused by Brn3b-null mutation rather than the apoptosis of RGCs, we asked whether the downregulation of Brn3a occurs prior to the onset of RGC apoptosis in developing Brn3b-null retinas. In situ hybridization of wild-type retinas showed the onset of Brn3a and Brn3b expression at E12.5 and E11.5, respectively (Fig. 2A,B). In Brn3b-null retinas, Brn3a expression was greatly reduced starting at E12.5 (Fig. 2C). As the apoptosis of 70% Brn3b-null RGCs does not start until E14.5 in Brn3b-null mice (Gan et al., 1999; Xiang, 1998), downregulation of Brn3a is unlikely to be due to the loss of RGCs. Rather, the initiation of Brn3a expression in normal retina depends on Brn3b expression and the loss of Brn3b directly causes the decreased Brn3a expression.

**Expression of Brn3a in developing retina**

We have previously shown that lacZ and AP knock-in reporters in the Brn3b locus are expressed in a spatiotemporal manner identical to that of endogenous Brn3b (Gan et al., 1999). To verify whether the expression of Brn3a recapitulates that of endogenous Brn3, we compared the expression of Brn3a and Brn3b in Brn3a and Brn3b retinas. Compared with the late onset of endogenous Brn3a expression detected by Brn3a 3’ UTR probe
in the GCL of Brn3b<sup>−/−</sup> retina (Fig. 2E), the lacZ probe detected Brn3a<sup>ki</sup> expression at E11.5 in the NBL and GCL, which resembled the spatiotemporal expression patterns of Brn3b (Fig. 2B,F). Immunolabeling with Brn3a antibody demonstrated that the expression of Brn3a<sup>ki</sup> started at E11.5 and was clearly detectable in cells of the NBL and the GCL (Fig. 2G). Double-immunolabeling of Brn3a and Brn3b revealed completely overlapping expression patterns of Brn3a and endogenous Brn3b (insets, Fig. 2G-I). Thus, by using the knock-in approach, we were able to express Brn3a in all Brn3b-expressing RGCs and in the same spatiotemporal manners as those of Brn3b.

**Absence of retinal defects of in adult Brn3b<sup>3a</sup> knock-in mice**

To detect the effect of Brn3a<sup>ki</sup> on retinal development in Brn3b-null mice, we examined the potential defects in retinal structures and in RGC number and properties. Hematoxylin and eosin staining of retinal structure and X-Gal staining of nuclear Brn3b-lacZ activity (as the specific marker for all RGCs) showed a loss of ~70% RGCs in the GCL of Brn3b<sup>AP/lacZ</sup> retina in mice 6 weeks of age (Fig. 3B,E). Interestingly, the RGC number and the overall structure were indistinguishable in Brn3b<sup>AP/lacZ</sup> and wild-type or Brn3b<sup>lacZ/+</sup> retinas (compare Fig. 3A with 3C, and 3D with 3F). As another measure to detect the changes in RGCs, we immunolabeled retinas with mouse monoclonal antibody SMI-32, which predominantly labels the axons of large RGCs (Nixon et al., 1989). Brn3b<sup>AP/lacZ</sup> retinas showed significantly fewer and less fasciculated axon bundles projecting into the optic disc (OD) (Fig. 3H). Staining of Brn3b<sup>AP/lacZ</sup> (Fig. 3I) and wild-type or Brn3b<sup>lacZ/+</sup> (Fig. 3G).

![Fig. 2. Downregulation of Brn3a expression in Brn3b-null retinas and co-localization of Brn3a and Brn3b in heterozygous Brn3b<sup>3a/+</sup> retinas. (A-F) In situ hybridization using Brn3b, Brn3a<sup>3′UTR</sup> and lacZ probes to show the expression patterns of Brn3b, Brn3a and Brn3a<sup>ki</sup> mRNA in E11.5-15.5 retinas. (A) Expression of endogenous Brn3a in wild-type mice. (B) Brn3b expression in wild-type mice. (C) Reduced expression of Brn3a in Brn3b-null retinas. (D) Absence of Brn3b mRNA in Brn3b-null retinas. (E) Expression of endogenous Brn3a in Brn3b<sup>3a/+</sup> retinas. (F) Onset of Brn3a<sup>ki</sup> expression at E11.5 and the expression of Brn3a<sup>ki</sup> mRNA in the GCL and the NBL of Brn3b<sup>3a/+</sup> retinas. (G-I) Immunolabeling of Brn3b<sup>3a/+</sup> retinas at E11.5-15.5 with anti-Brn3a (red) and anti-Brn3b (green). (G) Detection of Brn3a protein in Brn3b<sup>3a/+</sup> retinas in the NBL and the GCL. (H) Brn3b expression in Brn3b<sup>3a/+</sup> retinas in the NBL and the GCL. (I) Complete overlap of Brn3a and Brn3b expression in cells in the NBL and the GCL. Insets show the enlarged view of the corresponding boxed regions and revealed overlap of Brn3b and Brn3a. NBL, neuroblast layer; GCL, ganglion cell layer; l, lens. Scale bar: 100 µm.
retinas revealed similarly dense and fasciculated RGC axons. RGCs are the only output retinal neurons whose axons exit the eye to form the optic nerve with each RGC contributing a single axon to the optic nerve. We examined the optic nerves of wild-type, Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} and Brn3a\textsuperscript{lacZ/\textsuperscript{AP}} mice as an alternative measurement of RGC numbers. When compared with those of wild-type or Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} (Fig. 3J,M), the optic nerves of Brn3a\textsuperscript{lacZ/\textsuperscript{AP}} (Fig. 3K,N) were greatly reduced in the cross-sectional area (12.7±4.6% of wild-type, n=10). Conversely, the expression of a single copy of Brn3a\textsuperscript{kii} (Fig. 3L,O) was sufficient to restore normal optic nerve size to a level comparable with those of wild-type mice (85.2±6.6% of wild-type, n=9). These data indicate that the knock-in of Brn3a into Brn3b locus is capable of rescuing the defects in retinal structure and RGC number of Brn3b-null retinas.

**Restoration of proper RGC axon pathfinding and survival by Brn3a\textsuperscript{kii} expression**

To determine if Brn3a\textsuperscript{kii} can restore the viability of Brn3b-null RGCs, we isolated retinas from E13.5 to P0 and compared the RGC survival rate in Brn3b\textsuperscript{lacZ/\textsuperscript{AP}}, Brn3a\textsuperscript{lacZ/\textsuperscript{AP}} and Brn3a\textsuperscript{lacZ/\textsuperscript{AP}} mice. X-Gal staining of retinal sections from all three mice at E13.5 showed comparable lacZ-positive RGCs in the NBL and GCL (Fig. 4A-C), indicating that the generation and migration of RGCs remain unchanged in all three mice. At E15.5, when the majority of Brn3b-null RGCs were generated and densely packed in the GCL of normal retina (Fig. 4D), the lacZ-positive RGCs in Brn3b-null retina began to show signs of RGC loss from programmed cell death (Fig. 4E). At E17.5, the loss of RGCs from apoptosis in Brn3b-null retina accelerated and propagated from central to peripheral retina (compare Fig. 4G with 4H). At birth, in contrast to the strong X-Gal-stained GCL in Brn3b-null retina (Fig. 4J), the weak lacZ activities of Brn3b-lacZ/\textsuperscript{AP} retina demonstrated that only a small number of Brn3b-lacZ-positive RGCs survived (Fig. 4K). On the contrary, no overt difference was observed in the GCL of Brn3b-lacZ/\textsuperscript{AP} and wild-type or Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} mice throughout the developmental stages (compare Fig. 4A,D,GJ with 4CF,IL, respectively). Thus, the replacement of Brn3b with Brn3a cDNA enables the Brn3b-null RGCs to survive during retinal development and form the GCL with a ganglion cell density similar to that of wild-type mice.

In order to test whether Brn3a\textsuperscript{kii} restores the normal pathfinding process of RGCs, we examined the growth and guidance of RGC axons at major choice points within retina and along the central visual pathways. In Brn3b-null retina, the majority of RGC axons failed to fasciculate and were unable to reach the OD (Erkman et al., 2000; Gan et al., 1999). As seen in Fig. 5G,I, a similar number of fasciculated axon bundles from RGCs of Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} and wild-type or Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} retina reached the OD, implying that the expression of Brn3a\textsuperscript{kii} prevented the early intraretinal pathfinding defects of RGC axons in Brn3b-null mice.

For animals with binocular vision, the RGC axons from different retinal regions segregate at the optic chiasm to form the contralateral and ipsilateral visual pathways (Fig. 5A). In mice, the ipsilaterally projected axons arise from the ventrotemporal retina and amount to ~3% of total RGC axons. As shown in Fig. 5B, anterograde DiI labeling of wild-type mice at E17.5, when the segregation of RGC axons is nearly completed, revealed that a majority of RGC axons projected contralaterally with a small percentage of axons projecting into the ipsilateral pathway. In Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} mice, although a large proportion of RGC axons formed the contralateral optic tract, an abnormally large proportion of axons exhibited severe pathfinding defects at the optic chiasm and projected into ipsilateral optic track or into the optic nerve of the opposite eye (Fig. 5C). Conversely, no apparent pathfinding defects were identified at the optic chiasm of Brn3b\textsuperscript{lacZ/\textsuperscript{AP}} mice (n=4) (Fig. 5D), indicating that Brn3a\textsuperscript{kii} fully substitutes Brn3b in regulating the pathfinding decision of RGC axons.

**Fig. 3. Rescue of retinal defects in adult Brn3b-null mice by Brn3a knock-in in Brn3b locus.** Top, middle and bottom panels are representative of wild-type (Brn3b\textsuperscript{lacZ/\textsuperscript{AP}}), Brn3b\textsuperscript{lacZ/\textsuperscript{AP}}\textsuperscript{kii} mutant and Brn3a\textsuperscript{lacZ/\textsuperscript{AP}}\textsuperscript{kii} mice, respectively. (A-C) Hematoxylin and Eosin staining of the retinal structure. (D-F) X-Gal staining of Brn3b-lacZ-positive RGCs. (G-I) SMI32 immunostaining of neurofilament. (J-L) Whole-mount optic nerves and tracks. (M-O) Hematoxylin and Eosin staining of optic nerve cross-sections cut at the broken lines in J-L. Arrowheads indicate optic nerves. RPE, retinal pigmented epithelium; OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 100 μm in A-F; 300 μm in J-O.
Activation of Brn3b downstream target genes by Brn3a<sup>ki</sup>

Brn3b probably executes its roles in RGCs by regulating the expression of its downstream target genes. Many Brn3b downstream target genes have been identified by representational difference analysis (Erkman et al., 2000; Mu et al., 2004), cDNA microarrays (Mu et al., 2004) and in situ hybridization screening (Z.Y. and L.G., unpublished). They include the transcription factor genes Brn3a, Irx4 and Irx6 (homeodomain), Ablim (LIM-domain), Gfi1 and Gli1 (zinc-finger), Isl2 (LIM-homeodomain), Olf1 (bHLH), and Dlx1 and Dlx2 (homeodomain). Some of these factors have known or postulated roles in retinal development. Ablim and Irx4 have previously been reported to regulate RGC axon pathfinding (Erkman et al., 2000; Jin et al., 2003). Shh is essential for the formation of the optic disc and the optic nerve (Dakubo et al., 2003). Gap43 and L1 NCAM are associated with RGC axon guidance (Demyanenko and Maness, 2003; Zhang et al., 2000).

To test whether Brn3a and Brn3b share the identical transcription activities, as well as to determine whether Brn3a<sup>ki</sup> rescues the RGCs by restoring the RGC expression of Brn3b downstream target genes, we compared the expression of these genes in E14.5 retinal sections of wild-type, Brn3b<sup>lacZ</sup>/AP and Brn3b<sup>3a/3a</sup> embryos. The control Brn3b probe confirmed the absence of Brn3b expression in Brn3b<sup>lacZ</sup>/AP and Brn3b<sup>3a/3a</sup> retinas (Fig. 6A). Brn3a ORF probe detected the reduced expression of endogenous Brn3a in Brn3b<sup>lacZ</sup>/AP retina and Brn3b-like expression pattern of Brn3a<sup>li</sup> in Brn3b<sup>3a/3a</sup> retina (Fig. 6B). Compared with wild-type controls, loss of Brn3b in Brn3b<sup>lacZ</sup>/AP mice resulted in the downregulation of Brn3a, Irx2, Irx6, Ablim, Gfi1, Gli1, Isl2, Olf1, L1, Gap43, Shh and Hermes (Rbpms – Mouse Genome Informatics) (Fig. 6C-D, G-P) and the upregulation of Dlx1 and Dlx2 expression (Fig. 6E,F). When retinas from Brn3b<sup>3a/3a</sup> knock-in mice were examined, the expression levels of all of above genes were restored to those observed in wild-type retinas (Fig. 6C-P). The expression analysis demonstrates the identical ability for Brn3a and Brn3b to activate or suppress in RGCs the expression of Brn3b downstream genes and support the notion that rescue of Brn3b-null phenotypes by Brn3a<sup>li</sup> is achieved by restoring the expression of Brn3b downstream genes. Furthermore, Brn3a<sup>li</sup> expression in Brn3b<sup>3a/3a</sup> retina lead to the activation of endogenous Brn3a expression (Fig. 6C), implying the presence of positive feedback regulation of Brn3a expression in the developing retina.

Fig. 4. Absence of programmed cell death of Brn3b-null RGCs in Brn3a<sup>ki</sup> knock-in mice during embryonic development. Cryosections of retinas at E13.5 to P0 were stained with X-Gal to reveal the RGCs by the expression of Brn3b-lacZ reporter gene at Brn3b locus. (A,D,G,J) Control heterozygous Brn3b<sup>3a/2</sup> mice. During retinal development, RGCs are generated and remained in the GCL. (B,E,H,K) Brn3b<sup>3a/2</sup> mice. The lacZ-expressing RGCs were initially formed normally in the GCL (E) but a majority of them degenerated before birth (E,H,K). (C,F,I,L) Brn3a<sup>3a/2</sup> mice. Replacement of Brn3b with Brn3a<sup>ki</sup> prevents the apoptosis of Brn3b-null RGCs marked by the strong X-Gal staining in the GCL. RPE, retinal pigmented epithelium; NBL, neuroblast layer; GCL, ganglion cell layer; l, lens. Scale bar: 100 µm.

Fig. 5. Normal retinal axon projections in Brn3b<sup>3a/3a</sup> knock-in mice. Lipophilic dye tracings of the proximal visual pathway in E17.5 mice. (A) Schematic view of the visual system at the ventral surface of the brain. Anterior is towards the top, posterior towards the bottom. Contralateral pathway is shown in red and ipsilateral pathway in blue. The area within the broken lines is imaged with a Nikon fluorescent dissecting microscope. (B) Wild-type mouse. The majority of axons cross the optic chiasm towards the contralateral hemisphere; Anterior is towards the top, posterior towards the bottom. Contralateral pathway is shown in red and ipsilateral pathway is shown in blue. The area within the broken lines is imaged with a Nikon fluorescent dissecting microscope. (C) Brn3b-null mouse. Abnormally high percentage of axon fibers travel towards the ipsilateral optic tract and alongside the left optic nerve to the left eye. (D) Brn3b<sup>3a/3a</sup> knock-in mouse. Normal axon projection pattern is restored by Brn3a<sup>li</sup> expression in the absence of Brn3b. ON, optic nerve; OT, optic tract; Lksi, ipsilateral projection; Contra, contralateral projection. Asterisk indicates the optic chiasm. Scale bar: 200 µm.
Discussion

In this report, we have generated \textit{Brn3b}\textsuperscript{\textit{3a}} knock-in mice that express \textit{Brn3a}\textsuperscript{\textit{ki}} in a spatiotemporal pattern identical to that of endogenous \textit{Brn3b}. Our analyses of RGC development in \textit{Brn3b}\textsuperscript{\textit{3a/3a}} mice demonstrate that knocking-in \textit{Brn3a} in the \textit{Brn3b} locus is sufficient to rescue the RGC defects previously identified in the \textit{Brn3b}-null mice. The expression of \textit{Brn3a}\textsuperscript{\textit{ki}} corrects the RGC axon pathfinding errors within retina and along the visual pathway that are associated with the loss of \textit{Brn3b}. In addition, in the presence of \textit{Brn3a}\textsuperscript{\textit{ki}}, \textit{Brn3b}-null RGCs do not undergo the programmed cell death. Furthermore, we present the evidence that \textit{Brn3a}\textsuperscript{\textit{ki}} expression in the absence of \textit{Brn3b} restores the early developmental expression profiles of previously reported \textit{Brn3b} downstream target genes. Though further studies will be required to verify the phenotypic rescue in the physiological properties of RGCs and their target neurons in the brain, our data strongly argue that \textit{Brn3} transcription factors are functionally equivalent in regulating terminal differentiation genes during RGC development and suggest that a conserved \textit{Brn3} pathway could be present in the development and survival of various neuronal cell types.

**Functional equivalency of \textit{Brn3} factors**

The highly conserved \textit{Brn3} factors share a significantly high sequence homology, especially in the functionally important POU-domains and bind to the identical DNA sequences. Though the targeted deletion of each \textit{Brn3} gene results in unique defects in neuronal development, there is a tight correlation of \textit{Brn3} spatiotemporal expression patterns and their knockout phenotypes. In the developing retina, though the expression of \textit{Brn3a} and \textit{Brn3c} in RGCs mostly overlaps with that of \textit{Brn3b}, a comparison of temporal expression profiles of
Brn3 genes shows that Brn3b is expressed in retina 1 day earlier than Brn3a and Brn3c. Deletion of Brn3b causes the loss of RGCs and the downregulation of Brn3a and Brn3c expression in RGCs (Gan et al., 1996). The downregulation of Brn3a is detected at E12.5 and before the onset of RGCs apoptosis after E14.5 in Brn3b-null mice, suggesting that in a majority of RGCs, Brn3b acts upstream to activate the expression of Brn3a. Thus, it is probable that the loss of these RGCs in Brn3b-null mice is due to the requirement for early Brn3b expression during RGC development.

Similarly, the specific neuronal defects observed in Brn3a-null mice are closely linked to its distinctive expression in these neurons. In developing dorsal root ganglia, Brn3a expression starts at E9.5 and precedes those of Brn3b and Brn3c (McEvilly et al., 1996). Brn3a is also the only Brn3 family member to express in red nuclei and knockout of Brn3a specifically leads to apoptosis of neurons in both dorsal root ganglia and red nuclei (Xiang et al., 1996). In addition, in the caudal region of the inferior olivary nucleus, Brn3a and Brn3b are co-expressed in neurons at the ventral boundaries and Brn3a expression is further extended more dorsally. Removal of Brn3a in the knockout mice results in the loss of Brn3a-expressing neurons in regions dorsal to the ventral boundaries but not at the ventral boundaries where the expression of Brn3a and Brn3b overlaps (Xiang et al., 1996).

In inner ear, although both Brn3a and Brn3b are co-expressed in vestibular and cochlear ganglion cells of the developing inner ear, Brn3a expression starts at E9 and persists throughout development, and Brn3b expression does not start until E12.5. In agreement with their expression patterns, loss of Brn3a results in the degeneration of spiral and vestibular ganglion cells and the absence of Brn3b expression in these cells (Huang et al., 2001). Likewise, Brn3c is the only Brn3 gene to express in inner ear hair cells and the loss of hair cells in Brn3c knockout mice also reflects its unique spatial expression pattern (Xiang et al., 1997a).

In our present study, we express Brn3a in all Brn3b-expressing cells at a physiological level comparable with that of endogenous Brn3b by choosing a knock-in approach to replace Brn3b-coding regions with Brn3a cDNA. We have shown that Brn3a<sup>+</sup> can fully replace Brn3b and rescue the retinal defects associated with Brn3b-null mutation. Our in vivo studies demonstrate that Brn3 genes are functionally equivalent and that their distinctive roles in development are determined by their unique expression profiles. Consistent with our results, gain-of-function studies in chicken retinas have shown that all Brn3 proteins play a similar role to promote the development of RGCs (Liu et al., 2000). However, our findings appear different from other published in vitro studies. Those studies as such have shown that both Brn3a and Brn3b activate an iNOS promoter in BHK-21 fibroblast cells (Guy et al., 1998). However, the two genes exhibit antagonistic effects on the transcriptional regulation of human papilloma virus (HPV) type 16 and 18 E6 and E7 genes in cell lines of cervical origin (Ndissang et al., 1998) and on in vitro differentiation of ND7 cells (Smith et al., 1997). All of these in vitro studies are performed in culture of different cell lines and, sometimes, in lines derived from cells with no known Brn3 expression or function. In addition, these transactivation experiments have used strong viral promoters that express Brn3 factors at levels significantly higher than normal physiological concentrations. Thus, it is rather difficult to properly compare and to decipher the precise in vivo role of each Brn3 factor under such in vitro conditions.

As Brn3 factors are often expressed in the same cells, if Brn3 factors indeed possess unique biochemical properties and function antagonistically, disruption of the proper equilibriums of Brn3 factors within the same cells could have a detrimental effect on their development and survival. However, previously published studies have failed to show any developmental and survival defects in mice heterozygous for any of the three Brn3 mutations (Erkman et al., 1996; Gan et al., 1999; Gan et al., 1996; McEvilly et al., 1996; Xiang et al., 1997b; Xiang et al., 1996). Recent studies have shown that the autoregulation of Brn3a can compensate for the loss of one allele by increasing transcription from the remaining allele in trigeminal and dorsal root ganglia (Trieu et al., 2003). Such dose compensation mechanisms in retina have not been reported yet. Additional in vivo gene replacement experiments of Brn3 are needed to demonstrate whether the biochemical roles of all Brn3 factors are identical. Nonetheless, our results clearly provide the first in vivo evidence to support this theory.

**Brn3b regulatory pathway in the development and survival of retinal ganglion cells**

The postmitotic expression of Brn3b in retina is consistent with our previous findings that Brn3b acts downstream of bHLH-class transcription factor Math5 to regulate the terminal differentiation and survival of RGCs (Gan et al., 1999; Wang et al., 2001; Yang et al., 2003). In Brn3b-null mice, RGCs fail to project axons properly and die of programmed cell death. Brn3b probably controls these complicated processes by modulating the expression of genes essential for RGC differentiation and survival. Recently, a cDNA microarray analysis of gene expression profiles in wild-type and Brn3b-null retinas demonstrated that Brn3b regulates the expression of discrete sets of genes, including genes encoding transcription factors, secreted signaling molecules, and proteins for neuron integrity and function (Mu et al., 2004). Similarly, we have shown in this study that the expression of additional genes with known roles in axon growth and pathfinding are indeed altered in Brn3b-null retina.

It remains unclear whether the Brn3b downstream targets are primarily regulated by Brn3b or Brn3a in retina. Brn3b-null mutation leads to the diminished expression of Brn3a (Gan et al., 1996). A comparable phenomenon is observed in Brn3a-null mice, where Brn3b expression is drastically reduced in trigeminal, dorsal root, spiral and vestibular ganglia (Huang et al., 2001; McEvilly et al., 1996; Xiang et al., 1996). The lack of overt retinal phenotypes in Brn3a-null mice (McEvilly et al., 1996; Xiang et al., 1996) and the restoration of Brn3b downstream target gene expression by Brn3a<sup>+</sup> suggest that both Brn3a and Brn3b play equivalent roles to regulate these genes. Interestingly, ectopic expression of Brn3a<sup>+</sup> in Brn3b-deficient retina also activates the expression of endogenous Brn3a, suggesting a mutual, positive feedback regulation of Brn3a and Brn3b genes. Activation of Brn3b probably leads to the activation and maintenance of Brn3a and Brn3b expression in RGCs. Vice versa, initial activation of Brn3a could result in the activation and maintenance of Brn3a and Brn3b expression in other sensory neurons. The exact role of such feedback control in Brn3 gene expression is unclear. Nevertheless, as Brn3 factors function downstream of the initial differentiation
events initiated by bHLH transcription factors, such as a positive feedback mechanism would allow the irreversible activation of terminal differentiation programs of sensory neurons.

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