

# Subtype Specification of GABAergic Amacrine Cells by the Orphan Nuclear Receptor Nr4a2/Nurr1

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In the mammalian retina, amacrine cells (ACs) contain numerous subtypes with extremely diverse morphologies and physiological functions. To date, how these subtypes arise during retinogenesis remains largely unknown at the molecular level. The orphan nuclear receptor Nr4a2 plays an essential role in specifying ventral midbrain dopaminergic neurons, and its mutations are associated with familial Parkinson's disease. Here we show that Nr4a2 is also critically involved in the specification of AC subtype identity. During mouse retinogenesis, Nr4a2 is expressed in a subset of postmitotic GABAergic ACs and their precursors. Its targeted inactivation results in the loss of a subpopulation of GABAergic ACs that include all dopaminergic and p57Kip2<sup>+</sup> neurons as well as a simultaneous increase of calbindin<sup>+</sup> ACs. Misexpressed Nr4a2 can promote GABAergic AC differentiation and repress calbindin<sup>+</sup> ACs, whereas its dominant-negative form has the ability to suppress the GABAergic AC fate. Moreover, the expression of Nr4a2 is positively regulated by Foxn4 and negatively controlled by Brn3b, two retinogenic factors previously shown to promote and suppress GABAergic ACs, respectively. These data suggest that Nr4a2 is both necessary and sufficient to confer AC precursors with the identity of a GABAergic AC phenotype, and that it may network with multiple other retinogenic factors to ensure proper specification and differentiation of AC neurotransmitter subtypes.

## Introduction

The mammalian retina is a laminated sensorineural epithelium composed of six classes of neurons that include the rod, cone, bipolar, horizontal, amacrine (AC), and ganglion cells (RGCs), and one type of glia, the Müller cells. Of the six classes of neurons, ACs are most diversified and play major roles in the processing of visual information. They can be classified into at least 28 subtypes based on their morphologies, sublaminal positions, physiological properties, and functions (MacNeil and Masland, 1998; MacNeil et al., 1999; Masland, 2001b). The great majority of them contain either glycine or GABA inhibitory neurotransmitters, thereby forming two major groups—glycinergic and GABAergic—with each comprising ~40% of the total AC population (Vaney, 1990; Marquardt et al., 2001). All cells link the rod pathway to the cone circuitry by synapsing on both rod and cone bipolar cells (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975; Vaney, 1985; Strettoi et al., 1992). Dopaminergic neurons represent a GABAergic subtype that exerts multiple modulatory effects on the retinal circuitry. These include reducing gap-junction conductance between horizontal cells and altering the center-surround organi-

zation of ganglion cells (Jensen and Daw, 1984; Piccolino et al., 1987; Jensen, 1992; Gustincich et al., 1997).

During retinogenesis, all cell types, including ACs, are produced from multipotent progenitors in a developmental process requiring coordinated action of various intrinsic and extrinsic factors (Harris, 1997; Cepko, 1999; Livesey and Cepko, 2001). Several transcription factors act as intrinsic regulators to control the fate commitment and differentiation of ACs. These include Foxn4, which is required by progenitors to establish the competence state for AC generation (Li et al., 2004), and Math3, Neurod1, and Ptf1a, which are required for specifying the AC fate (Morrow et al., 1999; Inoue et al., 2002; Fujitani et al., 2006; Nakhai et al., 2007). The intrinsic mechanism underlying specification of the vast AC subtypes is largely unknown at present. Pax6 and Barhl2 have been implicated as playing a role in specifying glycinergic ACs (Marquardt et al., 2001; Mo et al., 2004), and Bhlhb5 and Isl1 in specifying GABAergic and cholinergic ACs, respectively (Feng et al., 2006; Elshatory et al., 2007).

Nr4a2/Nurr1 is an orphan nuclear receptor that functions in a ligand-independent manner (Wang et al., 2003). It is expressed predominantly by dopaminergic neurons of the ventral midbrain and has been demonstrated to play an essential role in dopaminergic neuron development (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998). *Nr4a2* inactivation causes a failure in the specification of midbrain dopaminergic neurons whereas its overexpression promotes the differentiation of dopaminergic neurons from stem cells (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998; Martinat et al., 2006). In the human, mutations in *NR4A2* are associated with familial Parkinson's disease (Le et al., 2003), reinforcing its potential in causing and treating

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neurodegenerative disorders. We have previously identified *Nr4a2* as a downstream gene repressed by *Brn3b* during mouse retinogenesis (Qiu et al., 2008), thereby leading to the speculation that *Brn3b* may inhibit the formation of dopaminergic ACs by negatively regulating *Nr4a2* expression. To test this hypothesis and to investigate whether *Nr4a2* plays any role during retinal development, we examined its expression and analyzed its biological function in the retina. Our results demonstrate a crucial role for *Nr4a2* in specifying a subset of GABAergic ACs that include all dopaminergic neurons.

## Materials and Methods

**Animals.** The *Nr4a2* knock-out mice were produced in and obtained from Dr. Orla Conneely's laboratory (Saucedo-Cardenas et al., 1998), and the *Foxn4* and *Brn3b* knock-out mice were generated and maintained in our laboratory (Gan et al., 1996; Li et al., 2004). The stage of mouse embryos was determined by taking the morning when the copulation plug was seen as embryonic day 0.5 (E0.5). All genotypes described were confirmed by PCR.

**Virus preparation and infection.** To construct the *Nr4a2*-GFP (green fluorescent protein) plasmid, a full-length human *Nr4a2* cDNA was bluntly ligated into the control-GFP viral vector (Mo et al., 2004). To construct the *Nr4a2*-EnR-GFP plasmid, a DNA fragment containing the *Nr4a2* DNA-binding domain (amino acids 94–365) fused in frame with the repressor domain of the *Drosophila* engrailed protein was ligated into the Control-GFP vector. Virus preparation and infection were performed as described previously (Li et al., 2004; Mo et al., 2004). Retinas were infected *in vivo* at postnatal day 0 (P0) with desired retroviruses and then harvested and analyzed at P21. Explanted retinas were infected at E14.5 and harvested for analysis after 12 d in culture.

**Immunofluorescence and quantification.** The preparation of retinal sections and immunofluorescent labeling of retinal sections and whole-mount retinas were performed as described previously (Li et al., 2004; Mo et al., 2004). The following primary antibodies were used: anti-tyrosine hydroxylase (TH) (rabbit polyclonal; Millipore Bioscience Research Reagents); anti-syntaxin (mouse monoclonal; Sigma); anti-GABA (rabbit polyclonal; Sigma); anti-calbindin-D28K (rabbit polyclonal; Swant); anti-Lim1/2 (mouse monoclonal; Developmental Studies Hybridoma Bank, University of Iowa); anti-*Brn3a* (mouse monoclonal; Millipore); anti-*Brn3b* (goat polyclonal; Santa Cruz Biotechnology); anti-Pax6 (rabbit polyclonal; Millipore); anti-GFP [rabbit polyclonal (MBL International); goat polyclonal (Abcam)]; anti-recoverin (rabbit polyclonal) (Dizhoor et al., 1991); anti-glycine transporter 1 (GLYT1) (goat polyclonal; Millipore); anti-Chx10 (sheep polyclonal; Exalpha Biologicals); anti-protein kinase C (PKC) (mouse monoclonal; GE Healthcare); anti-*Nr4a2* (rabbit polyclonal and goat polyclonal; Santa Cruz Biotechnology); anti-p57Kip2 (rabbit polyclonal; Santa Cruz Biotechnology); anti-aromatic L-amino acid-decarboxylase (AADC) (rabbit polyclonal; Abcam); anti-Bhlhb5 (goat polyclonal; Santa Cruz Biotechnology); anti-calretinin (mouse monoclonal; Millipore); anti-AP-2 $\alpha$  (mouse monoclonal; Santa Cruz Biotechnology); anti-Prox1 (rabbit polyclonal; Covance); anti-*Isl1* (mouse monoclonal; Developmental Studies Hybridoma Bank); anti-glutamine synthase (mouse monoclonal; Millipore); anti-GAD67 (mouse monoclonal; Millipore); anti-GAD65 (mouse monoclonal; BD), and anti-NF150 (rabbit polyclonal; Millipore). Alexa Fluor 488/594-conjugated donkey IgGs (anti-different species) were used as secondary antibodies (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) or TOPRO3 (Invitrogen) for nuclear counterstaining.

To quantify immunoreactive cells in P0 *Nr4a2* wild-type and mutant retinal explants, stained slides were masked and the number of marker-positive cells was scored on retinal sections of the intermediate region in an optical field using a reticule mounted on the microscope. Twelve fields were counted for each retina, and four independent retinas were scored for each type. All marker-positive cells were counted at a magnification of 1000 except for p57Kip2<sup>+</sup> ACs and calbindin<sup>+</sup> horizontal cells, which were counted at a magnification of 400, and TH<sup>+</sup> ACs, which were counted at a magnification of 100, due to the paucity of these positive

cells. To quantify GFP<sup>+</sup> cells and GFP<sup>+</sup> cells colocalized with cell type-specific markers on sections of retinas infected with control-GFP, *Nr4a2*-GFP, or *Nr4a2*-EnR-GFP viruses, >400 GFP<sup>+</sup> cells were usually scored for each retina, and at least three retinas were counted for each type. All data were tested for significance using two sample Student's *t* test with unequal variances.

**In situ hybridization, BrdU labeling, and TUNEL assay.** RNA *in situ* hybridization was performed as described using digoxigenin-labeled antisense riboprobes (Liu et al., 2001; Mo et al., 2004). The murine *Nr4a2* probe contains ~600 bp of the open reading frame (1436–2032 bp). BrdU labeling of dividing retinal progenitor cells was performed as previously described (Xiang, 1998).

TUNEL assay was performed using the *In Situ* Cell Death Detection Kit, TMR Red (Roche Diagnostics) following the manufacturer's protocol except that all sections were counterstained with 0.02  $\mu$ g/ml DAPI. Three to four independent retinas were collected and analyzed for each type at each stage. For each retina, fluorescence-positive cells were counted from 3–5 sections under the microscope, and images of entire sections were captured with a microscope-mounted digital camera. All data were tested for significance using two sample Student's *t* test with unequal variances.

**Retinal explant culture.** Retinal explants were cultured as previously described with modification (Morrow et al., 1999; Dyer and Cepko, 2000). P0 control and mutant retinas were dissected free from the surrounding tissue in prewarmed (37°C) explant culture medium [42.5% DMEM (Invitrogen), 42.5% F12 Nutrient Mix (Invitrogen), 15% fetal calf serum, penicillin/streptomycin/L-glutamine (Invitrogen), and 5  $\mu$ M forskolin (Sigma)]. Immediately following dissection, retinas were placed on Millicell filters (0.4  $\mu$ m pore size; Millipore) in explant culture medium at 37°C and 5% CO<sub>2</sub>. The medium was replaced every other day.

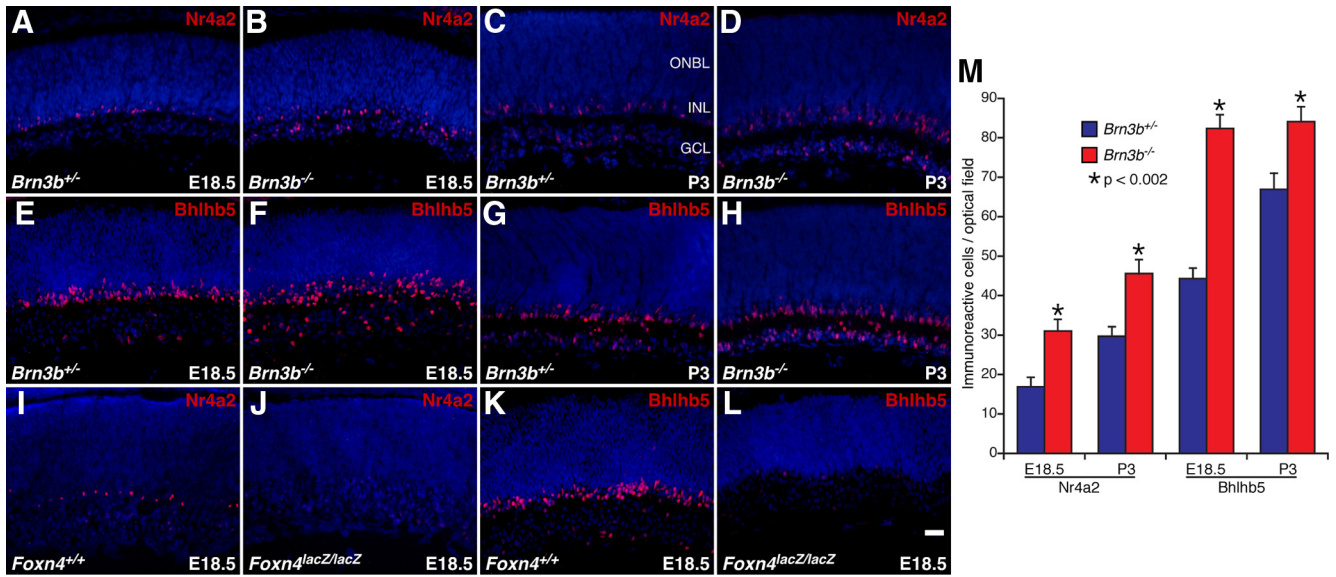
## Results

### Alteration of *Nr4a2* expression in *Foxn4* and *Brn3b* mutant retinas

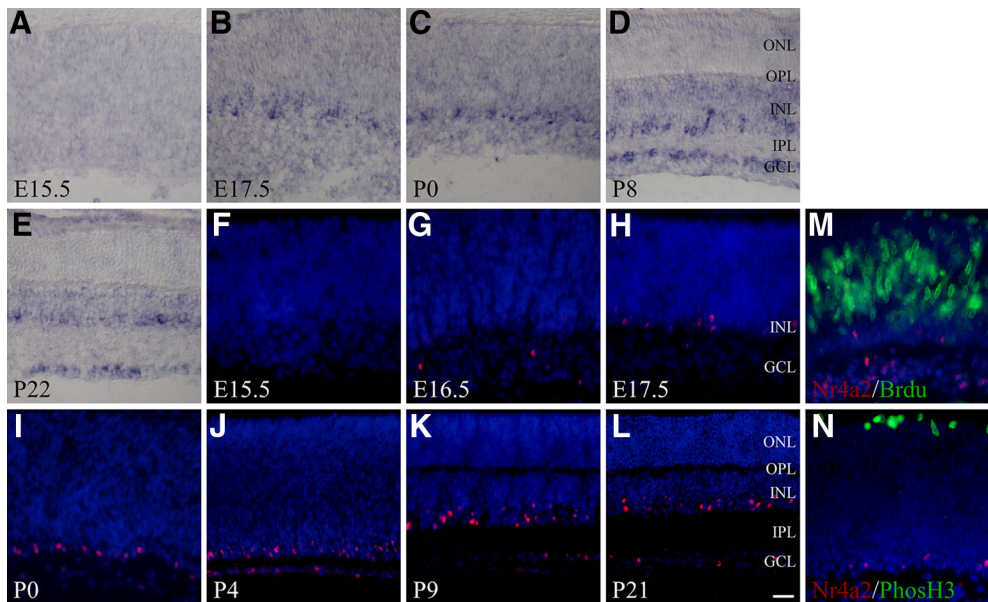
We have reported previously that targeted inactivation of *Brn3b* in mice leads to increased dopaminergic ACs as well as upregulation of *Nr4a2* transcription in embryonic retinas (Qiu et al., 2008). In this study, we further examined *Nr4a2* protein expression in *Brn3b*-null mutant retinas by immunofluorescence with an anti-*Nr4a2* antibody. Compared with control retinas, there was a 50–80% increase in the number of *Nr4a2*-immunoreactive cells in E18.5 and P3 *Brn3b*<sup>-/-</sup> retinas (Fig. 1*A–D,M*). The correlative relationship between *Nr4a2*, a midbrain dopaminergic neuron specifier factor (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998), and dopaminergic ACs in *Brn3b* mutant retinas thus suggests a possibility that *Nr4a2* may play an important role in the differentiation of ACs, in particular, dopaminergic ACs. If so, we would expect to observe a downregulation of *Nr4a2* expression in *Foxn4* mutant retinas since *Foxn4* is critically involved in competence acquisition and fate commitment of ACs (Li et al., 2004). Indeed, we found that *Nr4a2* expression nearly disappeared in E18.5 *Foxn4*<sup>lacZ/lacZ</sup> retinas (Fig. 1*I,J*), consistent with the idea that *Nr4a2* may act downstream of *Foxn4* to play a role in AC differentiation. Similarly, we observed a significant increase and a dramatic decrease of Bhlhb5-immunoreactive cells in *Brn3b*<sup>-/-</sup> and *Foxn4*<sup>lacZ/lacZ</sup> retinas, respectively (Fig. 1*E–H,K–M*), in agreement with the previous demonstration of a crucial role for Bhlhb5 in development of a subset of GABAergic ACs (Feng et al., 2006).

### Expression of *Nr4a2* in a subset of GABAergic amacrine cells during retinal development

As a first step to determine whether *Nr4a2* has a role in AC development, we performed RNA *in situ* hybridization and immunostaining analyses to investigate its spatiotemporal expres-



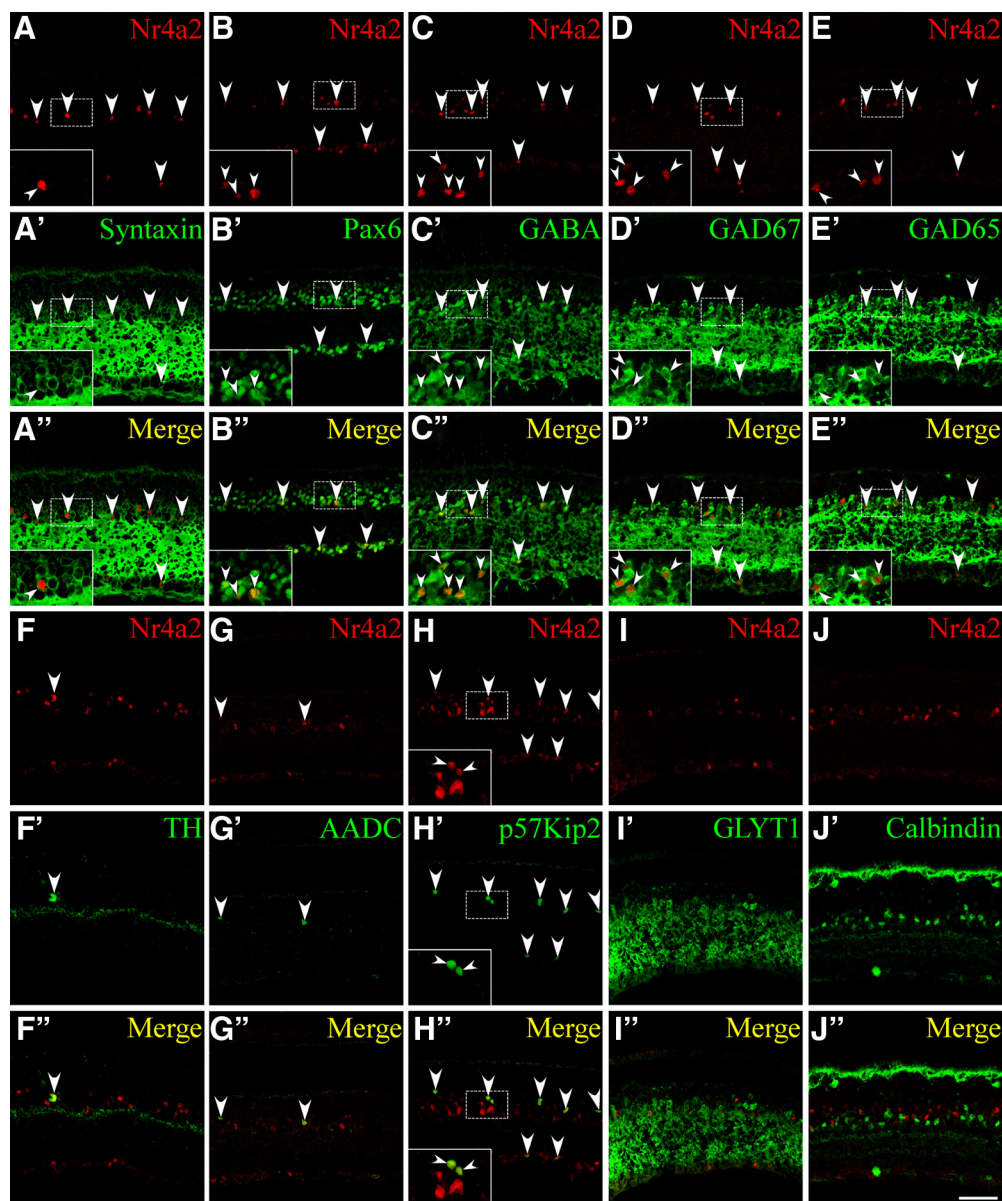
**Figure 1.** Altered expression of Nr4a2 and Bhlhb5 in *Brn3b* and *Foxn4*-null mutant retinas. **A–L**, Retinal sections from *Brn3b*<sup>+/+</sup> (**A, C, E, G**), *Brn3b*<sup>-/-</sup> (**B, D, F, H**), *Foxn4*<sup>+/+</sup> (**I, K**), and *Foxn4*<sup>lacZ/lacZ</sup> (**J, L**) mice at the indicated stages were immunostained with antibodies against Nr4a2 (**A–D, I, J**) or Bhlhb5 (**E–H, K, L**), and weakly counterstained with DAPI. There is a significant increase of Nr4a2- and Bhlhb5-immunoreactive cells but a dramatic decrease of these cells in *Brn3b*<sup>-/-</sup> and *Foxn4*<sup>lacZ/lacZ</sup> retinas, respectively. **M**, Quantitation of Nr4a2- and Bhlhb5-immunoreactive cells in *Brn3b*<sup>+/+</sup> and *Brn3b*<sup>-/-</sup> retinas. Each histogram represents the mean ± SD for three retinas. ONBL, Outer neuroblastic layer. Scale bar: **A–L**, 25 μm.



**Figure 2.** Expression pattern of Nr4a2 during mouse retinogenesis. **A–L**, Retinal sections from the indicated developmental stages were *in situ* hybridized with a *Nr4a2* probe (**A–E**) or immunostained with an anti-Nr4a2 antibody (red) (**F–L**). *Nr4a2 in situ* signal is seen in one row of cells at E17.5, then gradually expands to two rows of cells within the INL and the GCL in late postnatal retinas (**A–E**). Similarly, Nr4a2-immunoreactive cells are undetectable at E15.5 but gradually increase from E16.5 to postnatal retinas, where they are maintained within the inner edge of the INL as well as in the GCL (**F–L**). **M, N**, Retinal sections from E18.5 embryos pulse labeled with BrdU were double immunostained with antibodies against Nr4a2 and BrdU (**M**), or E18.5 retinal sections were double immunostained with antibodies against Nr4a2 and phosphorylated histone 3 (PhosH3) (**N**). There is no colocalization between Nr4a2 and BrdU or PhosH3. IPL, Inner plexiform layer; OPL, outer plexiform layer. Scale bar: **A–E, J–L**, 25 μm; **F–I, M, N**, 16.7 μm.

sion pattern in the developing mouse retina. *Nr4a2 in situ* signal was not observed until E16.5–E17.5, when one row of cells was seen to express it in the inner retina (Fig. 2*A, B*). It then gradually expanded over time into two rows of cells located within the inner nuclear layer (INL) and ganglion cell layer (GCL) (Fig. 2*B–D*). In late postnatal and adult stages (Fig. 2*E*), the expressing cells were maintained in the inner half of the INL, where ACs reside, as well as in the GCL, where both ganglion cells and displaced ACs are located. Similarly, Nr4a2-immunoreactive cells were undetect-

able at E15.5 in the retina but gradually increased from E16.5 to P0 (Fig. 2*F–I*). In more postnatal retinas, Nr4a2 was clearly expressed in a subset of neurons located within the inner edge of the INL as well as in the GCL (Fig. 2*J–L*), suggesting expression in a subpopulation of ACs and displaced ACs and/or ganglion cells. All Nr4a2-expressing cells are postmitotic, as none of them could be labeled by short pulses of BrdU, an S-phase marker, or by an antibody against phosphorylated histone H3, an M-phase marker (Fig. 2*M, N*).

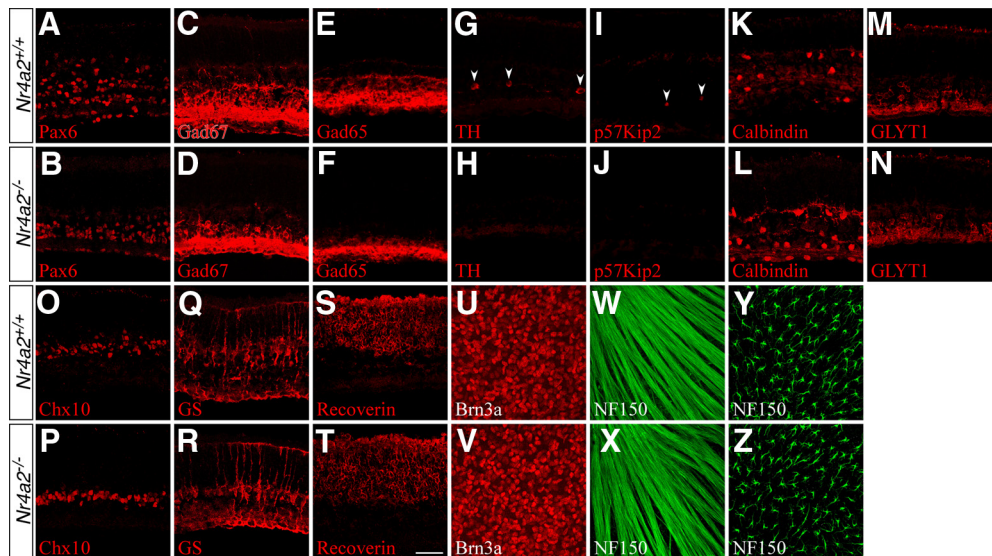


**Figure 3.** Expression of Nr4a2 in a subset of GABAergic ACs. *A–J''*, Sections from P21 mouse retinas were double immunolabeled with an anti-Nr4a2 antibody and those against the indicated cell type-specific markers. *A–A''*, *B–B''*, All Nr4a2<sup>+</sup> cells are syntaxin<sup>+</sup> and Pax6<sup>+</sup> ACs. *C–C''*, *D–D''*, *E–E''*, Nr4a2 is expressed in a subpopulation of GABA<sup>+</sup>, GAD67<sup>+</sup>, or GAD65<sup>+</sup> ACs. *F–F''*, *G–G''*, *H–H''*, All TH<sup>+</sup>, AADC<sup>+</sup>, or p57Kip2<sup>+</sup> neurons coexpress Nr4a2. *I–I''*, Nr4a2 is not expressed in GLYT1<sup>+</sup> glycinergic ACs. *J–J''*, Nr4a2 is not colocalized with calbindin in ACs or horizontal cells. Arrowheads point to representative colocalized cells, and insets show corresponding outlined regions at a higher magnification. Scale bar: *A–J''*, 47.6  $\mu$ m.

To identify the types of cells that express Nr4a2, we performed a series of double-immunofluorescence labeling with a variety of cell type-specific markers and used confocal microscopy to determine colocalized cells. Consistent with it being expressed by ACs, Nr4a2 is colocalized with syntaxin and Pax6, two pan-AC protein markers, in all of Nr4a2-expressing cells (Fig. 3*A–A''*, *B–B''*); additionally, most Nr4a2-expressing cells also express other AC markers including calretinin and AP-2 $\alpha$  (supplemental Fig. S1*A–A''*, *B–B''*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (Bassett et al., 2007). There appears to be a complete colocalization between Nr4a2 and GABA, GAD67 (glutamic decarboxylase 67) and GAD65 in Nr4a2-expressing cells (Fig. 3*C–C''*, *D–D''*, *E–E''*), indicating the expression of Nr4a2 in a subset of GABAergic amacrine neurons. In agreement with its expression in dopaminergic neurons elsewhere, Nr4a2 is found in all dopaminergic ACs that are immunoreactive for TH and AADC (Fig.

3*F–F''*, *G–G''*). Additionally, Nr4a2 is seen in all cells immunoreactive for p57Kip2 (Fig. 3*H–H''*), a cyclin-dependent kinase inhibitor that marks a subpopulation of GABAergic ACs that exclude the dopaminergic neurons (Dyer and Cepko, 2000, 2001).

Bhlhb5 marks another subset of GABAergic ACs (Feng et al., 2006), but its expression and that of Nr4a2 are mutually exclusive (supplemental Fig. S1*C–C''*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). There is no expression of Nr4a2 in glycinergic ACs immunoreactive for GLYT1 (glycine transporter 1), nor is it expressed in ACs immunoreactive for calbindin or Isl1, which label retinal cells that include cholinergic amacrine neurons (Fig. 3*I–I''*, *J–J''*; supplemental Fig. S1*J–J''*, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (Haverkamp and Wässle, 2000; Elshatory et al., 2007). Nr4a2 is not expressed in ganglion cells immunoreactive for Brn3a, Brn3b, or Isl1 (supplemental Fig. S1*D–D''*, *E–E''*, *J–J''*, available at [www.jneurosci.org](http://www.jneurosci.org) as supple-



**Figure 4.** Effect of targeted *Nr4a2* deletion on the formation of different retinal cell types. **A–T**, Sections from P0 *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> retinal explants that were cultured for 2 weeks were immunostained with antibodies against the indicated cell type-specific markers. Loss of *Nr4a2* function results in a significant decrease of ACs immunoreactive for Pax6 (**A, B**), Gad67 (**C, D**), or Gad65 (**E, F**) as well as a complete loss of TH<sup>+</sup> dopaminergic ACs (**G, H**) and p57Kip2<sup>+</sup> ACs (**I, J**). In addition, it causes a significant increase of calbindin<sup>+</sup> ACs (**K, L**). However, there is no difference between *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> retinas in the number of glycinergic ACs immunoreactive for GLYT1 (**M, N**), bipolar cells immunoreactive for Chx10 (**O, P**), Müller cells immunoreactive for glutamine synthase (GS) (**Q, R**), and photoreceptor cells immunoreactive for recoverin (**S, T**). **U–Z**, P0 *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> whole-mount retinas were immunostained with anti-Brn3a (**U, V**) or anti-NF150 (**W–Z**) antibodies. The confocal images in **W** and **X** were focused on the nerve fiber layer and in **Y** and **Z** on the horizontal cell layer. In *Nr4a2*<sup>-/-</sup> retinas, there is no significant change in the number of Brn3a<sup>+</sup> ganglion cells and NF150<sup>+</sup> nerve fibers and horizontal cells. Arrowheads in **G** and **I** point to TH<sup>+</sup> and p57Kip2<sup>+</sup> ACs. Scale bar: **A–T**, 29.8  $\mu$ m; **U–Z**, 47.6  $\mu$ m.

mental material), bipolar cells immunoreactive for PKC, Chx10, or Prox1 (supplemental Fig. S1F–F', G–G', H–H', available at www.jneurosci.org as supplemental material), horizontal cells immunoreactive for Lim1, calbindin, or Prox1 (Fig. 3J–J'; supplemental Fig. S1H–H', I–I', available at www.jneurosci.org as supplemental material), or Müller cells immunoreactive for glutamine synthase (supplemental Fig. S1K–K', available at www.jneurosci.org as supplemental material).

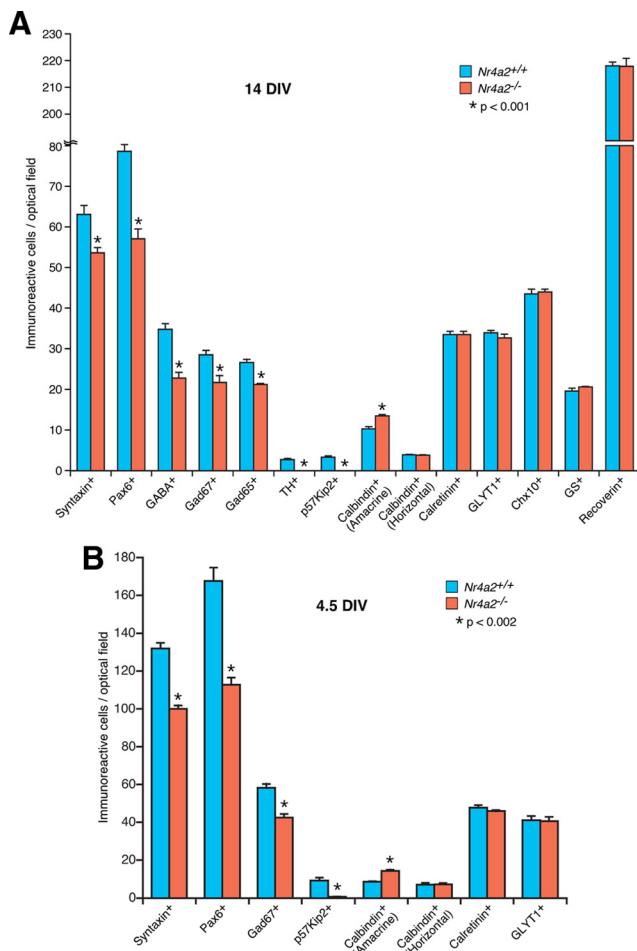
Quantification of colocalized cells revealed that the proportions of *Nr4a2*-expressing cells in the syntaxin<sup>+</sup>, Pax6<sup>+</sup>, GABA<sup>+</sup>, Gad67<sup>+</sup>, and Gad65<sup>+</sup> populations are only 13.1%, 10.9%, 27.5%, 20%, and 21.9%, respectively, whereas 100% of p57Kip2<sup>+</sup> and TH<sup>+</sup> cells coexpress *Nr4a2* (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Thus, *Nr4a2* is expressed only by a subset of GABAergic ACs that include all dopaminergic neurons and the population that express p57Kip2.

#### Loss of GABAergic amacrine cells in *Nr4a2*-null retinas

To understand the role of *Nr4a2* during mouse retinogenesis, we investigated the effect of its inactivation on retinal cell development. Due to perinatal lethality of *Nr4a2*-null mutant mice (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998), P0 retinal explants were cultured for 2 weeks [14 DIV (days *in vitro*)] for analysis of various retinal cell types in control and mutant retinas. We found that the number of Pax6-, GABA-, Gad67-, and Gad65-immunoreactive ACs decreased by 27%, 34%, 24%, and 20%, respectively, in mutant retinas compared with the control (Figs. 4A–F, 5A), consistent with the expression of *Nr4a2* in GABAergic ACs. In addition, the TH-immunoreactive dopaminergic neurons and p57Kip2-immunoreactive GABAergic ACs all disappeared in the null retina (Figs. 4G–J, 5A), in agreement with the observation that *Nr4a2* is localized in all TH- and p57Kip2-expressing cells. In contrast, calbindin-immunoreactive ACs increased by 31% in the *Nr4a2* mutant retina (Figs. 4K, L, 5A),

suggesting a possible fate change and consistent with the significant increase seen in *p57Kip2*<sup>-/-</sup> retinas (Dyer and Cepko, 2000). In *Nr4a2*<sup>-/-</sup> retinas, no change was observed in the number of calretinin-immunoreactive ACs and GLYT1-immunoreactive glycinergic ACs (Figs. 4M, N, 5A). Similarly, there was no alteration in the number of horizontal cells immunoreactive for calbindin, bipolar cells immunoreactive for Chx10, Müller cells immunoreactive for glutamine synthase, and photoreceptors immunoreactive for recoverin (Figs. 4K, L, O–T, 5A). In P0 control and *Nr4a2*<sup>-/-</sup> retinas, there were similar numbers of ganglion cells immunoreactive for Brn3a, horizontal cells immunoreactive for NF150 as well as nerve fibers labeled for NF150 (Fig. 4U–Z). Thus, the absence of *Nr4a2* leads to selective loss of GABAergic ACs including all dopaminergic neurons and p57Kip2<sup>+</sup> ACs while increasing the calbindin<sup>+</sup> ACs.

It is possible that all GABAergic ACs might be initially specified in *Nr4a2*-null mutant retinas but then gradually degenerate later during development. To test this possibility, we cultured P0 retinas for only 4.5 d (4.5 DIV) before analyses with various AC markers. Similar to retinas at 14 DIV, the number of syntaxin-, Pax6-, Gad67-, and p57Kip2-immunoreactive ACs diminished by 24%, 33%, 27%, and 92%, respectively, in mutant retinas at 4.5 DIV; whereas calbindin-immunoreactive ACs increased by 66% (supplemental Fig. S3A–N, available at www.jneurosci.org as supplemental material; Fig. 5B). There were similar numbers of GLYT1- or calretinin-immunoreactive ACs and calbindin-immunoreactive horizontal cells in wild-type and mutant retinas at 4.5 DIV (supplemental Fig. S3M–R, available at www.jneurosci.org as supplemental material; Fig. 5B). Therefore, it appears that a subset of GABAergic ACs fail to generate and at least some of them are misspecified into calbindin<sup>+</sup> ACs in the absence of *Nr4a2*. Given that *Bhlhb5* is expressed in and required for specifying a subset of GABAergic ACs (Feng et al., 2006), we examined its expression in the null retina and found no significant change (supplemental Fig. S3S, T, available at



**Figure 5.** Quantitation of cells that are immunoreactive for various cell type-specific markers in P0 *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> retinal explants cultured for 14 (**A**) and 4.5 (**B**) DIV (days *in vitro*). Each histogram represents the mean  $\pm$  SD for four retinas. Note that all marker-positive cells were counted under a fluorescent microscope at a magnification of 1000 except for p57Kip2<sup>+</sup> ACs and calbindin<sup>+</sup> horizontal cells, which were counted at a magnification of 400, and TH<sup>+</sup> ACs, which were counted at a magnification of 100, due to the paucity of these positive cells.

www.jneurosci.org as supplemental material), consistent with the nonoverlapping expression pattern between *Nr4a2* and *Bhlhb5* (supplemental Fig. S1C–C', available at www.jneurosci.org as supplemental material) and suggesting that *Nr4a2* controls GABAergic AC development independent of *Bhlhb5*.

### Enhanced cell death in *Nr4a2*<sup>-/-</sup> retinas

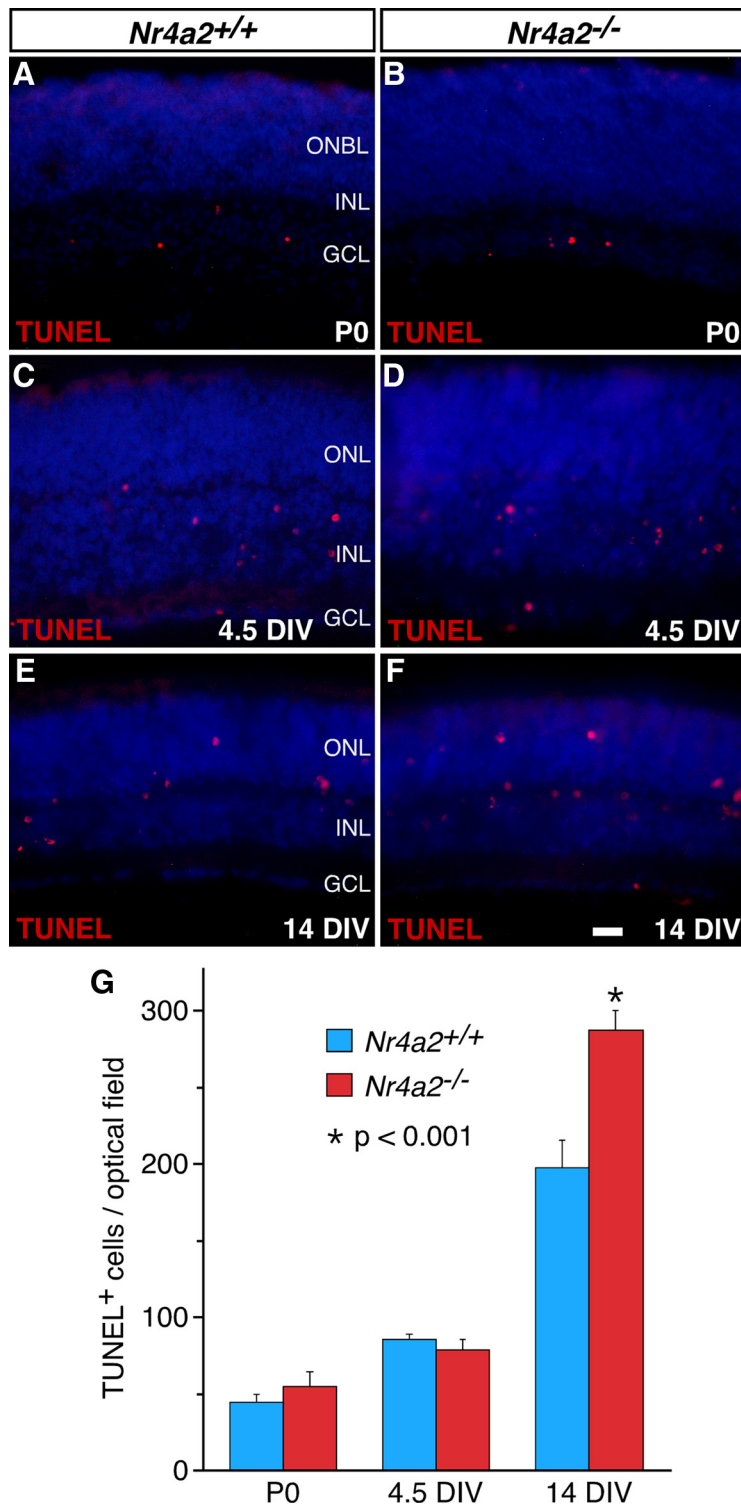
The improper specification of GABAergic ACs from retinal precursors in *Nr4a2*<sup>-/-</sup> retinas may result in primary and/or secondary retinal cell degeneration by apoptosis. To test this, we measured apoptotic cell death in wild-type and mutant retinas. As determined by TUNEL, no significant difference was observed in the number of labeled cells in P0 mutant retinas or in P0 mutant retinas cultured for 4.5 DIV (Fig. 6A–D,G). In contrast, P0 null retinas cultured for 14 DIV displayed a 45% increase of apoptotic cell death (Fig. 6E–G). In *Nr4a2*<sup>-/-</sup> retinas at 14 DIV, most cells undergoing apoptosis were seen within the inner edge of the outer nuclear layer (ONL) rather than in the INL (Fig. 6F), suggesting that some inappropriately differentiated cells or photoreceptors instead of ACs undergo enhanced apoptosis. This is consistent with the idea that a subpopulation of GABAergic ACs

fail to generate or are improperly specified in the absence of *Nr4a2*.

### *Nr4a2* misexpression promotes the GABAergic amacrine cell fate

Given its requirement in specifying GABAergic ACs, we next tested whether *Nr4a2* was sufficient to promote this particular cell fate from retinal precursors by a gain-of-function approach using a replication-incompetent murine retroviral vector that carries a GFP reporter (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material) (Li et al., 2004; Mo et al., 2004). Retinal progenitors were infected at P0 by subretinal injection of *Nr4a2*-GFP or control-GFP viruses, and we analyzed the laminar position and morphology of GFP<sup>+</sup> cells in infected retinas at P21. In control retinas, the great majority of GFP<sup>+</sup> cells differentiated as photoreceptors randomly distributed within the ONL, with 43.9% of their cell bodies located in the outer half of the ONL and 41.6% in the inner half of the ONL (supplemental Fig. S4B,E, available at www.jneurosci.org as supplemental material). In retinas infected with *Nr4a2*-GFP viruses, however, 53.9% of GFP<sup>+</sup> cells were seen in the outer half of the ONL and only 14.4% in the inner half of the ONL (supplemental Fig. S4C,E, available at www.jneurosci.org as supplemental material). Moreover, most of the GFP<sup>+</sup> cells in the ONL lacked the inner and outer segments characteristic of photoreceptors seen in control retinas (supplemental Fig. S4B,C, available at www.jneurosci.org as supplemental material). In the INL, the fraction of GFP<sup>+</sup> cells increased from 15.8% in the control to 30.4% in retinas infected with *Nr4a2*-GFP viruses (supplemental Fig. S4B,C,E, available at www.jneurosci.org as supplemental material). Similarly, there was a significant increase in the number of GFP<sup>+</sup> cells distributed in the GCL of retinas infected with *Nr4a2*-GFP viruses (supplemental Fig. S4B,C,E, available at www.jneurosci.org as supplemental material). Thus, *Nr4a2* misexpression in progenitors substantially changes the proportions of progeny distributed in different retinal cell layers.

The increased GFP<sup>+</sup> cells in the INL and GCL of retinas infected with *Nr4a2*-GFP viruses could represent more amacrine, ganglion, horizontal, bipolar, and/or Müller cells. To distinguish these possibilities, we used a series of cell type-specific markers to analyze the types of cells that were differentiated from the virus-infected progenitors. First, misexpressed *Nr4a2* increased 3–4-fold the number of GFP<sup>+</sup> ACs that were immunoreactive for Pax6, GABA, or GAD67 (Figs. 7A, B, D, E, G, H, 8), whereas it reduced calbindin-immunoreactive ACs by 45% and had no effect on glycinergic (GLYT1<sup>+</sup>) ACs (Figs. 7J, K, M, N, 8). This is in agreement with the specific loss of GABAergic ACs and significant increase of calbindin<sup>+</sup> ACs in *Nr4a2*-null retinas. Second, there was no effect exerted by misexpressed *Nr4a2* on the number of Chx10-immunoreactive bipolar cells, calbindin-immunoreactive horizontal cells, Brn3a-immunoreactive ganglion cells, or glutamine synthase-immunoreactive Müller cells (Figs. 7P, Q, S, T, 8; data not shown). Finally, misexpressed *Nr4a2* reduced the proportion of recoverin<sup>+</sup> photoreceptor cells from 83% to 18.7% (Figs. 7V, W, 8). In retinas infected with *Nr4a2*-GFP viruses, most of the GFP<sup>+</sup> cells (59.6% of all) were located within the outer half of the ONL, nonimmunoreactive for the above-tested markers, and were additionally negative for red/green opsin, blue opsin, nestin, NeuN, and *Otx2* (Fig. 8; supplemental Fig. S4B,C, available at www.jneurosci.org as supplemental material; data not shown), suggesting them as some kind of immature cells whose differentiation is prevented by misexpressed *Nr4a2*. The formation of these unidentified cells in large quantity as well as more



**Figure 6.** Increased cell death in *Nr4a2*<sup>-/-</sup> retinas. **A–F**, Cells undergoing apoptosis were TUNEL-labeled in sections from P0 *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> retinas (**A, B**) and from P0 retinal explants cultured for 4.5 (**C, D**) and 14 (**E, F**) DIV (*days in vitro*). In null mutant retinas, there is a significant increase of TUNEL-labeled cells at 14 DIV but no change in the number of these cells at P0 and 4.5 DIV. **G**, Quantitation of apoptotic cell death in P0 *Nr4a2*<sup>+/+</sup> and *Nr4a2*<sup>-/-</sup> retinas and retinal explants. Each histogram represents the mean ± SD for four retinas. ONBL, Outer neuroblastic layer. Scale bar: **A, B**, 25 μm; **C–F**, 16.7 μm.

GABAergic ACs may cause a depletion of virus-transduced retinal progenitors which in turn may result in the observed dramatic reduction of photoreceptor cells.

When P0 retinas were infected with either control-GFP or Nr4a2-GFP viruses, few, if any, GFP<sup>+</sup> cells became p57Kip2<sup>+</sup>

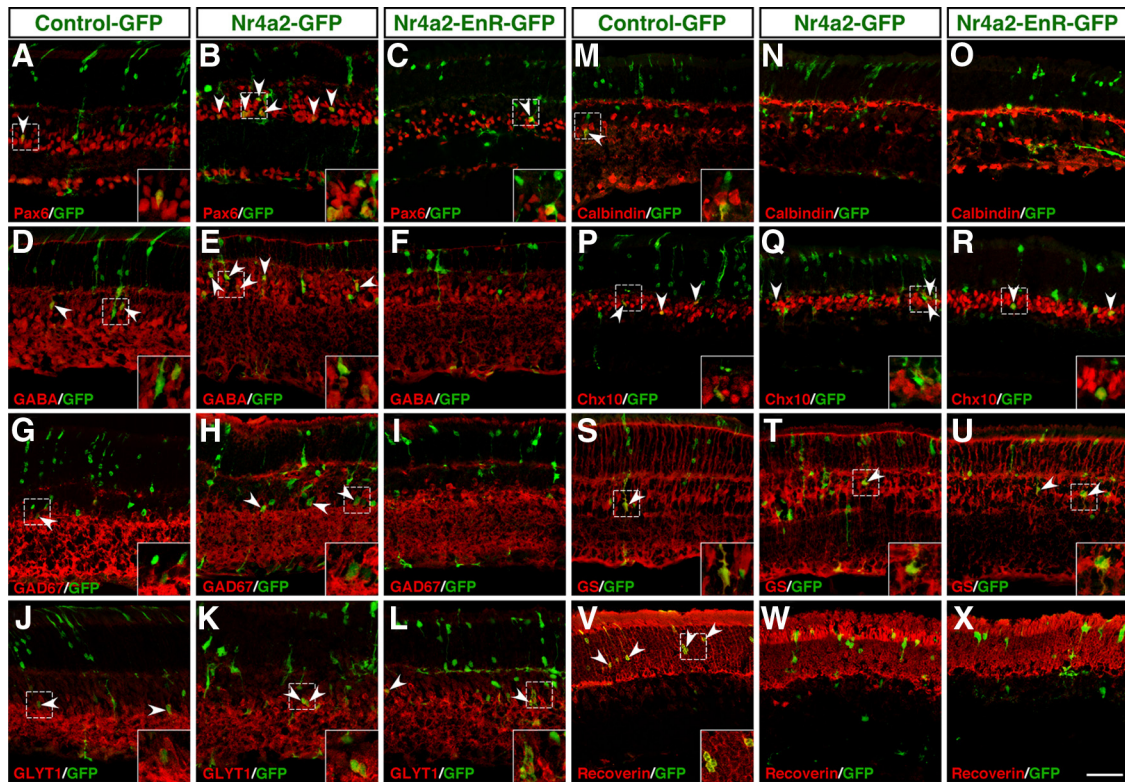
ACs perhaps because postnatal retinal progenitors are incompetent of generating this subpopulation of cells. To test this speculation, we infected retinal explants at E14.5 and harvested them after 12 d in culture for analysis. We found that in retinas infected with Nr4a2-GFP viruses, there were ~4.5% of GFP<sup>+</sup> cells coexpressing p57Kip2, whereas in control retinas, cells coexpressing GFP and p57Kip2 were negligible (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). Thus, these misexpression data together demonstrate the ability of Nr4a2 to bias retinal precursors toward the GABAergic AC fate while suppressing the differentiation of calbindin<sup>+</sup> ACs and photoreceptors.

**A dominant-negative form of Nr4a2 suppresses GABAergic amacrine cell differentiation**

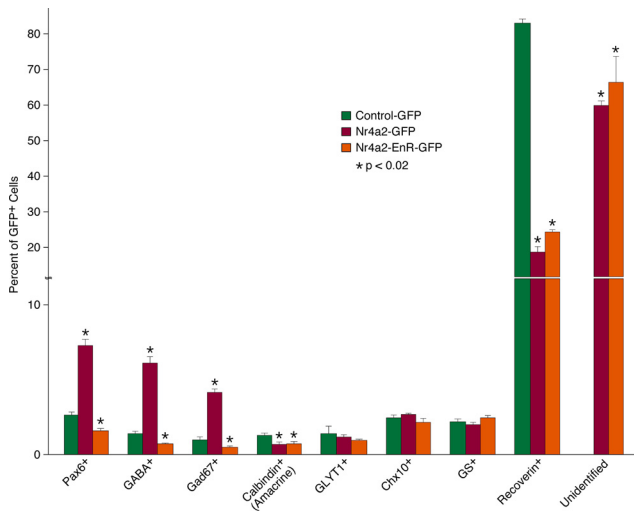
To ask whether Nr4a2 acts as a transcriptional activator for the specification of GABAergic ACs, we constructed a dominant-negative viral plasmid, Nr4a2-EnR-GFP, by fusing the repressor domain of the *Drosophila* engrailed protein to a Nr4a2 N-terminal region that contains the DNA-binding domain (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material). Compared with control retinas, we found that the fractions of GFP<sup>+</sup> cells immunoreactive for Pax6, GABA, or Gad67 were all reduced by 40–50% in retinas infected with Nr4a2-EnR-GFP viruses (Figs. 7A,C,D, F, G, I, 8), consistent with Nr4a2 being an activator for GABAergic AC differentiation. Consistent also with the notion that Nr4a2 acts as a repressor for the differentiation of calbindin<sup>+</sup> ACs and photoreceptor cells, misexpressed Nr4a2-EnR diminished the fractions of GFP<sup>+</sup> cells that became immunoreactive for calbindin or recoverin (Figs. 7M, O, V, X, 8). Similar to wild-type Nr4a2, misexpressed Nr4a2-EnR resulted in no changes in the number of GLYT1-immunoreactive ACs, Chx10-immunoreactive bipolar cells, calbindin-immunoreactive horizontal cells, Brn3a-immunoreactive ganglion cells, or glutamine synthase-immunoreactive Müller cells (Figs. 7J, L, P, R, S, U, 8; data not shown). Thus, Nr4a2 appears to act as a transcriptional activator to promote GABAergic neuron specification but as a repressor to suppress the differentiation of calbindin<sup>+</sup> ACs and photoreceptor cells.

**Discussion**

Among the seven classes of retinal cell types, ACs stand out in that they consist of numerous subtypes with extremely diverse mor-



**Figure 7.** Effect of misexpressed Nr4a2 and its dominant-negative form on the formation of different retinal cell types. **A–X**, Sections from retinas infected with control-GFP, Nr4a2-GFP, or Nr4a2-EnR-GFP viruses were double immunostained with an anti-GFP antibody and antibodies against Pax6, GABA, GAD67, GLYT1, calbindin, Chx10, glutamine synthase (GS), or recoverin. Forced expression of wild-type Nr4a2 results in an increase in the number of GABAergic ACs immunoreactive for Pax6, GABA, or GAD67, whereas the dominant-negative form has the opposite effect (**A–I**); however, both the wild-type and dominant-negative forms of Nr4a2 suppress the formation of calbindin<sup>+</sup> ACs (**M–O**) and photoreceptor cells immunoreactive for recoverin (**V–X**). They have no effect on glycinergic<sup>+</sup> ACs immunoreactive for GLYT1 (**J–L**), bipolar cells immunoreactive for Chx10 (**P–R**), and Müller cells immunoreactive for GS (**S–U**). Arrowheads point to representative colocalized cells, and insets show corresponding outlined regions at a higher magnification. Scale bar: **A–X**, 39.7 μm.



**Figure 8.** Quantitation of virus-transduced retinal cells that become immunoreactive for a series of cell type-specific markers. Each histogram represents the mean ± SD for three retinas. The number of GFP<sup>+</sup> cells scored in each retina ranges from 432 to 1333. The unidentified cells are those that are located at the outer edge of the outer nuclear layer but negative for recoverin.

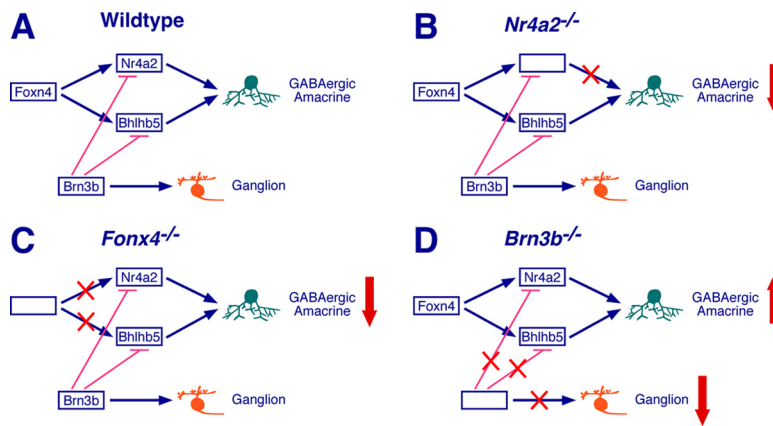
phologies and physiological functions (MacNeil and Masland, 1998; MacNeil et al., 1999; Masland, 2001a,b). At present, little is known about the intrinsic mechanism that underlies the specification of these distinct AC subtypes. Here, we demonstrate a role for the orphan nuclear receptor Nr4a2 in the specification of a subset of GABAergic ACs. We find that Nr4a2 colocalizes with a

number of GABAergic markers in a subset of ACs that are much more numerous than the dopaminergic AC subset. In *Nr4a2*<sup>-/-</sup> retinas, there is a significant loss of GABAergic ACs that include all dopaminergic ACs and p57Kip2<sup>+</sup> ACs as well as a simultaneous increase of calbindin<sup>+</sup> ACs. Gain-of-function studies show that misexpressed Nr4a2 can specifically promote the differentiation of GABAergic ACs while inhibiting that of calbindin<sup>+</sup> ACs, whereas its dominant-negative form has the ability to suppress the GABAergic AC fate. Our data together thus implicate a role for Nr4a2 in the specification of a subset of GABAergic ACs that include all dopaminergic neurons during mammalian retinogenesis, and further suggest distinct molecular pathways by which other retinogenic factors may promote and suppress GABAergic ACs to ensure proper specification of retinal cell types and subtypes. Compared with previous studies focused on the role of Nr4a2 only in dopaminergic neuron development, our results reveal a function for Nr4a2 that is much broader than just specifying dopaminergic ACs.

**Nr4a2 specifies a subset of GABAergic amacrine cells during retinogenesis**

Due to the loss of dopaminergic neurons in the ventral tegmental area and substantia nigra in *Nr4a2*-null mutant mice and its relevance to Parkinson’s disease (Zetterström et al., 1997; Saucedo-Cardenas et al., 1998; Le et al., 2003), numerous previous reports have focused only on the function of Nr4a2 in dopaminergic neuron development. In this work, we have provided several lines of evidence to demonstrate that at least during retinogenesis Nr4a2 plays a much broader role in the specification of neuro-





**Figure 9.** Schematics illustrating the genetic relationship between *Nr4a2* and other retinogenic factors involved in GABAergic AC specification. **A**, *Foxn4* is required for directly and/or indirectly activating the expression of *Nr4a2* and *Bhlhb5*, which act in parallel to specify GABAergic ACs. While promoting ganglion cell differentiation, *Brn3b* inhibits GABAergic AC development by repressing the expression of *Nr4a2* and *Bhlhb5*. **B**, *Nr4a2* inactivation blocks the *Nr4a2*-dependent pathway to GABAergic AC specification, leading to reduced GABAergic ACs. **C**, *Fonx4* inactivation blocks both the *Nr4a2*- and *Bhlhb5*-dependent pathways to GABAergic AC specification, resulting in dramatically reduced GABAergic ACs. **D**, *Brn3b* inactivation blocks the *Brn3b*-dependent pathway to ganglion cell specification as well as causes derepression of *Nr4a2* and *Bhlhb5* expression, which in turn leads to more GABAergic ACs.

transmitter phenotypes: (1) the onset of *Nr4a2* expression (at ~E16.5) greatly precedes the appearance of TH-expressing dopaminergic ACs (at ~P12) during retinal development; (2) *Nr4a2*-expressing cells dramatically outnumber those that express TH and AADC in the mature retina; (3) besides colocalization with TH and AADC, *Nr4a2* colocalizes with GABAergic markers GABA, GAD65, GAD67, and p57Kip2 in many more ACs; (4) apart from a complete loss of dopaminergic ACs, there is a significant loss of GABAergic ACs and in particular, near complete absence of p57Kip2 ACs in *Nr4a2*<sup>-/-</sup> retinas; and (5) misexpressed *Nr4a2* is capable of specifically promoting GABAergic AC differentiation, whereas its dominant-negative form has the opposite effect. All these data together suggest that *Nr4a2* may act to specify a subset of GABAergic ACs that include all dopaminergic neurons during mammalian retinogenesis. In fact, TH-immunoreactive cells constitute also only a minor fraction of *Nr4a2*-expressing ACs in the zebrafish retina (Filippi et al., 2007); hence fish *Nr4a2* homologs may be similarly involved in GABAergic AC development.

*Nr4a2* appears to confer the GABAergic identity to a subset of postmitotic AC precursor cells but not likely to be required for the maturation and maintenance of differentiated GABAergic ACs. This is because (1) *Nr4a2* expression is found exclusively in postmitotic cells; and (2) in P0 *Nr4a2*<sup>-/-</sup> retinas cultured for only 4.5 DIV when GABAergic ACs are freshly produced, a subpopulation of GABAergic ACs are already missing, indicating that most likely they are not even specified in the first place in the absence of *Nr4a2*. Thus, all GABAergic AC precursors may be initially generated in the developing mutant retina but some of them may be unable to differentiate and some of them may even change their fates to improperly differentiate as other AC subtypes. Indeed, a fate switch in precursor cells seems likely as we observed a significant increase of calbindin-immunoreactive ACs in the mutant retina. The increased calbindin<sup>+</sup> cells may result from the near complete downregulation of p57Kip2 expression in the null retina as targeted inactivation of *p57Kip2* has been shown to cause a similar elevation of calbindin<sup>+</sup> ACs (Dyer and Cepko, 2000). Alternatively, p57Kip2<sup>+</sup> and/or TH<sup>+</sup> ACs may non-cell-autonomously regulate the cell number of calbindin<sup>+</sup>

AC population, and vice versa, to ensure appropriate cell number ratios. The loss of p57Kip2<sup>+</sup> and TH<sup>+</sup> cells in the mutant would then break the cell number control mechanism, leading to an increase of calbindin<sup>+</sup> cells. In *Nr4a2* mutant retinas, the AC precursors that fail to differentiate and/or misspecified calbindin<sup>+</sup> ACs may degenerate later during development. Consistent with this notion, in cultured mutant retinal explants, we observed increased apoptosis at 14 DIV and the proportion of calbindin<sup>+</sup> ACs was reduced by half at 14 DIV compared with that at 4.5 DIV (Figs. 5, 6). In the midbrain, *Nr4a2* is found also to be required for specifying dopaminergic neurons from postmitotic precursors. In the mutant brain, dopaminergic neuron precursors are initially generated but fail to differentiate into mature neurons, and they eventually degenerate by apoptosis (Saucedo-Cardenas et al., 1998; Wallén et al., 1999).

Our data in this report demonstrate that development of dopaminergic neurons in the midbrain and those of retina share some common molecular events. This may provide an explanation why some patients with Parkinson's disease display visual abnormalities, including delayed visual evoked potential and loss of visual contrast sensitivity (Bodis-Wollner and Yahr, 1978; Regan and Maxner, 1987; Raskin et al., 1990; Tagliati et al., 1996). For instance, in patients with familial Parkinson's disease caused by mutations in the *NR4A2* locus (Le et al., 2003), abnormal vision may result from the loss of all dopaminergic ACs as well as many other GABAergic ACs.

### Distinct molecular pathways leading to the specification of GABAergic amacrine cells

All amacrine cells are determined and differentiated from multipotent retinal progenitors by the concerted action of a series of intrinsic transcriptional regulators. *Foxn4* confers retinal progenitors with AC-producing competence by activating the expression of *Math3*, *Neurod1*, and *Ptf1a*, which are subsequently required for the specification of ACs and their differentiation (Inoue et al., 2002; Li et al., 2004; Fujitani et al., 2006). Once the AC precursors are generated, other more select transcription factors such as *Nr4a2*, *Bhlhb5*, *Barhl2*, and *Isl1* may then come into play to further specify them into numerous subtypes (Mo et al., 2004; Feng et al., 2006; Elshatory et al., 2007). The molecular bases underlying subtype specification of neurotransmitter phenotypes is poorly understood at present but our work and other studies implicate distinct molecular pathways. For instance, *Pax6* and *Barhl2* may be involved in the specification and/or differentiation of glycinergic ACs, whereas *Isl1* may be involved in specifying cholinergic ACs (Marquardt et al., 2001; Mo et al., 2004; Elshatory et al., 2007).

Given the previous demonstration of *Bhlhb5* in specifying GABAergic ACs (Feng et al., 2006), our current work suggests that *Nr4a2* and *Bhlhb5* may independently and parallelly specify two nonoverlapping subsets of GABAergic ACs during retinogenesis. First, *Nr4a2* and *Bhlhb5* were found not to be colocalized in the same cells (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), implying that they are expressed in two mutually exclusive subpopulations of GABAer-

gic ACs. Second, there was no change in the number of Bhlhb5-immunoreactive cells in *Nr4a2*-null retinas (supplemental Fig. S3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), indicating that the subset of GABAergic ACs that express Bhlhb5 remains intact in the mutant despite significant loss of other GABAergic ACs. Therefore, there exist at least two molecular pathways independently controlled by *Nr4a2* and *Bhlhb5* that lead to the differentiation of GABAergic ACs during mouse retinal development (Fig. 9A). The functional nonredundancy between *Nr4a2* and *Bhlhb5* ensures that inactivating either gene would result in the loss of a subset of GABAergic ACs, which is exactly what occurs in either mutant (Fig. 9B) (Feng et al., 2006). Apart from the pathways regulated by *Nr4a2* and *Bhlhb5*, there might be other ones leading to the GABAergic phenotype. In the *Xenopus* retina, *Ptf1a* has been shown recently to specifically determine the GABAergic AC fate (Dullin et al., 2007). However, this does not hold true in the mouse retina, where *Ptf1a* is essential for differentiation of both GABAergic and glycinergic ACs (Fujitani et al., 2006; Nakhai et al., 2007).

We have shown previously that targeted disruption of *Foxn4* and *Brn3b* in mice causes a dramatic loss and increase of GABAergic ACs, respectively (Li et al., 2004; Qiu et al., 2008). This and other studies delineating the molecular events leading to GABAergic ACs enable us to better understand these mutant phenotypes. In addition to activating the expression of *Math3*, *Neurod1*, and *Ptf1a* (Inoue et al., 2002; Li et al., 2004; Fujitani et al., 2006; Nakhai et al., 2007), all of which are pan-AC specifier genes, *Foxn4* appears to directly and/or indirectly activate the expression of *Nr4a2* and *Bhlhb5* as well for GABAergic AC specification (Figs. 1, 9A). Loss of *Foxn4* function would disable both differentiation pathways controlled by *Nr4a2* and *Bhlhb5*, thereby resulting in a drastic loss of GABAergic ACs (Fig. 9C). On the contrary, *Brn3b* normally represses the expression of *Nr4a2* and *Bhlhb5* besides specifying ganglion cells and promoting their differentiation (Figs. 1, 9A). The absence of *Brn3b* would cause ganglion cell loss and derepression of *Nr4a2* and *Bhlhb5*, which in turn would specify more GABAergic ACs (Fig. 9D). Furthermore, the upregulation of *Nr4a2* and *Bhlhb5* rather than *Barhl2*, a glycinergic AC specifier gene (Mo et al., 2004), can explain why only GABAergic ACs increase but glycinergic ACs do not change in *Brn3b*-null retinas (Qiu et al., 2008). Thus, *Nr4a2* appears to network with a number of other retinogenic factors to ensure proper specification and differentiation of retinal cell types and subtypes.

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