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Requirements for *Neurogenin2* during mouse postnatal retinal neurogenesis

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ABSTRACT

During embryonic retinal development, the bHLH factor *Neurog2* regulates the temporal progression of neurogenesis, but no role has been assigned for this gene in the postnatal retina. Using *Neurog2* conditional mutants, we found that *Neurog2* is necessary for the development of an early, embryonic cohort of rod photoreceptors, but also required by both a subset of cone bipolar subtypes, and rod bipolars. Using transcriptomics, we identified a subset of downregulated genes in P2 *Neurog2* mutants, which act during rod differentiation, outer segment morphogenesis or visual processing. We also uncovered defects in neuronal cell culling, which suggests that the rod and bipolar cell phenotypes may arise via more complex mechanisms rather than a simple cell fate shift. However, given an overall phenotypic resemblance between *Neurog2* and *Blimp1* mutants, we explored the relationship between these two factors. We found that *Blimp1* is downregulated between E12-birth in *Neurog2* mutants, which probably reflects a dependence on *Neurog2* in embryonic progenitor cells. Overall, we conclude that the *Neurog2* gene is expressed and active prior to birth, but also exerts an influence on postnatal retinal neuron differentiation.

1. Introduction

The vertebrate eye contains a precise retinal circuitry for the detection and processing of visual stimuli. Failure to produce and maintain the correct proportions of each retinal cell type contributes to vision impairment and blindness. These retinal cell types (six neuronal and one glial) are derived from a common pool of multipotent progenitor cells that arise in an overlapping temporal sequence (Sidman, 1961; Young, 1985). Mouse retinal development occurs over a nearly six week period of time, during which retinal ganglion cells (RGCs), horizontal cells, cone photoreceptors, some amacrines and a subset of rod photoreceptors differentiate before birth, while the remainder of amacrines, the majority of rods, bipolar cells and Müller glia differentiate postnatally (Sidman, 1961; Young, 1985). In mice, retinal neurogenesis begins at the center of the optic cup at embryonic day 11 (E11) and expands peripherally, reaching the distal-most region by E16 (Hinds, 1968; Sidman, 1961). Retinal progenitor cells (RPCs) are generally instructed by intrinsic factors about their cell fate, and by extrinsic signals regarding temporal competence states and the overall proportion of each cell class (Cepko, 2014; Cepko et al., 1996).

Basic helix-loop-helix (bHLH) transcription factors are well established intrinsic regulators of neurogenesis. Two bHLH factors, *Atoh7* and *Neurogenin2* (*Neurog2*), are expressed by RPCs that produce the first RGCs (Brown et al., 1998, 2001b; Gradwohl et al., 1996; Sommer et al., 1996; Wang et al., 2001; Yan et al., 2001). Previously *Neurog2* was shown to activate *Atoh7* transcription directly, plus control the spatiotemporal progression of the initial wave of retinal neurogenesis (Hufnagel et al., 2010; Skowronska-Krawczyk et al., 2009). However, *Neurog2* does not instruct early cell fates per se, given that in E18.5 *Neurog2* germline mutants there was only a 2% increase in RGCs, and no impact on the proportions of RPCs, cone photoreceptor, amacrine or horizontal neurons (Hufnagel et al., 2010). However, the requirements for this gene in the postnatal retina have not been explored, since germline mutants die at birth (Fode et al., 1998).

Here we assessed the role of *Neurog2* during the later phases or retinal development, using a conditional allele and two retinal Cre drivers (Hand et al., 2005; Prasov and Glaser, 2012; Rowan and Cepko, 2004). We found that only the earliest differentiating rod photoreceptors require *Neurog2*, consistent with the *Neurog2* retinal lineage producing mostly rods at E17.5 (Brzezinski et al., 2011), and the complete downregulation of this gene soon after birth. Although the proportion

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of rods that depend on *Neurog2* is relatively small, our postnatal day 2 (P2) transcriptomic analysis of *Neurog2* conditional mutants revealed that the main class of down-regulated transcripts are rod-specific. Somewhat paradoxically, we also found additional retinal phenotypes that arose after *Neurog2* expression is normally abolished. These included a significant loss of several cone bipolar subtypes, an increase in rod bipolar neurons, and defects in neuronal culling. Our data also show that the photoreceptor-bipolar fate determinant, *Prdm1/Blimp1* (Brzezinski et al., 2010, 2013; Katoh et al., 2010; Mills et al., 2017; Wang et al., 2014) is downregulated embryonically in *Neurog2* mutants, well ahead of the rod and bipolar phenotypes. Although the cell classes affected are similar between *Neurog2* and *Blimp1* retinal mutants, the *Neurog2* phenotypes were considerably milder and seem to primarily impact differentiation. Overall, we conclude that *Neurog2* is required during postnatal retinal differentiation, but that other molecular pathway(s) likely act redundantly with *Neurog2*. Our findings provide an entry point for better elucidation of the gene networks that comprehensively regulate rod, cone and rod bipolar development.

2. Materials and methods

2.1. Mice and BrdU injections

Mouse lines used in this study were a *Neurog2*^{GFP} germline allele (*Neurog2*^{tm4Fgu}), maintained on an ICR background (Seibt et al., 2003), *Neurog2*^{CKO} allele (*Neurog2*$^{tm5(Neurog2)Fgu}$) maintained on a CD-1 background (Hand et al., 2005), *Chx10-Cre* transgenic line (*Tg(Chx10-EGFP/cre;-ALPP)2Clc*) maintained on a CD-1 background (Rowan and Cepko, 2004), and a BAC-Tg *CrxCre* mouse (*Tg(Crx-cre)352Gla*) maintained on a C57BL/6 background (Prasov and Glaser, 2012). PCR genotyping was performed as described (Hand et al., 2005; Prasov and Glaser, 2012; Rowan and Cepko, 2004; Seibt et al., 2003). Timed matings were used to determine embryonic age, with E0.5 as the date of the vaginal plug was noted. In retinal birthdating experiments, a 10 mg/ml BrdU solution (0.1 mg/g body weight in 0.9 M NaCl) was injected in pregnant dams carrying E17.5 litters and P21 eyes collected for analysis. Postnatal P3 pups were injected intraperitoneally with 40 μls of the same BrdU solution and eyes collected at P21. All mice were housed and cared for in accordance with the guidelines provided by the National Institutes of Health, Bethesda, Maryland, and the Association for Research in Vision and Ophthalmology, and conducted with approval and oversight from the UC Davis Institutional Animal Care and Use Committee.

2.2. Immunohistochemistry

Embryonic and postnatal tissues were fixed in 4% paraformaldehyde for 30 min at 4 °C and processed, embedded, sectioned and antibody labeled as in Mastick and Andrews (2001). Anti-BrdU labeling conditions were as in Le et al. (2006). Primary antibodies used were rabbit anti-Arrestin 3/Cone Arrestin (1:7000, Millipore Cat#: AB15282), rat anti-Blimp1/Prdm1 (1:100, Santa Cruz Cat#: sc-47732), rat anti-BrdU (1:100, AbD Serotec Cat#: OBT0030), sheep anti-Chx10/Vsx2 (1:700, Abcam Cat#: AB16141), rabbit anti-cleaved PARP (1:500, Cell Signaling Cat#:9544), rabbit anti-Cre (1:500, Covance Cat#: MMS-106R), rabbit anti-Crx (1:1000, a gift from Cheryl Craft, USC; Fig. 1 and Suppl Fig. 3), rabbit anti-Crx (1:500, Santa Cruz Cat#: sc-30150 (discontinued); Suppl Fig. 2), chick anti-GFP (1:3000, Aves Cat#: GFP-1020), mouse anti-Neurog2 (1:1000, R & D Systems Cat#: MAB3314), mouse anti-Nr2e3 (1:200, R & D Systems Cat#: PP-H7223-00), goat anti-Otx2 (1:200, R & D Systems Cat#: BAF1979), mouse anti-Pax6 (1:200, Santa Cruz Cat#: sc-32766), rabbit anti-Pax6 (1:1000, Covance Cat#: PRB-278P), mouse anti-protein kinase C α (1:200, Sigma-Aldrich Cat#:P5704), rabbit anti-Sox9 (1:200, Millipore Cat#: AB5535), and rabbit anti-Vsx1 (1:200, Clark et al., 2008). Sections were then incubated with directly conjugated Alexafluor secondary antibodies (1:400, Jackson ImmunoResearch or Molecular Probes) or biotinylated secondary antibodies (1:500, Jackson ImmunoResearch or ThermoScientific) followed by Alexafluor conjugated streptavidin (1:500, Jackson ImmunoResearch). In Fig. 5D–F, rabbit anti-cPARP was directly conjugated with a Zenon Rabbit IgG Alexa Fluor 594 Labeling Kit (Molecular Probes Cat: Z-25307). Nuclei were labeled with DAPI (1:1000 dilution of a 1 mg/ml solution, Sigma-Aldrich Cat#:28718-90-3).

2.3. Microscopy and cell counting

Microscopy was performed with either a Zeiss fluorescent microscope, Zeiss camera and Apotome deconvolution device or a Leica DM5500 microscope, equipped with a SPEII solid state confocal and processed using Leica LASAF and Adobe Photoshop (CS4) software programs. All digital micrographs were equivalently adjusted among genotypes for brightness, contrast and pseudo-coloring.

For quantification of marker labeled cells, ≥ 3 individuals per genotype were analyzed using at least 2 sections per individual. Equivalent anatomical depth in the retina was determined by proximity to the optic nerve. Cell counts were performed using the count tool in Adobe Photoshop CS4. Statistical significance was determined using IBM SPSS Statistics (v. 24) with either an unpaired 2 sample T-Test with a Welsh correction or one-way ANOVA with Welsh's correction and a Tukey post hoc test.

2.4. Western blots

P2 retinal pairs were sonicated in RIPA buffer with protease inhibitors (Complete, Sigma Cat# 11697498001) and processed as described (Prasov et al., 2010). Total retinal protein (25 μg/lane) was loaded on a NuPage 4–12% Bis-Tris gel (Invitrogen Cat#: NP0322BOX), electrophoresed and transferred to a nitrocellulose membrane (Invitrogen Cat#: LC2000). Blots were blocked in 4% milk/0.1 M Tris (pH 7.4)/0.15 M NaCl/0.1% Tween20, probed with rat anti-Blimp1/Prdm1 (1:100, Santa Cruz Cat#: sc-47732) and mouse anti-β-actin (1:4000, Sigma-Aldrich Cat#: A1978), and visualized with IRDye 800CW (1:15,000, Li-Cor Cat#:926-32219) and IRDye 680RD (1:20,000, Li-Cor Cat#:926-68022), respectively, on the Li-Cor Odyssey Clx Imaging System. Densitometric analysis was performed using the Image Studio Lite software (v. 5.2).

2.5. RNA-sequencing and quantitative PCR

RNA-sequencing was performed on *Neurog2*$^{CKO/CKO}$, Chx10-Cre;*Neurog2*$^{CKO/+}$ and Chx10-Cre;*Neurog2*$^{CKO/CKO}$ P2 retinas (n = 5 biologic replicates/genotype). Total RNA was extracted from individual pairs of retinal tissue using the Zymo Research Quick RNA miniprep kit (Cat#: R1055). RNA concentrations were determined with a Qubit 3.0 Fluorometer and Molecular Probes Qubit RNA HS Assay kit (Cat#: Q32852). Fifteen samples with RIN values ≥ 7.9 were sent to the CCHMC DNA sequencing Core for library preparation and paired end, poly-A stranded RNA sequencing on an Illumina HiSeq. 2500, at a 30 million read depth. Reads were aligned with BWA and Bowtie programs to the mm10 genome. Aligned reads were then analyzed for differentially expressed transcripts using the CuffDiff program in the Galaxy online bioinformatics package (www.usegalaxy.org). Differentially expressed transcripts were initially evaluated with an adjusted p-value cutoff of $q \leq 0.05$. Analysis was broadened to a significance of $p \leq 0.05$ for some transcripts with the requirement of validation. Transcripts were grouped by ontology using PANTHER (www.geneontology.org) and ranked for fold enrichment, which is the proportion of genes in a particular functional group, compared to the number of genes in that group expected in a random list of genes. For genes showing significant changes, sequence reads were aligned to mm10 were visualized with the Integrative Genomics Viewer (IGV) browser (v. 2.3) (Robinson et al., 2011; Thorvaldsdottir et al., 2013).