Neurod6 expression defines novel retinal amacrine cell subtypes and regulates their fate

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Supplementary Figures and Tables

Figure S1

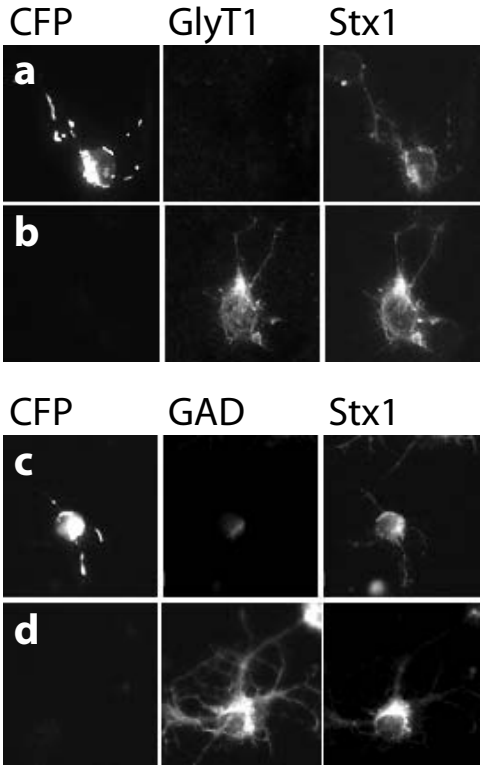

Figure S1: MP-CFP$^+$ ACs in dissociated cultures do not express GlyT1 or GAD65/67

Dissociated retinal cells triple-stained for CFP (using anti-GFP), Syntaxin-1 (a pan-AC marker), and either GlyT1 (**a**,**b**) or GAD (**c**,**d**). CFP$^+$ cells are Stx1$^+$ but are neither GlyT1$^+$ (**a**) nor GAD$^+$ (**c**). Conversely, glycinergic (**b**) and GABAergic (**d**) ACs (Stx1$^+$) are not CFP$^+$.

Figure S2

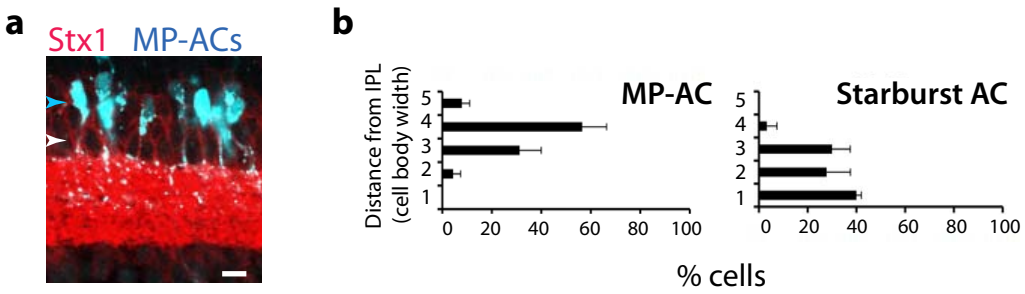

Figure S2: nGnG amacrines have a characteristic position within the INL

a: nGnG ACs in the MP line are CFP$^+$ (blue); the amacrine zone of the INL is marked with anti-Stx1 (red). ACs close to the IPL are never CFP$^+$ (white arrowhead). Instead, CFP$^+$ cell bodies are found in the outermost sublayer of AC cell bodies (blue arrowhead). Scale bar = 10 μm.

b: Quantification of cell body position. As expected from **a**, nGnG ACs labeled with MP-CFP are located in the outermost part of the INL AC zone (left graph). For comparison, the INL location of a second AC subtype, Starburst ACs, is replotted from ref. 12. Starbursts are closer to the IPL than nGnG ACs. ($n > 50$ cells for each subtype; P15 mice. Error bars = S.E.M.)

Figure S3

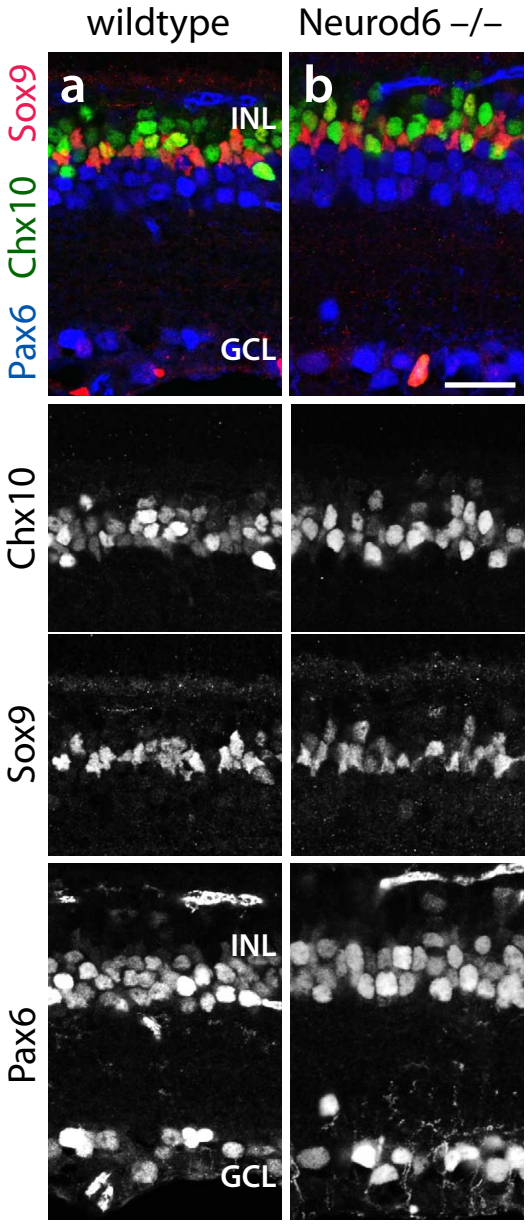

Figure S3: Normal layers and cell positions in Neurod6 mutant retina

Neurod6 mutants (**b**) have normal numbers of Müller glia, bipolar cells, ACs, and RGCs relative to littermate controls (**a**). Immunostaining for Pax6 (blue) labels ACs in the INL, and RGCs and displaced ACs in the ganglion cell layer (GCL). Antibodies against Chx10 (green) and Sox9 (red) label bipolar and Müller cells, respectively. There are no obvious differences between wild-type and mutant in the number or position of cells expressing these markers. All sections from P15 animals. Scale bar = 25 μm.

Figure S4

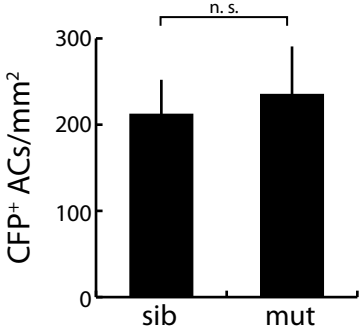

Figure S4: No effect of Neurod6 mutation on size of MP-CFP⁺ population

Neurod6 mutants (mut) carrying the MP transgene have similar numbers of CFP⁺ cells as sibling controls (sib). Cell density was quantified in 20μm sections through central retina from P15 animals. N = 5 fields (317μm^2) from 2 mice of each genotype.

Figure S5

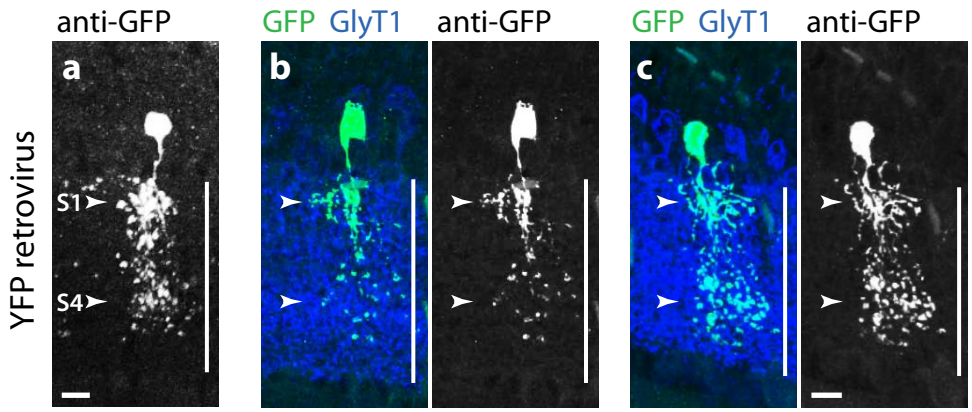

Figure S5: SEG ACs are narrow-field ACs with bistratified projections to ON and OFF IPL.

a–**c**: Retinal sections showing morphology of three individual SEG amacrine cells from P20 mice. Cells were labeled by neonatal injection of low-titer YFP retrovirus as in **Figs. 3d** and **5f**, and were identified as SEG ACs using Ebf3 and GlyT1 immunostaining (not shown). We only imaged cells that were well-isolated from neighboring YFP$^+$ cells in the same and adjacent sections. Anti-GFP staining (green) shows YFP$^+$ cells. GlyT1 counterstain (blue) reveals the IPL (white vertical bar). Each of these GlyT1$^+$ cells has similar narrow-field morphology, with projections to IPL sublaminae S1 and S4 (arrowheads). These cells are representative of the most common morphology seen for SEG cells, although narrow-field cells with different IPL projection patterns were occasionally observed. The cell in (**a**) is the same as that shown in **Fig. 5f**, but it is displayed here without counterstain to better show the cell's fine morphology.

Figure S6

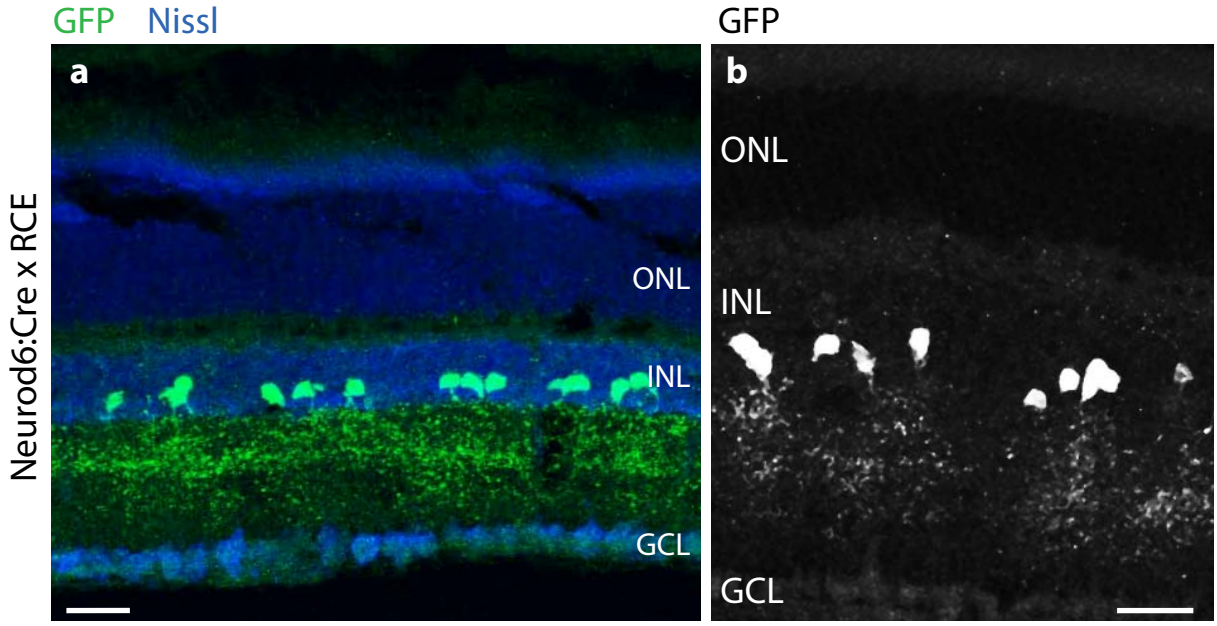

Figure S6: Neurod6:Cre-mediates recombination in postmitotic retinal neurons, but not in progenitors.

a,**b**: Retinal sections from Neurod6:Cre x RCE mice. The RCE reporter[43] expresses GFP in a Cre-dependent manner under control of the Rosa26 locus, which provides for robust expression in most retinal cells. If Neurod6 were expressed in progenitor cells, clones of photoreceptors, bipolar cells, and Müller glia would have been labeled. Instead, Cre-dependent reporter gene (GFP, green) was expressed only by ACs. Blue = Nissl counter-stain. **b**: Higher-magnification view without counterstain to show GFP expression in ACs, and absence of GFP signal in outer retinal cells. Similar results were obtained with the Thy1 reporter line (robust expression by inner retinal cells) used in **Fig. 3**, except that in this case a few RGCs were also labeled. Thus, we conclude that Neurod6 is not expressed in progenitors. Images from adult (P37) retina. Scale bars = 25 μm.

Figure S7

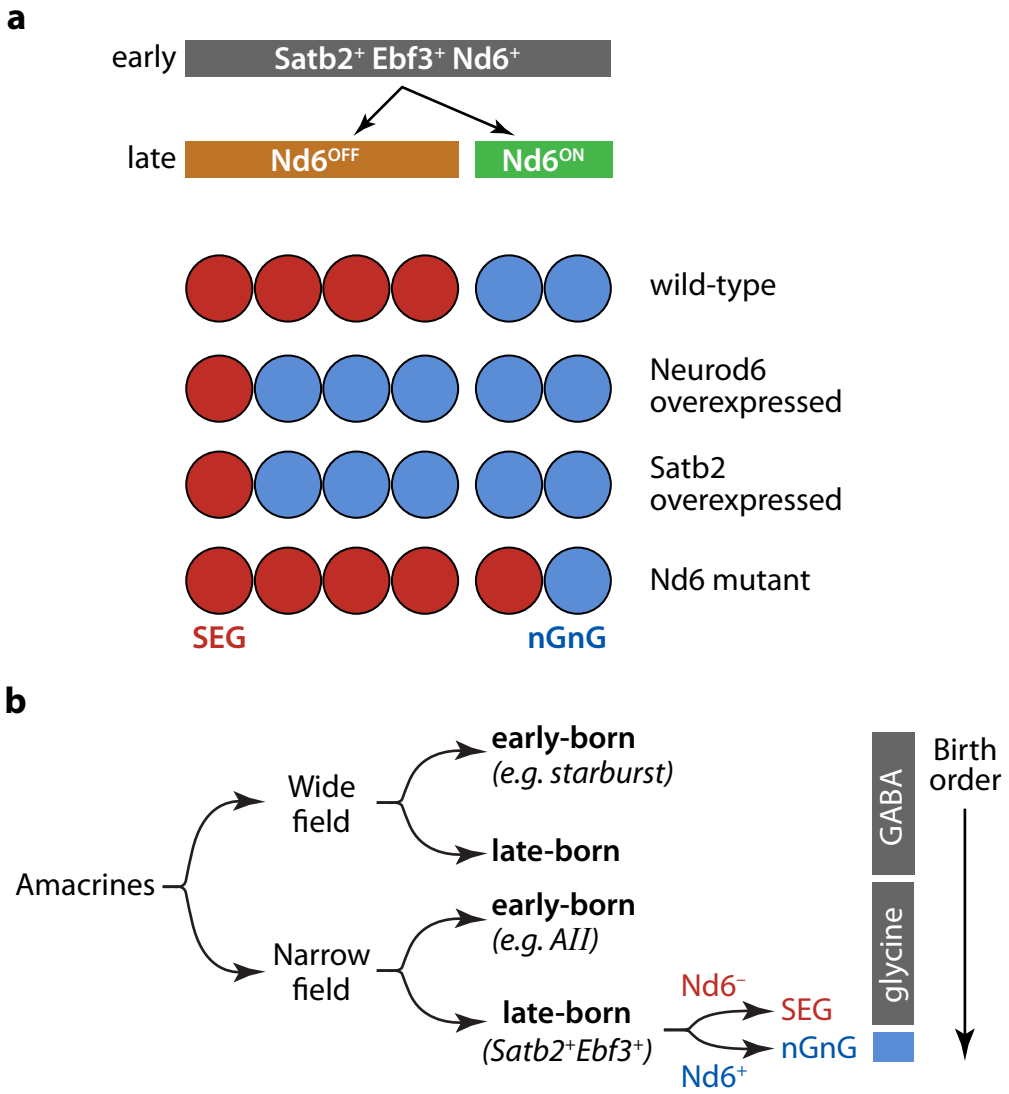

Figure S7: Model for diversification of AC subtypes

a: Satb2, Ebf3, and Neurod6 (Nd6) expression and their effects on AC subtype fate. Regulation of Neurod6 expression subdivides the postmitotic Satb2$^+$Ebf3$^+$ AC population into those that become GlyT1$^+$ (SEG) ACs or nGnG ACs. Overexpression of Neurod6 or Satb2 increases the fraction of Satb2$^+$Ebf3$^+$ cells that become nGnGs. Loss of Neurod6 function, by contrast, biases the Satb2$^+$Ebf3$^+$ population towards the SEG fate.

b: Model for diversification of ACs via progenitor-based and postmitotic mechanisms. Wide-field and narrow-field ACs, as well as subsets of these groups, have characteristic birth orders, suggesting changes in progenitor competence over time. Late-born narrow-field ACs express Satb2 and Ebf3, and are subdivided postmitotically into glycinergic SEG or nGnG subtypes.

Supplementary Table 1:

MP-AC marker genes identified by microarray

			MP-AC fold change over other cell types:					
Gene	Affy Code	MP-AC expression intensity	SAC	Pax	AII	J-AC	ON-BC	MP-BC
6430573F11Rik	1443902_at	132.9	16.1	235.9	28.0	12.4	14.5	10.0
6430573F11Rik	1456096_at	106.0	26.5	41.4	21.9	16.4	10.4	7.6
Ebf3	1428349_s_at	1224.4	57.3	33.3	150.7	2.9	85.8	48.9
Ebf3	1460666_a_at	496.7	9.5	8.3	56.2	4.0	30.9	11.0
Frem1	1455280_at	756.6	168.2	14.2	47.7	3.0	99.2	35.0
Galr2	1422942_at	203.8	9.7	11.2	39.3	8.3	15.3	23.9
Neurod6	1418047_at	1602.0	790.2	268.2	507.3	6.2	618.0	281.9
Pde5a	1455970_at	372.4	13.1	10.9	60.2	13.0	205.6	6.4
Pde5a	1458341_x_at	460.5	6.7	12.4	48.8	6.9	21.3	5.4
Pkdcc	1454838_s_at	1698.8	7.1	16.3	130.2	4.1	5.6	24.9
Pkdcc	1460411_s_at	376.8	5.0	9.5	45.2	3.8	4.7	12.6
Satb2	1453245_at	198.0	300.0	24.7	41.7	6.4	101.9	39.2
Satb2	1427017_at	204.5	12.5	8.7	23.5	4.6	6.3	11.7

Supplementary Table 2:

Molecular phenotype of nGnG and SEG ACs

AC population	Marker(s)	% of population expressing marker(s)	S.E.M.	sample size*
$Ebf3^+$	$Satb2^+$	99.3	0.4	> 250
$Satb2^+$	$Ebf3^+$	96.4	0.5	> 250
$MP\text{-}CFP^+$	$Ebf3^+$	100	–	> 200
$MP\text{-}CFP^+$	$Satb2^+$	100	–	> 200
$Ebf3^+$	$MP\text{-}CFP^+$	24.0	1.1	> 500
$Ebf3^+$	$GlyT1^+$	76.0	1.4	> 700
$Ebf3^+$	$MP\text{-}CFP^+GlyT1^+$	0	–	> 500
$Ebf3^+$	$Gad65/67^+$	0	–	> 100
$MP\text{-}CFP^+$	$Ebf3^+GlyT1^-$	100	–	> 150
$Ebf3^+GlyT1^-$	$MP\text{-}CFP^+$	96.7	1.6	> 150
$Neurod6(Cre)^+$	$MP\text{-}CFP^+$	98.4	1.4	> 60
$MP\text{-}CFP^+$	$Neurod6(Cre)^+$	73.5	4.8	> 60
$Neurod6(Cre)^+$	$Ebf3^+$	100	–	> 60

*Number of cells counted. All counts were performed on at least 3 images from at least 2 different animals.