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Characterization of retinal ganglion cell, horizontal cell, and amacrine cell types expressing the neurotrophic receptor tyrosine kinase Ret

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Abstract

We report the retinal expression pattern of Ret, a receptor tyrosine kinase for the glial derived neurotrophic factor (GDNF) family ligands (GFLs), during development and in the adult mouse. Ret is initially expressed in retinal ganglion cells (RGCs), followed by horizontal cells (HCs) and amacrine cells (ACs), beginning with the early stages of postmitotic development. Ret expression persists in all three classes of neurons in the adult. Using RNA sequencing, immunostaining and random sparse recombination, we show that Ret is expressed in at least three distinct types of ACs, and ten types of RGCs. Using intersectional genetics, we describe the dendritic arbor morphologies of RGC types expressing Ret in combination with each of the three members of the POU4f/Brn3 family of transcription factors. Ret expression overlaps with Brn3a in 4 RGC types, with Brn3b in 5 RGC types, and with Brn3c in one RGC type, respectively. Ret⁺ RGCs project to the lateral geniculate nucleus (LGN), pretectal area (PTA) and superior colliculus (SC), and avoid the suprachiasmatic nucleus and accessory optic system. Brn3a⁺ Ret⁺ and Brn3c⁺ Ret⁺ RGCs project preferentially to contralateral retinorecipient areas, while Brn3b⁺ Ret⁺ RGCs shows minor ipsilateral projections to the olivary pretectal nucleus and the LGN. Our findings establish intersectional genetic approaches for the anatomic and developmental characterization of individual Ret⁺ RGC types. In addition, they provide necessary information for addressing the potential interplay between GDNF neurotrophic signaling and transcriptional regulation in RGC type specification.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Keywords

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1 | INTRODUCTION

Retinas are the sensory organ for light detection and visual perception. Within the retina, the photon flux is captured by photoreceptors, and the various features of visual stimuli are computed and extracted by relay neurons called bipolar cells and interneurons called horizontal cells (HCs) and amacrine cells (ACs) (Dowling, 2012; Rodieck, 1998). Their output converges onto the dendritic arbors of about 20 distinct retinal ganglion cells (RGC) types, which are the projection sensory neurons and transmit the different features of the visual stimulus to the pertinent retinorecipient areas of the brain (Levick, 1975; Masland, 2012; Wässle, 2004). Classification criteria for RGC types include dendrite morphology, brain projections, response properties to visual stimuli, functions within the visual circuit and more recently, molecular markers (Badea, Cahill, Ecker, Hattar, & Nathans, 2009; Badea & Nathans, 2004, 2011; Baden et al., 2016; Carciari, Jacobs, & Nirenberg, 2003; Cleland & Levick, 1974a,b; Coombs, van der List, Wang, & Chalupa, 2006; Farrow & Masland, 2011; Helmstaedter et al., 2013; Hong, Kim, & Sanes, 2011; Martersteck et al., 2017; Sajgo et al., 2017; S. Siegert et al., 2012; Sun, Li, & He, 2002; Volgyi, Chheda, & Bloomfield, 2009). The integration of different classification schemes and the assignment of different features to individual cell types are significantly facilitated by molecular genetic approaches that allow for the labeling and characterization of RGC subpopulations or subtypes. However, since few genetic markers perfectly overlap with individual RGC types, successful genetic approaches typically require random sparse recombination, transgenic integration effects, or combinatorial genetics involving more than one locus or combinations of genetic and viral drivers (Badea & Nathans, 2004, 2011; Badea, Wang, & Nathans, 2003; Feng et al., 2000; Madisen et al., 2015; Martersteck et al., 2017; Siegert et al., 2009; Zhu, Xu, Hauswirth, & DeVries, 2014).

Tightly connected to the characterization of neuronal cell types, is the question of how these neurons develop, differentiate, and acquire their final distinctive features. One key mechanism involves transcription factors (TFs) that regulate the developmental programs of cell fate specification. The transcriptional cascade for RGC specification involves cell fate determination genes, typically active in the still proliferating neuroblasts (*Atoh7/Math5*) as well as RGC determining factors, such as *Isl1* and *Pou4f2/Brn3b* (Brown, Patel, Brzezinski, & Glaser, 2001; Gan et al., 1996; Wang et al., 2001; Wang et al., 2002). Previous work has established that three members of the *Pou4f* family, including *Pou4f1/Brn3a*, *Brn3b*, and *Pou4f3/Brn3c*, participate in a combinatorial code of RGC type specification, in collaboration with several other TFs (Badea, Cahill, et al., 2009; Badea & Nathans, 2011; Shi et al., 2013). The three *Brn3* factors are expressed in partially overlapping populations of RGC types, and regulate distinct morphological and molecular features of these cells, either alone or in combination. In addition, *Pou4f/Brn3* TFs are also responsible for specifying other types of projection sensory neurons, involved in somatosensation, the vestibular and

auditory senses (Badea et al., 2012; Erkman et al., 1996; Erkman et al., 2000; Gan et al., 1996; Wang et al., 2002; Xiang et al., 1997; Xiang, Gao, Hasson, & Shin, 1998).

In addition to transcriptional control, the development and specification of sensory neurons requires neurotrophic support, provided in large part by ligands from target derived neurotrophins (NTs) and glial derived neurotrophic factor (GDNF) families, and their receptors. Mice carrying loss of function mutations in a variety of neurotrophins or their receptors exhibit loss of sensory neuron populations or specific types (Airaksinen & Saarma, 2002; Ernsberger, 2008; Marmigere & Ernfors, 2007). The exact role of these neurotrophic factors in RGC development, however, is relatively poorly understood. All major neurotrophin receptors (TrkA, TrkB, TrkC, and P75) are expressed by RGCs in various stages of development. In particular, neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), and the BDNF receptor, TrkB, have been implicated in the development of dendrites, axons and physiological properties of RGCs (Cantrell, Cang, Troy, & Liu, 2010; Grishanin et al., 2008; Liu et al., 2007; Liu et al., 2009; Llamas, Cernuda-Cernuda, Huerta, Vega, & Garcia-Fernandez, 1997).

The roles of the GDNF family neurotrophins and their receptors in RGC development are essentially unexplored. Glial family ligands (GFLs) consist of four ligands (GDNF, Artemin, Neurturin, and Persephin) that bind selectively to four co-receptors, “GDNF family receptor- α ” (GFR α 1–4), which are attached to the plasma membrane via a Glycosyl Phosphatidylinositol (GPI) anchor (Airaksinen & Saarma, 2002). The signaling of the ligands through the GFR α receptors requires the receptor tyrosine kinase RET. Mice lacking RET have severe defects in kidney morphogenesis, megacolon (Hirschsprung disease) as a result of defects in the specification and correct migration of the enteric neurons, severe defects in sympathetic ganglion formation, and defects in specific somatosensory neuron subtypes, that is, pain receptors (nonpeptidergic nociceptors) and touch receptors (rapidly adapting mechanoreceptors) (Enomoto et al., 2001; Golden et al., 2010; Luo, Enomoto, Rice, Milbrandt, & Ginty, 2009; Luo et al., 2007; Ohgami et al., 2010; Uesaka, Nagashimada, Yonemura, & Enomoto, 2008).

GFR α 2 and RET are expressed in HCs, and subpopulations of ACs and RGCs, while GFR α 1 is expressed in subpopulations of ACs and RGCs. Mice carrying hypomorphic (or Dominant Negative) mutations in RET or loss of function mutations in Neurturin show diminished photoreceptor light responses (smaller ERG “a” and subsequently “b” waves) and have morphological defects in the outer plexiform layer, at the level of synapses between photoreceptors, bipolar, and HCs. However, no defects in ACs or RGCs have been reported, and the exact AC and RGC types expressing RET have not been determined (Brantley, Jain, Barr, Johnson, & Milbrandt, 2008).

Previous work had established an interplay between transcriptional regulation and neurotrophic support in somatosensory neurons of the Dorsal Root Ganglion and Trigeminal Ganglion, and for vestibular and auditory neurons of the Spiral and Vestibular Ganglion (de Melo et al., 2008; Huang et al., 2001; Huang et al., 1999). Cross-talk of neurotrophic signaling and Brn3s or other TFs during RGC development still remain to be explored.

We now use gene expression profiling of RGCs, double immunostaining with a variety of markers, and sparse recombination in order to reveal the cell type distribution of RET⁺ Horizontal, Amacrine, and RGCs. Using a previously generated CreERT2 allele targeted at the *Ret* locus and Cre dependent histochemical reporters targeted at the *Brn3a*, *Brn3b*, and *Brn3c* loci, we are able to identify the RGC types expressing *Ret*, and their overlap with the previously reported expression domain of the *Brn3* genes. We find that, during development, *Ret* is first expressed in RGCs, followed by HCs and finally ACs. In addition, RGC types expressing *Ret* cover about ten distinct cell types, which also express *Brn3* TFs with varying degrees of overlap.

2 | MATERIALS AND METHODS

2.1 | Mouse lines and crosses

The Cre reporter allele *ROSA26^{AP}* locus contains the histochemical reporter Alkaline Phosphatase (AP) targeted at the ubiquitously expressed *ROSA26* gene (Badea, Hua et al., 2009). AP is interrupted by an intron, and the second half of its open reading frame (ORF) is cloned in reverse orientation and flanked by inverted loxP sites. Upon Cre mediated recombination, the second exon is inverted, resulting in the correct splicing of the two exons into one complete cDNA containing the AP ORF. The conditional knock-in reporter alleles *Brn3a^{CKOAP}*, *Brn3b^{CKOAP}*, and *Brn3c^{CKOAP}* (Badea, Cahill, et al., 2009; Badea & Nathans, 2011; Badea et al., 2012) contain loxP sites flanking the *Brn3* (*Pou4f*) genes and a strong transcriptional STOP following the endogenous gene. An AP cDNA is inserted after the second loxP site. Upon Cre mediated recombination, the endogenous gene is deleted and replaced by the AP reporter that is now expressed under the control of the endogenous locus. The *Ret^{CFP}* allele is a conditional minigene knock-in (Uesaka et al., 2008), containing the full *Ret* cDNA flanked by loxP sites and followed by a Cyan Fluorescent Protein (CFP) cDNA, knocked-in to exon 1 of the *Ret* gene. Cre recombination results in the loss of the *Ret* cDNA, and expression of CFP under the control of the *Ret* regulatory elements, faithfully reproducing the endogenous expression pattern of the *Ret* gene (Uesaka et al., 2008). For this study, we used a germline-recombined allele, generated previously by crossing the conditional CFP line, *Ret^{CKOCFP}* with *Sox2^{Cre}*, a Cre driver expressed in the germline. Tissues were derived from these germline recombined heterozygote *Ret^{CFP/WT}* mice. The *Ret^{CreERT2}* knockin allele was generated by inserting the CreERT2 coding sequence in the first exon of the *Ret* gene, and results in Cre recombination in *Ret* positive neurons (Luo et al., 2009). The colocalization of *Ret* with CFP in *Ret^{CFP/WT}* mice and Cre in *Ret^{CreERT2/WT}* mice was confirmed by double immunostaining (Supporting information Figure S1). Careful review of the literature has failed to reveal any phenotypic changes in *Ret^{CFP/WT}* or *Ret^{CreERT2/WT}* mice (Jain et al., 2006; Uesaka et al., 2008). *Pax6α:Cre* is a transgenic line expressing Cre exclusively in the retina (Marquardt et al., 2001).

To describe the morphologies of RET⁺ neurons, we have crossed *Ret^{CreERT2/WT}* males × *ROSA26^{AP/AP}* females resulting in *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* pups. We then induced recombination and AP expression by injecting various amounts of Tamoxifen either by gavage or intraperitoneal injection (I.P.). To describe the morphologies of RGCs expressing *Ret* in combination with *Brn3a*, *Brn3b*, or *Brn3c*, we have crossed *Ret^{CreERT2}* males with

Brn3a^{CKOAP/CKOAP}, *Brn3b^{CKOAP/CKOAP}*, and *Brn3c^{CKOAP/CKOAP}* females. Double positive offspring (*Ret^{CreERT2/WT}; Brn3^{CKOAP/WT}*) were injected with 4 Hydroxytamoxifen (4HT) I.P. to achieve random sparse recombination. Alternatively, to induce unilateral recombination, 4 HT was administered intravitreally in one eye. All mice were on C57/Bl6–SV129 mixed background. All animal procedures were approved by the National Eye Institute (NEI) Animal Care and Use Committee under protocol NEI640.

2.2 | AP histochemistry and morphometric analysis

Retina whole mounts and brain sections were stained, processed, and imaged, and RGC dendrites and axons were traced and quantified as previously described (Badea, Cahill et al., 2009; Badea & Nathans, 2004; Shi et al., 2013). In brief, mice were anesthetized, fixed by intracardiac perfusion with 4% Paraformaldehyde (PFA), retinas were dissected and flat mounted, and 200 µm thick coronal brain sections were generated using a vibratome. Retinal flat mounts and brain sections were heat inactivated in a water bath at 65°C for one hour, and then the AP histochemical stain developed. Low resolution images of brain sections were acquired on a Zeiss Discovery V8 Stereomicroscope, using a Zeiss Axiocam MRC color camera, and Axiovision software. Color images of retinal flatmounts and DIC grayscale image stacks of individual RGC dendritic arbors were acquired using a Zeiss Imager.Z2. Morphologic parameters were measured using ImageJ as described in Figures 5 and 6 and references (Badea & Nathans, 2004, 2011; Shi et al., 2013). To orient the relative stratification levels of dendritic arbors in the IPL, plots describing the stratification measurements in Figures 6–7, and 11 are labeled with the previously reported lamination levels for ON and OFF Starburst ACs and the delineation between the ON and OFF subdivisions of the IPL, as inferred from the stratification of axon terminals of ON bipolar cells (mGlu6:GFP positive) (Morgan, Dhingra, Vardi, & Wong, 2006). Neuronal arbor reconstructions were done using Neuromantic (Darren Myat, <http://www.reading.ac.uk/neuromantic>) and projections were generated using a Matlab (Mathworks, Inc.) script (Shi et al., 2013). For each genotypic combination and condition, at least three mice were analyzed.

2.3 | Indirect immunofluorescence

For immunofluorescence, retina sections were processed and immunostained as previously described (Shi et al., 2013). Briefly, retinas were immersion fixed for 30 min in 2% paraformaldehyde, cryoprotected in OCT, and sectioned at 14 µm thickness on a cryostat. For each genotype, retinas from three distinct animals were imbedded collectively in the same OCT block, and all staining procedures were carried out collectively on these slides. Images (20×) were captured on a Zeiss Imager.Z2 fitted with an Apotome for fluorescent imaging and Axiovision software. Number of analyzed retinas and collected images are indicated in each figure legend.

Primary antibodies used are listed in Table 1. *Brn3b* antibody was generated in our lab: A DNA sequence encoding Brn3b protein aa 184–252 was fused to Glutathione S-transferase (GST). The resulting fusion protein was overexpressed in *Escherichia coli*, purified and used to immunize rabbits. Antibody specificity was shown using immunocytochemistry by lack of staining in Brn3b knockout tissues. In addition, specificity for Brn3b versus Brn3a and Brn3c was checked by Western blot against fusion proteins between Brn3a, Brn3b, and

Brn3c peptides and Maltose Binding Protein. All other antibodies were obtained commercially. *Brn3a*: Mouse monoclonal anti-Brn3a (Millipore; MAB1585, RRID: AB_94166, clone 5A3.2) antibody was raised against amino acids (aa) 186–224 of human Brn3a fused to T7 gene 10 protein (Xiang et al., 1995). According to the manufacturer's technical information, the antibody shows no reactivity to Brn3a knockout mice (Xiang et al., 1996). On Western blotting, the antibody does not detect Brn3b or Brn3c. In rodent and monkey, the antibody labels the nuclei of Brn3a-positive RGCs and DRG neurons. *Ret*: Rabbit polyclonal (IBL, cat # R787), was raised against an affinity-purified synthetic peptide for c-terminus of human c-Ret (STWIENKLYGRISHAFTRF). This antibody is confirmed using immunostaining antibody stains Ret-ERT2 genetically labeled DRG neurons (Luo et al. 2009), and does not label knockout tissues (Uesaka et al., 2008). *Isl1*: Anti-Isl1 goat polyclonal antibody (Neuromics; GT15051, RRID:AB_2126323) was raised against recombinant human islet-1. Specificity was tested by Western blot by the manufacturer and in immunohistochemistry by colocalization with the mouse monoclonal anti-Isl1 (DSHB, 39.4D5, RRID:AB_2314744). *Lim1*: Mouse monoclonal (Developmental Hybridoma Studies Bank created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242., clone 4F2, contributed by Jessell and Brenner-Morton), raised against recombinant rat Lim2, but detecting both Lim1 and Lim2 by western blot and immunostaining. In the retina it labels characteristically HC bodies. *Prox1*: Rabbit polyclonal to Prox1 (abcam; ab38692) was raised against a synthetic peptide conjugated to KLH derived from within residues 150–250 of Human Prox1, detects a 95 kDa band on Western Blotting of HepG2 (Human) and SHSY-5Y (Human neuroblastoma cell line) but not HEK293 (human) cell lines whole cell lysates. It shows the previously reported expression pattern (HCs, Bipolar Cells, ACs) for Prox1. *Calbindin*: Rabbit polyclonal anti-calbindin D-28K antibody (CB38a; Swant, Bellinzona, Switzerland) detects a band of approximately 27–28 kDa in western blots on brain extracts from mouse. The specificity of the antibody in the rodent retina, including mouse, was proved by several studies to be in the horizontal and ACs. *Choline Acetyl-Transferase (ChAT)*: Goat polyclonal anti-ChAT (RRID:AB_11214092) shows bands of 68 kDa on western blots on brain extracts from several species (rodents, birds, primates). In the retina of the rodents, the antibody detects the cholinergic/starburst ACs. *Tyrosine Hydroxylase (TH)*: The rabbit polyclonal anti-TH antibody detects a 62-kDa band on lysate of PC12 cells on western blots. In the retina it detects DOPaminergic ACs (Haverkamp and Wässle, 2000). *Glycine*: Rabbit Purified Polyclonal Antibody against Glycine-glutaraldehyde-BSA (Chemicon/Millipore, ab5020). Specificity was tested by detection against a panel of other aminoacids, as well as Glycine incorporated in peptides or proteins. Retinal staining reflects previously reported pattern with many positive ACs in the INL, and fewer positive cell bodies in the GCL. *GABA*: Affinity purified Rabbit PolyclonalAnti-GABA (γ -AMINOBUTYRIC ACID), (Sigma, A 2052), shows positive binding with GABA, and GABA-KLH in a dot blot assay, and negative binding with BSA. *Neurofilament Light Chain (NFL)*: When used for western blots, 1:1,000 dilution detects NFL on 10 mg of mouse brain lysates. Retina expression is restricted to RGCs. *Alkaline Phosphatase (AP)*: (a) Affinity purified mouse monoclonal antibody (VEB Gent, Belgium, E6 clone) recognizing only the human placental Alkaline Phosphatase isoform. (b) Sheep Polyclonal (American Research products, 13–2355) raised against human PLAP protein. Both have been used extensively to label transgenic lines

containing the AP reporter, and faithfully reproduces the expression pattern observed by AP histochemistry in the same type of tissue. *Green Fluorescent Protein (GFP)*: Anti-GFP antibody (Abcam, Cambridge, MA; ab13970, RRID: AB_300798) was raised against full-length GFP protein. According to the manufacturer's technical information on Western blotting, the antibody recognizes a 27–30-kDa band specific for GFP. *Cre Recombinase (Cre)*: (Millipore cat # MAB3120) Mouse monoclonal raised against amino acids 77 to 343 of recombinant Cre recombinase recognizing a 38.5 kDa band on Western Blots of recombinant Cre. The antibody was validated by immunofluorescence labeling of NIH3T3 and HeLa cells transfected with Cre recombinase. Alexa-Fluor conjugated Donkey polyclonal secondary antibodies were from Molecular Probes/Life Sciences.

2.4 | Gene expression profiling

For technical details regarding RNA expression profiling data we refer the reader to Sajgo et al. (2017). In brief, *Pax6a:Cre; Brn3a^{WT/KO}* or *Pax6a:Cre; Brn3b^{WT/KO}* males were crossed to *Brn3a^{CKOAP/CKOAP}* or *Brn3b^{CKOAP/CKOAP}* females. We then isolated retinas from E15 or P3 offspring. As a result of Cre recombination, RGCs in the offspring are either *Brn3a^{AP/WT}* (Brn3a WT) or *Brn3a^{AP/KO}* (Brn3a KO) respectively *Brn3b^{AP/WT}* (Brn3b WT) or *Brn3b^{AP/KO}* (Brn3b KO). Retinas were dissociated, and RGCs were separated from the retinal supernatant ("Retina" in Figure 1a), using an immunomagnetic protocol based on antibodies against AP (Sajgo et al., 2017). Multiple RGC samples of same age and genotype were pooled and RNA extraction and library preparation performed. Sequencing was done on an Illumina platform, and gene and transcript level alignments performed (Sajgo et al., 2017). Data in Figure 1a reflects gene level analysis. Number of samples and statistical significance tests are reported in the Figure 1 legend.

3 | RESULTS

3.1 | Ret is sequentially expressed in three retinal cell classes

We use several approaches to follow the expression of Ret in HCs, ACs, and RGCs during development. Previously generated RNAseq data (Sajgo et al., 2017) reveal that both *Brn3a^{AP}* and *Brn3b^{AP}* RGCs express significantly higher amounts of Ret when compared to the whole retina, at both embryonic (E15) and early postnatal stages (P3) of development (Figure 1a, compare Retina to RGC samples). However, neither of the two TFs significantly regulate *Ret*, since its RNA expression levels are only mildly reduced in *Brn3^{AP/KO}* (Brn3 "KO") versus *Brn3^{AP/WT}* (Brn3 "WT") RGCs (Figure 1a, RGC samples). Consistently, Ret expression in the newly forming RGCs can be seen at E14.5 by immunofluorescence using a *Ret^{CFP}* knock-in allele (Figure 1b, arrow). Interestingly, very few cells are observed in the neuroblast layer of the retina, suggesting that Ret is expressed in RGCs once they become postmitotic and migrate into their final position at the vitreal edge. By P3, CFP⁺ cells with morphologies and positions characteristic of HCs and ACs can be seen, in addition to ganglion cell layer (GCL) positioned cells (Figure 1c). This pattern is fully developed by P28, with HCs occupying the OPL, ACs the vitreal layers of the INL, and potential RGCs and ACs in the GCL (Figure 1d). At both P3 and P28, CFP⁺ sublaminae can be seen in the Inner Plexiform Layer (IPL), suggesting Ret expression in specific populations of ACs and RGCs.

In order to better define the cell type distribution of Ret⁺ retinal neurons in the adult, we labeled sections from *Ret*^{CFP/WT} mice in conjunction with a variety of retinal cell type markers (Figure 2). CFP⁺ HCs can be identified by the expression of TFs Prox1 and Lim1 (Dyer, Livesey, Cepko, & Oliver, 2003; Liu, Wang, & Xiang, 2000; Poché et al., 2007) (Figure 2a, b). Among putative ACs in the inner INL, about 18% are GABAergic (Figure 2c, i), and 26% are glycinergic (Figure 2e, j), but none are cholinergic (ChAT⁺, Isl1⁺) or DOPaminergic (TH⁺) (Figure 2d, f, k). This marker distribution leaves about 54% of INL ACs with an uncharacterized neurotransmitter signature. All CFP⁺ cells located in the GCL appear to be RGCs (positive for Neurofilament Light Chain, NFL⁺, Figure 2g, l), and may belong to at least four distinct cell types, based on triple labeling with Brn3a and Brn3b (Figure 2h, m). About 60% are Brn3a⁺ Brn3b⁺, 17% Brn3a⁺, 13% Brn3b⁺, and 10% Brn3a⁺ Brn3b⁺. In conjunction with previous data on cell type distribution in Brn3⁺ RGCs, these results suggest significant cell type diversity for RGCs expressing Ret (Badea & Nathans, 2011). We note that some CFP⁺ RGCs in the GCL are also Isl1 positive (Figure 2f). However, since the RGC type distribution amongst Isl1⁺ cells has not been explored, we did not further characterize this population.

The temporal dynamic of Ret expression described above is reminiscent of the dynamic expression of Ret in distinct neuronal populations in the DRG (early in mechanoreceptors versus late in nociceptors (Golden et al., 2010; Luo et al., 2009; Luo et al., 2007; Molliver et al., 1997)). We therefore defined the temporal profile of Ret induction during late embryogenesis and early postnatal stages of retinal development (Figures 1–3). No potential RET⁺ HCs can be observed at E14.5 (Figure 1b) in the outer retina. Correctly positioned RET⁺ HC cell bodies (Calbindin⁺, Lim1⁺, Prox1⁺, Figure 3a, b, g) can be identified at E17, and incipient neuronal arbors start to emerge at P1 (Figure 3c, d, i). By P3, far more elaborate HC arbors can be observed with both CFP and Calbindin (Figures 1c, 3e, f, k, l), suggesting the first steps of OPL lamination. Essentially no Ret⁺ ACs are apparent at the inner aspect of the neuroblast layer up to E17 (Figure 3a, b, g, h). This is despite the presence of Calbindin⁺, Prox1⁺, and Isl1⁺ cells at this age, suggesting that some AC types begin to be specified (Figure 3a, g, h). However, at P1 and more strikingly at P3, Ret⁺/Prox1⁺ ACs can be seen at the inner limit of the INL, thus placing the initial expression of Ret in ACs at around P1 (Figure 3c, d, i, j). Ret⁺ RGCs expressing either Brn3b, Isl1 or both can be clearly seen already at E17, and increase in numbers through P3 (Figure 3b, d, f, h, j, l). Thus, Ret is first expressed in RGCs, followed by HCs and finally ACs. This pattern is consistent with the order in which these retinal cell classes are thought to be specified and develop their neuronal arbors.

3.2 | Morphological characterization of Ret⁺ neurons

Although retinal cell type definition based on molecular markers is constantly improving, it is still lagging behind their classification based on neuronal arbor morphology. To further characterize Ret⁺ retinal neurons based on morphology, we crossed a previously described *Ret*^{CreERT2} allele with our *ROSA26*^{AP} histochemical Cre reporter line, and induced variable degrees of sparse recombination by Tamoxifen administration using several combinations of dosage, timing, and administration routes (Figure 4). Retinas were then flat mounted, and the AP histochemical stain developed as described. Besides the expected characteristic

morphologies of RGCs and HCs, a large number of small field, dense dendritic arbor ACs were observed (Figures 4a, 6 b1 for high magnification).

3.3 | Morphological characteristics of *Ret^{CreERT2}; ROSA26^{AP}* horizontal cells

Sparse recombination offered us a unique opportunity to characterize the relative distribution of HC axonal and dendritic arbors (Figure 5). HCs display a dendritic arbor emerging from the cell body and centered on it, an axonal process that is typically unbranched but ends in a dramatically dense and branched axonal arbor (Figure 5a, c). We imaged and quantified several morphometric parameters for 139 HCs. We find that axon arbors are nearly twice as large in area compared to dendritic arbors (Figure 5e, f), and that the axonal process connecting them is 11 times longer than the dendritic arbor radius (compare Figure 5h, “A1”, median length = 279 μm to Figure 5f, median dendrite area = 1,940 μm^2 , corresponding to a radius of about 25 μm). Surprisingly, the axonal process rarely follows a straight trajectory between the dendrite and axon arbors (Figure 4a, arrowhead, Figure 5a, c), and more often loops around, thus bringing the two arbors in close proximity (Figure 5d), or even resulting in their partial overlap (Figure 5b). Expressed differently, the axonal process length is, in most cases, larger than the distance between the centers of mass of dendrite and axon arbor (Figure 5g, h).

3.4 | Dendritic morphologies of *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* RGCs

The most diverse retinal population labeled in the *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* retinas were RGCs. We imaged a total of 241 RGCs from retinas in which sparse recombination was induced at P14 and histological preparations were made in the adult (P21 and beyond, Figure 4b, c, summarized in Table 2). Out of these, 138 RGCs had monostратified (Figure 6) and 103 had bistratified dendritic morphologies (Figure 7). It should be noted that, throughout the text we use a nomenclature borrowed from combined physiological–morphological studies, where available, and that our names should be taken under advisement of this fact. Monostратified RGCs could be further classified in six types based on dendritic arbor area and depth of lamination in the IPL (Figure 6d–h). The most numerous RGC type had the characteristic ON-alpha morphology, with large dendritic arbor areas ($n = 74$, area median = 29,142 μm^2 , range = 11,829–66,272 μm^2), and very narrow, ON lamination, centered on the inner ChAT band (Figure 6a–a2, f–h). OFF-beta RGCs, with small area, dense and thick dendritic arbors spanning multiple IPL sublaminae, from about 0.55 IPL to the INL, were the second-most numerous type ($n = 31$, area median = 5,799 μm^2 , range = 3,713–10,310 μm^2 , Figure 6c–c2, f–h). The third most numerous class was represented by multistratified RGCs, with thick and very dense dendritic arbors, spanning the ON-OFF boundary of the IPL, and dendritic areas of medium size ($n = 22$, area median = 19,576 μm^2 , range = 13,095–34,715 μm^2 , Figure 6b–b2, f–h). Finally, a few ($n = 6$) ON-beta RGCs, with small area (median = 6,259 μm^2 , range = 3,290–8,186 μm^2), dense and thick dendritic arbors confined typically between the ChAT bands were observed (Figure 6f–h, for examples see Figure 10d–d3). Only one OFF-DS RGC, with the characteristic, sloping, sector shaped dendritic arbor, and four OFF-alpha RGCs (medium to large size dendrites, laminated in a relatively flat way close to the INL) were found (Figure 6d–h, examples shown in Figure 10b–b2 and c–c2, respectively).

Bistratified RGCs could be subdivided in the characteristic ON-OFF-DS type ($n = 81$, Figure 7b–b3), with dendritic arbors of roughly equal area (Figure 7e, ON arbor median = $16,626 \mu\text{m}^2$, range = $2,911$ – $32,279 \mu\text{m}^2$, OFF arbor median = $10,727 \mu\text{m}^2$, range = $2,454$ – $29,299 \mu\text{m}^2$) and lamination centered on the ON and OFF ChAT bands (Figure 7d), and the Small Bistratified (SB, $n = 21$, Figure 7a–a3), with larger ON arbors (median = $18,787 \mu\text{m}^2$, range = $13,417$ – $25,047 \mu\text{m}^2$), laminating scleral to the ON ChAT band, and sparser, smaller OFF dendrites (median = $6,105 \mu\text{m}^2$, range = $1,734$ – $14,178 \mu\text{m}^2$), reaching all the way to the INL (Figure 7a–a3, c, e). One instance of a large bistratified RGC, was also observed (not shown here, however see below description of $\text{Ret}^+ \text{Brn3b}^+$ bistratified RGCs).

3.5 | Dendritic morphologies of $\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3}^{\text{CKOAP/WT}}$ RGCs

As described above, *Ret* expression in the GCL seems to be restricted to RGCs, and its cell type distribution is partially overlapping with the *Brn3* TFs. In order to identify the RGC types that express *Ret* in conjunction with *Brn3a*, *Brn3b*, or *Brn3c*, we crossed the $\text{Ret}^{\text{CreERT2}}$ allele to our previously generated *Brn3* conditional knock-in alleles. We induced random sparse recombination by intraperitoneal administration of 4-HT in adult mice of either $\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3a}^{\text{CKOAP/WT}}$, $\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3b}^{\text{CKOAP/WT}}$ or $\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3c}^{\text{CKOAP/WT}}$ genotype, and then performed AP histochemistry on retinal flat mounts (Figure 8a–c). We then imaged, measured, and reconstructed dendritic arbors from the identified RGC types in the three crosses (Figures 9–11, Table 2), and identified the retinorecipient areas to which these RGC subpopulations project (Figure 12).

$\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3a}^{\text{CKOAP/WT}}$ RGCs fall in at least four distinct cell types. The two bistratified cell types are the same as those observed in $\text{Ret}^{\text{CreERT2/WT}}; \text{ROSA26}^{\text{AP/WT}}$ RGCs (Figure 7): ON-OFF-DS ($n = 57$; inner arbor area median = $20,297 \mu\text{m}^2$, range = $6,850$ – $37,167 \mu\text{m}^2$; outer arbor area median = $14,487 \mu\text{m}^2$, range = $1,162$ – $36,912 \mu\text{m}^2$; Figures 9b–b3, 11b, c), SB ($n = 4$; inner arbor area median = $23,279 \mu\text{m}^2$, range = $15,106$ – $30,490 \mu\text{m}^2$; outer arbor area median = $12,385 \mu\text{m}^2$, range = $7,459$ – $14,323 \mu\text{m}^2$; Figures 9a–a3, 11b, c). The monostatified RGCs are dominated by morphologies of the beta type, including ON and OFF, treated here as a single population, as their dendritic arbors are too thick and deep in the vertical (z) dimension, and hence cannot be differentiated into two clusters ($n = 43$; arbor area median = $7,744 \mu\text{m}^2$, range = $4,779$ – $12,587 \mu\text{m}^2$; example of an OFF-beta shown in Figure 9e–e2; morphometric parameters in Figure 11f–i). In addition, ON-spiny cells can be observed ($n = 12$; arbor area median = $10,628 \mu\text{m}^2$, range = $6,023$ – $16,386 \mu\text{m}^2$; Figure 9d–d2; morphometric parameters in Figure 11f–i). We also observed one instance of an RGC with lamination at the boundary between the ON and OFF layers, and relatively unbranched, medium sized dendritic arbor (Figure 9c–c2). This is a cell morphology that we have rarely picked up in any of our previous work, so we provide this one observed example.

$\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3b}^{\text{CKOAP/WT}}$ RGCs can also be grouped in at least five major types (Figures 10–11a, d, e, j–m). Monostatified $\text{Ret}^{\text{CreERT2/WT}}; \text{Brn3b}^{\text{CKOAP/WT}}$ RGC morphologies were predominantly ON-beta ($n = 101$, median area = $6,476 \mu\text{m}^2$, range = $4,065$ – $13,320 \mu\text{m}^2$, Figure 10b–b3), ON-alpha ($n = 39$, median area = $38,717 \mu\text{m}^2$, range = $9,879$ – $76,855 \mu\text{m}^2$, Figure 10c–c2), OFF-DS ($n = 7$, median area = $19,011 \mu\text{m}^2$, range = $14,033$ – $27,167 \mu\text{m}^2$, Figure 10a–a2) and OFF-alpha ($n = 11$, median area = $20,717 \mu\text{m}^2$,

range = 15,723–26,803 μm^2 , Figure 10d–d2). Morphometric parameters for all monostратified RGCs, coded by cell type, are shown in Figure 11j–m. The multistratified RGC morphology, often seen in the *Ret^{CreERT2/WT}; ROSA26^{AP}* retinas (Figure 6b–b2), was seen only once in this dataset including 170 RGCs (morphometric parameters in Figure 11j–m). Two types of *Ret⁺ Brn3b⁺* bistratified cell morphologies were observed. A first morphology has an ON arbor with properties indistinguishable from the ON-alpha, with large regularly branched dendrites, from which a few fairly localized, small OFF laminating branches (“tufts”) emerge ($n = 6$; inner arbor area median = 34,290 μm^2 , range = 16,510–43,680 μm^2 ; outer arbor area - range = 4,166–19,177 μm^2 , median = 10,614 μm^2 ; Figures 10e–e3, 11d, e). The second morphology has large, very sparse dendritic arbors in both the ON and OFF layer ($n = 5$; inner arbor area median = 21,728 μm^2 , range = 18,213–35,731 μm^2 ; outer arbor area median = 33,979 μm^2 , range = 27,551–37,748 μm^2 ; Figures 10f–f3, 11d, e). Given its arbor area, and lamination of the inner and outer arbor close to the GCL and INL, respectively, we have assigned this morphology the large bistratified label.

Interestingly, *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* RGCs belonged to only one morphological type, the monostратified ON-spiny cells ($n = 31$, median area = 11,405 μm^2 , range = 8,475–18,499 μm^2 ; Figures 8c, 9f–f2, 11n–q). This cell morphology is characterized by a very dense, flat dendritic arbor, laminating between the inner ChAT band and the limit of ON and OFF IPL sublaminae (Figure 11n, o) and can also be observed in *Ret^{CreERT2}; Brn3a^{CKOAP/WT}* RGCs (compare Figure 9f–f2 and d–d2).

Although the retina flat mounts were not oriented with respect to the dorsoventral and temporonasal axes, most RGC morphologies collected in this study show an eccentricity effect, that is, an increase in area correlated to the distance from the optic nerve (Figure 11i, m, q).

3.6 | Brain projections of *Ret^{CreERT2/WT}; Brn3^{CKOAP/WT}* RGCs

The retinorecipient areas targeted by the three RGC subpopulations were distinct but showed partial overlap (Figure 12). *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* RGC projections were restricted to LGN, superior colliculus (SC), and to very little extent the pretectal area (PTA), (Figure 12a1–a3, d1–d3). Within the LGN, most terminal axon arbors exhibited a superficial lamination (dorsolateral) in the dLGN and vLGN, combined with fewer projections in the ventromedial aspect of the dLGN. This distinct targeting can be observed under conditions of sparse labeling (Figure 12a1) or full labeling (Figure 12d1). Within the SC, terminal arbors seem to segregate in two sublaminae, easier to distinguish under conditions of sparse labeling (Figure 12a3). No projections were observed into the SCN, olivary pretectal nucleus (OPN), IGL, MTN, and AOT.

In contrast, *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* RGCs targeted a much broader set of nuclei (Figure 12b–b4, e–e4). In the suprachiasmatic region, the two dorsolateral aspects of the SCN are labeled, but the SCN proper is devoid of projections, a pattern we have previously reported for full labeling of all *Brn3b^{AP}* RGCs (Figure 12b, e) (Badea, Cahill et al., 2009; Shi et al., 2013). The OPN (Figure 12b1, e1, as well as ventral aspects of the more caudal PTA (Figure 12b2, e2) receive substantial projections. The dLGN and vLGN are diffusely innervated, but a more intense set of projections is seen at the medioventral edge of the

dLGN, particularly in more densely recombined preparations (Figure 12b1, e1). Projections into the IGL are clearly observed (Figure 12b1, e1). Axonal terminals can be seen in multiple layers of the SC (Figure 12b3, e3), however, the MTN and AOT are fully devoid of projections (Figure 12b4, e4).

The unique *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* RGC population projects to LGN, PTA, and SC (Figure 12c, f). Within LGN, the IGL has no projections, while axons projecting to the dLGN and vLGN exhibit a superficial, latero-dorsal lamination, not unlike the one seen for *Ret^{CreERT2}; Brn3a^{CKOAP/WT}* RGCs (Figure 12c1). However, at a more caudal plane of section (Figure 12f1), the projection areas into the dLGN and vLGN are extending further medially. The axonal arbors projecting to the SC exhibit a broad lamination (Figure 12c3, f3), and the pretectal projection appears diffuse, placed at the laterodorsal aspect of the PTA (Figure 12c2, f2), in a complementary pattern to the *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* RGC projection.

For the unilateral intraocular 4HT injections into eyes of *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* and *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* mice, RGC axons project mostly to the contralateral side. This is illustrated by the essentially unstained ipsilateral (left) Optic Tracts (Figure 12d, f), and SCs (Figure 12d3, f3). However, for unilateral inductions in *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* mice, the ipsilateral OT shows significant amounts of staining, as does the dorsolateral aspect of the SCN (Figure 12e). These ipsilateral projecting RGCs seem to target mostly the OPN, and the “ipsi patches” of the dLGN and vLGN (insets in Figure 12e1), whereas the ipsilateral SC is largely devoid of projections (Figure 12e3).

4 | DISCUSSION

In summary, we use immunohistochemistry and random sparse recombination coupled with histochemical AP staining to provide an in-depth characterization of HCs, ACs, and RGCs expressing the neurotrophic receptor tyrosine kinase RET. The partial overlap of expression between Ret and the three Brn3 TFs with regard to RGCs allows us a detailed description of their dendritic arbor morphologies and brain projections. We found that the intersection of Brn3c and RET results in the labeling of one RGC type, thus opening the way to genetic manipulations for the characterization of this cell. Finally, *Ret* is expressed in HCs, ACs, and RGCs close to the developmental stages in which these cells can be identified, and persisting into the adult age, suggesting a role in the postmitotic stages of development and maintenance of specific retinal cell types. Our results suggest an interplay of neurotrophic support and transcriptional codes in retinal cell type specification. Sparse random recombination combined with intersectional genetics allowed the description of the neuroanatomic features in the targeted cells.

4.1 | Horizontal cell anatomy

Work from many labs had previously reported meandering morphologies of HC axonal processes (Boycott, Peichl, & Wässle, 1978; Kolb, Mariani, & Gallego, 1980; Nelson, von Litzow, Kolb, & Gouras, 1975; Peichl & Gonzalez-Soriano, 1994; Vaney, 1993). To our knowledge this is the first large scale survey of intact HC arbor anatomies in the mouse. Our analysis of the relative size and position of HC dendritic arbors reveals interesting

morphological features that are relevant to HC physiology. Thus, instead of connecting the two arbors on the shortest path, the axonal process seems to wander around for some distance, even if the dendrite and axonal arbors are in close proximity. This could be due to differential interleaving of retinal neuroblast columns between dendrite and axon arbors distorting the trajectory of the axon process randomly (Poche et al., 2008; Reese, Harvey, & Tan, 1995). However it could also have physiological implications, as it has been suggested that the axon and dendrite arbor are electrically isolated from each other by the axonal process (Nelson et al., 1975). Effective electrical isolation would require a minimal distance between the two arbors, in order to prevent passive current propagation. We did not detect any correlation between retinal distribution of HCs (e.g., distance from the center) and the size or relative position of axon and dendritic arbor, further supporting the impression that these two compartments function in an uncorrelated fashion, as previously proposed. If crossed into a Cre-dependent GCAMP reporter mouse, the sparse labeling of HCs could be used to center visual stimuli over either the dendritic or axonal arbor and record Ca events from the two arbors as well as Ca^{2+} spread across the axonal process, thus addressing the question of the electrophysiological isolation of the two arbors.

4.2 | Amacrine cell type distribution

Our study confirms the previous finding that Ret is expressed in ACs. We further characterize the Ret^+ AC populations, and find that it can be subdivided in GABAergic, Glycinergic and a third population using some other neurotransmitter to be identified. Interestingly, a recently identified population of RET^+ spinal cord dorsal horn interneurons also uses GABA and Glycine as neurotransmitters (Cui et al., 2016). Ret^+ ACs are neither DOPaminergic nor Cholinergic, and exhibit small area, dense dendritic arbors (Figures 4, 6b1), reminiscent of the Glycinergic small size AC morphology (Menger, Pow, & Wassle, 1998; Pang, Gao, & Wu, 2012). A population of RET^+ non-Gaba non-Glycinergic ACs with similar small field arbors, has been previously reported (Cherry et al., 2011; Kay, Voinescu, Chu, & Sanes, 2011). The density and dendritic arbor size of Ret^+ ACs argues against them being the recently identified Vesicular Glutamate transporter 3 - positive AC population (Grimes, Seal, Oesch, Edwards, & Diamond, 2011; Johnson et al., 2004).

4.3 | Ret^+ RGC types

We present evidence that about one third of all RGCs are Ret^+ (as judged by NFL staining, Figure 2g, l), distributed amongst at least ten distinct RGC types (Table 2). We note that our cell-type nomenclature is based on combined physiological-anatomic studies in mice if available or from other species. The frequency with which each Ret^+ RGC type was observed in the *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* sample (241 RGCs), was not correlated with the expected frequency of individual types, based on their dendritic arbor area and tiling factor. As an example, assuming tiling and coverage factor of 1, large ON-alpha RGC numbers are expected to represent about 1/5 of small field beta cells, since their dendritic arbors areas are five times as large. However, in our data set, the frequency of ON-alpha RGCs was about twice the one of small beta cells (Table 2). This difference in frequency could be explained by the levels of expression of *Ret* in the different cell types, which in turn translates to larger amounts of CreERT2 protein, and hence higher likelihood of recombination for the same amount of available 4HT. Essentially all identified

morphological cell types have been previously described by us and others for the mouse (Badea & Nathans, 2004, 2011; Coombs et al., 2006; Helmstaedter et al., 2013; Sumbul et al., 2014; Sun et al., 2002; Volgyi et al., 2009), and by many groups in other species (Table 2). There is no obvious common feature of the Ret⁺ RGC subpopulation, other than the conspicuous absence of M1 and M2 ipRGCs (very large, sparse ON or OFF dendritic arbors lamination in close proximity to the GCL or INL) and ON-Direction Selective RGCs. Accordingly, the SCN and AOS (Accessory Optic System) are devoid of any projections. In addition to defining RET⁺ RGCs, the intersection of the *Ret^{CreERT2}* locus with the three *Brn3^{CKOAP}* alleles brings further definition to the expression domain of the three Brn3 TFS amongst RGCs. In the following, we will compare the findings in this study to our previous understanding of the Brn3 RGC subpopulations, and to other recent characterizations of mouse RGC types.

Multistratified RGCs: A somewhat surprising finding were the numerous (9% of the 241 Ret⁺ RGCs) multistratified RGCs, which we previously observed in random populations generated by sparse recombination using ubiquitous loci (*Rosa26^{CreERT}*; Z/AP, fig. 15, Cluster 5a) in (Badea & Nathans, 2004), but never observed using any of the Brn3 reporter mice. In fact, only one example was observed in this study amongst 170 *Ret^{CreERT2}*; *Brn3b^{CKOAP}* RGCs and none in the Brn3a⁺ or Brn3c⁺ populations. These multistratified RGCs could represent the RET⁺Brn3a⁻Brn3b⁻ RGC population observed by immunostaining, and illustrate an example of Brn3 negative RGCs.

ON-alpha cells (Krieger, Qiao, Rousso, Sanes, & Meister, 2017; Pang, Gao, & Wu, 2003; van Wyk, Wässle, & Taylor, 2009) represented a large fraction of both the general *Ret^{CreERT2/WT}*; *ROSA26^{AP/WT}* population (31%), as well as the *Ret^{CreERT2/WT}*; *Brn3b^{CKOAP/WT}* subset (22%). Whereas the *Ret^{CreERT2/WT}*; *ROSA26^{AP/WT}* ON-alpha RGC dendrites stratified on both sides of the ON ChAT band landmark, the Brn3b⁺ subset was mostly confined to the laminae between the GCL and ON-ChAT landmark, suggesting that the recently described ON-transient alpha cell (Krieger et al., 2017) might be RET⁺Brn3b⁻ (compare Figure 6f,g and Figure 11j,k). Interestingly, a bistratified ON-alpha morphology was observed in the Brn3b⁺ subset, characterized by small OFF dendritic tufts emerging from the otherwise typical ON arbor (Figure 10e). Although very rare (3.5% of *Ret^{CreERT2/WT}*; *Brn3b^{CKOAP/WT}* RGCs or 15% of ON-alpha RGCs in this genotype), they were consistent enough to raise the possibility that these OFF arbors are more than a developmental artifact. Interestingly, our previous analysis of *Brn3b^{CKOAP}* RGCs had failed to highlight the ON-alpha morphology, although they may have been included in the larger group of ON-laminated large dendritic arbor RGCs that stratify between the ON ChAT band and the GCL (Badea 2011, figs. 2 and 3) (Badea & Nathans, 2011). As previously reported, ON-alpha morphologies were not observed in the Brn3a or Brn3c subsets.

OFF-alpha cells stratifying in close proximity to the INL (Krieger et al., 2017; Pang et al., 2003; van Wyk et al., 2009) were represented in small numbers in both the *Ret^{CreERT2/WT}*; *ROSA26^{AP/WT}* (1.7%), and *Ret^{CreERT2/WT}*; *Brn3b^{CKOAP/WT}* sets (6.5%), but were absent from either Brn3a⁺ and Brn3c⁺ populations. This is somewhat surprising, since this population was found in the previously reported general Brn3a⁺ positive population (Badea 2011, fig. 2c cell “b”) (Badea & Nathans, 2011). The alpha denomination is somewhat

tentative, as they appear to be smaller in area than their ON homologues, and have somewhat denser dendritic arbor morphologies.

OFF-DS morphologies (Kim, Zhang, Yamagata, Meister, & Sanes, 2008) represented a small fraction of both *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* (0.4%), and *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* sets (4.1%), but were not observed amongst either Brn3a or Brn3c RGCs. This is again in contradiction with our previous reporting of Brn3a⁺ OFF-DS RGCs (Badea & Nathans, 2011).

Betta morphologies are defined in this study as small dense dendritic arbor RGCs with lamination in multiple IPL sublaminae. Such morphologies have been reported in the transgenically labeled W3 (Y. Zhang, Kim, Sanes, & Meister, 2012) population, and are quite similar to some of the cells recently reported to be FoxP2 positive (Rousso et al., 2016). Collectively the betta description is incomplete and most likely includes multiple cell types. As can be appreciated from examples in Figures 6c1, 9e1, and 10b1, the density and multi-lamination of the dendritic arbor makes it difficult to reliably define the lamination boundaries. Moreover, in many instances, small individual dendrites descend from the main arbor towards the INL (illustrated in Figure 10b2, and the side projection in 10b3), thus making the call of OD - Outer Distance somewhat arbitrary. Nevertheless, the preference for ON laminated betta cells in Brn3b⁺ RGCs, and the inclusion of both ON and OFF laminating betta morphologies seen in the Brn3a⁺ subset is consistent with our previous report of Brn3 RGCs (Badea & Nathans, 2011).

ON-OFF DS morphologies (Huberman et al., 2009; Kay et al., 2011; Rivlin-Etzion et al., 2011; Trenholm, Johnson, Li, Smith, & Awatramani, 2011; Weng, Sun, & He, 2005) represented an important fraction of both *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* (34%), and *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* sets (48%), most likely reflecting high expression levels of Ret in these RGCs. As previously reported, ON-OFF-DS RGCs are neither Brn3b nor Brn3c positive.

A second bistratified RGC population, reminiscent of morphologies reported as small bistratified or color opponent in the monkey (Crook et al., 2009; Dacey & Lee, 1994), uniformity detectors, or suppressed by contrast cells (Mani & Schwartz, 2017; Tien, Pearson, Heller, Demas, & Kerschensteiner, 2015) was present amongst *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* (8.7%), and *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* sets (3.4%). It is characterized by a smaller, sparser OFF dendritic arbor that laminates close to the INL. We had previously identified this RGC morphology in the general sparse recombination set (*Rosa26^{CreERT}; Z/AP*, Badea & Nathans 2004, fig. 15, Cluster 5b, c) (Badea & Nathans, 2004), but it is unclear how close it is to the Brn3a⁺Brn3b⁺ morphology (fig. 2C-e in Badea 2011) or distinct from the Brn3a⁻Brn3b⁻Brn3c⁺ bistratified cell (Badea 2011, fig. 2C-m). Although the stratification levels of both ON and OFF arbors are consistent with the aforementioned “e” cell, the density and size of the OFF arbor is highly variable, based on the number of dendrites leaving the ON arbor and their degree of branching (see Figures 7a2–a3, 9a2–a3). In addition, both cells “e” and “m” in the 2011 study had a large degree of recursive dendrites, defined as stratifying in the ON arbor, but being derived from branches in the OFF arbor. By comparison, the reconstructions in the current study (Figures 7a–a3,

9a–a3) show only a limited amount of recursiveness, and the OFF dendrites are many times limited to simple branches, that descend vertically towards the INL without spreading widely in the OFF lamina. It should be noted that, in guinea pigs, which, like mice, exhibit cone photopigment gradients across the retina this type of morphology was shown not to possess strong color opponency (Yin, Smith, Sterling, & Brainard, 2009).

A further large bistratified RGC population was represented by only six occurrences in the current study, one in the general Ret^+ population, and five in $Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}$ RGCs, consistent with our previous findings (Badea 2011, fig. 2Ch) (Badea & Nathans, 2011). The closest morphology we are aware of is the M3, Melanopsin positive ipRGC, (Ecker et al., 2010; Schmidt & Kofuji, 2011). Interestingly, the M1 and M2 ipRGC morphologies were not observed in either the general Ret^+ dataset (241 RGCs) or in the intersections with any of the $Brn3^+$ populations (some 300 further RGCs), nor were they observed in the further many retinas derived from these genotypes, that we stained and imaged but do not report here. This fact is significant, as a set of retinorecipient targets classically associated with ipRGCs can be observed in the coronal sections of $Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}$ RGCs, namely the dorsolateral aspect of the SCN, the OPN, and the IGL (Chen, Badea, & Hattar, 2011; Hattar et al., 2006; Hattar, Liao, Takao, Berson, & Yau, 2002). Therefore, the large bistratified, putative M3 cells would be candidates for innervating the three targets. However, it should be noted that Melanopsin expression is thought to also occur in ON alpha RGCs (also known as M4), at least in crosses of $Opm4Cre \times Z/AP$ (or Z/EG) mice (Ecker et al., 2010; Estevez et al., 2012), and ON-alpha cells are one of the major populations labeled in both the general Ret^+ and Ret^+Brn3b^+ populations.

The only RGC type labeled in $Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}$ retinas is the ON-spiny morphology, an RGC type which was previously reported and now confirmed to be $Brn3a^+Brn3c^+$. Similar morphologies were previously reported in other species (Berson, Pu, & Famiglietti, 1998; Puller, Manookin, Neitz, Rieke, & Neitz, 2015; van Wyk, Taylor, & Vaney, 2006). RGCs with similar dendritic arbor size and density were recently described as Foxp2 positive cells (Rousso et al., 2016) and/or sensitive to various aspects of object motion (Jacoby & Schwartz, 2017), however the lamination reported in these studies is somewhat more scleral than ours, perhaps in the lamina comprised between the ON-OFF IPL limit, and the OFF ChAT band. Since this ON-Spiny RGC is uniquely labeled in the $Ret^{CreERT2}; Brn3c^{CKOAP}$ cross, we can give an unambiguous characterization of its brain projection. We note that its LGN lamination is reminiscent to the one seen for the ON-OFF-DS population (outer shell of the dLGN and vLGN), especially in the more anterior aspects of the LGN, while the SC axons have a broader pattern compared to the projections of the ON-OFF-DS and betta RGCs (compare Figure 12a3–c3). Somewhat unexpected is the strong but very diffuse staining in the Pretectal Area (PTA; Figure 12f2). The physiological characteristics assigned to RGCs with similar dendritic morphologies in mice and other species range from Local Edge Detector to visual pursuit error signaling, but seem to generally be related to motion detection (Berson et al., 1998; Jacoby & Schwartz, 2017; Puller et al., 2015; Rousso et al., 2016; van Wyk et al., 2006). The axonal projection into SC, PTA and the outer shell of the LGN would therefore be appropriate, as there is evidence that this subdivision of the LGN is involved in motion processing. Our genetic intersection tools

should give us access to the more complete characterization of this RGC type, and the study of its circuit function by ablation studies.

Finally we note that, whereas Brn3a⁺ and Brn3c⁺ RGCs send their axons largely to the contralateral side, a subpopulation of Brn3b⁺ RGCs is innervating the dorsolateral SCN, the OPN, the IGL and characteristic patches of the LGN on the ipsilateral side. This could suggest that, aside from the previously described functions in RGC survival and dendrite and axon formation, Brn3s could also be involved in distinguishing ipsi- versus contralateral projecting RGCs, as previously shown for other TFs such as Zic2, Isl2, and SoxC (Herrera et al., 2003; Kuwajima, Soares, Sitko, Lefebvre, & Mason, 2017; Pak, Hindges, Lim, Pfaff, & O'leary, 2004).

Thus, expression of Ret partially overlaps with that of all three Pou4f/Brn3 TFs in RGCs. What common feature could Ret expression convey to the diversity of characterized RGC cell types? No obvious common property of the dendritic arbors is obvious, as large and small, mono- and bi-stratified, ON and OFF dendritic arbors can be observed. However two classes of RGCs are missing from this survey: (a) M1/M2 ipRGCs, projecting to the SCN and required for circadian photoentrainment, and (b) ON-direction selective RGCs, projecting into the Accessory Optic System, and required for image stabilization during body, head, and eye motion. It does thus appear that most Ret⁺ RGCs project to either the LGN or the SC, and exclude non-image-forming projection areas. This pattern is broken by projections of (some?) Ret⁺Brn3b⁺ RGCs into the OPN and PTA, and the significant branches provided by the Ret⁺Brn3c⁺ RGCs to the PTA. It is possible that these RGCs target primarily the LGN/SC and provide additional side branches to the mentioned PA neurons, as is clearly the case for the ON-Spiny Brn3c neuron. It is therefore conceivable that RET provides chemotactic/axon guidance support during development followed by survival/neurotrophic support in the adult for RGCs using the main Optic Tract and targeting the LGN/SC, as opposed to the AOT and SCN alternative pathways.

4.4 | RET and other neurotrophic signaling involvement in retinal cell type specification

Combinatorial Pou4f/Brn3 transcription factor expression is characteristic for the projection sensory neurons of visual, auditory, vestibular, and somatosensory systems, while neurons in the olfactory and taste pathways do not seem to express or require these TFs (Badea & Nathans, 2011; Badea et al., 2012; Dykes, Lanier, Eng, & Turner, 2010; Erkman et al., 1996; Sajgo et al., 2017; Zou, Li, Klein, & Xiang, 2012). Work done mostly in the somatosensory system suggests an important role for neurotrophic support in fate determination, survival, and neurite development of neuronal cell types (Airaksinen & Saarma, 2002; Ernsberger, 2008; Marmigere & Ernfors, 2007). Loss of function studies in mice suggest that Pou4f/Brn3 and other TFs can function both up and downstream of the neurotrophic signaling (de Melo et al., 2008; Huang et al., 2001; Huang et al., 1999; Ma, Lei, Eng, Turner, & Parada, 2003; Wiggins et al., 2004). Brn3a plays an important role in the development of projection somatosensory neurons of the head, located in the Trigeminal Ganglion (TG). In *Brn3a*^{KO/KO} mice, expression of neurotrophin receptors (TrkA, TrkB, and TrkC) is heavily impaired. TrkC expression is drastically reduced from early stages of development, while TrkA and TrkB are initially expressed but eventually get turned off, concurrent to a massive

loss of TG neurons. Only about 30% of TG somatosensory neurons survive, most of whom express Ret. Loss of Brn3a from DRG somatosensory neurons has more subtle effects consisting of quantitative changes in the representation of the various sensory modalities (Badea et al., 2012; Ichikawa, Mo, Xiang, & Sugimoto, 2002, 2004; Ichikawa, Mo, Xiang, & Sugimoto, 2005; Ichikawa et al., 2002; Ichikawa, Yamaai, Mo, Xiang, & Sugimoto, 2002; Zou et al. 2012). It is thought that Brn3a is directly regulating TrkA expression (Huang et al., 1999; Wiggins et al., 2004).

In the retina, the Dlx1 TF, co-operates with Brn3b in the establishment of RGC and AC cell fates and regulates TrkB expression, and therefore could modulate the role that TrkB, and its ligand, BDNF, are playing in RGC dendrite specification and hence light response properties (de Melo et al., 2008; Liu et al., 2007; Zhang et al., 2017). In the somatosensory system, RET and some of its GFR α co-receptors are known to be required for the specification of nonpeptidergic nociceptors and some mechanoreceptor subtypes (Golden et al., 2010; Luo et al., 2009; Luo et al., 2007). A thorough study of retinal Ret and GFR α expression revealed localization of Ret in HCs, ACs and RGCs and demonstrated a functional requirement for Ret in the synapses formed at the level of the OPL between photoreceptors, bipolar cells and HCs (Brantley et al., 2008). We now find that Ret has a very specific pattern of expression amongst subtypes of both ACs and RGCs in the adult retina. Loss of Brn3a or Brn3b from Brn3a^{AP} or Brn3b^{AP} RGCs does not significantly affect Ret expression in the surviving cells, however it remains possible that Ret depends on Brn3s in RGCs normally eliminated in Brn3 KO mice. RET is turned on sequentially in RGCs, HCs, and ACs, in the same temporal order in which these cell classes are thought to become postmitotic, but with some delay relative to their respective birth dates. Therefore Ret could play a role in the development of neuronal arbors and potentially synaptic input. Our findings argue for a thorough analysis of RGC type distribution, and Brn3 expression in *Ret*^{KO/KO} retinas, and for an assignment of GFR α 1–4 co-receptors to the specific RGC types expressing them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Ret is dynamically expressed in the retina. (a) RNASeq data from RGCs and retina. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) levels for *Ret* gene expression in RGCs and retina of distinct genotypes at E15 and P3. Brn3a^{AP} or Brn3b^{AP} RGCs of indicated genotypes were purified by immunomagnetic isolation, and compared to retinal supernatants (Sajgo et al., 2017). For both retina (top, individual samples) and RGCs (bottom, medians of two samples) ages and genotypes are indicated. RGC samples represent means of two biological replicates, and retinal samples are individual biological replicates. Stars indicate the following comparisons: Brn3b^{AP} E15 RGCs vs. retina supernatant (***, fold change = 2.67; *p* value = .00052), Brn3b^{AP} P3 RGCs vs. retina supernatant (*, fold change = 2.14; *p* value = .00855) and Brn3a^{AP} P3 RGCs vs. retina supernatant (***, fold change = 3.56; *p* value = 3.65E-07). Significance values are derived using the DESeq algorithm. For a complete description of data collection and analysis, see Sajgo et al. (2017). (b–d) Sections through E14.5 (b), P3 (c) and adult (d) *Ret*^{CFP/WT} retinas, showing anti-CFP immunofluorescence (red) and DAPI nuclear stain. Potential RGCs are indicated with arrows, Horizontal Cells with arrowheads, and Amacrine cells with stars. ONL = outer nuclear layer; OPL = outer plexiform layer; INL = Inner nuclear layer; IPL = inner plexiform layer; GCL = Ganglion Cell layer. Scale bars: (b) = 100 μ m, and (c, d) = 25 μ m [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 2.

Ret is expressed in horizontal cells, amacrine cells, and subpopulations of RGCs. (a–h) Immunostaining of vertical sections through retinas from *Ret^{CFP/WT}* mice, using anti-GFP antibodies (red) in conjunction with antibodies against retinal cell type markers (green or white), and DAPI nuclear stain. (a) Prox1 (green) is a marker for HC, AC, and subpopulations of RGCs. b, Lim1 (green) is a marker for HCs, and Brn3b (white) is a marker for RGCs. (c, e) GABA and Glycine (green) label subpopulations of ACs respectively. (d) ChAT (green) and TH (white) are markers for Cholinergic or Dopaminergic

Amacrine cells respectively. (f) Brn3b (green) and Isl1 (white) label distinct, partially overlapping subpopulations of RGCs. Isl1 also labels bipolar cells and ChAT positive ACs. (g) NFL (green) is a general RGC marker. (h) Brn3b (green) and Brn3a (white) label distinct, partially overlapping subpopulations of RGCs. Arrowheads indicate double and triple labeled cells respectively. (i–k) CFP⁺ cells in the inner INL are negative for Isl1 and only partially overlap with GABA and Glycine. l, All CFP⁺ cells in the GCL are NFL positive. m, Half of GCL CFP⁺ cells are also Brn3a⁺/Brn3b⁺, while CFP⁺/Brn3a⁺, CFP⁺/Brn3b⁺, and single labeled CFP⁺ cells are also visible. Pie charts represent quantitation of the immunostainings and total number of cells are presented next to the pie chart markers. For each combination of markers, 3–5 sections from 3 distinct animals were stained and 4–5 images were counted. Scale bar (h) = 25 μ m [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3.

Dynamic expression of the *Ret* gene during developmental stages in RGCs, HCs, and ACs. Retinal sections from *Ret*^{CFP/WT} E17 embryos (a, b, g, h), P1 (c, d, i, j) and P3 pups (e, f, k, l) were stained for Calbindin (green, a, c, e), Prox1 (green, g, i, k), Lim1 and Brn3b (green and white, b, d, f) and Brn3b and Isl 1 (green and white, h, j, l). For marker specificity see Figure 2. Note that *Ret* is present in RGCs from E17 (b, d, f, h, j, l), in HCs (a–k) beginning with E17, and in ACs at P1 and P3 (c, e, i, k). For each marker combination, 3–5 sections

from three distinct animals were stained. Scale bar (l) = 25 μ m [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 4.

AP histochemistry reveals drug-inducible recombination in retinas from *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* mice. (a–d) Retinal flat mounts processed for AP histochemistry reveal various degrees of recombination in response to distinct dosages, routes and ages of tamoxifen administration, and increasing copy number of the *Rosa26^{AP/WT}* allele. a, *Ret^{CreERT2/WT}; Rosa26^{AP/WT}* mouse, gavaged with 0.1 mg Tamoxifen at P28, stained at P37. (b, c) Retinas from *Ret^{CreERT2/WT}; Rosa26^{AP/WT}* (b) and *Ret^{CreERT2/WT}; Rosa26^{AP/AP}* (c) mice, injected i.p. at P14 with 0.1 mg Tamoxifen, and stained at P28. (d) *Ret^{CreERT2/WT}*;

Rosa26^{AP/AP} mouse, injected i.p. at P14 with 0.25 mg Tamoxifen, and stained at P21. AP positive neurons are HCs (black arrowhead), RGCs (black arrow) or ACs (red arrow). Scale bar (d) = 500 μ m [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 5.

Morphology of RET⁺ Horizontal Cells (HC). (a, b, d) Examples of AP labeled Ret⁺ HCs. (a) In this HC, the axon arbor (black arrowhead) is placed at a distance from the dendritic arbor (black arrow, centered on the cell body, red arrow), connected by a relatively straight axonal process (star). (b) HC example in which the dendritic and axon arbors are partially overlapping, and connected by a looping axonal process. (d) Four examples of HCs, showing proximity between dendritic and axonal arbors, and distinct levels of axon process looping. (c) Tracing and parameters measured for the HC in (a). Areas and center of mass were determined for dendritic (red) and axon (green) arbors. The distance between arbors was computed as the distance (di) between the centers of mass. The length of axonal process (al) was measured by tracing it. The segments of the axon process included in the dendrite or axon arbors were estimated by the radii of the two arbors (rD and rA, respectively). The total length of the axon process (Al) was then calculated as $Al = al + rD + rA$. (e, f) HC Axon arbor areas (y axis, median = 3,360 μm^2) are larger than corresponding dendritic (x axis, median = 1,940 μm^2) arbors (highly significant by Kolmogorov-Smirnov 2 test, $p = 2.81 \times 10^{-24}$). (g, h) Axon lengths (Al, median = 279.27 μm) are larger than the arbor centers distance (di, median = 176.8 μm ; highly significant, Kolmogorov-Smirnov 2 test, $p = 2.12 \times 10^{-25}$). Measurements are derived from 139 HCs. Scale bars for (a), (b) and (d) = 50 μm [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 6.

Morphological characterization of *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* monostratified RGCs. (a–c2) The most frequently encountered RGC morphologies in *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* retinas were ON alpha (a–a2), multistratified (b–b2) and OFF beta (c–c2). For each cell, focal planes at the level of the cell body and axon (a, b, c) and dendritic arbor (a1, b1, c1) are shown in flat mount (en face) perspective. Scale bars (c1) = 25 μm . Arrows in (b1) point to the dendritic arbor of two small field amacrine cells. (a2, b2, c2) represent 3D reconstructions with flat mount (en face) perspective (top) and vertical projection (bottom). The horizontal bars on the sides of each vertical projection represent the boundaries of the IPL, and are 25 μm long. Axons are green, vitreal (ON) dendrites are blue, and scleral (OFF) dendrites are red. (d, e) RGC dendritic arbor parameters. Inner distance (ID) and outer distance (OD), normalized to the IPL thickness, define the boundaries of dendritic arbor stratification, and are set to 0 at the GCL and 1 at the INL. (e) Arbor area of the bounding polygon is determined from the flat mount perspective. (f–h) Scatter grams of morphological parameters for all monostratified RGCs as defined in (d, e). (f) Normalized ID (ID/IPL, x) versus area (y). (g) Normalized ID (ID/IPL, x) versus OD (OD/IPL, y). (h) Dendritic arbor

thickness ((OD – ID)/IPL, x axis) versus Area (y axis). The two vertical bars in (f) and (g) represent the theoretical lamination levels of ON and OFF starburst ACs, and the gray box represents the OFF sublaminae of the IPL, as derived from the absence of mGluR6^{GFP} positive axons (Morgan et al., 2006). The putative morphological types are assigned based on previous work and correspondence to literature, color coded in the scatter plots, and indicated at the bottom [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 7.

Two distinct types of bistratified *Ret^{CreERT2/WT}; ROSA26^{AP/WT}* RGCs. Bistratified RGCs belonged either to Small Bistratified (SB, a–a3), or ON-OFF direction selective (ON-OFF DS, b–b3) types. Individual RGCs were imaged, measured, and traced as described in Figure 6. For each cell, focal planes through the axon and cell body (a, b) and the ON (a1, b1) and OFF (a2, b2) dendritic arbors are shown. Reconstructions, using the same conventions as in Figure 6, are shown in (a3, b3). Scale bar in (b2) is 50 μm , and horizontal bars delineating the IPL in (a3) and (b3) are 25 μm . Axons are green, vitreal (ON) dendrites are blue, and scleral (OFF) dendrites are red. Recursive dendrites, laminating in the ON arbor, but branching from the OFF arbor, are labeled in gray in a3 (arrowheads in a1 and a3). Note that the reconstruction in a3 is somewhat rotated counterclockwise compared to the original image. (c, d) Scatter plots for normalized ID (x) versus OD (y) measurements for either the inner (ON, circles) or outer (OFF, squares) dendritic arbors for SBs (c) and ON-OFF-DS (d) cells. (e) Area measurements for ON vs. OFF dendritic arbors. For parameter definition, see Figure 6 [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 8.

Sparse recombination reveals distinct RGC types in *Ret^{CreERt2/WT}; Brn3^{CKOAP/WT}* retinas. (a–c) Flat mount AP histochemistry of retinas from *Ret^{CreERt2/WT}; Brn3a^{CKOAP/WT}* (a), *Ret^{CreERt2/WT}; Brn3b^{CKOAP/WT}* (b), and *Ret^{CreERt2/WT}; Brn3c^{CKOAP/WT}* (c) mice in which sparse recombination was induced by adult IP injections of 4HT. Injection conditions were: 50 µg 4HT at P22 (a), 500 µg 4HT at P45 (b), 1500 µg 4HT at P52 (c). Histochemical analysis was performed at least 2 weeks after induction. Scale bar in (c) = 500 µm [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 9.

Dendrite morphologies of RGCs stained in *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* and *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* retinas. RGC dendritic arbor morphologies identified in *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* (a–e2) and *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* (f–f2) retinas are illustrated by DIC (nomarsky) images and 3D reconstructions. (a–a2) and (b–b2) illustrate focal planes through cell body and axon (a, b), ON dendritic arbor (a1, b1) and OFF dendritic arbor (a2, b2) for two bistratified RGC types, Small Bistratified (a–a3) and ON-OFF DS (b–b3). (a3) and (b3) show “en face” and vertical (z plane) projections of the 3D traces of these cells. Blue dendrites represent the ON arbor and red dendrites the OFF arbor. Axon is in green. Yellow dendrites in (a3) represent recursive dendrites, derived from the OFF plane but laminating to the ON plane (arrowheads in a1 and a3). For monostратified RGC types, focal planes through the axon/cell body (c–f) and dendritic arbor (c1, d1, e1 and f1) are shown, while projections of the 3D reconstructions are shown in (c2) through (f2). (d–d2) and (f–f2) ON-spiny RGCs from *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* or *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* retinas, respectively. (e–e2) OFF-beta RGC. Scale bar for all DIC images is in (f1) = 50 μm. For traces, limits of the IPL are indicated by the black horizontal lines (50 μm in length) in the bottom (z-plane) projections. Note that IPL thickness varies for the different cells. Traces and DIC images are scaled identically

FIGURE 10.

Dendrite morphologies of RGCs stained in *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* retinas. Six distinct dendritic arbor morphologies were revealed in *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* retinas: OFF-DS (a–a2), ON-beta, (b–b3), ON alpha (c–c2), OFF-alpha (d–d2), a bistratified version of the ON-alpha (e–e3), and a Large Bistratified (f–f3). Focal planes for DIC, projections of 3D traces, scaling and marker conventions are as in Figure 9. Note that the small field RGC in (b–b3) appears to have a nearly bistratified aspect. Red arrowheads in (e2) point to the small OFF dendritic tufts. Scale bar for all DIC images is in (f2) = 50 μ m.

For traces, IPL boundaries are indicated in black in the vertical projections (bottom), and horizontal bars are 50 μm [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 11.

Morphological parameters of RGCs stained in *Ret^{CreERT2/WT}; Brn3^{CKOAP}* retinas.

Morphological parameters for the RGC types described in Figures 8–10. Measurements, quantified parameters and scatter plot conventions are as described in Figures 6 and 7. (a) Proportions of monostratified (red) to bistratified (blue) *Ret^{CreERT2/WT}; Brn3^{CKOAP}* RGCs. Numbers represent the total number of quantified RGCs for each genotype and category. No bistratified RGCs were observed in *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* retinas. (b–e) Stratification levels and dendritic arbor areas for bistratified RGCs. In (b, c), *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* RGCs, blue markers refer to ON-OFF DS RGCs, while red markers refer to SB RGCs. In (d, e), *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* RGCs, blue markers refer to ON-alpha – bistratified RGCs, while red markers refer to LB RGCs. In (b, d), circles mark the inner dendritic arbor, while squares mark the outer dendritic arbor. (f–q) Morphometric parameters for *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* (f–i), *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* (j–m) and *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* (n–q) monostratified RGCs. (f, j, n) represent the inner

(x axis, ID/IPL) and outer (y axis, OD/IPL) lamination boundaries. (g, k, o) plot the inner (x axis, ID/IPL) lamination boundary versus “en face” dendritic arbor area (y axis). (h, l, p) Dendritic arbor thickness across IPL sublaminae (x axis, (OD-ID)/IPL) vs. area (axis). (i, m, q) Correlation of dendritic arbor area (y axis) with distance from the optic nerve (x axis). For each genotype (column), the color legends in (h, l, and p) indicate the corresponding RGC types. At least six retinas (three mice) were stained and imaged for each genotype, however single cell measurements were performed on three retinas (three mice) for Brn3a and Brn3b and the two retinas of one mouse for Brn3c [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 12.

Ret^{CreERT2/WT}; Brn3^{CKOAP} RGCs target partially overlapping retinorecipient areas. Coronal sections through brains from *Ret^{CreERT2/WT}; Brn3a^{CKOAP/WT}* (a–a4, d–d4), *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* (b–b4, e–e4), and *Ret^{CreERT2/WT}; Brn3c^{CKOAP/WT}* (c–c4, f–f4) mice.

Sections are oriented with dorsal at the top, ventral at the bottom. For (a–c4) sparse recombination was induced by adult I.P. injections of 4HT. For (d–f4), full recombination was induced by injecting 1 μ l (4 μ g) of 4HT intraocularly in one eye. Sections (d1–f1), (d2–f2), and (d4–f4) show retinorecipient areas from hemispheres contralateral to the injected

eye. Positions of the retinorecipient nuclei or tracts are indicated in (b–b4): Optic Tract (OT), Suprachiasmatic Nucleus (SCN), Olivary Pretectal Nucleus (OPN), dorsal and ventral Lateral Geniculate Nucleus (dLGN and vLGN), Intergeniculate Leaflet (IGL), Pretectal Area (PTA), Superior Colliculus (SC), Medial Terminal Nucleus (MTN), and Accessory Optic Tract (AOT). Red arrowheads in (a3) indicate the lateral and medial line of separation between the two axonal projection sublaminae. Note that the SCN, MTN, and AOT are not stained in any of the three genetic backgrounds, however *Ret^{CreERT2/WT}; Brn3b^{CKOAP/WT}* RGCs send a projection into the dorsolateral region of the SCN. Insets in (e1) represent the OPN (top) and LGN (bottom) of the hemisphere ipsilateral to the injected eye, reflected laterally to match the orientations of the equivalent contralateral regions. Scale bar for all images: (f4) = 1,000 μm ; scale bar for insets in (e1) = 500 μm [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Primary antibodies and dilutions used for immunohistochemistry. See also Immunostaining section in material and methods

Antigen	Immunogen	Manufacturer, Species, type	Catalogue number	Dilution
Bm3a	Human Bm3a aa186-224 - protein 10 fusion (pGEMEX)	Millipore, Mouse monoclonal, MAB1585 (RRID:AB_94166), clone 5A3.2		1:20
Bm3b	Human Bm3b aa184-252 - GST fusion	Rabbit polyclonal, made in house		1:200
Isl1	Recombinant human Isllet-1	Neuromics, Goat polyclonal, GT15051 (RRID:AB_2126323)		1:500
GFP	Recombinant GFP	Abcam, Chicken polyclonal, ab13970 (RRID:AB_300798)		1:500
Ret	synthetic peptide from human c-Ret (STWIENKLYGRISHAFTRF)	IBL, Rabbit polyclonal, R787		1:50
Cre	aminoacids 77 to 343 of recombinant Cre	Millipore, MAB 3120		1:40
Lim1	Rat Lim2	Developmental Studies Hybridoma Bank, clone 4F2, recognizes Lim1 + Lim2, donated by Thomas Jessell		1:10
Prox1		Abcam, Rabbit Polyclonal, AB11941		1:1,000
Chat	Human placental choline acetyltransferase	Chemicon, Goat polyclonal, AB 144P (RRID:AB_11214092)		1:100
Alkaline Phosphatase	Human placental alkaline phosphatase	VIB Gent, Mouse monoclonal, E6 clone		1:100
Alkaline Phosphatase	Human placental alkaline phosphatase	American research products, Sheep Polyclonal, 13-2355		1:200
Tyrosine Hydroxylase	Rat TH	Chemicon - Millipore, Rabbit polyclonal, AB152		1:200
Calbindin	recombinant rat CB	Swant, Rabbit Polyclonal, CB-38a		1:2,000
Glycine	Aminoacid Glycine	Chemicon, Rabbit Polyclonal, AB5020		1:200
GABA	Aminoacid Gamma-aminobutyric acid	Sigma, Rabbit Polyclonal, A2052		1:200
NFL	Neurofilament light chain	Chemicon, Rabbit Polyclonal, AB9568		1:500

TABLE 2

Summary of RET⁺ RGC types observed in this study

RGC Type	ON-OFF DS	SB	LB	ONs alpha	ONt alpha	OFFs	On Spiny	Multi	ON beta	OFF beta	OFF DS
Badea 2004	none	cluster 5b,c	cluster 2	cluster 9a	none	cluster 7c	cluster 2b	cluster 5a?	cluster 4	none	cluster 6
Coombs 2006	13	12	None	9ON/10	7ON	9OFF	1	none	11	none	5
Vogly 2009	G17	none	G12	G1	G2/G10	none	G8/G13	none	G5	G18?	G15
Badea 2011	1	none	H	none	none	b	n	none	d	j	c
Genetic label	yes	no	Yes	yes	yes	yes	no	no	ref	none	yes
Physio	yes	ref	Yes	yes	yes	yes	unclear	no	ref	none	yes
Rosa26AP	33.6	8.7	0.4	30.7 (not separated)		1.66	0	9.1	2.5	12.9	0.4
Bm3a	49.1	3.4	0	0	0	0	10.3	0	37 (not sperated)		0
Bm3b	0	0	3	23.8	0	6.7	0	0.6	61.6	0	4.3
Bm3c	0	0	0	0	0	0	100	0	0	0	0

First row indicates the RGC type name used in this study. Rows 2–5 indicate closest equivalent from previous surveys of RGC morphology, with closest equivalent. For Badea et al. (2004), cluster numbers refer to clusters and examples shown in figs. 12–18. For Coombs (2006), numbers indicate the cluster numbers as presented in figs. 2–3, and 6. “G” numbers for Volgyi et al. (2009) refer to clusters presented in figs. 13 and 14. Finally, Badea 2011 small letters refer to fig. 2c, showing the various Bm3 positive cells observed by random sparse recombination. Genetic labeling indicates RGC types for which genetic drivers with more or less specificity are available. Physiology field indicates whether we are aware of previous recordings done from these cells. References are indicated in the text. Last four rows indicate the ratios (percent of total number of RGCs imaged) of cell type occurrences in either the Rosa26 general reporter background, or in the Bm3a^{Cre}CKOAP⁺, Bm3b^{Cre}CKOAP⁺ or Bm3c^{Cre}CKOAP⁺ crosses.