Ancient origin of the rod bipolar cell pathway in the vertebrate retina

Vertebrates rely on rod photoreceptors for vision in low-light conditions. The specialized downstream circuit for rod signalling, called the primary rod pathway, is well characterized in mammals, but circuitry for rod signalling in non-mammals is largely unknown. Here we demonstrate that the mammalian primary rod pathway is conserved in zebrafish, which diverged from extant mammals ~400 million years ago. Using single-cell RNA sequencing, we identified two bipolar cell types in zebrafish that are related to mammalian rod bipolar cell (RBCs), the only bipolar type that directly carries rod signals from the outer to the inner retina in the primary rod pathway. By combining electrophysiology, histology and ultrastructural reconstruction of the zebrafish RBCs, we found that, similar to mammalian RBCs, both zebrafish RBC types connect with all rods in their dendritic territory and provide output largely onto amacrine cells. The wiring pattern of the amacrine cells postsynaptic to one RBC type is strikingly similar to that of mammalian RBCs and their amacrine partners, suggesting that the cell types and circuit design of the primary rod pathway emerged before the divergence of teleost fish and mammals. The second RBC type, which forms separate pathways, was either lost in mammals or emerged in fish.

Rod photoreceptors of the vertebrate retina are capable of detecting very dim light, down to individual photons~. The mammalian cell types and circuitry that convey rod-driven signals, called the primary rod pathway, were identified and defined in the cat retina about 50 years ago. Since its initial characterization, this rod pathway has been examined extensively in many mammalian species including humans. Across species, it consistently uses homologous cell types and connectivity patterns: rod bipolar cells (RBCs), as well as A2 and A17 amacrine cells (ACs)~. The RBC is a molecularly, structurally and functionally distinct retinal bipolar cell (BC) type that receives input from all rods within its dendritic field and is predominantly driven by rods~. In mammals, all other BC types receive most of their photoreceptor input from cones, which operate in daylight conditions. In contrast, the circuitry for rod signalling is less clear in non-mammals. Although some BCs...
in non-mammals are predominantly driven by rods and exhibit light sensitivity close to that of mammalian RBCs, but the ratios of rod to cone inputs are graded among BCs and less distinct than those in mammals. In addition, the downstream circuitry of the rod-dominant BCs in non-mammals is unclear. It is possible that rod and cone signals are solely merged at the level of the BC dendrites and processed by the conventional cone pathway in non-mammals. The lack of evidence of the cell types of the mammalian primary rod pathway (for example, RBC, A2 and A17 AC) in non-mammals has even led to the speculation that the BC pathway is unique to mammals and evolved separately in mammals. However, since the molecular, structural and functional signatures that together define BC type diversity in mammals are largely unknown in non-mammals, it remains unclear whether the signatures characteristic of the mammalian RBC and its downstream pathway are absent or present in non-mammals. To address this issue, we focused on zebrafish, a species in which neuronal populations can be labelled transgenically, to analyse single-cell transcriptomics, histology, physiology and circuit reconstructions of retinal BCs.

There are more than a dozen BC types in mammals that are diverse in morphology, connectivity and molecular profiles but can be classified into two main groups: ON BCs that depolarize and OFF BCs that hyperpolarize in response to increases in luminance. RBCs are a type of ON BC that is distinct in many ways from all other BCs, which mainly connect with cones and are called cone BCs (CBCs) here. Transcriptionally, mammalian RBCs can be distinguished from CBCs by the expression of protein kinase C-alpha (PKCa). Morphologically, the axon terminals of RBCs are generally larger than those of CBCs and end in the innermost layer of the inner plexiform layer (IPL). The synaptic arrangement of the RBC axons differs from the common synaptic arrangement of most CBCs. Whereas CBC axons directly synapse onto the retinal output neurons, retinal ganglion cells (RGCs), RBCs predominantly form a ‘dyadic’ synapse with two types of inhibitory amacrine cell: small-field A2 (or A-B) and large-field A17 ACs. The A17 AC almost exclusively makes reciprocal feedback synapses onto RBC axon terminals. By contrast, A2 ACs receive numerous synapses from RBCs (~40 synapses per RBC in mice), but do not provide feedback onto the RBCs. These RBC to A2 AC synapses are the critical sites for the amplification and gain control of rod signals. Rod signals are eventually relayed to RGCs by connections from A2 ACs on CBCs, which split rod signals into ON and OFF channels via sign-conserving gap junctions with ON CBCs and sign-inverting inhibitory synapses with OFF CBCs.

Here, by analysing single-cell transcriptomic profiles of zebrafish BCs, we discovered two BC types, RBC1 and RBC2, with molecular signatures similar to those of mammalian RBCs. Using transgenic zebrafish lines that express a fluorescent protein in RBC1 or RBC2 cells, we identified the inputs and outputs of RBC1 and RBC2. We found that both zebrafish RBC types connect with all rods and red cones (or long-wavelength-sensitive cones) within their dendritic fields. We further reconstructed the downstream circuits of both BC types using serial block-face electron microscopy and found that RBC1 predominantly synapses onto three morphological types of AC. The circuit diagrams and synaptic arrangements of two of the ACs closely resemble those of the mammalian A2 and A17 ACs. By contrast, RBC2 mainly connects to a different set of ACs, which does not include A2-like ACs. We conclude that (1) the RBC1 pathway is homologous to the mammalian primary rod pathway and the predecessor to both emerged >400 million years ago, before the divergence of teleosts and mammals in the Devonian period, and (2) the second pathway, through RBC2, was either lost in mammals or emerged in fish.

**Results**

**Transcriptomics identifies two RBC types in zebrafish**

We first determined the transcriptional similarity between each zebrafish BC type and the mammalian RBCs by using single-cell RNA sequencing (scRNA-seq) in adult zebrafish. BCs were isolated using a fluorescent marker in the Tg(oxs1:GFP) transgenic line, in which all BCs express green fluorescent protein (GFP). Clustering analysis of 19,492 high-quality single-cell transcriptomes identified 23 molecularly distinct BC clusters (Fig. 1b,c). To identify the clusters most similar to mammalian RBCs, we performed a hierarchical clustering analysis based on average transcriptomic profiles (Fig. 1d) and combined this with the expression patterns of marker genes identified in mice to tentatively annotate each cluster as ON CBC, OFF CBC or RBC (Fig. 1e). In mice, RBCs are clearly separated from CBCs at the first dendrogram bifurcation. Similarly, the first dendrogram bifurcation separates two BCs from the other BCs in zebrafish. In contrast to mice, however, the zebrafish RBC clade contains two molecularly distinct clusters (Fig. 1f). We observed that prka (the gene encoding PKCa), a common marker of mammalian RBCs, is only highly expressed in c14. However, both c14 and c19 specifically express grand1b, which is an RBC-specific marker in mice. In addition to these genetic similarities similar to mammalian RBCs, both c14 and c19 clusters express neurotransmitter receptors, grim6a and grim6b, and their downstream signalling molecules, trpo1a, trpo1b, nyx and rgs11, which are essential for mediating rod inputs in mammals. (Extended Data Fig. 1). Therefore, we hypothesized that zebrafish, unlike mice, may possess two RBC types, which we call RBC1 (c14) and RBC2 (c19).

**RBC1 and RBC2 morphologies resemble mammalian RBCs**

We next determined the morphological similarities of RBC1 and RBC2 with mammalian RBCs. In mammals, RBC axons arborize in the innermost layer of the IPL. By screening our zebrafish transgenic lines, we identified two lines, Tg(oxs1:memCerulae) and Tg(oxs2:memCerulae) and Tg(oxs2:memCerulae) and Tg(oxs1:memCerulae), that each label BCs with axon terminals in the innermost layer of the IPL (Fig. 2). Fluorescence in situ hybridization for the identified gene markers, si00a10b and uts1, which are selectively expressed by RBC1 and RBC2 (Fig. 1c), revealed that oxs1:memCer and oxs2:memCer label RBC1 and RBC2, respectively (Fig. 2a,b). We also observed that dendritic arbours of both RBC1 and RBC2 cover the retina in a non-overlapping manner, an arrangement called ‘tiling’ that is considered a hallmark of a BC type. Therefore, both RBC1 and RBC2 represent single BC types that transcriptionally and morphologically resemble mammalian RBCs.

We observed slight variations in morphology and molecular expression between RBC1 and RBC2. The axon terminal of RBC1 is relatively spherical, similar to mammalian RBCs, in contrast to the ‘flat-footed’ axonal ending of RBC2 (Fig. 2c,d). RBC1s were immunoreactive for PKCo (Fig. 2c), whereas RBC2s were not (Fig. 2d), consistent with the difference in their prka expression (Fig. 1d). In addition, their abundance differed: RBC1s were more densely packed than RBC2s (P = 0.0052, Mann–Whitney two-tailed U-test) (Fig. 2g,i). This difference in densities is unlikely due to regional variations as RBC1s outnumbered RBC2s in both the dorsal and ventrotemporal retina (Fig. 2g,j and Extended Data Fig. 2). The dendritic field sizes of the two RBC types were inversely related to their cell density, consistent with their tiling arrangement (Fig. 2g–j).

**Both RBC1 and RBC2 connect with rods and red cones**

If RBC1 and RBC2 are authentic RBCs, they should synapse preferentially with rods. Using 4C12 antibodies to label rods, we found that most of the dendritic tips of both RBC types (RBC1: 84 ± 3.9%, RBC2: 78 ± 2.9%) were associated with rods and red cones (Fig. 3a–d), which is an RBC-specific marker in mice. In addition to these genetic similarities similar to mammalian RBCs, both c14 and c19 clusters express neurotransmitter receptors, grim6a and grim6b, and their downstream signalling molecules, trpo1a, trpo1b, nyx and rgs11, which are essential for mediating rod inputs in mammals. (Extended Data Fig. 1). Therefore, we hypothesized that zebrafish, unlike mice, may possess two RBC types, which we call RBC1 (c14) and RBC2 (c19).
Fig. 1 | Comparison of single-cell gene expression identified two possible rod bipolar cells in zebrafish. 

a, Schematic representation of retinal circuits (left) and an image of a retinal slice from Tg(vsx1:GFP)nns5 transgenic adult zebrafish (right). GFP expression in BCs. Nuclei were stained with DAPI. HC, horizontal cell; PR, photoreceptor. 

b, 2D visualization of single-cell clusters using uniform manifold approximation (UMAP)\textsuperscript{97}. Individual points correspond to single cells coloured according to cluster identity as indicated in d. c, Marker genes for each cluster. d, Agglomerative hierarchical clustering of average gene signatures of clusters using the correlation metric and complete linkage. BC subclasses (colours) were assigned on the basis of the known marker expressions shown in e. 

e, Gene expression patterns of known BC subclass markers in BC clusters. The size of each circle depicts the percentage of cells in the cluster in which the marker was detected (≥1 unique molecular identifier, UMI), and its contrast depicts the scaled average expression level of cells within the cluster in c and e. 

Data for mouse are from ref. 28.
in zebrafish include green, blue and ultraviolet cones. Therefore, both RBC1 and 2 receive predominant rod input and share specificity for red cones among cones (Fig. 3e,g). This connectivity pattern is also shared in mice RBCs (see Discussion)

Interestingly, the dendritic tips of RBC1 and RBC2 terminating in rod spherules differed in structure (Fig. 3c,d). Specifically, the dendrites of RBC1 invaginating rod spherules appeared to form a horseshoe or ‘doughnut’ ending, whereas those of RBC2 ended in a simpler arrangement (Fig. 3c,d). The larger surface area of RBC1 dendritic tips at rod terminals may increase the sensitivity to rod inputs in this BC type compared with RBC2. We also observed differences in the distal axonal boutons of RBC1 and RBC2 (Fig. 3i,j). While axons of both types terminate close to the ganglion cells in the IPL, RBC2 axons have a bouton in the OFF layer of the IPL, next to the boundary with the ON

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Fig. 2 | Transgenic labelling of clusters 14 and 19 revealed morphological features of these BCs. a, b, En face view of retinal flat mount at the inner nuclear layer level. Cerulean fluorescent expression (coloured yellow) transgenic lines, Tg(vsx1:memCerulean)q19 in a and Tg(vsx2:memCerulean)wst01 in b. vxs1:memCer and vxs2:memCer BCs are positive for cluster specific gene expressions, s100a10b and uts1, respectively, which are detected using in situ hybridization chain reaction. c, d, Side views of the labelled cells and the distribution patterns of their axon terminals in en face views of retinal flat mounts for RBC1 (c) and RBC2 (d). Immunolabelling for PKCa is in magenta. Note that not all PKCa-immunoreactive cells are apparent in this image of the vxs1:memCer line due to the incomplete labelling of this line. Other ON stratifying BC axon terminals are also labelled by PKCa. e, f, Dendritic tiling of RBC1 (e) and RBC2 (f) in en face view of retinal flat mounts at the outer plexiform layer level. Dendritic territories are marked by the red boundaries. g–j, Mean cell densities of RBC1 (g, n = 3 and 4 biologically independent animals for dorsal (D) and ventrotemporal (VT), respectively.) and RBC2 (l, n = 3 for both D and VT) BCs in different regions of the retina. Box and violin plots (centre line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range) of dendritic field sizes of RBC1 (h, n = 37 cells and 33 cells for D and VT, respectively) and RBC2 (j, n = 40 cells and 41 cells for D and VT, respectively) BCs. White circles are medians, grey circles indicate individual cells.
These distal boutons are probably presynaptic sites as they contain the presynaptic protein, Ribeye (Fig. 3). These differences in the dendritic tip and axon bouton shapes between RBC1 and RBC2 suggest that, while both BCs receive input from the same combination of photoreceptor types, they may contact different postsynaptic targets and serve distinct visual functions.

RBC1 receives rod inputs via mGluR6 receptors

We next asked whether zebrafish RBCs receive functional rod input via the metabotropic glutamate receptor mGluR6 as seen in mammalian RBCs. We first investigated the expression of mGluR6 in RBC1 and RBC2 dendritic tips at rod spherules. Super-resolution imaging of mGluR6 immunolabelling in vsx1:memCerulean and vsx2:memCerulean retinas...
showed that the dendritic tips of RBC1, but not RBC2, robustly overlapped with mGluR6 immunoreactivity at contacts with rod spherules (Fig. 4a,b). These findings are consistent with the transcriptional profiles, which showed that RBC1 expresses higher mRNA levels of grm6a and grm6b (which encode mGluR6) than RBC2 (Extended Data Fig. 1).

We prepared retinal whole mounts that preserve synaptic connections in the outer retina and performed whole-cell patch-clamp recordings on the axonal terminals of RBC1 and RBC2 (Fig. 4c and Extended Data Fig. 3). Both RBC1 (n = 10) and RBC2 cells (n = 3) exhibited ON responses to a cone-activating flash (red light-emitting diodes (LED)), confirming the successful patch-clamp recordings of light responses in these BCS and demonstrating that both cell types are ON cells (Extended Data Fig. 3), consistent with the position of their axonal arbours (Fig. 2c,d).

We then used electrophysiological recordings to ask whether mGluR6 mediates rod input to the zebrafish RBC1. Recording rod-mediated responses from RBC2 was infasible for technical reasons (Methods). We presented rod-isolating dim blue flashes (10 ms) before and after introducing the mGluR6 receptor agonist 6-(2-aminopropyl)benzofuran (APB) to the perfusion solution. To isolate excitatory inputs to the cell, all recordings were performed near the reversal potential for chloride-mediated conductances (approximately −60 mV, as set by the Cl− concentrations in the internal and external solutions) and in the presence of inhibitory receptor blockers, gabazine, strychnine and (1,2,5,6-tetrahydropyridin-4-yl)methylphosphonic acid (TPMPA). Nearly all rod input was blocked in the presence of APB, indicating that, similar to mammalian RBCs, mGluR6 mediates rod input to RBC1 (Fig. 4e).

Both RBCs primarily synapse onto amacrine cells
To determine the synaptic targets of RBC1 and RBC2, we reconstructed their connectomes using serial block-face scanning electron microscopy (SBFSEM). In the reconstructions, we observed an array of large BC axon terminals in the innermost layer of the IPL; these axon terminals resembled those of RBC1s and RBC2s (Extended Data Fig. 4a,b). To confirm that these large axon terminals belong to RBC1 and RBC2, we reconstructed dendrites of two RBC1s and two RBC2s (Fig. 5a). Consistent with our observations in light microscopic experiments (Fig. 3a–d), the large axon BCS predominantly connect with rods.

We then reconstructed all the large axons in the SBFSEM image volume. To distinguish RBC2 from RBC1, we used the containing an axonal distal bouton in the OFF layer as a proxy for RBC2 (Fig. 3i,j and Extended Data Fig. 3c). These reconstructions revealed the regular mosaic arrangements of both presumed RBC1 and RBC2 (Extended Data Fig. 4d), indicating that we identified most, if not all, presumed RBC1s and RBC2s in the EM volume. Using this criterion, we also verified that dendritic tips of RBC1 are doughnut shaped, whereas those of RBC2 ended in a simple tip within the rod spherule, consistent with our light microscopy data (Figs. 3c,d and 5a).

We then focused on one RBC1 and one RBC2 in the central area of the volume and traced all of their postsynaptic neuronal processes (Extended Data Fig. 4e,f). ACs, unlike RGCs, make output synapses within the retina. Hence, we identified the neuronal class (for example, AC or RGC) for the majority (28/32) of the RBC1 postsynaptic processes and over half (18/31) of the RBC2 postsynaptic processes on the basis of the presence or absence of presynaptic structures. We found that both RBC1 and RBC2 predominantly synapse on ACs (Fig. 5b,g). The majority of the postsynaptic processes received 4 or fewer ribbon synapses from one RBC1 or RBC2, except for one process, which received 14 inputs from one RBC1 (Fig. 5c,h).

We further morphologically classified the postsynaptic ACs that we traced, comprising 14 cells for RBC1 (Extended Data Figs. 5–7) and 10 cells for RBC2 (Extended Data Figs. 8 and 9). Most ACs extended their dendrites within a single sublamina in the IPL (Fig. 5d,i). Among these mono-stratifying ACs for RBC1, we identified two groups on the basis of their dendritic stratification depth within the IPL (Fig. 5e,f). These two groups of ACs differed in their synaptic arrangement. ACs stratifying in the lower layer formed reciprocal synapses—a synaptic arrangement that includes both input and output synapses with a BC axon— with RBC1, whereas ACs stratifying in the upper layer did not (Fig. 5e,f, and Extended Data Figs. 5 and 6). In addition to the mono-stratifying ACs, RBC1 formed an exceptionally high number of ribbon connections with one bi-stratifying AC (marked in red in Fig. 5c,d, morphology in Fig. 6d,e and Extended Data Fig. 7). For RBC2, all but one AC (1 out of 8) formed local reciprocal synapses (Fig. 5) and Extended Data Figs. 8 and 9). RBC2 does not have a postsynaptic partner with extensive synapses.
Fig. 5 | Identification of RBC1 and RBC2, and their postsynaptic neuron types in an SBFSEM volume. 

**a**, Reconstructions of an RBC1 and an RBC2, and zoomed-in images of their dendritic tips at rod and cone terminals. Ribbons in the rods and cones are painted red. 

**b**, Quantification of morphological parameters of neurons postsynaptic to one of the RBC1s in the EM volume (open arrowhead in Extended Data Fig. 4d). One postsynaptic neuron contained an exceptionally higher number of synapses (14) with the RBC1 (marked in red in **c** and **d**). Dendritic stratification is normalized to 0 and 1 at the lower and upper ends of the RBC1 axon terminals, respectively in **e, f**, Mono-stratifying ACs with (red) or without (blue) reciprocal synapses with RBC1s in the volume. The axon of the presynaptic RBC1 is also shown in the side view. Individual cells are colour coded. 

**g**, Quantification of morphological parameters for neurons postsynaptic to one of the RBC2s in the EM volume (closed arrowhead in Extended Data Fig. 4d).
Fig. 6 | The circuit diagram of RBC1 is similar to the mammalian RBC pathway. a, An example mono-stratified amacrine cell (reciprocal synapse (RS) AC) making a reciprocal synapse with RBC1. b, A mouse A17 AC (taken from ref. 72). c, d, A2-like ACs that are postsynaptic to two neighbouring RBC1s. e, f, Locations and distributions of synaptic sites and non-synaptic contacts with BCs in zebrafish bi-stratifying AC (e) and in rabbit A2 ACs (taken from ref. 33) (f). Note that synapses or non-synaptic contacts with AC and RGC are not included in e, and inputs from CBC are not included in f. g-i, Examples of SEM images of output (open arrowheads in g) and ribbon synapse input (open arrowhead in h) synapses, and non-synaptic contacts (i). An example reconstruction of A2-like AC dendrites forming extensive contact with ON BC axon terminals is shown in i. The A2-like AC boutons in the OFF layer often contain mitochondria (black arrowheads in g). j, k, Schematic diagrams of zebrafish (j) and mammalian (k) RBC pathways. Mouse A17 and rabbit A2 images are from refs. 33,72, respectively, used with permission from John Wiley & Sons and Elsevier. Data for the distributions of synaptic sites within mammalian A2 ACs across the IPL were taken from ref. 53.
Together, these results demonstrated that RBC1 allocated the majority (91%) of synaptic outputs to 3 types of AC: 14% to non-RS ACs, 37% to reciprocal synapse ACs and 40% to one bi-stratifying AC. By contrast, the RBC2 we reconstructed synapsed primarily onto mono-stratifying ACs (68%). Among 24 ACs that we traced throughout the volume, only 5 were shared between RBC1 and RBC2 (Extended Data Figs. 5–9). Therefore, the downstream circuits of these BC types are largely separate at least at the AC level.

RBC1 wiring diagram resembles the mammalian RBC pathway

Finally, we compared the targets of RBC1 and RBC2 with those known to comprise the primary rod pathway in the mammalian retina. Mammalian RBC1 synapse onto the mono-stratified A17 AC and the bi-stratified A2 AC22,33. The A17 ACs are wide-field ACs that synapse exclusively with RBCs and form reciprocal synapses with them33,47, whereas A2 ACs are narrow-field ACs that receive numerous (~40) synapses from RBCs but do not form reciprocal synapses with them43,43. Instead, A2 ACs form gap junctions with ON BCs through dendrites in the ON layer and output synapses onto OFF BCs through the bouton structures in the OFF layer called lobular appendages33.

We found that zebrafish RBC1s synapse onto both wide-field ACs with reciprocal synapses, resembling mammalian A17s, and narrow-field bi-stratifying ACs with extensive synaptic connections, resembling mammalian A2s (Fig. 6 and Extended Data Figs. 5 and 7). By marking synaptic sites of reciprocal synapse ACs throughout their dendrites, we found that reciprocal synapse ACs are dedicated to the RBC1 pathway; synapsing predominantly (both input and output) with RBC1 and to a lesser extent with RBC2 (Extended Data Fig. 5), with no synapses with other BC types (n = 7 cells). This synaptic specificity and the reciprocal synapse arrangement in reciprocal synapse ACs mirror those of mammalian A17 ACs33.

Next, we examined the synaptic arrangements of RBC1 with the A2-like ACs. First, we confirmed that this type of AC is common to other RBCs. By tracing the postsynaptic processes of neighbouring RBCs, we found another A2-like AC, which received a high number of ribbon inputs from the neighbouring RBC1 (Fig. 6c,d). We marked the locations of synapses with all BCs for those two ACs (Fig. 6e). Mammalian A2 ACs transfer rod signals to ON cone-BCs via gap junctions containing connexin 36 (cx36)48. Although gap junctions are too small to be resolved in our SBFSEM images, we observed that the dendrites of A2-like ACs often form extensive contacts with the cone-BC axon terminals without forming apparent chemical synapses (Fig. 6i). We marked these ‘non-synaptic contacts’ between A2-like AC dendrites and ON BC axon terminals as sites of potential gap junctions (Fig. 6e). Consistent with this possibility, connexin 35 (cx35, the orthologue of mammalian cx36)49–51 puncta are highly enriched in two layers where A2-like ACs stratify their dendrites (Extended Data Fig. 9a,b). Furthermore, many of the cx35 puncta co-localize with PKCα-positive ON cone-BC axon terminals (Extended Data Fig. 10c).

We found a striking similarity in the distribution patterns of synapses and (potential) gap-junction sites across the IPL layers between this AC type in zebrafish and mammalian A2 ACs (Fig. 6e,f), including the bouton structures in the OFF layer that contain large presynaptic sites and mitochondria22,44 (Fig. 6g). Together, we conclude that the circuit diagram among mammalian RBC, A2 and A17 ACs is conserved in the zebrafish RBC1 pathway (Fig. 6g,h).

In contrast to the targets of RBC1, RBC2 formed synapses exclusively with wide-field ACs (Extended Data Figs. 8 and 9) and lack a synaptic partner with extensive synapses. Thus, RBC2 participates in a circuit that differs from that of mammalian RBCs.

Discussion

Understanding the similarities and differences of neural circuits across species provides valuable insights into the evolutionary origins of neuronal cell types and circuitry, highlights crucial neural circuits that are commonly used and aids in selecting animal models for studying human diseases. Although comparative scRNA-seq has been used to match cell types (for example, ref. 55), applying this approach beyond mammals has proven challenging (but see ref. 56). Furthermore, whether the circuitries of the matched cell types are conserved is unclear. By combining scRNA-seq, electrophysiology, and light and electron microscopic circuit reconstructions, we were able to show that cell types and circuitry for the mammalian rod signalling pathway are conserved in zebrafish (Fig. 6j,k). Our study emphasizes the importance of integrating molecular, functional and structural information to identify conserved neural circuits.

The number of BC types

In this study, we found 23 molecular types in adult zebrafish BCs. However, a previous morphological characterization of zebrafish BCs, based on their dendritic connectivity with photoreceptors and axon stratifications, identified 32 anatomical types32. The discrepancy in the number of BC types between morphological and transcriptional characterizations may arise from the regional specializations that have been documented in the larval zebrafish retina46–48. In any event, it is clear that the adult zebrafish retina contains at least 23 BC types.

The number of molecular types of BCs in zebrafish, as identified in this study, is higher than that found in mammals investigated so far (14–15 across mammals65,66,67), but similar to that found in chick retina (22 molecular and 15 morphological BC types)61,68. The higher number of BC types in zebrafish and chicken is not surprising, given that these species have higher numbers of photoreceptor types: 5 in fish and 7 in chicks, compared with ≥3 in mammals57,69. We demonstrate here one source of the increase: a single type of BC carries most of the input from rods in mammals, whereas zebrafish have two RBC types.

The number of RBC types

Previous morphological characterization of zebrafish BCs found only one BC type that connects rods and red cones. Axons of these BCs terminate in the innermost layer of the IPL10. We speculate that this type includes both RBC1 and RBC2, which were combined owing to their high morphological similarity (Figs. 2 and 3). Consistent with this hypothesis, studies in goldfish have reported two morphologically distinct ‘mixed’ BC types that receive dominant inputs from rods35. They have large axon terminals at the bottom of the IPL, but theaxon of one mixed BC type contains a smaller axonal distal bouton in the OFF layer, similar to RBC2 (ref. 66). Immunostaining for PKC only labels mixed BCs without an axonal distal bouton, similar to RBC1 (ref. 67). The presence of these features in goldfish suggests that RBC1 and RBC2 are conserved among teleost fish.

The functional differences between RBC1 and RBC2 remain to be determined. However, the structural differences of their dendritic tips at rod spherules hint at distinct roles for RBC1 and RBC2 in rod signalling. RBC1 forms a doughnut-shaped dendritic ending, which provides a larger surface area at synaptic sites compared with the simpler dendritic tip of RBC2. Furthermore, the doughnut endings of RBC1 are located farther from thecentre. This results in RBC1 receiving stronger rod drive compared with RBC2. The location of dendritic tips relative to synaptic release sites also differs between RBC1 and RBC2. RBC2 tips terminate near the centre of the invagination (Fig. 4b), placing them closer to the release site, whereas the doughnut endings of RBC1 are located farther from the centre. This difference in the location of dendritic tips may result in different response kinetics between RBC1 and RBC2. For example, in squirrel, OFF BCs with dendritic ending near the synaptic release sites have a transient response at light off, whereas other OFF BCs with dendritic ending away from the release sites show more sustained responses66. Therefore, the observed variation in dendritic tip shapes and locations between RBC1 and RBC2 suggests that RBC1 may be more sensitive to weak rod inputs, whereas RBC2, although possibly less sensitive, could
transmit faster rod signals. However, light sensitivity and response kinetics are determined not only by the dendritic structures but also by many other factors such as neurotransmitter receptor expression patterns, and further studies are required to understand the functional difference between RBC1 and RBC2.

A2- and A17-like ACs may also be conserved in goldfish. Paired electrophysiological recordings between goldfish RBC1 and ACs revealed that RBC1 provides synaptic inputs to two morphological types of AC: wide-field mono-stratifying and narrow-field bi-stratifying AC types. The dendrites of the bi-stratifying ACs wrap around the RBC axon terminals, similar to A2-ACs in zebrafish and mammals (Fig. 6c). Goldfish RBC1 receives GABAergic reciprocal feedback at its axon terminals, similar to mammalian RBC. It is not known whether these two AC types in goldfish exhibit similar synaptic connectivity patterns to mammalian A2 and A17 ACs. Nonetheless, the findings in goldfish are consistent with the idea that the primary rod pathway, including A2 and A17 ACs, is conserved in goldfish. Some differences in physiological properties between mammalian RBC and goldfish RBC1 were also found. First, goldfish RBC1 receives GABAergic lateral inhibition. The exact cell types that provide this inhibition are unclear, but it is likely coming from the wide-field mono-stratifying ACs, as their dendrites extend laterally. In contrast, mammalian A17 ACs do not provide lateral inhibition onto RBCs, as each A17 varicosity provides independent feedback to a single RBC axon terminal. Second, goldfish RBC1 exhibits spikes, whereas spikes are only found in cone BCs in mammals. Nonetheless, the absolute visual sensitivity of goldfish is comparable to that of mammals, suggesting that the primary rod pathway we discovered in teleosts is capable of transmitting information evoked by a single photon.

In mammals, morphological, molecular, and functional studies have identified only a single RBC type. We speculate that either RBC2 evolved after the divergence between teleost fish and mammals, or mammals lost this pathway. The number of RBCs is unclear in other non-mammalian vertebrates. In salmonid, one type of mixed ON BC exhibits sensitivity close to that of rods. They terminate their axons at the bottom of the IPL, similar to teleosts and mammals, but it is unknown whether they express the RBC marker PKC. Furthermore, the anatomical connections of BCs with rods are not yet comprehensively studied in salmonid. In birds, PKC labels some ON layer stratifying BC types strongly. scRNA-seq of chick BCs revealed a BC type that is transcriptomically similar to mammalian RBCs. However, the physiological properties and connections of these BCs are unknown. Moreover, a connectomic survey of chick BC types failed to identify BCs that connect with all rods in their dendritic field. Thus, it remains unclear whether RBC2 orthologues are present in species other than zebrafish. Unlike the species mentioned above, the presumed RBCs in sea lamprey are of the OFF type. Therefore, the conventional rod ON BC pathway may not be conserved in jawless vertebrates.

Roles of cone inputs in RBCs
Cone inputs onto rod-dominant mixed BCs have been proposed to broaden the dynamic range of light intensities to which they can respond. Consistent with this idea, we found that both RBC1 and RBC2 are selective for red cones which, with their broad spectral sensitivity, are suited for encoding achromatic luminance information.

Because rods evolved from cones, we speculate that RBCs may have emerged from red-cone-specific BCs. Red-cone selectivity is also conserved in at least one of the mixed rod-dominant BC types in goldfish. Although cone selectivity is unknown in salmonid rod-driven mixed BCs, their spectral sensitivity curve is broader at longer wavelengths than that of rods, indicating that they may connect to red cones.

Electrophysiological recordings from rod-driven BCs in giant Danio, a teleost fish species, showed that rod and cone inputs onto rod-dominant BCs are mediated by different mechanisms: rod inputs through mGluR6 and cone inputs through both mGluR6 and excitatory amino acid transporter (EAAT) in this BC type. mGluR6 and EAAT-mediated inputs suppress each other, probably to allow this cell to respond to both rod and cone dynamic ranges. Electrophysiological recordings in zebrafish found that some of the ON BCs responded to glutamate via both mGluR6 and a glutamate-gated chloride conductance increase mechanism, which is probably through EAATs. Whether EAAT activity contributes to cone responses in zebrafish RBC1 and RBC2 is unknown.

While the study in giant Danio suggested that mixed inputs expand the dynamic range of rod-dominant BCs, electrophysiological recordings in goldfish and salamander have found that the dynamic range of rod-dominant BCs is similar to that of rods and that cone contributions to the light response are small. Therefore, the roles of red-cone inputs to RBCs remain to be determined.

Unifying mixed BCs and RBCs
In mammals, it was initially thought that RBCs exclusively synapse with rods. However, several recent studies have demonstrated convincingly that RBCs also receive synapses from cones, at least in mice and rabbits; so, such cone contacts have not been observed in primates. Mouse RBCs contact the majority of cones (80%) in their dendritic territories. RBCs were probably thought to be exclusive to rods because of the high ratio of rods to cones in the outer nuclear layer in mice and rabbits. As a consequence, only a few cones, generally three or fewer, synapse on a mouse RBC, compared with inputs from around 35 rods. M-cones are much more numerous than S-cones in mice; hence the majority of cone inputs to mouse RBCs are M-cones even though mouse RBCs contact both M- and S-cones.

The rod-driven BCs in non-mammals are classically called ‘mixed’ BCs because they connect with both rods and cones. However, as argued above, this mixed connectivity is conserved in the mammalian RBCs. Moreover, the dendritic connectivity of RBCs is conserved in mice and zebrafish. In both species, RBCs connect with all rods and the majority of red cones (or M-cones) in their dendritic fields. Finally, together with the striking similarity in the downstream circuitry of RBCs between zebrafish and mammals, we conclude that zebrafish RBC1 is transcriptomically, anatomically and functionally equivalent to mammalian RBC and that they share the same evolutionary origin.

Methods
Animals
All procedures were performed in accordance with the University of Washington Institutional Animal Care and Use Committee (IACUC) guidelines, the Harvard University/Faculty of Arts and Sciences Standing Committee on the Use of Animals in Research and Teaching, and the UK Animals (Scientific Procedures) Act 1986 and approved by the animal welfare committee of the University of Sussex. For all experiments, we used adult zebrafish (age 6–18 months) of either sex, were kept at 28 °C in a room with a normal 14:10 h light cycles.

The following previously published transgenic lines were used: Tg(osx1:GFP)90 (ref. 91), Tg(osx1:memCerulean)91 (ref. 92) and Tg(br2:tdTomato)112 (ref. 93). In the larva Tg(osx1:memCerulean)110, a subpopulation of OFF layer stratifying BCs are labelled. In adults, while OFF stratifying BCs are still weakly labelled, Cerulean is now strongly expressed in RBC1 (Fig. 2a–c). In addition, a Tg(osx1:memCerulean)110 line was generated by injecting pBH-vsxx2-memCerulean-pA plasmid into single-cell-stage eggs. Plasmid was diluted in 1x Danieau’s solution to a concentration of 50 ng ml⁻¹. Plasmid solution was loaded into a
BC scRNA sequencing
BC purification and sequencing. The raw and processed scRNA-seq data reported in this paper were previously published as a part of cross-species comparison of BC transcriptomics: the Gene Expression Omnibus (GEO) entry GSE237215 (subseries GSE237214)\(^9\). We detail the protocols for zebrafish in this paper. Adult zebrafish carrying the Tg(glx3a:GFP)\(^\text{promoter}\) transgene were used to isolate BCs for scRNA-seq. Retinas were dissected and digested in papain solution containing 20 U ml\(^{-1}\) papain, 80 U ml\(^{-1}\) DNasel and 1.5 mM l-cysteine in oxygenated Ames solution at 28 °C for 45 min. The digestion was stopped by replacing the papain solution with a papain inhibitor solution containing 15 mg ml\(^{-1}\) ovomucoid and 15 mg ml\(^{-1}\) BSA. The tissue was gently dissociated by trituration using a flame glass pipette and washed with oxygenated Ames containing 0.4% BSA. The resulting cell suspension was filtered through a 30 μm strainer and fluorescence-activated cell sorting (FACS) was performed. Non-transgenic wild-type retinas were used to determine background fluorescence levels and adjust sorting gates. Live BCs were distinguished using calcein blue. Cells were washed, resuspended in PBS with 0.04% BSA, and loaded onto the microfluidic device within 45 min after FACS enrichment. Droplet RNA-sequencing experiments were conducted on the 10X Chromium platform according to manufacturer instructions with no modifications. Up to 16 retinas from up to 8 fish per batch were dissected and dissociated. Eight cDNA libraries were generated across 4 experiments with 2 replicates each. The cDNA libraries were sequenced on an Illumina HiSeq 2500 to a depth of ~30,000 reads per cell.

Single-cell transcriptomics data analysis. We performed the initial preprocessing using the cellranger software suite (v.2.1.0, 10X Genomics), following steps described previously in our study of zebrafish RGCS\(^9\). The sequencing reads were demultiplexed using ‘cellranger mkfastq’ to obtain a separate set of fastq.gz files for each of the 8 samples, which were distributed across 4 biological replicates. Reads for each sample were aligned to the zebrafish reference transcriptome (ENSEMBL zv10, release 82) using ‘cellranger count’ with default parameters to obtain a binary alignment file and a filtered gene expression matrix (GEM) for each sample. To account for intronic reads, the binary alignment files were processed using velocito with default parameters\(^-\), producing a loom file containing a gene expression matrix (GEM) for exonic reads and a separate matrix for intronic reads. The matrices were combined for each sample, resulting in a total GEM (genes × cells) summarizing transcript counts. We used the Seurat R package to combine the GEMs from different channels and analysed them for each of the biological replicates, unless otherwise stated, with default parameter values. In addition, we evaluated the robustness of our clustering results to variations in select parameters. The full details of our analyses are documented in markdown scripts, which are available on GitHub at https://github.com/shekharlab/ZebrafishBC.

Preprocessing and batch integration. The combined GEM was filtered to remove genes expressed in <25 cells and cells expressing <50 genes, resulting in 25,233 genes and 19,492 cells. In brief, each cell was normalized to a total library size of 10,000 and the normalized counts were log-transformed using the function Seurat::NormalizeData. We used Seurat::FindVariableFeatures with option ‘selection.method=vst’ to identify the top 2,000 highly variable genes (HVGs)\(^9\) in each batch. Next, we performed scRNA-seq integration. We used Seurat::FindIntegrationAnchors and Seurat::IntegrateData, both with options ‘dims=1:40’ to perform canonical correlation analysis-based batch correction on the reduced expression matrix consisting of the HVGs. The integrated expression values were combined across replicates and used for dimensionality reduction and clustering.

Dimensionality reduction, clustering and visualization. To remove scale disparities between genes arising from differences in average expression levels, the integrated expression values for each HVG were z-scored across the cells using Seurat::ScaleData. Next, we performed principal component analysis on the scaled matrix and used Seurat::ElbowPlot to select principal components (PCs). Using the top 20 PCs, we built a k-nearest-neighbour graph using Seurat::FindNeighbors and identified transcriptionally distinct clusters using Seurat::FindClusters, using a resolution parameter of 0.5. Using the top 20 PCs, we also embedded the cells onto a two-dimensional (2D) embedding using uniform manifold approximation\(^-\) with the Seurat function RunUMAP.

Identification of BCs and filtering contaminant classes. BC clusters were identified on the basis of expression of the pan-BC markers vsx1, and other cell classes were filtered on the basis of well-known gene markers. Examples of such genes include rlbpl1a and apoeb for Muller glia\(^9\), rbpms2b for retinal ganglion cells\(^9\), gad1 and gad2 for amacrine cells\(^9\), pdes for photoreceptors\(^9\), and cldn19 for endothelial cells\(^9\). A total of 155 cells corresponding to these cell classes were removed.

Hierarchical clustering. To identify transcriptional relationships between BC clusters, we used Seurat::FindVariableFeatures to recalculate the top 2,000 most variable genes. The average expression values of genes in each cluster were used as input for hierarchical clustering, performed using pvclust with parameters ‘method.hclust = ‘complete’ and ‘method.dist = ‘correlation’. The resulting output was visualized as a dendrogram.

Plasmid construction
Plasmid pH-Vxs2-membrane-Cerulean-pA was made using the Gateway system (ThermoFisher, 12538120) with combinations of entry and destination plasmids as follows: pTol2CG2 (ref. 103), pSE-vxs2, pME-membrane-Cerulean, p3E-pA (ref. 103). Plasmid pSE-vxs2 was generated by inserting a PCR-amplified vxs2 promoter genomic fragment into pSE plasmid using BP clonase (ThermoFisher, 11759013). PCR reaction was performed using the primers: 5′-GGGGACAACTTTG and 3′-GGACTGCTTTTTTGTACAAACTTGGCCTCTGAGACTATT TATAGAAAAGTTGATGCTAAACAACTTCAAACGACCAA and 5′-GGGGACAACTTTG and 3′-GGACTGCTTTTTTGTACAAACTTGGCCTCTGAGACTATT. A total of 155 cells corresponding to these cell classes were removed.

Immunostaining and light microscopy imaging
Adult zebrafish were humanely euthanized in ice-chilled fish water. After decapsulation, retinal tissues were dissected from the enucleated whole eyes by removing cornea, lens and epithelial layer in 1x PBS. The tissues were immediately fixed in 4% paraformaldehyde (Agar Scientific, AGR1026) in PBS for 20 min at r.t., followed by three washes in PBS. For retinal slice preparation, the tissues were mounted in 2% agarose in PBS and sliced at 100 or 200 μm thickness using vibratome (TPI1000). For rod staining, the tissues were sliced horizontally, parallel to the outer plexiform layer (OPL), to facilitate antibody penetration in the tissue.

Primary antibodies were 4C12 antibody (mouse; 1:50; provided by J. Fadool\(^9\)), anti-mGluR6b antibody (rabbit, 1:500; provided by A. McGarvey\(^9\), anti-apoB antibody (rabbit, 1:500; provided by N. Purohit\(^9\), anti-glutamate receptor antibody (rabbit, 1:50; provided by J. Fadool\(^9\)), anti-cldn19 antibody (rabbit, 1:500; provided by B. Liu\(^9\)), and anti-cadherin antibody (rabbit, 1:500; provided by M. Ginn\(^9\)).

Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG (Jackson ImmunoResearch, 115–095–003) and Alexa Fluor 568 goat anti-rabbit IgG (Jackson ImmunoResearch, 115–701–004). For nuclear staining, the tissues were sliced horizontally, parallel to the outer plexiform layer (OPL), to facilitate antibody penetration in the tissue.

Among the various antibodies, we found that anti-apoB antibody worked best for the detection of Muller glia.
The dendritic field was defined by tracing the extent of a given cell's dendrites with the polygonal select tool and removing any concavity using Fiji (Extended Data Fig. 2). The dendritic arbor area was then obtained by calculating the area enclosed by the polygon. Because the dendritic tips of some neighbouring cells of the same type overlapped and could not be distinguished readily, one investigator repeatedly traced (3 to 4 measurements for a single cell) the dendrite boundary and obtained the respective area for a given cell until at least 3 measurements were within ±2.5% of the average of all previous measurements for that cell. Confocal images from three fish were used, with images of RBC1 and RBC2 cells acquired from the dorsal and ventral regions of the retina: 10 to 17 cells per fish were measured for each location and cell type, resulting in a total of 33 to 41 cells measured for each location and cell type.

Photoreceptor connectivity. Dendritic contacts with photoreceptors were defined via the co-localization of dendritic tips extending towards outer nucleus layer and photoreceptor terminals by scrolling through confocal image stacks in Fiji. The percentage contacted was computed by dividing the number of photoreceptors contacted by a given BC by the number of photoreceptors within the dendritic field of the BC.

Electrophysiology. Fish (3–6 months old) used in physiology experiments were dark adapted for at least 2 h, and the retinas were isolated under infrared light following procedures approved by the Administrative Panel on Laboratory Animal Care at the University of Washington. Retinas were continuously superfused (~8 ml min⁻¹) with oxygenated (95% O₂, 5% CO₂) bicarbonate-buffered Ames solution (Sigma) maintained at 25–28 °C. Recordings were conducted in a flat-mount preparation with photoreceptors facing down, and BCs were patched at the axon terminals. To access BC terminals for recording, small groups of ganglion cells were suctioned off the top of the retina to expose the inner plexiform layer. Terminals of RBC1 could be targeted for recording using only infrared illumination, whereas RBC2 terminals, which were not easily visible without fluorescence imaging, were targeted using a custom-built two-photon microscope. As a result, measuring rod-mediated responses in RBC2 was unfeasible due to the compromise of rod responses by two-photon imaging.

Whole-cell voltage-clamp recordings were obtained using patch pipettes filled with a Cs⁺-based internal solution. This internal solution also included Alexa Fluor 594, which was used for two-photon imaging of each cell after recording to confirm its type by morphology. To isolate excitatory postsynaptic currents, we voltage-clamped cells near the reversal potential for chloride-mediated conductances (~60 mV). In addition, to block inhibitory synaptic transmission, we added the GABA receptor antagonist gabazine (20 μM), the GABAC receptor antagonist TPMPA (50 μM) and the glycine receptor antagonist strychnine (3 μM) to the superfusion solution. In experiments in which mGluR6-mediated input was blocked, the mGlur6 receptor agonist APB (10 μM) was also added to the superfusion solution.

Light from blue or red LEDs (peak output ~ 470 nm and 640 nm, respectively) was delivered to the recording chamber via fibre optic cable positioned beneath the microscope’s condenser lens. The light uniformly illuminated a circular area through an aperture 0.5 mm in diameter centred on the recorded cell. Protocols for light stimulation were designed to either activate rods only (using the blue LED) or both rods and cones (using the red LED).

EM data acquisition, reconstruction and annotation. Dissected retinal tissues from wild-type adult zebrafish were immediately transferred into a 1.5 ml tube with the fixative (4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4)) and incubated overnight on a shaker at r.t. Subsequently, the tissue was washed three times in 0.1 M cacodylate buffer (pH 7.4) and incubated in a solution containing 1.5% potassium ferrocyanide and 2% osmium tetroxide (OsO₄) in 0.1 M cacodylate buffer (0.66% lead in 0.03 M aspartic acid (pH 5.5)) for 1 h. After washing, the tissue was placed in a freshly made thio-carbohydrazide (TCH) solution (0.1 g TCH in 10 ml of double-distilled H₂O heated to 60 °C for 1 h) for 20 min at r.t. After another rinse at r.t., the tissue was incubated in 2% OsO₄ for 30 min at r.t. The samples were rinsed again and stained en bloc in 1% uranyl acetate overnight at 40 °C, washed and stained with Walton’s lead aspartate for 30 min. After a final wash, the retinal pieces were dehydrated in a graded ice-cold alcohol series and placed in propylene oxide at r.t. for 10 min. Last, the sample was embedded in Durcupan resin. The block was then trimmed and mounted in a serial block-face scanning electron microscope (Gatan/Zeiss, 3View). The location of the retinal piece was not recorded. One thousand serial sections were cut at a thickness of 70 nm and imaged at an xy resolution of 7 nm. Six tiles, each ~40 μm × 40 μm with an overlap of ~10%, covering from the outer nuclear layer to the ganglion cell layer in a side view were obtained. Retinal location was not recorded. The image stacks were concatenated and aligned using TrakEM2 (NIH). Neurons were traced or painted using the tracing and painting tools in TrakEM2.

Statistics and reproducibility. Mann–Whitney U-test (two-sided) was used to determine the P value for comparing dendritic field sizes.

One sample was prepared for Fig. 1a. Figures 2a–f, 3a–d,i,j and 4a,b, and Extended Data Figs. 2a,b and 10 show representative images or traces from at least 3 independently repeated experiments. Examples from two cells each are shown in Fig. 5a. Figure 6g–i are examples from at least 20 synaptic sites. Extended Data Fig. 4a,b is an example section from one sample.

Reporting summary. Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability. No new scRNA-seq data were generated in this paper.
References

**Acknowledgements**

We thank the Vision Core at the University of Washington for processing zebrafish retina samples and acquiring serial images for SBFSEM, and R. N. Swanstrom for helping with cell tracing of the EM processing zebrafish retina samples and acquiring serial images for Tyrannus.

**Author contributions**

A.M.H., P.M., J.H., Y.K., K.S., J.R.S., H.B., T.B., R.O.W. and T.Y. designed the study; Y.K. performed scRNA-seq under the supervision of H.B. and J.R.S. with guidance from K.S.; J.H. processed and analysed the scRNA-seq data under the supervision of K.S.; the data were further interpreted by J.H., K.S., H.B. and J.R.S.; T.Y.; S.C.S. and L.G. generated new plasmids; T.Y. and F.D.D. generated novel lines; T.Y. performed experiments, collected and analysed the data for light microscopy with help from O.L.; P.M. and F.R. performed whole-cell patch recordings and interpreted the data; F.D.D. prepared the sample for SBFSEM; A.M.H., O.L. and T.Y. traced the EM images; T.Y. analysed and interpreted the EM data; all results including transcriptional, physiological and anatomical analysis were further interpreted by K.S., J.R.S., H.B., T.B., R.O.W. and T.Y.; T.Y. wrote the manuscript with input from all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Extended data** is available for this paper at https://doi.org/10.1038/s41559-024-02404-w.

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41559-024-02404-w.

**Correspondence and requests for materials** should be addressed to Takeshi Yoshimatsu.

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Extended Data Fig. 1 | Expression patterns of the identified marker genes in BC clusters. The size of each circle depicts the percentage of cells in the cluster in which the marker was detected (≥1 UMI), and its grey scale depicts the scaled average expression level of cells within the cluster.
Extended Data Fig. 2 | Dendritic tiling of RBC1 and RBC2 BCs across the retina. a, b, Confocal images of retinal flat mount at outer plexiform layer level from Tg(vsx1:memCerulean)q19 (vsx1:memCer) (a) and Tg(vsx2:memCerulean)wst01 (vsx2:memCer) (b). Note that the vsx1:memCer line occasionally labels OFF BCs. These BCs were distinguished by tracing the cells to the axon terminals in the confocal image volumes.
Extended Data Fig. 3 | RBC1 and RBC2 are ON BCs. Example traces of voltage responses of RBC1 and RBC2 after a cone activating light flash (arrow heads). Inhibitory neurotransmitter receptors were blocked (inh lock) by a bath application of gabazine, strychnine, and TPMPA ((1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid).
Extended Data Fig. 4 | Identification of RBC1 and RBC2 postsynaptic neurons in SBFSEM volume. a, A partial image of an example SEM image of an adult zebrafish retina. OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer). b, Magnified image of the region within the black box in a at the bottom layer of the IPL. Characteristic large bipolar cell axons are painted in light yellow and green.

c, Ribbon synapse distributions in a RBC1 and a RBC2. The locations of ribbon synapses are marked in red. Arrow heads indicate the locations of example ribbon synapses (arrows) shown in the insets. d, Reconstruction of all RBC1s and RBC2s in the EM volume. Postsynaptic neurons of a centrally located RBC1 (open arrow head) and RBC2 (closed arrow head) were reconstructed in Figs. 5, 6, and S5–9. e, f, Traces of neuronal processes and the location of somas of cells that are post-synaptic to RBC1 and RBC2 cells. Individual cells were color coded. IPL: inner plexiform layer.
Extended Data Fig. 5 | Gallery of mono-stratifying AC making reciprocal synapses with RBC1. En face and side views of individual cells. The numbers of input (open bar) and output (closed bar) synapses with each RBC1 (blue) and RBC2 (red) terminal are indicated as the height of the bars.
Extended Data Fig. 6 | Gallery of mono-stratifying AC without reciprocal synapses with RBC1. En face and side views of individual cells. The numbers of input (open bar) and output (closed bar) synapses with each RBC1 (blue) and RBC2 (red) terminal are indicated as the height of the bars.
Extended Data Fig. 7 | Gallery of bi-stratifying AC and RGC contacted to RBC1. En face and side views of individual cells. The numbers of input (open bar) and output (closed bar) synapses with each RBC1 (blue) and RBC2 (red) terminal are indicated as the height of the bars.
Extended Data Fig. 8 | Gallery of mono-stratifying AC connected to RBC2. En face and side views of individual cells. The numbers of input (open bar) and output (closed bar) synapses with each RBC1 (blue) and RBC2 (red) terminal are indicated as the height of the bars.
**Extended Data Fig. 9 | Gallery of bi-stratifying AC and RGC connected to RBC2.** En face and side views of individual cells. The numbers of input (open bar) and output (closed bar) synapses with each RBC1 (blue) and RBC2 (red) terminal are indicated as the height of the bars.
Extended Data Fig. 10 | Cx35 is highly expressed in two layers of the IPL and co-localizes with ON stratifying BC axon terminals. 

**a**, Confocal images of retinal slices from Tg(vsx1:memCerulean)q19 (vsx1:memCer). Immunolabeling for Connexin35 (Cx35) and PKCα are in cyan and magenta, respectively. IPL: inner plexiform layer. 

**b**, Distribution patterns of Cx35 immunostaining across the IPL. Cx35 labeling is enriched in the layers where A2-like AC dendrites stratify (grey shades). The blue thick line is mean values and shades are S.E.M. n=3 animals. 

**c**, Distribution of Cx35 puncta in the PKCα positive ON BC axons. Cx35 labeling outside the axons was digitally masked (removed) in the image on the right.
Reported Summary

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- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. mean) or other basic estimates (e.g. regression coefficient) and variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values wherever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code.

Data collection: We did not use any software for data collection.

Data analysis: For single cell transcriptomics data analysis, we used the cellranger software suite (version 2.1.0, 10X Genomics). The sequencing reads were demultiplexed using “cellranger mkfastq”. Reads for each sample were aligned to the zebrafish reference transcriptome (ENSEMBL zv10, release 82) using “cellranger count” with default parameters. To account for intronic reads, the binary alignment files were processed using velvetCo with default parameters. We used the Seurat R package to combine the GEMs from different channels and analyzed them for each of the biological replicates, unless otherwise stated, with default parameter values. The full details of our analyses are documented in markdown scripts, which are available at https://github.com/Shekharlal/ZebrafishBC

For EM data analysis, we used TrackEM2 package in Image.

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Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third-party data, please ensure that the statement adheres to our policy.

Computational scripts detailing scRNA-seq analysis reported in this paper are available at https://github.com/shekharlab/Zebrafish1C. We have also provided R markdown (Rmd) files that show step-by-step reproduction of the key results at https://github.com/shekharlab/Zebrafish1C. The raw and processed scRNA-seq data reported in this paper was obtained from the Gene Expression Omnibus (GEO) entry GSE237215 (subseries GSE237214).

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

| Reporting on sex and gender | n/a |
| Reporting on race, ethnicity, or other socially relevant groupings | n/a |
| Population characteristics | n/a |
| Recruitment | n/a |
| Ethics oversight | n/a |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We did not perform sample-size calculation. The sample size for single cell transcriptomics was chose based on our previous similar studies in mice (Shekhar K, et al., 2016 Cell), primate (Peng YF, et al., 2019 Cell), and zebrafish (Kölsch Y, et al., 2021 Neuron), which provided robust clustering of cell types. The sample size for the RBC1 and 2 morphology and connectivity were determined based on our previous similar studies in zebrafish (Yoshimatsu T, et al., 2014 Nat Comms; and Yoshimatsu T, et al., 2016 Nat Comms). For the EM analysis, due to the amount of task, we used one sample. |
| Data exclusions | No data was excluded in the analysis. |
| Replication | To verify the reproducibility in single cell transcriptomics, we confirmed that the data from each sample distributes into all clusters. For the EM analysis, we used one sample. |
| Randomization | n/a |
| Blinding | The analysis of the morphology and connectivity of RBC1/2 were performed by blinding the samples. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
### Materials & experimental systems

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### Methods

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### Antibodies

**Antibodies used**

Primary antibodies were 4C12 antibody (mouse, 1:50; kindly provided by Jim Fadoo 93), anti-mGluR6b antibody (rabbit, 1:500; kindly provided by Stephan CF Neuhaus 94), PKCo antibody (rabbit, 1:100; P4334 Sigma), and CIBP2 (Ribeeye) antibody (rabbit, 1:50; 10346-1-AP Proteintech). Secondary antibodies were Alexafluor 594 anti-rabbit (donkey, 1:500; Jackson Immunoresearch Laboratories 711-586-152) and Dylight647 anti-mouse (donkey, 1:500; Jackson Immunoresearch Laboratories 715-606-150).

**Validation**

4C12, mGluR6b, and PKCo antibodies were verified previously 4C12: Morris AC, et al., 2010 Dev Neurobiol, mGluR6b: Huang YY, et al., 2012 PLoS One, PKCo: Hau MF, et al., 2019 Histocherm Cell Biol. For CIBP2, we verified the same distribution pattern of the ribbon synapse by EM.

### Animals and other research organisms

**Policy information about studies involving animals, ARRIVE guidelines** recommended for reporting animal research, and Sex and Gender in Research

**Laboratory animals**

For all experiments, we used adult zebrafish, age 6-18 months.

**Wild animals**

n/a

**Reporting on sex**

We used either sex. We did not perform sex-based analyses as there was no previous reports about the differences in visual performance between sexes in zebrafish.

**Field-collected samples**

n/a

**Ethics oversight**

All procedures were performed in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines, the Harvard University/Faculty of Arts & Sciences Standing Committee on the Use of Animals in Research and Teaching (IACUC), and the UK Animals (Scientific Procedures) act 1986 and approved by the animal welfare committee of the University of Sussex.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

### Plants

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