# ARTICLE

# The functional diversity of retinal ganglion cells in the mouse

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In the vertebrate visual system, all output of the retina is carried by retinal ganglion cells. Each type encodes distinct visual features in parallel for transmission to the brain. How many such 'output channels' exist and what each encodes are areas of intense debate. In the mouse, anatomical estimates range from 15 to 20 channels, and only a handful are functionally understood. By combining two-photon calcium imaging to obtain dense retinal recordings and unsupervised clustering of the resulting sample of more than 11,000 cells, here we show that the mouse retina harbours substantially more than 30 functional output channels. These include all known and several new ganglion cell types, as verified by genetic and anatomical criteria. Therefore, information channels from the mouse eye to the mouse brain are considerably more diverse than shown thus far by anatomical studies, suggesting an encoding strategy resembling that used in state-of-the-art artificial vision systems.

Visual processing begins in the retina (reviewed in ref. 1). Here, photoreceptors feed into bipolar cells<sup>2</sup>, which provide input to a diverse set of retinal ganglion cells (RGCs). Each type of RGC tiles the retinal surface and extracts specific features of the visual scene for transmission to the brain. However, it is still unclear how many such parallel retinal 'feature channels' exist, and what they encode.

Early studies classified cells into ON, OFF or ON–OFF and transient or sustained types (for example, refs 3, 4) based on the response of individual RGCs to light stimulation. These studies also identified RGC types selective for local motion, motion direction or uniform illumination<sup>3,5–7</sup>. In the most complete physiological survey to date, Farrow and Masland<sup>8</sup> clustered ~450 mouse RGCs by their light responses into at least 12 functional types using multi-electrode array (MEA) recordings, suggesting a similar number of feature channels in the retina. In contrast, anatomical classifications of RGC dendritic morphologies estimated around 15–20 types (for example, refs 9–12). Recently, Sümbül and co-workers<sup>10</sup> found at least 16 types using unsupervised clustering together with genetic markers. If each of these anatomically distinct types performed one function, there should be no more than ~20 retinal output channels.

Commonly, RGCs of the same 'genuine' type are thought to share the same physiology, morphology, intra-retinal connectivity, retinal mosaic, immunohistochemical profile and genetic markers. Whether these features suffice to define a type and how classification schemes should be organized is a matter of long-standing debate<sup>13–16</sup>. For example, if also axonal projections were considered type-specific, this could result in a much greater variety of retinal output channels. In zebrafish, RGCs show at least 50 unique combinations of 'dendro-axonal RGC morphologies' targeting a total of ten anatomically defined projection fields<sup>17</sup>. RGCs in mice project to at least 40 targets<sup>18</sup>, suggesting that there may be an even larger number of mouse RGC types.

#### Reliably recording from all RGC types

Here, we sought to test this idea and determine the number of functional output channels of the mouse retina, to obtain a complete picture of what the mouse's eye tells the mouse's brain. We used two-photon Ca<sup>2+</sup> imaging to record light-evoked activity in all cells within a patch of the ganglion cell layer (GCL). Cells were loaded with the fluorescent Ca<sup>2+</sup> indicator Oregon-Green BAPTA-1 (OGB-1) by bulk electroporation<sup>19</sup> (Fig. 1a). This approach resulted in near-complete (>92%) staining of GCL cells, with less than 1% damaged cells<sup>20</sup>. To acquire a patch of several hundreds of cells, we recorded up to nine neighbouring 110 × 110 µm fields (at 7.8 Hz), each containing 80 ± 20 GCL somata (Fig. 1a and Supplementary Video 1). In total, more than 11,000 cells were sampled.

We presented four light stimuli (Fig. 1b): (i) a full-field 'chirp' stimulus to characterize polarity, kinetics and the preference for temporal frequencies and contrasts; (ii) a moving bar to probe for direction and orientation selectivity; (iii) binary dense noise to estimate receptive fields; and (iv) an alternating long- or short-wavelength ('green/blue') full-field stimulus to probe for chromatic preference (Methods). This set of stimuli was chosen to cover a large stimulus space that distinguishes different response types.

We performed recordings in the ventral retina, as verified by the mean chromatic response preference<sup>21,22</sup> of each imaged field (Extended Data Fig. 1e), to control for retinotopic sources of variability<sup>23–25</sup>. In addition, we always presented stimuli at the same light levels in the low photopic regime (Methods).

Our approach allowed us to determine the soma size and position of each recorded cell. Immunohistochemistry (Methods) was used in a subset of experiments: displaced amacrine cells (dACs), which are GABAergic and/or cholinergic and represent a substantial fraction of GCL somata<sup>26</sup>, were labelled for GAD67 and ChAT (Fig. 1c; n = 522 of 1,584 cells; double-labelled: n = 96). Melanopsin-labelling<sup>27,28</sup> identified strongly melanopsin-expressing intrinsically photosensitive RGCs (ipRGCs, n = 18 of 905 cells). SMI-32 labelled a small set of GCL cells (n = 85 of 1,912 cells), including starburst dACs<sup>29</sup>, and was used to identify alpha RGCs (large, strongly labelled somata<sup>9,23</sup>). In addition, we performed recordings in two transgenic lines, Pvalb (PV) and Pcp2 (Methods; Fig. 1d), to relate individual recorded cells to known genetically defined populations.

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**Figure 1** | **Data collection. a**, Whole-mounted mouse retina, electroporated with OGB-1 and recorded with a two-photon microscope ( $64 \times 64$  pixels at 7.8 Hz) in the GCL. Scan fields (left;  $110 \times 110 \mu$ m) comprised  $80 \pm 20$  cells. Regions of interest (ROIs) (right), were placed semi-automatically. Bottom, montage of nine consecutively recorded fields (rectangles; top panels indicated by red dashed line). **b**, Ca<sup>2+</sup> signals from seven regions of interest colour-coded in **a**. Single trials in grey, averages of n = 4 (chirp, green/blue) or 24 (moving bars) trials in black. Responses to four visual stimuli: full-field chirp, bright bars moving in eight directions, full-field alternating green/blue and binary noise for spacetime kernels. Rightmost column, direction- and orientation-selectivity: traces by motion direction; polar plot of peak response, vector sum in red.

Finally, we made electrical single-cell recordings from RGCs (n=84) followed by dye filling to reconstruct their dendritic morphology (Fig. 1e). For all cells with Ca<sup>2+</sup> and spike activity recorded simultaneously (n=17), Ca<sup>2+</sup> responses estimated from spike trains closely resembled measured Ca<sup>2+</sup> responses (Fig. 1f; Extended Data Fig. 1a–d).

#### A probabilistic clustering framework

Combining locally complete optical population recordings, genetic and immunohistochemical labels, as well as electrical measurements, yielded a comprehensive data set of GCL light responses to a set of standardized visual stimuli. This provided the unprecedented opportunity for an unbiased characterization of the retinal output. Since the data set (11,210 cells, n = 50 retinas; Extended Data Fig. 2) is too complex to be interpreted manually (for discussion, ref. 30), we used a clustering approach, making our analysis as objective and quantitative as possible.

In the first step, we used an automatic unsupervised clustering procedure to identify response prototypes of GCL cells (Extended Data Fig. 2d, f; Methods). Specifically, we used sparse principal component analysis (sPCA) to extract features from the light-driven  $Ca^{2+}$  signals of the GCL cells (Methods), which identified many classically used temporal response features such as ON and OFF responses with different kinetics or selectivity to different temporal frequencies. We then used a Mixture of Gaussian model on this feature set for clustering (Methods). In the second step, we post-processed the clustered data

c, Left, experiment in (a) immunostained for GAD67 (green; GABAergic ACs) and ChAT (red; starburst ACs). Right, from (a); both images show same colour-coded regions of interest (left, dots; right, region of interest outlines) and starburst ACs (white dots): cell 6 is GAD67-positive, cell 7 is a starburst AC. d, OGB-1 (green) electroporated retina from transgenic mice with tdTomato (red) expressed in sets of RGCs (top, PV (Pvalb); bottom, Pcp2). e, f, Simultaneous Ca<sup>2+</sup> imaging and electrical recording: dye-filled, anatomically reconstructed cell (e, top, whole-mount; bottom, profile, lines mark ChAT bands). Light responses (f) from top to bottom: spike raster and rate (20-ms bins), recorded (black) and reconstructed (orange) Ca<sup>2+</sup> signal. Scale bars: 50 µm unless otherwise indicated.

to make it accessible for interpretation, including the identification of clusters corresponding to dAC types based on GAD67 staining and isolation of alpha RGCs<sup>9</sup> with large somata from similarly responding cells (Extended Data Fig. 2g, h–j). The validity of this step was verified in detail below (see section 'Example RGC types').

Finally, we arranged the clusters according to a hierarchical tree based on their functional similarity (Methods) and suggest a grouping scheme based on available domain knowledge (Fig. 2a–c). Some groups span different branches, as the tree was solely based on the functional response features.

This framework yielded a total of 46 groups (n = 7,982 cells, 71.2% of all cells, Extended Data Fig. 2h), divided into 32 RGC groups (n = 5,024, 62.9% of grouped cells; including 4 groups,  $G_{29-32}$ , from 'uncertain' clusters) and 14 dAC groups (n = 2,958, 37.1% of grouped cells). The estimated fraction of dACs (between 37.1% and 50.6%, if including all uncertain groups) is within the expected range<sup>26</sup>. We did not analyse dACs in detail (see Extended Data Fig. 3a–c; Supplementary Video 2, Supplementary Figures 1: 50–75, and Supplementary Discussion).

#### A minimum of 32 mouse RGC types

The identified 32 RGC groups comprised non-direction-selective (9 OFF, 12 ON, 3 ON–OFF) and direction-selective (2 ON–OFF, 4 ON, 2 OFF) groups (Fig. 2a–c; Supplementary Figures 2: 1–32) and accounted for all known RGC types in the mouse retina<sup>15</sup>. This includes groups corresponding to three alpha types<sup>31</sup> (G<sub>5,8,24</sub>,





see also below), ON–OFF<sup>32</sup> (G<sub>12,13</sub>) and ON direction-selective types<sup>33</sup> (G<sub>16,25,26,29</sub>), JAM-B (ref. 24) (G<sub>6</sub>) and W3 cells<sup>25</sup> (G<sub>10</sub>), and the OFF 'suppressed-by-contrast' cell<sup>34</sup> (G<sub>31,32</sub>). The allocation of cells to individual groups was uniform across space (Fig. 2d) and the fraction of the population accounted for by broad response types was consistent across experiments (Extended Data Fig. 3e).

As each RGC type is thought to tile the retina, we calculated each group's functional coverage factor based on its average receptive field size and its relative abundance (Extended Data Fig. 4; Methods). A single RGC type would yield a coverage factor of ~1 without receptive field overlap, and a coverage factor of ~2 with 30% overlap. A coverage factor  $\ll 1$  may indicate that a type has been artificially split.

The average coverage factor across all RGC groups was  $2.0 \pm 0.7$  (mean  $\pm$  s.d. of Gaussian fit, Fig. 2e), broadly consistent with reported coverage factors for mouse RGCs (roughly 2–3; see Supplementary Table 1 and Supplementary Discussion). Coverage factors higher than two may indicate groups consisting of multiple types. For example, G<sub>12</sub> corresponding to ON–OFF direction-selective cells<sup>32</sup>, has a coverage factor of 7.7, consistent with four ON–OFF direction-selective types, each preferring a different motion direction<sup>35</sup> (see below).

The mixed non-direction-selective groups  $G_{17}$  and  $G_{31}$  probably contain more than one type, as supported by multiple distinct morphologies and genetic identities (for example,  $G_{31,32}$ , Extended Data Fig. 5) or response properties (for example,  $G_{17}$ , see below).

Taken together, our coverage factor analysis suggests that the number of unique functional RGC types in the mouse is substantially above 32, probably as high as 40, in particular since classical ipRGCs (that is, M1) as well as at least one PV-positive small-field RGC type were largely discarded based on their low signal-to-noise ratio (S/N) response to our stimuli (see Supplementary Discussion). This is about three times the highest number of physiologically defined RGC types to date<sup>8</sup> and about twice the highest anatomical diversity reported in mouse<sup>11</sup> (Supplementary Table 2).

#### **Example RGC types**

There are three types of alpha cells known in the mouse retina<sup>31</sup>: the sustained ( $G_5$ ) and transient OFF alpha ( $G_8$ ), and the ON alpha ( $G_{24}$ ). These cells are characterized by their large somata, a feature that we used during the post-processing step (Extended Data Fig. 2i–j). For the transient OFF and the ON alpha we found similarly responding cells

### **RESEARCH** ARTICLE



#### Figure 3 | Classical alpha RGCs and their 'mini' counterparts. **a**, **b**, Functional 'fingerprint' of classical transient OFF alpha cells (G<sub>8a,b</sub>). **a**, Light-evoked Ca<sup>2+</sup> responses of n = 80 cells: heat maps (top) of individual responses, response averages (with 1 s.d.) and firing rates estimated from Ca<sup>2+</sup> signals below (cf. Extended Data Fig. 1). b, Left, G<sub>8a,b</sub> somata (yellow) and receptive fields (dotted) indicated in example experiment. Grey circles mark cells with receptive fields above quality criterion (Methods). Right, sample morphology of a G<sub>8a,b</sub> cell filled after electrical single-cell recording. For details, see Supplementary Figure 2: 8. c, G<sub>9</sub> RGCs (n = 68), dubbed OFF mini alpha transient because of their similarity in light response to G<sub>8a,b</sub> RGCs (compare with **a**). **e**, **f**, G<sub>24</sub> RGCs (n = 44), identified as classical ON alpha. **g**, **h**, G<sub>23</sub> RGCs (n = 113), dubbed ON mini alpha (compare with e). Mini alphas have smaller receptive field diameters than classical alphas (median in $\mu$ m, 95% confidence interval): G<sub>9</sub>, 280 (270–293) versus G<sub>8</sub>, 306 (294–315) with P = 0.01232, and G<sub>23</sub>, 236 (218–256) versus $G_{24}$ , 319 (290–352) with P = 0.00026 (rank-sum test). i, overlay of OGB-1-stained cells (green) and SMI-32 (magenta). SMI-32-positive RGCs include classical alphas (solid contours; n.r. indicates a non-responsive cell), one large-soma non-alpha cell (green, dotted contour) as well as weakly-labelled ON-OFF direction-selective cells (dashed contours) and starburst ACs (asterisks). Mini alpha cells (blue, dotted contours) are SMI-32-negative. j, Chirp-evoked Ca<sup>2-</sup> responses for five cells in **i**. **k**, SMI-32 statistics (OFF tr.: alpha, n = 16, mini, n = 3; ON: alpha, n = 6, mini, n = 15; OFF sus. alpha, n = 14; ON tr. large, n = 7; other, n = 957; means with 95% confidence intervals; $**P \le 0.01; ****P \le 0.0001$ , logistic regression).

with smaller somata, which we named transient OFF 'mini' ( $G_9$ ) and ON 'mini' alpha cells ( $G_{23}$ ), respectively (Fig. 3a–h). For the sustained OFF alpha ( $G_5$ ) we did not identify an obvious 'mini' version.

We tested whether these pairs consisted of distinct types using SMI-32 immunohistochemistry on a subset of cells (n = 1,912, Fig. 3i–k). These immunolabels were not used during post-processing (Extended Data Fig. 2g). In an example field, all 'classical' alpha cells were SMI-32positive (numbers 1–3, Fig. 3i, j). In contrast, two ON mini alphas (no. 4) had smaller somata and were SMI-32-negative, despite a response profile similar to the ON alpha. Across our entire set of stained cells, transient OFF and ON alpha cells were consistently SMI-32-positive, while their respective mini counterparts were not (Fig. 3k, P < 0.01, logistic regression, Methods). In addition, ON alphas, unlike their mini counterparts, were PV-positive ( $n_{ONalpha} = 16/26$  versus  $n_{ONal}$ pha-mini = 1/37, Supplementary Figures 2: 23, 24). Finally, alpha and mini alpha types formed independent mosaics (Fig. 3b, d, f, h). Transient OFF mini alpha cells (n = 2) stratified similarly to transient OFF alpha cells (n = 5), but with smaller dendritic arbors, consistent with their smaller receptive fields (Fig. 2c; Supplementary Figures 2: 8, 9; for statistics, see Fig. 3 legend). The same was the case for ON alpha and mini alpha RGCs (Supplementary Figures 2: 23, 24). Together, these data suggest that alphas and mini alphas are distinct cell types. The OFF sustained alpha was only moderately SMI-32-immunoreactive (Fig. 3k), consistent with previous reports<sup>23</sup>. An additional, weakly SMI-32-positive RGC group with large somata (no. 5 in Fig. 3i, j) was dubbed 'ON transient, large' (G<sub>19</sub>).

The classical 'local edge detector' (ref. 36) or W3 cell<sup>25</sup> probably corresponds to  $G_{10}$  (Extended Data Fig. 6a–f). This type had small somata and receptive fields, and responded poorly to full-field stimuli. Instead,  $G_{10}$  cells reliably responded to moving bars with a brisk burst at the leading and trailing edges, consistent with previous reports<sup>25</sup>. Intracellular filling confirmed the cell's morphological identity (Extended Data Fig. 6c). A second group,  $G_{11}$ , had similar response properties with a reduced leading-edge response, potentially corresponding to a W3 variant<sup>25</sup>.

Contrast-suppressed RGCs (for example, see refs 37, 38) occur in many species and a type of OFF supressed-by-contrast (Sbc) cell has recently been described in mice<sup>34</sup> (G<sub>31</sub>). We found a new ON Sbc cell type (G<sub>28</sub>), which responded slowly to a full-field increase in light level but was suppressed by temporal contrast (Extended Data Fig. 6g–1). One candidate morphology diffusively stratified across the entire inner plexiform layer (IPL), while another stratified exclusively in the ON layer.

#### **Direction and orientation selectivity**

Our RGC groups contained n = 1,757 direction-selective cells (35% of RGCs; Extended Data Fig. 7a–e; Methods). Most direction-selective cells (70%) were sorted into eight groups (G<sub>2,6,12,13,16,25,26,29</sub>). This high functional diversity among direction-selective cells is in agreement with studies of direction-selective RGC-specific transgenic mouse lines<sup>39</sup>. Further direction-selective clusters were grouped with functionally similar non-direction-selective counterparts (G<sub>4,18,28</sub>), as they contained less cells than the matching non-direction-selective clusters. These may include RGCs with slightly asymmetric dendritic arbors (for example, ref. 10) that could lead to directional bias in response to motion. Classically, these cells are not considered direction-selective.

To identify the motion axes of direction-selective groups, we registered the orientation of the retina in a subset of experiments (n = 3,830 cells, n = 12 retinas, Fig. 4; Extended Data Fig. 7f, g). Three direction-selective groups largely preferred one direction: the ON direction-selective transient (G<sub>16</sub>), the JAM-B RGC<sup>24</sup> (G<sub>6</sub>) and one of the sustained ON direction-selective cells (G<sub>25</sub>) preferred backward, upward and forward motion, respectively. The other direction-selective groups responded to more than one direction. The two groups of ON–OFF direction-selective cells (G<sub>12,13</sub>) contain the four classical subtypes<sup>32</sup>, with room for additional ones, as suggested by genetic analyses and central projections (for example, ref. 40; Supplementary Discussion). The three classical sustained ON direction-selective types<sup>33</sup> are included in G<sub>25,26,29</sub>.

We found two OFF direction-selective groups ( $G_{2,6}$ ), with  $G_6$  containing JAM-B RGCs<sup>24</sup>. These cells responded weakly to ON and strongly to OFF light changes, consistent with the JAM-B's polarity switch with stimulus size. In addition, we identified a new OFF direction-selective cell ( $G_2$ , Extended Data Fig. 6m–r). This group did not co-stratify with the ChAT bands, which play a role in most known direction-selective circuits (reviewed in ref. 39). Morphology, response profile, and directional preference of  $G_2$  suggests that these cells are not the JAM-B RGCs (see above).

As predicted by recordings from mouse lateral geniculate nucleus<sup>41</sup> and superior colliculus<sup>42</sup>, we identified n = 730 (14.5%) orientation-selective RGCs (Fig. 4b). While most RGC groups contained only few



**Figure 4** | **Direction and orientation selectivity. a**, Pairs of retinocentric polar plots showing distributions of preferred motion directions of selected direction-selective (DS) RGC groups (V, ventral; N, nasal). Top plot of each pair: preferred directions, with length representing direction-selective index and grey level  $p_{DS}$  (Methods). Bottom plot of

orientation-selective cells,  $G_{1,14,17,30}$  contained disproportionately more (~30% each). (ON–)OFF orientation-selective cells ( $G_{14}$ ) were selective for vertical and horizontal orientations, whereas ON transient orientation-selective cells ( $G_{17}$ ) included many preferred orientations, consistent with its coverage factor of seven. Additional experiments with contrast inversed moving and stationary bars (n = 826 cells) revealed further functional diversity in response to these stimuli among  $G_{17}$  cells (Extended Data Fig. 7h–s; Supplementary Discussion).

#### Genetic and anatomical RGC types

To link RGC groups to genetically-defined populations, we performed a subset of our experiments in the PV<sup>43</sup> and Pcp2 (ref. 44) transgenic mouse lines (Extended Data Figs 5, 8;  $n_{PV} = 173$  cells in 24 retinas,  $n_{Pcp2} = 15$  cells in 3 retinas). PV- and Pcp2-positive cells were sorted into 20 (PV<sub>a-t</sub>) and 6 (Pcp2<sub>a-f</sub>) groups (Extended Data Fig. 5b–d), with 14 and 3 of the groups containing  $n \ge 3$  genetically labelled cells, respectively. In the case of the PV line, many matches were very robust (for example, the ON alpha cells: G<sub>24</sub> or PV<sub>r</sub> or 'PV1' from ref. 45). Nevertheless, the count of  $\ge 14$  functional types in the PV line is much higher than the previously described eight PV types<sup>45</sup>, suggesting higher functional diversity than appreciated in earlier studies (Supplementary Discussion).

Next, we used our comparatively small sample of n = 84 morphologically reconstructed cells to link functional groups to anatomically defined types. In many cases, it was possible to identify likely morphologies for functional RGC groups (Extended Data Fig. 9; Supplementary Discussion). To generate an approximate mapping of functional groups to dendritic depth profiles in the IPL (Fig. 5), we averaged the stratification profiles of all reconstructed cells, weighted by the correlation coefficient between each cell's light response and the functional group average (to full-field chirp and moving bars; Methods).

The resulting map reproduced many known principles of inner retinal organization. For example, OFF ( $G_{1-9}$ ) and sustained ON ( $G_{21-24;27,28,30}$ ) groups mostly stratified in the upper and lower half of the IPL<sup>4</sup> and groups with more transient responses closer to the centre

each pair: circular area-normalized histogram. **b**, As for **a**, but for selected orientation-selective (OS) RGCs. Further direction-selective/orientation-selective groups detailed in Extended Data Fig. 7. **c**, Motion directions in the visual space of the mouse.

(for example,  $G_{1,2,8-10,20}$ )<sup>46,47</sup>. Most groups corresponding to known types had expected stratification profiles (for example, W3, ON–OFF direction-selective and classical alphas) with few exceptions (for example,  $G_{6,11,18,19}$ ). Our prediction was for the JAM-B ( $G_6$ ) to stratify mainly below the ChAT band, while the cell is known to stratify above it, as it also does in our individual examples (Extended Data Fig. 9a). This is probably caused by many cell types with responses comparable to those of the JAM-B confounding the  $G_6$  IPL profile.

#### Conclusions

We found that a minimum of 32 different functional types of RGCs could be distinguished based on their light responses and basic anatomical criteria. The unusually high abundance of some of these functional types and evidence from immunohistochemistry suggests that further sub-divisions are needed. Accordingly, the number of distinct visual feature channels available to the mouse brain appears to be two- to threefold that of previous estimates.

Taken together, our RGC groups cover a broad range of 'classical' features such as polarity, receptive field size, frequency and contrast sensitivity (Extended Data Fig. 10). In particular, RGC groups broadly span feature dimensions such as response polarity and their preference for global versus local stimuli. Less balanced is the temporal frequency selectivity, with only a few groups preferring high frequencies, particularly for groups with low contrast preference.

We verified our suggested functional classification by showing that: (i) most functionally defined types (ii) exhibited a similar coverage factor; (iii) some could be linked to genetically defined populations; and (iv) types had consistent morphology/dendritic stratification profiles in the IPL. Nonetheless, our definitions certainly remain incomplete; the classification of mouse RGCs will need to be refined by the expansion of the probed stimulus space, the use of cell-type-selective genetic lines<sup>40</sup> or single-cell transcriptomics<sup>48</sup>, and integration with data from largescale electron microscopy<sup>49</sup>. However, even our comparatively basic analysis already reveals a large diversity in feature coding by mouse RGCs, very different from how digital cameras encode images, rather



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profiles indicate deviation from known stratification pattern ( $G_6$ ) or an unexpected pattern given a potentially novel group's response polarity ( $G_{11,18,19}$ ). a.u., arbitrary units, DS, direction-selective; OS, orientation-selective.

resembling an encoding strategy used in state-of-the-art artificial vision systems<sup>50</sup>. In the future, the 'fingerprint' of different functional RGC types introduced here can provide a frame of reference for systematic investigations of feature coding by RGCs and for detecting functional changes in degenerated retina.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Data (original data, clustering and grouping results) as well as Matlab code for visualization are available from http://www.retinal-functomics.org. Reprints and permissions information is available at www. nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.E. (thomas.euler@cin.uni-tuebingen.de).

#### **METHODS**

No statistical methods were used to predetermine sample size.

Animals and tissue preparation. All procedures were performed in accordance with the law on animal protection issued by the German Federal Government (Tierschutzgesetz) and approved by the institutional animal welfare committee of the University of Tübingen. For all experiments, we used 4- to 8-week-old mice of either sex. In addition to C57Bl6 (wild-type) mice, we used the transgenic lines Pvalb<sup>Cre</sup> ('PV', JAX 008069, The Jackson Laboratory; ref. 43), Pcp2<sup>Cre</sup> ('Pcp2', JAX 006207; ref. 44) and ChAT<sup>Cre</sup> (JAX 006410; ref. 51), cross-bred with the red fluorescence Cre-dependent reporter line Ai9:tdTomato (JAX 007905). Owing to the exploratory nature of our study, we did not use randomization and blinding.

Animals were housed under a standard 12h day/night rhythm. For activity recordings, animals were dark-adapted for >1 h, then anaesthetized with isoflurane (Baxter) and killed by cervical dislocation. The eyes were enucleated and hemisected in carboxygenated (95% O2, 5% CO2) artificial cerebral spinal fluid (ACSF) solution containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 20 glucose, and 0.5 L-glutamine (pH 7.4). Bulk electroporation of the fluorescent Ca<sup>2+</sup> indicator Oregon-Green BAPTA-1 (OGB-1) into the ganglion cell layer (GCL) was carried out as described before<sup>19,47</sup>. In brief, the retina was dissected from the eyecup, flat-mounted onto an Anodisc (#13, 0.2 µm pore size, GE Healthcare) with the GCL facing up, and placed between two 4-mm horizontal plate electrodes (CUY700P4E/L, Nepagene/Xceltis). A 10µl drop of 5 mM OGB-1 (hexapotassium salt; Life Technologies) in ACSF was suspended from the upper electrode and lowered onto the retina. After application of 10-12 pulses (+9 V, 100 ms pulse width, at 1 Hz) from a pulse generator/wide-band amplifier combination (TGP110 and WA301, Thurlby handar/Farnell), the tissue was moved to the recording chamber of the microscope, where it was continuously perfused with carboxygenated ACSF at  $\sim$  37 °C and left to recover for  $\sim$  60 min before the recordings started. In all experiments with wild-type mice, ACSF contained  $\sim 0.1 \,\mu M$ Sulforhodamine-101 (SR101, Invitrogen) to reveal blood vessels and any damaged cells in the red fluorescence channel<sup>20</sup>. All procedures were carried out under very dim red (>650 nm) light.

**Two-photon Ca<sup>2+</sup> imaging and light stimulation.** We used a MOM-type two-photon microscope (designed by W. Denk, MPI, Martinsried; purchased from Sutter Instruments/Science Products). Design and procedures were described previously<sup>20</sup>. In brief, the system was equipped with a mode-locked Ti:Sapphire laser (MaiTai-HP DeepSee, Newport Spectra-Physics) tuned to 927 nm, two fluorescence detection channels for OGB-1 (HQ 510/84, AHF/Chroma) and SR101 (HQ 630/60, AHF), and a water immersion objective (W Plan-Apochromat 20x/1,0 DIC M27, Zeiss). For image acquisition, we used custom-made software (ScanM, by M. Müller, MPI, Martinsried, and T.E.) running under IGOR Pro 6.3 for Windows (Wavemetrics), taking 64 × 64 pixel image sequences (7.8 frames per s) for activity scans or 512 × 512 pixel images for high-resolution morphology scans.

For light stimulation, we focused a DLP projector (K11, Acer) through the objective, fitted with band-pass-filtered light-emitting diodes (LEDs) ('green', 578 BP 10; and 'blue', HC 405 BP 10, AHF/Croma) that roughly match the spectral sensitivity of moose M- and S-opsins. LEDs were synchronized with the microscope's scan retrace. Stimulator intensity (as photoisomerisation rate, 10<sup>3</sup> P\* per s per cone) was calibrated as described previously<sup>52</sup> to range from 0.6 and 0.7 (black image) to 18.8 and 20.3 for M- and S-opsins, respectively. Owing to two-photon excitation of photopigments, an additional, steady illumination component of  $\sim 10^4$ P\* per s per cone was present during the recordings (for detailed discussion, see ref. 22). For all experiments, the tissue was kept at a constant intensity level (see stimuli below) for at least 30s after the laser scanning started before light stimuli were presented. Four types of light stimulus were used (Fig. 1b, top): (i) full-field 'chirp' stimulus consisting of a bright step and two sinusoidal intensity modulations, one with increasing frequency and one with increasing contrast; (ii)  $0.3 \times 1$  mm bright bar moving at 1 mm s<sup>-1</sup> in eight directions<sup>19</sup>; (iii) alternating blue and green 3-s flashes; and (iv) binary dense noise, a  $20 \times 15$  matrix with  $40 \,\mu$ m pixel-side length; each pixel displayed an independent, perfectly balanced random sequence at 5 Hz yielding a total running time of 5 min for receptive field (RF) mapping. In some experiments, we used in addition dark moving bars (like (ii) but contrast-inversed), and stationary bright or dark  $0.2 \times 0.8$  mm bars flashed for 1 s in six orientations (see Extended Data Fig. 7h-s). Except for (iii), stimuli were achromatic, with matched photo-isomerization rates for M- and S-opsins.

Single-cell electrophysiology, dye filling and morphological reconstruction. TdTomato- or OGB-1-labelled RGCs were targeted using two-photon imaging for juxtacellular recordings using borosilicate electrodes (4–6 M $\Omega$ ) filled with ACSF with added SR101 (250  $\mu$ M). Data were acquired using an Axoclamp-900A or Axopatch 200A amplifier in combination with a Digidata 1440 (all: Molecular Devices), digitized at 10 kHz and analysed offline using IGOR Pro. We presented the same light stimuli as for the Ca<sup>2+</sup> imaging. In some experiments, electrical recordings and Ca<sup>2+</sup> imaging was performed simultaneously. After the recording,

the membrane under the electrode was opened using a voltage 'buzz' to let the cell fill with dye by diffusion for approximately 30 min; then two-photon image stacks were acquired to document the cell's morphology.

Filled cells were traced semi-automatically offline using the Simple Neurite Tracer plugin implemented in Fiji (http://fiji.sc/Fiji), yielding cell skeletons. If necessary, we used the original image stack to correct the skeletons for any warping of the IPL using custom-written scripts in IGOR Pro. To this end, we employed the SR101-stained blood vessel plexuses on either side of the IPL as landmarks to define the IPL borders (see below). Only cells where the filling quality allowed full anatomical reconstruction were used for analysis (see below).

**Immunohistochemistry.** Following Ca<sup>2+</sup> imaging, retinas were mounted onto filter paper ( $0.8 \mu$ m pore size, Millipore) and fixed in 4% paraformaldehyde (in PBS) for 15 min at 4°C. Immunolabelling was performed using antibodies against ChAT (choline-acetyltransferase; goat anti-ChAT, 1:100, AB144P, Millipore), GAD67 (glutamate decarboxylase; mouse anti-GAD67, 1:100, MAB5046, Millipore), SMI-32 (mouse anti-SMI32, 1:100, SMI-32R-100, Covance), and melanopsin (rabbit anti-melanopsin, 1:4000, AB-N38, Advanced Targeting Systems) for 4 days. Secondary antibodies were Alexa Fluor conjugates (1:750, 16h, Life Technologies). For each retina, the recorded region was identified by the local blood vessel pattern and confirmed by comparing size and position of individual somata in the GCL. Image stacks were acquired on a confocal microscope (Nikon Eclipse C1) equipped with a  $\times 60$  oil objective (1.4 NA). The degree of immunolabelling of GCL cells was evaluate and rated (from 0, negative, to 4 positive) using *z*-stacks. Attribution of labelled somata to recorded ones was performed manually using ImageJ (http://imagej.nih.gov/ij) and IGOR Pro.

**Data analysis.** Data analysis was performed using Matlab 2012 and 2014a (The Mathworks Inc.), and IGOR Pro. Data were organized in a custom written schema using the DataJoint for Matlab framework (http://datajoint.github.io/; D. Yatsenko, Tolias lab, Baylor College of Medicine).

**Pre-processing.** Regions of interest (ROIs), corresponding to somata in the GCL, were defined semi-automatically by custom software (D. Velychko, CIN) based on a high-resolution (512 × 512 pixels) image stack of the recorded field. Then, the Ca<sup>2+</sup> traces for each ROI were extracted (as  $\Delta F/F$ ) using the IGOR Probased image analysis toolbox SARFIA (http://www.igorexchange.com/project/SARFIA). A stimulus time marker embedded in the recording data served to align the Ca<sup>2+</sup> traces relative to the visual stimulus with a temporal precision of 2 ms. Stimulus-aligned Ca<sup>2+</sup> traces for each ROI were imported into Matlab for further analysis.

First, we de-trended the Ca<sup>2+</sup> traces by high-pass filtering above ~0.1 Hz. For all stimuli except the dense noise (for RF mapping), we then subtracted the baseline (median of first eight samples), computed the median activity r(t) across stimulus repetitions (typically three to five repetitions) and normalized it such that  $\max(|r(t)|) = 1$ .

**Receptive field mapping.** We mapped the linear RFs of the neurons by computing the Ca<sup>2+</sup> transient-triggered average. To this end, we resampled the temporal derivative of the Ca<sup>2+</sup> response  $\dot{c}(t)$  at 10-times the stimulus frequency and used Matlab's findpeaks function to detect the times  $t_i$  at which Ca<sup>2+</sup> transients occurred. We set the minimum peak height to 1 s.d., where the s.d. was robustly estimated using:

$$\overline{\sigma} = \frac{\text{median}(|\dot{r}(t)|)}{0.6745}$$

We then computed the Ca<sup>2+</sup> transient-triggered average stimulus, weighting each sample by the steepness of the transient:

$$F(x, y, \tau) = \frac{1}{M} \sum_{i=1}^{M} \dot{\boldsymbol{c}}(t_i) \boldsymbol{S}(x, y, t_i + \tau)$$

Here, S(x, y, t) is the stimulus,  $\tau$  is the time lag (ranging from approximately -320 to 1,380 ms) and M is the number of Ca<sup>2+</sup> events. We smoothed this raw RF estimate using a 5 × 5 pixel Gaussian window for each time lag separately. RF maps shown correspond to a s.d. map, where the s.d. is calculated over time lags  $\tau$ :

$$F_{\text{map}}(x, y) = \text{s.d.}[F(x, y, \tau)]_{\tau}$$

To extract the RF's position and scale, we fitted it with a 2D Gaussian function using Matlab's lsqcurvefit. The time course of the receptive field  $F_{tc}(\tau)$  was estimated by the average of the eight pixels closest to the fitted RF centre (according to the Mahalanobis distance) weighted by a Gaussian profile. RF quality ( $QI_{RF}$ ) was measured as one minus the fraction of variance explained by the Gaussian fit  $\overline{F}_{map}$ ,

$$QI_{
m RF} = 1 - rac{
m Var}{
m Var}[F_{
m map} - F_{
m map}]}{
m Var}[F_{
m map}]$$

**Direction and orientation selectivity.** To extract time course and directional tuning of the Ca<sup>2+</sup> response to the moving bar stimulus, we performed a singular value decomposition (SVD) on the *T* by *D* normalized mean response matrix *M* (times samples by number of directions; T=32; D=8; Extended Data Fig. 7a, b):

$$[U, S, V] = \operatorname{svd}(M)$$

This procedure decomposes the response into a temporal component in the first column of U and a direction dependent component or tuning curve in the first column of V, such that the response matrix can be approximated as an outer product of the two:

$$\boldsymbol{M} \approx \boldsymbol{S}_{11} \boldsymbol{U}_{:1} \boldsymbol{V}_{:1}^T$$

An advantage of this procedure is that it does not require manual selection of time bins for computing direction tuning, but extracts the direction tuning curve given the varying temporal dynamics of different neurons.

To measure direction selectivity (DS) and its significance, we projected the tuning curve  $V_1$  on a complex exponential  $\phi_k = \exp(i\alpha_k)$ , where  $\alpha_k$  is the direction in the *k*th condition:

$$K = \phi^T V_{:1}$$

This is mathematically equivalent to computing the vector sum in the 2D plane or computing the power in the first Fourier component. We computed a DS index as the resulting vector length

$$DSi = |K|$$

correcting for the direction spacing. We additionally assessed the statistical significance of direction tuning using a permutation test<sup>53</sup>. To this end, we created surrogate trials (that is, stimulus repetitions) by shuffling the trial labels (that is, destroying any relationship between condition and response), computed the tuning curve for each surrogate trial and projected it on the complex exponential  $\phi$ . Carrying out the procedure 1,000 times generated a null distribution for *K*, assuming no direction tuning. We used the percentile of the true *K* as the *P* value for direction tuning (Extended Data Fig. 7c). Importantly, a large DSi does not necessarily result in a small *P* value, for example, in the case of large trial to trial variability. As a result, the DSi distributions of significantly and not significantly direction tuned neurons show substantial overlap (Extended Data Fig. 7d, e). Therefore, a simple threshold as a DS criterion (for example, and thers.

Orientation selectivity (OS) was assessed in an analogous way. However, we used the complex exponential  $\phi_k = \exp(2i\alpha_k)$ , corresponding to the second Fourier component.

**Response quality index.** To measure how well a cell responded to a stimulus (chirp, moving bar, colour), we computed the signal-to-noise ratio

$$QI = \frac{\operatorname{Var}[\langle \boldsymbol{C} \rangle_r]_t}{\langle \operatorname{Var}[\boldsymbol{C}]_t \rangle_r}$$

where *C* is the *T* by *R* response matrix (time samples by stimulus repetitions) and  $\langle \rangle_x$  and Var[]<sub>x</sub> denote the mean and variance across the indicated dimension, respectively. If all trials are identical such that the mean response is a perfect representative of the response, *QI* is equal to 1. If all trials are completely random with fixed variance (so that the mean response is not informative about the individual trial responses at all), *QI* is proportional to 1/R.

For further analysis, we used only cells that responded well to the chirp and/or the moving bar stimulus ( $QI_{chirp} > 0.45$  or  $QI_{DS} > 0.6$ ). **Full-field index.** The full-field index was computed as

$$FFi = \frac{QI_{DS} - QI_{chirp}}{QI_{DS} + QI_{chirp}}$$

comparing the response quality to a local stimulus (moving bar) and a global stimulus (chirp).

ON-OFF index. ON-OFF preference was measured as

$$OOi = \frac{\langle r_{ON} \rangle_t - \langle r_{OFF} \rangle_t}{\langle r_{ON} \rangle_t + \langle r_{OFF} \rangle_t}$$

where  $r_{\rm ON}$  and  $r_{\rm OFF}$  are defined as the activity during the response to the leading edge of the moving bar (the first 400 ms of the ON response) and the trailing edge of the moving bar (the first 400 ms of the OFF response).

Colour selectivity index. Colour selectivity was measured for the ON response using

$$GB_{ON} = \frac{\max(r_{ON,green}^2) - \max(r_{ON,blue}^2)}{\max(r_{ON,green}^2) + \max(r_{ON,blue}^2)}$$

and for the OFF response using an analogous definition. Here,  $r_{\rm ON,green}$  and  $r_{\rm ON,blue}$  are the responses in a time window of 1,280 ms after onset of the green and blue stimulus, respectively.

**Feature extraction.** We used sparse principle component analysis<sup>54</sup>, as implemented in the SpaSM toolbox by K. Sjöstrang *et al.* (http://www2.imm.dtu.dk/ projects/spasm/), to extract sparse response features from the responses to the chirp, colour, and moving bar stimulus, resulting in features which use only a small number of time bins. The extracted features are localized in time and therefore readily interpretable (for example, 'high-frequency feature'), although this constraint was not explicitly enforced by the algorithm (Extended Data Fig. 2e). We also explored alternative feature extraction techniques such as regular PCA, but these resulted in inferior cluster quality. In addition, they required manually defining regions corresponding to specific parts of the stimulus (for example, frequency chirp) to yield localized and interpretable features.

We extracted 20 features with 10 non-zero time bins from the mean response to the chirp (averaging across trials) and 6 features with 10 non-zero time bins from the mean response to the colour stimulus. For the moving bar stimulus, we extracted 8 features with 5 non-zero time bins from the response time course (see above) and 4 features with 6 non-zero time bins from its temporal derivative. All features were in the temporal domain, ensuring spatial invariance. In addition, we used two features from the time course of the RF, extracted with regular PCA. Overall, this procedure resulted in a 40 dimensional feature vector for each cell. Before clustering, we standardized each feature separately across the population of cells.

**Clustering.** DS and non-DS cells were clustered independently, classifying cells as DS if the permutation test resulted in P < 0.05 (see above). We fit each data set with a Mixture of Gaussians model using the expectation-maximization algorithm (Matlab's gmdistribution object). We constrained the covariance matrix for each component to be diagonal, resulting in 80 parameters per component (40 for the mean, 40 for the variances). We further regularized the covariance matrix by adding a constant ( $10^{-5}$ ) to the diagonal. To find the optimal number of clusters, we evaluated the Bayesian information criterion<sup>55</sup>

$$BIC = -2\log[L] + M\log[N]$$

where *L* is the log-likelihood of the model, *N* is the number of cells and *M* is the number of parameters in the model, that is, M = 81C - 1 where C is the number of clusters and the contributions that arose from means, variances and mixture proportions (which have to add to 1). Although other choices such as the Aikaike information criterion (AIC) would have been possible, we found the BIC to yield a good compromise between model complexity and quality, since the AIC is known to find too many clusters for large sample sizes. We also computed log Bayes factors as  $2\Delta$ BIC for each candidate cluster number to test how strong the evidence for further splitting is. Values >6 were treated as strong evidence in favour of further splitting. The minimum of the BIC coincided well with the number of clusters after which there was no strong evidence for further adding more clusters. To avoid local minima, we restarted the EM algorithm 20 times per candidate number of clusters and used the solution with the largest likelihood. This procedure resulted in 24 and 48 clusters for DS and non-DS cells, respectively (Extended Data Fig. 2a).

To evaluate cluster quality, we rank-ordered the posterior probabilities for cluster assignment for each cluster, normalized for cluster size and averaged across clusters for non-DS and DS cells separately (Extended Data Fig. 2b). The steep decays of the sigmoidal functions indicate good cluster separability. To check how consistent the clustering was against subsampling of the data, we created 20 surrogate data sets containing random selections of 90% of the cells. We fit these surrogate data sets with a Mixture of Gaussians model with the optimal number of clusters determined on the original data set. For each cluster mean in these models, we computed the correlations with the most similar cluster for the model fit on the original data set. To summarize the similarity of clusterings, we computed the surrogate and the original data set were very similar (usterings obtained on the surrogate and the original data set were very similar (mean median correlation:  $0.96 \pm 0.19$  and  $0.97 \pm 0.01$ ; mean  $\pm$  s.d.; for DS and non-DS cells separately).

In addition, we performed an alternative clustering version, where we did not split the data in DS and non-DS cells but added DSi, OSi, soma and receptive field size as features. The identified clusters were very similar, but this strategy failed to identify most DS types as separate clusters, except for the ON-OFF DS

cell. Therefore, we decided to first isolate significant DS cells and cluster them separately, before merging similarly responding DS and non-DS clusters (see below), if we did not find a reason to keep the DS group as a separate RGC type. Nevertheless, a strategy equally justified as ours could start with the alternative clustering and then split those clusters containing large fractions of DS cells.

Automatic identification of RGCs and ACs. A subset of cells was stained against GAD67 to identify dACs (see above). The intensity of this staining was manually rated as follows: -2 (absent), -1 (probably absent), 0 (uncertain), 1 (probably present), and 2 (present). For each cluster, we computed the average staining from the labelled cells (average number of cells with GAD67 information per cluster:  $16.8 \pm 10.0$ , mean  $\pm$  s.d.). Clusters with an average staining <-0.2 were labelled RGCs (n = 30 clusters), those with average staining >0.2 were labelled ACs (n = 26). Clusters with average staining in-between those values (n = 5), or those that contained 6 or less cells with GAD67 information (n = 8) were labelled as uncertain, unless other clear criteria such as soma size or genetic labels indicated that they are ACs or RGCs. In this case they were manually allocated to RGC or AC (n = 3 and n = 2, respectively). Two clusters automatically classified as AC were included in the uncertain group due to their functional similarity with the OFF-suppressed types (G<sub>31</sub>). This procedure resulted in 33 RGC clusters, 10 uncertain clusters and 26 AC clusters.

Identification of alpha-RGCs. We extracted all cells with large cell bodies (>136µm<sup>2</sup>; mean + 1 s.d. of total soma size distribution; Extended Data Fig. 2i, j) from RGC and uncertain clusters. Predominantly, these cells had been assigned to nine of the clusters. We re-clustered those cells using a Mixture of Gaussians model as described above, resulting in 16 clusters (Extended Data Fig. 2j). Receptive field size was not used in this process. Five of these clusters could be clearly associated with the three known alpha-RGC types and their response profiles<sup>31</sup> (trans. OFF alpha, 2; sust. OFF alpha, 2; ON alpha, 1). Cells in these clusters were SMI-32-positive, as expected from alpha RGCs (Fig. 3i, k). Probably, this procedure missed some alpha cells, as somata close to the edge of scan fields were cut and we thus underestimate the soma size of these cells (for example, G<sub>5</sub>c, see Fig. 2a-c). Remaining cells were kept in their original cluster. Logistic regression was used to assess the effect cell type (alpha vs. mini) on SMI-32 staining (absent vs. present). We used the Matlab implementation fitglm with a binomial nonlinearity. 95%-confidence intervals on the proportion of SMI32-positive cells were computed using bootstrapping with 1,000 samples.

**Dendrogram.** We used a standard linkage algorithm on the means of the RGC groups in the standardized feature space with correlation distance  $d_{ij} = 1 - \operatorname{corr}(m_i, m_j)$  and average unweighted distance and plotted the result as a dendrogram (using Matlab functions linkage and dendrogram). The leaf order was optimized using the Matlab function optimalleaforder and modified for clarity of presentation.

Calculation of coverage factor. We calculated each group's coverage factor (CF)

$$\mathrm{CF} = \frac{n_{\mathrm{Cells}} \cdot A_{\mathrm{RF,median}}}{A_{\mathrm{Scan}}}$$

with the number of cells in a group ( $n_{\text{Cells}}$ ), the median RF size ( $A_{\text{RF,median}}$ ) within a group counting only cells that surpassed a RF quality criterion of 0.3, and the total scan area across all experiments ( $A_{\text{Scan}}$ ). We corrected  $n_{\text{Cells}}$  for 29% cells discarded

by our quality criterion as well as an empirically estimated 8% of cells that did not yield a ROI in the first place due to weak or absent labelling. In addition,  $A_{\text{Scan}}$  was corrected for an empirically estimated 34.8% RF overhang (that is, where a cell's RF exceeds the scan field edge). This procedure yielded a CF of  $2.0 \pm 0.7$  for most RGC groups (Gaussian fit; see Fig. 2e, right). However, differences between studies in approaches to measure RFs (for example, checkerboards vs. bars), in the assumptions used for RF fitting (for example, homogenous RFs best fitted by Gaussians), or in the methods to estimate dendritic arbor area can easily yield different absolute estimates of CF (see also Supplementary Table 1).

**Estimation of IPL stratification profiles.** To determine a cell's IPL stratification profile, we calculated dendritic density as described previously<sup>10</sup> with spatial smoothing of 1  $\mu$ m<sup>3</sup>. The resultant 3D density cloud was projected on the *z* axis to estimate the mean IPL depth profile. The relationship between the depth profiles and the two ChAT bands was estimated in independent experiments using mice that express tdTomato in cholinergic ACs (ChAT<sup>Cre</sup> × Ai9:tdTomato). We compared the IPL depth of the tdTomato-labelled dendritic plexi to the two SR101-labelled blood vessel planes that line the inner retina. We estimated the error to be ~1.5  $\mu$ m (s.d.), corresponding to 3–4% IPL depth (*n*=13 measurements in 2 mice).

To relate each cell's IPL profile to functional groups we calculated the mean correlation coefficient between a cell's response to the chirp and moving bar stimuli and each group's mean response. The correlation coefficient (-1...1) for each pair was then multiplied with the cell's depth profile and a correlation-rank based weighting factor  $W = 0.9^{rank-1}$ . Thus, each individual recording yielded a complete two-dimensional map, with IPL depth on one axis and functional group on the other. Next, we averaged across the maps for those cells that passed our response quality criterion ( $n_{\text{non-DS}} = 31/51$ ;  $n_{\text{DS}} = 24/33$ ; see above). The resultant matrices were normalized in two steps: First, we divided each group's IPL depth profile by the mean depth profile of all included cells to eliminate any bias in sampling depth. Second, we divided each depth profile by its own maximum. This resulted in an automatic and unbiased estimate of dendritic stratification depth for all RGC groups (Fig. 5). Note that this automated approach is based on a relatively small sample of reconstructed cells and therefore can only provide an approximate prediction of stratification levels. This approach is invariant to differences in lateral dendritic field dimensions that may be associated with retinal position (for example, refs 10,23).

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Extended Data Figure 1 | Linking electrophysiology and imaging data (related to Fig. 1). a, Simultaneously recorded RGC Ca<sup>2+</sup> (top) and spiking (bottom) activity in response to binary spatial dense noise stimulation. b, Average Ca<sup>2+</sup> event triggered by a single spike, averaged across n = 6 cells (shading indicates 1 s.d.); event decay was fitted (red) using a single exponential (for time constant  $\tau$ , see inset, mean  $\pm$  1 s.d.) to yield an estimated impulse response. c, A linear prediction of Ca<sup>2+</sup>

mean BGi

(calculated by convolution of the impulse response with binarized spike traces) was compared to measured values to estimate the mean nonlinearity. **d**,  $Ca^{2+}$  (top) and spiking (bottom) response to the full-field chirp stimulus (Methods) simultaneously recorded in an RGC (red trace,  $Ca^{2+}$  signal predicted from spiking response). **e**, Number of scan fields as a function of blue/green index (BGi, see Methods) averaged over all ROIs in each field (Fig. 1a).



Extended Data Figure 2 | See next page for figure caption.



Extended Data Figure 2 | Clustering and grouping (related to Fig. 2). **a**-**c**, Selection of cluster size and cluster quality/consistency analysis. a, Normalized Bayesian information criterion (BIC) curves for non-DS (black) and DS (blue) cells. Arrows indicate the optimal numbers of clusters. b, Rank-ordered posterior probability curves indicating cluster quality. Curves were normalized for cluster size and averaged for non-DS (black) and DS (blue) clusters separately. Shaded area indicates 1 s.d. across clusters. c, Histogram of median correlation between the original clusters and clusters identified on 20 surrogate data sets, created by repeated subsampling of 90% of the original data set (bootstrapping); for each cluster, the best matching cluster from the original clustering was selected. d, Heat maps of Ca<sup>2+</sup> responses to the four visual stimuli (see Fig. 1) of n = 11,210 cells from 50 retinas. Shown are raw data sorted by the response to the colour stimulus. Each line represents responses of a single cell with activity colour-coded such that warmer colours represent increased activity. e, Temporal features were extracted from the cells' light responses (Methods) and used for automatic clustering (d to f). f, Heat

maps showing clustered data (n = 72 clusters plus cells discarded based on signal-to-noise (S/N) ratio), with block height representing the number of included cells. g, Distributions of S/N (top) and GAD67 labelling (bottom) used to discard clusters and sort the remaining ones into retinal ganglion cells (RGCs), 'uncertain' RGCs and displaced amacrine cells (dACs). **h**, Heat maps showing n = 46 groups (divided into n = 32 RGC groups, including n = 4 'uncertain' ones, and n = 14 dAC groups; sorted by response similarities) after re-clustering of large-soma cells (alpha cell post-processing, see panels i, j). i, Distribution of region of interest (ROI) area (as proxy for soma size) for all cells classified as RGCs and 'uncertain' (g). Inset, same distribution but on a log-scale. Dashed line marks threshold to separating large-soma cells (Methods). j, Results of re-clustering of large-soma cells (from i): heat maps show light-evoked Ca<sup>2+</sup> responses to the four visual stimuli (see Fig. 1b). Clusters that resulted in new RGC groups are indicated; the remaining cells stayed with their original clusters.



Extended Data Figure 3 | Group overview—functional groups classified as 'uncertain' RGCs and displaced amacrine cells (dACs) in the mouse retina (related to Fig. 2). a, Clusters organized according to hierarchical trees (dendrograms, see Methods) and grouped based on functional similarity (see main text for details), resulting in n = 4 'uncertain' RGCs (top) and n = 14 dAC groups (bottom). b, Mean Ca<sup>2+</sup> responses to the four stimuli (see Fig. 1b) for each cluster. c, Histograms of selected properties, from left to right: ROI (soma) area, receptive field (RF) diameter (2 s.d. from Gaussian fit; see Fig. 1b and Extended Data Fig. 4),

DS and OS indices (DSi and OSi, respectively, Methods). For details on each cluster, see also Supplementary Figures 1: 40–49 ('uncertain'), and Supplementary Figures 1: 50–75 (dACs). **d**, Example experiment (left, from Fig. 1a); centre, dACs (lilac) and 'uncertain' RGCs (blue); right, colour-coded by broad categories, as in **e**. **e**, Total number of cells (top) and percentage of cells in sets of groups (bottom) per experiment (only experiments with  $\geq$ 198 cells) illustrating consistency across experiments. Scale bar, 50 µm.



**Extended Data Figure 4** | **Relationship between RGC receptive field centres and their dendritic arbors (related to Fig. 2). a**, **b**, Receptive field (RF) centre maps of a G<sub>8</sub> transient OFF alpha RGC (**a**) and a G<sub>2</sub> small-field RGC (**b**), with their reconstructed morphologies overlaid. 1- and 2-s.d.

contours of RF centres fitted with 2D Gaussians are indicated by blue and red ovals, respectively. **c**, Area of RF centre fits from **a**, **b** as function of dendritic arbor area (n = 18 RGCs). Scale bars, 100 µm.



**Extended Data Figure 5** | Mapping RGC groups onto genetic types functional diversity of PV- and Pcp2-positive RGCs (related to Fig. 2). a, b, Diversity of PV-positive RGCs (red) in a PV:tdTomato mouse retina electroporated with OGB-1 (a, green).  $Ca^{2+}$  responses and receptive fields (b) from six PV-positive cells in exemplary field are shown (black, mean response, grey, single trials). The top four cells could be clearly matched to RGC groups (see Fig. 2), whereas the remaining two (x<sub>1</sub>, x<sub>2</sub>) were discarded due to the lack of responses to both full-field and moving bar stimuli; note, however that both cells yielded a clear RF. c,  $Ca^{2+}$  responses of functionally distinct PV-RGC groups (20 response types  $PV_{a-b}$  thereof 14 with  $n \ge 3$  cells). Traces colour-coded by group assignment (colours as in Fig. 2) represent mean responses, with individual cell responses in grey. d, Same for Pcp2-positive (six response types  $Pcp2_{a-b}$  thereof three with  $n \ge 3$  cells) RGC groups. e, Table illustrating the relationship between RGC groups (Fig. 2) and functional PV- and Pcp2-positive RGC types from (c, d). Numbers represent the total cell count of each allocation. Names in quotes (for example, "PV5") refer to the cell's original names (see PV (ref. 45) and Pcp2 studies (ref. 56)).

# **RESEARCH ARTICLE**





**d**–**f**, Electrical single-cell recording of a G<sub>10</sub> cell: spiking responses as raster plots and mean spike rates for chirp, moving bar and blue/green stimuli as well as time kernel derived from noise stimulus (**d**), polar plot of responses to moving bar (**e**) and RF map (**f**). **g**–**i**, G<sub>28a,b</sub> (*n* = 100) contrast-suppressed ON RGCs with sample morphology (**i**; G<sub>28a,b</sub> cell dye-injected after Ca<sup>2+</sup> imaging). **j**–**l**, Electrical single-cell recording of a contrast-suppressed ON RGC with different morphology (**l** vs. **i**). **m**–**r**, G<sub>2</sub> direction-selective OFF RGCs (*n* = 162) that stratify between the ChAT bands (**o**), as fingerprint (**m**, **n**) and exemplary electrical single-cell recording (**p**–**r**). Scale bars, 50 µm; grey lines in **c**, **i**, **l**, **o**, ChAT bands.



**Extended Data Figure 7** | See next page for figure caption.



Extended Data Figure 7 | Direction and orientation selectivity (related to Fig. 4). a, Stimulus direction vs. time map for an exemplary directionselective RGC with temporal (top) and directional (right) activation profiles shown; singular value decomposition (SVD) was used to estimate the time course and tuning function; individual stimulus repeats in grey, average in black. **b**, Reconstruction of direction vs. time map based on time course and tuning function of extracted by SVD. c, Statistical significance testing for direction selectivity (DS) or orientation selectivity (OS) was performed by projecting the direction/orientation profile on a single (for DS) or double (for OS) period cosine (blue) and the magnitude of the projection to the distribution of projections obtained by randomly permuting tuning angles from the original data (grey; bootstrapping). The *P* value is obtained by computing the percentile of the data (blue) in the bootstrap distribution (grey). **d**, **e**, *P* values for direction (**d**) and orientation (e) tuning as a function of the respective selectivity index (top, scatter plot; bottom, histogram; black, non-DS cells; light blue, DS cells; dark blue, OS cells). Note that tuning probability ( $p_{DS}$ ,  $p_{OS}$ ) only partially

predicted tuning strength (DSi, OSi). f, Pairs of polar plots showing the distribution of preferred motion directions for all direction-selective (DS) RGCs together and for all DS RGC groups not shown in Fig. 4, (V, ventral; N, nasal direction; same group colour code as in Fig. 2). Top, plot of each pair: the cells' individual preferred directions, with line length representing DSi and line grey level  $p_{DS}$  (Methods). Bottom, plot of each pair: circular histogram of preferred direction. g, As for f but for orientation-selective (OS) RGCs. h-s, exemplary OS RGCs, illustrating the functional diversity within G17 (local ON trans. OS cells); none of them display strong full-field responses (h, l, p). A 'vertically tuned' ON OS cell (i, *left*) that shows little tuning to a dark moving bar (i, right; j, another example). Note the lobular structures bracketing the RF centre (coloured RF maps in k). m-o, Two examples for 'horizontally tuned' ON OS cells (m, n) with their respective RF maps (o). p-s, ON OS cell that shows weak tuning to bright moving bars (q) but strong OS to stationary bright and dark bars (r, left and right, respectively; Methods).



Extended Data Figure 8 | Retinal distribution of PV-positive cells in the PV<sup>Cre</sup> × Ai9<sup>tdTomato</sup> mouse line (related to Fig. 2). a, b, Density map (a) and magnified sample areas (b) illustrate PV-labelling anisotropy.

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**Extended Data Figure 9** | **Mapping RGC groups to morphologies. a-c**, Exemplary morphologies of RGCs filled after electrical recording or Ca<sup>2+</sup> imaging and subsequently clustered/sorted into specific RGC groups or discarded (c, right) based on their light-response S/N. Scale bars, 50 µm.





**Extended Data Figure 10 | RGC groups cover a basic feature space. a**, **b**, Relationship of four basic response indices of RGC groups. Disc area shows group size. Indices capture preference for stimulus polarity (ON–OFF index; Methods), for high vs. low temporal frequencies and contrasts (see below), as well as the full-field index (FFi; Methods), which reflects response preference for global (full-field chirp) versus local (moving bar)

stimulation. Contrast and frequency indices represent contrasts of feature activation  $((F_j - F_k) / (F_j + F_k))$  at respective time points during the full-field chirp stimulus, with j = 12, k = 9 for frequency, and j = 17, k = 15 for contrast. Before calculating ratios, feature activation (*F*) was normalized (0...1) by passing values through a cumulative normal distribution.