### Abstract

Comprehensive high-resolution structural maps are central to functional exploration and understanding in biology. For the nervous system, in which high resolution and large spatial extent are both needed, such maps are scarce as they challenge data acquisition and analysis capabilities. Here we present for the mouse inner plexiform layer—the main computational neuropil region in the mammalian retina—the dense reconstruction of 950 neurons and their mutual contacts. This was achieved by applying a combination of crowd-sourced manual annotation and machine-learning-based volume segmentation to serial black-face electron microscopy data. We characterize a new type of...
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ABSTRACT
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Connectomic reconstruction of the inner plexiform layer in the mouse retina

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Comprehensive high-resolution structural maps are central to functional exploration and understanding in biology. For the nervous system, in which high resolution and large spatial extent are both needed, such maps are scarce as they challenge data acquisition and analysis capabilities. Here we present for the mouse inner plexiform layer—the main computational neuropil region in the mammalian retina—the dense reconstruction of 950 neurons and their mutual contacts. This was achieved by applying a combination of crowd-sourced manual annotation and machine-learning-based volume segmentation to serial block-face electron microscopy data. We characterize a new type of retinal bipolar interneuron and show that we can subdivide a known type based on connectivity. Circuit motifs that emerge from our data indicate a functional mechanism for a known cellular response in a ganglion cell that detects localized motion, and predict that another ganglion cell is motion sensitive.

Information about neuronal wiring has long been the basis of formulating and testing ideas about how computation is performed by neural circuits. Complete and partial wiring diagrams are being used where available. Whether such diagrams can be created by statistical extrapolation or whether higher order connectivity is functionally important is highly controversial. The assumption that mingling neurites connect (Peters’ rule) allows connectivity to be inferred from light microscopic observations of sparsely stained tissue, but is frequently violated, showing that connectivity must be explicitly tested rather than inferred from proximity. Simultaneous electrical recordings from several cells can determine and quantify their synaptic connectivity, but do not allow a comprehensive sampling of connections.

Unlike light microscopy, electron microscopy can follow even the thin nest neurites through densely stained neuropil, and can detect unambiguously whether two cells touch and over which area. Serial section transmission electron microscopy was, for example, used to reconstruct the complete wiring diagram of the roundworm Caenorhabditis elegans and to study synaptic connectivity in the retina. Volume electron microscopy data sets hundreds of micrometres in extent have been used to reconstruct guided by previous functional imaging specific neural circuits.

The retina performs a variety of image processing tasks and is one of the best studied parts of the central nervous system. But, only in few cases, such as for direction sensitivity, has a volume of more than 1 million μm³, includes all layers that contain intra retinal synaptic connections, and was stained to enhance plasma membrane visibility, further facilitating traceability and automated segmentation.

Because completely labelling such a volume by hand would be prohibitively expensive (about US$10 million), we tried to establish an entirely automatic reconstruction pipeline. Our SBEM data can be automatically segmented into objects that represent the local cellular geometry accurately. But even at voxel error rates of a few per cent, cells get fragmented into many pieces. We used SBEM because a superior resolution and lack of image distortions makes SBEM data sets more easily traced by humans and segmented by computers. The main data set used in this study has a volume of more than 1 million μm³, includes all layers that contain intra retinal synaptic connections, and was stained to enhance plasma membrane visibility, further facilitating traceability and automated segmentation.

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Skeletors were created by a team of trained human annotators, which included, over time, more than 224 different students. First, the annotators identified all somata and classified them as photoreceptor (n > 2,000), glial (n = 173), horizontal (n = 33), bipolar (n = 496), amacrine (n = 407) or ganglion (n = 47) cells, based on soma location and emerging neurites (Fig. 1a). Starting from the soma, the annotators skeletonized the neurites of all glial, bipolar (Fig. 1b), amacrine and ganglion cells using the KNOSSOS program—http://www.knossostool.org. Multiple tracings by different annotators (average redundancies: 6,
4 and 4 for ganglion, amacrine and bipolar cells, respectively), were automatically consolidated<sup>34</sup>, visually inspected and, in a few cases, manually corrected. A total of >20,000 annotator hours yielded 2.6 m of skeletons, representing 0.64 m of neurite, with estimated<sup>35</sup> error rates of 9, 12 and 6 per ganglion, amacrine and bipolar cell, respectively.

**Cell types**

We classified all neurons into cell types by visual inspection of the bare skeletons, with a focus on the IPL. We found 459 almost complete bipolar cells (Fig. 1c; all reconstructed types and cells are shown in Supplementary Data 1 and 6, respectively). Most bipolar cells clearly belonged to one of the 10 types described previously<sup>36</sup> (Fig. 1c). However, particularly for OFF cone bipolar cells (CBCs) (1-4), some classification ambiguity remained, even after taking into account tiling. A random re-examination of 59 ON CBCs (CBCS 9) found one error.

Seven cells showed no similarity to any of the ten bipolar types<sup>36</sup>, but shared a distinct morphology and were designated as XBCs (Fig. 1d and Supplementary Data 2a). XBC axons stratify more narrowly but at the same average depth as CBCS (Fig. 1d, e). Laterally, XBC axons roam widely, similar to CBC9, but their dendrites are comparatively compact, different from CBC9 (Supplementary data 2b), and their depth suggests that they contact cones.

The dendrites of all ganglion cells and of many amacrine cells extended beyond the data set volume. Many ganglion and amacrine cells could nevertheless be grouped by inspecting their neurites (12 ganglion cell types, Fig. 2a; 12 narrow field amacrine cell types, Fig. 2c; d; 33 medium/wide field amacrine cell types, including 6 displaced types, Fig. 2e, f and Supplementary Data 1 and 6). We used the type averaged (for individual variations see Supplementary Data 1) neurite density over depth in the IPL (Fig. 2) to create for all amacrine and ganglion cell types unique identifiers (ac64 73, for example, is an amacrine cell type with first and third quartiles at 64% and 73% IPL depth, respectively). Prominent among cell types previously known (see Supplementary Data 7 for a complete listing) are gc30 63, ac25 31 and ac60 65, corresponding to ON/Off direction selective ganglion cells<sup>29</sup> (DSGs; Fig. 2a) and ON and OFF starburst amacrine cells<sup>16</sup> (SACs; Fig. 2e), respectively.

**Contact detection**

We next combined the skeletons with an automatic segmentation (Fig. 3), created by first training a convolutional network to detect cell boundaries<sup>37</sup>, followed by several grow and merge steps (Fig. 3a). The final volume consolidation into a representation of the cellular geometry was performed by combining for each cell all segments overlapping its skeleton (Fig. 3b, typically several hundred segments; total estimated volume error rate about 3%, see Methods).

Of 1,123 fully volume reconstructed cells, 173 were glia, 110 were orphans (one of a kind cells or cells without a reasonable neurite morphology), and 840 were the neurons used in the analysis. All contacts (n = 579,724) between them were automatically detected and quantified (Fig. 3c, Supplementary Data 5 and Methods). When testing...
Figure 3 | Automatic segmentation and contact detection. a, From left: raw data (offset and contrast adjusted), edge classifier (synapse average), initial and iterated segmentations (see also Supplementary Data 3e, g). b, From top: bare skeleton, skeleton with overlapping segmentation objects, and the resulting volume representation for a CBC6 axon. c, Automatically detected contact (red arrow) between a CBC5 cell and a DSGC (gc30 63). d, Cross sections through a non-synaptic contact (left, Supplementary Data 3a, b) and a ribbon synapse (right) from data set k563, coloured by hand. e, Frequencies (bottom) of non-synaptic (red, n = 63) and synaptic (green, n = 30) cell–cell contacts versus contact area and the Gaussian fits to them (thin lines, centre/width: 0.18/0.38 and 0.22/1.13, all in μm²) and the resulting synapse probability (syn. prob.) estimate (top). Scale bars, 1 μm (a), 500 nm (d).

Connectivity-based type classification

We next explored whether comprehensive contact information contained in the cell–cell matrix can be used to discriminate between otherwise very similar cell types. When we searched for a way to divide the CBC5s, which fall into two molecularly distinguishable classes in rats and are too numerous for a single class in mouse, by using their connectivities to ganglion cells and amacrine cells, gc31 56 and gc36 51 emerged as potential discriminators (Fig. 5a). A reasonably complete tiling pattern resulted (Fig. 5b) when including only cells (n = 22) contacting gc31 56 more strongly than gc36 51 (the exception was a single cell, which was near that threshold but was not included to avoid strong axonal overlap, asterisk in Fig. 5a). This group of cells, ‘CBC5A’, also shows a strong repulsion between their dendritic centroids (Fig. 5c), indicating a mosaic and hence a pure type, and is specifically avoided by ac43 49 (Fig. 5a). The remaining 37 cells (‘CBC5SR’) still show strong axonal overlap, lack a mosaic (Fig. 5b, c), and are thus probably a mixture of types for which we did not, however, find a connectivity-based discriminator. The depth profiles of CBC5A (first and third quartiles: 54% and 61%) and CBC5SR (50% and 59%; Fig. 5d) seem to be different. Ten cells did not overlap the dendrites of both ganglion cell types (Fig. 5b) and were therefore collected into a separate group (‘CBC5X’).

XBC circuits

We next investigated how the XBC is integrated into the IPL circuitry (Fig. 6a, c). Like RBC and CBC7, XBC devotes less of its contact area to ganglion cells than the average bipolar cell (Fig. 6a). XBC strongly contacts (Supplementary Data 2b) medium-wide field amacrine cells ac38 56 (15.5%) and ac53 59 (7.1%), of which ac53 59 shares the XBC sharp depth profile (Fig. 6b) and, in turn, makes contact with gc31 56 (3.5%) and gc47 57 (4.2%). Those ganglion cells, however, receive only minimal amounts (0.9% and 0.4%) of their contacts directly from the XBC, even though their dendrites strongly overlap XBC axons in depth (Fig. 6b). Instead gc31 56 receives direct bipolar cell contacts mainly from CBC5A (7.0%) and gc47 57 from CBC5R (12.0%), ac38 56 is bistratified, overlapping in the ON stratum with the XBC and in the OFF stratum with gc35 41 (Fig. 6b, c), which is clearly an OFF cell (contacting CBCs 3A, 3B and 4, with 5.4%, 6.3% and 5.4%, respectively; all other CBCs are at most 0.5%) and receives 10.0% of its contacts from ac38 56.

ON/OFF ganglion cell circuits

Some of the best studied ganglion cells respond to both ON and OFF stimuli. We therefore analysed the connection patterns onto several...
ganglion cells that ramify in both ON and OFF layers (Fig. 6d f). Among those, gc36 51 ("W3a") and gc44 52 ("W3b") are consistent with cells labelled in the TYW3 mouse. Either or both are likely to be homologous to what is called the 'local edge detector' in rabbit (Fig. 6d). Their contact patterns with CBCs are mostly similar (gc36 51/gc44 52: CBC5R, 7.5%/11.5%; CBC5A, 1.3%/0.8%; CBC4, 3.0%/3.5%; CBC3A, 1.7%/1.8%; and CB3B, 3.2%/1.7%; Supplementary Data 1), with the exception of the outermost part of the inner nuclear layer (INL) (CBC2, 1.5%/0.1%, and CBC1, 1.6%/0.1%). Substantial contacts are made by gc36 51 and gc44 52 with several narrow field amacrine cells, ac52 90 (6.0%/2.8% (ref. 37), A2), ac21 67 (3.8%/2.1%), ac51 70 (3.5%/5.0%) and ac21 44 (3.3%/2.2%). The strongest amacrine cell contact made by gc36 51 is with ac43 49 (6.8%), which straddles the boundary between ON and OFF layers (Supplementary Data 1), and also substantially contacts ac43 49 (5.6%) as well as ON and OFF bipolar cells (CBC5R, 9.3%, and CBC4, 5.0%). ac43 49 is one of two medium/ wide field amacrine cells that degrade most of their contacts to gc36 51 and gc44 52 (Supplementary Data 1). The second is ac45 54 (7.0%/6.2%), a cell dominated by ON CBCs (7.9% with CBC5R compared to 1.3%, 2.0% and 1.2%, with CBCs 3A, 3B and 4, respectively).

The ON/OFF DSGC (gc30 63, Fig. 6f), as expected, strongly contacts SAGs (9.2% and 11.4%, for gc21 35 (OFF SAC) and gc50 65 (ON SAC)), but substantial contacts from other medium/ wide field amacrine cells are conspicuously absent (ac34 84, 2.5%, all others < 1.6%). Like gc36 51/gc44 52 (W3a/b), the ON/OFF DSGC prefers CBC5R (6.9%) to CBC5A (1.9%, all other ON CBCs at most 1.1%). Its main OFF 'input' comes from CBC3 (4.2%) and CBC5 (A/B, 3.0%/2.7%). SACs make most contacts (Fig. 6e) among themselves (26.6% and 21.4% for ON and OFF). They discriminate less than the DSGC between CBC5R (9.7%) and CBC5A (5.0%), but, most notably, contact...
Figure 6 | Circuits originating from the XBC and ON/OFF cells. a, IPL circuitry from the XBC (a-c) and from three ON/OFF cells (d-f). a, Fractional contact areas between all ganglion cells and each bipolar cell. b, Depth profiles. c, XBC circuit schematic. d, One gc36 51/W3a (cell 16), one ac43-49 (cell 307), one CBCSR (cell 578) and all the detected contacts between (cyan spheres, volume proportional to contact area). e, Normalized contact areas for amacrines and bipolar cells with both SACs (ac25 31, blue; ac60 65, red). f, Circuit diagrams. Arrow width in circuit diagrams proportional to (total contact area between types)**. Only connections with areas per type > 30 μm² shown.

**CBC7 (5.1%), which is largely ignored by the DSGC (1.1%, Fig. 6f). Similar differences are seen for the OFF sublamina: DSGC and SAC contact strengths to CBC1/CBC2 are 1.4%/0.5% and 4.7%/3.1%, respectively.

Our last example is the analysis of a cell not associated with any known type in mouse but possibly homologous to a rabbit retina³⁸. ON/OFF ganglion cell gc31 56 is an ON/OFF cell by stratification (Fig. 2a, b), filling the space between the SAC bands (Fig. 2a, e) and ‘connects’ strongly to both SACs (ac60 65, 5.4% and ac25 31, 7.1%). Surprising is the strong imbalance between ON and OFF bipolar cell ‘input’ (7.0%/3.7% for CBC5A, but only 0.8%, 0.7%, 0.9%, 1.5% and 1.2%, for CBC1, 2, 3A, 3B and 4).

**Discussion**

Our comprehensive analysis of the bipolar cells confirmed the existence of the ten bipolar cell types previously identified³⁸, and revealed the existence of the XBC, which had not emerged even in large genetic screens³⁸. Although sharp stratification and large size (Fig. 1d, e and Supplementary Data 2a, also note the similarity to cluster 6 in ref. 40) suggest homology between the XBC and the giant bipolar cell described recently in the primate retina, the small size of the XBC dendrites relative to its axonal arbour argues against it. The functional role of the XBC is unclear. Its sparseness suggests low spatial resolution and its small dendritic fields suggest that it does not collect signals from all cells of one cone type, thus potentially forgoing some amount of signal. Curious is the absence of a bipolar cell with a similarly sharp stratification on the OFF side. Instead, we find an inter layer connection via the symmetrically bistriated ac38 56 (Fig. 6b, c). One might speculate that the XBC is part of a luminance adaptation pathway.

Dense sampling and the complete high resolution reconstruction of neurites as is only possible with three dimensional electron microscopy data, contributes in several ways to cell type classification. First, when all cells of a class, for example, all bipolar cells, are reconstructed, no type will be missed and the prevalence of different types can be determined precisely (Fig. 1e, inset). Second, differences in neurite geometry can be compared for cells within the same piece of tissue. For almost all bipolar cells and a substantial fraction of ganglion and amacrine cells, it was thus possible to establish a correspondence to cell types described in the literature (Supplementary Data 7). We generally erred on the side of splitting groups and expect that some groups actually belong to the same type (for example, the similar connectivity to the XBC suggests that ac38 56 and ac37 52 could be the same type; Supplementary Data 4). Third, even if they cannot be selectively stained and imaged, tiling and mosaic formation (both used to assess purity of type³⁸) can be easily assessed (Figs 2b and 5b and Supplementary Data 1). Fourth, complete contact information can confirm or refine the definition of types (Fig. 5), and may ultimately become sufficient for classification all by itself⁴².

Because of the constrained size of our data set, many amacrine cell and all ganglion cell neurites are truncated, and many larger neuron types are presumably completely missed³⁸. Advances in volume electron microscopy technology now make it possible to acquire volumes with a lateral extent of at least 500 μm. One might then, using the same tools and a similar manual annotation effort as were used in our study, densely reconstruct a central region of 100 μm in extent and trace neurites of passage far enough into the periphery to determine their cell type.

Although our analysis provides contact areas and not synaptic strength, the absence of contact always indicates a lack of synaptic connection. The absence of contacts between some cell types, for example, XBC and gc35 41 as well as CBC7 and DSGC, the neurites of which mingle extensively, confirms that Peters’ rule is routinely violated. Furthermore, it seems that large contacts are quite likely to be synaptic (at least between bipolar cells and ganglion cells; Figs 3e and 4d). Although we have not used them here, other geometric parameters describing contact shape might provide enough additional
information to identify actual contacts with near certainty for many types of synapses.

It has been our consistent experience that selectively enhancing cell surface contrasts simplifies manual tracing and enables automatic volume segmentation. If recent results that suggest that even conventionally stained tissue can be reliably traced by hand (K.L.B. and M.H.J., unpublished observations) and automatically segmented (M. Berning and M.H.J., personal communication) are confirmed it may no longer be necessary to trade traceability for synapse identification.

The reliability of the entries in the contact matrix depends on several factors. Likely dominant are neurite continuity errors, which occur roughly six times per bipolar cell but presumably mostly in the periphery and thus should cause only a small fractional loss (or false addition) of synapses. Local volume reconstruction seems to be fairly reliable. Finally, although not all contacts are synaptic, there are, typically, many contacts between any actually connected pair of cells, making it unlikely that any strong connection is spurious. The connectivity estimate between C5CR (38 cells) and W3a (gc36 51, 3 cells), for example, is based on the areas of 1,358 observed contacts, for which our simulation predicts between 278 and 705 synaptic contacts (fifth and ninety-fifth percentiles, respectively) with a median of 483 contacts, that is, 13 per bipolar cell and 161 per ganglion cell. The direction of a potential synaptic connection can in most cases not be determined by visually inspecting the e2006 data set but contacts onto a ganglion cell, for example, are presumably never postsynaptic.

Our analysis of three ON/OFF layer cell types has several concrete functional implications, which, at the very least, will guide further concentrated on the addition) of synapses. Local volume reconstruction seems to be fairly reliable. Finally, although not all contacts are synaptic, there are, typically, many contacts between any actually connected pair of cells, making it unlikely that any strong connection is spurious. The connectivity estimate between C5CR (38 cells) and W3a (gc36 51, 3 cells), for example, is based on the areas of 1,358 observed contacts, for which our simulation predicts between 278 and 705 synaptic contacts (fifth and ninety-fifth percentiles, respectively) with a median of 483 contacts, that is, 13 per bipolar cell and 161 per ganglion cell. The direction of a potential synaptic connection can in most cases not be determined by visually inspecting the e2006 data set but contacts onto a ganglion cell, for example, are presumably never postsynaptic.

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METHODS SUMMARY

Tissue preparation for SBEM. The retinae for the e2006 and k563 data sets were described as prepared previously. SBEM imaging and data analysis. The sample was mounted in a custom built ultra microtome operating inside the chamber of a field emission scanning electron microscope (FEI Quanta200), and serial block face imaged under 130 Pa hydrogen, at 3keV landing energy, a dose of 14 electrons per nm², and a resolution of 16.5 x 16.5 x 25 nm (for the conventionally stained sample, see Methods). A custom designed back scattered electron detector was used. SBEM data were aligned and stitches using custom Matlab routines. Skeletons were manually traced by trained student annotators using custom written software (KNOSOS, http://www.knosostool.org) and consolidated using RESCOP. Volumes were traced using KLEF (M.H. et al., manuscript in preparation). Boundary classification was with a five hidden layer convolutional neural network that was trained with the MAJS procedure (S.C.T. et al., manuscript in preparation). Segmentation used a 15 step iterative growth procedure, followed by a 6 step merging procedure. Data visualization was in KLEF, Knossos, Matlab, Mathab, matica and Amira.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.


Author Contributions M.H. and W.D. designed the study. KLB produced the samples and acquired the data using a microtome designed by W.D. W.D. analysed the data with minor contributions from W.D. and Y.R. M.H. developed the boundary classifier. M.H., KLB and W.D. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this paper. Correspondence and requests for materials should be addressed to M.H. (mheilmstaedter@neuro.mpg.de).
provide translation between, respectively, the different indices for individual cells and between type indices, type identifiers, and common type names.

The classification of the cells proceeded as follows. The neurite ramification pattern in the IPL, particularly its distribution along the light axis and its overall lateral size, was used first. We did not usually comment when cells obviously clustered into a type by those criteria (for example, types 9, 33 and 51). Unless otherwise indicated, percentage numbers represent position along the light axis. As the boundaries of the IPL (0%, 100%), we defined the points where the total skeleton density falls below 15% of its maximum. We use the point where the skeleton densities of ON and OFF bipolar cells cross over (46.5%), as the ON/OFF boundary. In some cases (types 58-62, corresponding to CBC4, and in one case for the CBC5A versus CBC5RS distinction) we used, in addition, tilting (the lateral overlap between neurites in the plane of the retina).

First, we identified the ON and OFF SACs (types 33 and 51). We next considered all remaining cells that had their somata in the GCL. Because we were initially not sure how reliably the axon could be detected, we did not use the presence of an axon as a criterion to distinguish ganglion cells from displaced amacrine cells. In all but one of the cells classified as ganglion cells an axon was found eventually. We begin with the actual ganglion cells (types 12), postponing the discussion of displaced amacrine cells (types 27, 43, 51 and 56-57).

There are three clearly bistratified ganglion cell types (2, 8 and 9) that extensively ramify in both ON and OFF layers. Only type 2 has one of its branches only adjacent to the IPL, whereas the lowest branch of type 8 is well separated from the INL. Additional discrimination was provided by bands at 50% and 70% for types 2 and 8, respectively. Only one of the type 8 cells shows all aspects of the dendritic tree, whereas the other two cells are presumably missing parts of the dendrite inside the reconstructed volume but share enough features to put them into the same class. Type 9 is the ON/OFF DSGC.

Type 6 could be called bistratified but the space between the bands still contains a lot of neurite. The two bands are just inside the choline acetyltransferase (ChAT) bands, which is where the SACs (types 33 and 51) and DSGCs (type 9) ramify. Types 7 and 10 both have only one band straddling the ON/OFF border, but 7 has numerous branches going all the way to the INL. Types 7 and 10 probably correspond to the two subtypes labelled in the TYW3 montage.

Next we considered cells that ramify mostly in the OFF (types 1, 3, 4 and 5) or the ON layer (types 11 and 12). Among those, only types 1 and 3 ramify all the way up to the INL (a slender dendritic resemblance to type 27, a displaced amacrine cell, can be resolved by looking at the lateral (in plane) branching pattern, which is much more tortuous for 27). Type 1 has multiple branches emerging directly from the soma but type 3 only has a single one. Type 5 has a much denser in plane branching pattern than type 4. Type 11 ramifies further towards the GCL than type 5 and is broader than type 10. Type 12 is the only ganglion cell ramifying in a single band adjacent to the GCL.

Among the amacrine cells we started with the narrow field types (13-24). Types 18, 20 and 24 reach deep into the ON layer and have bands in both the ON and the OFF layers, which was used to separate them from 23 and 22, with no bands in the OFF layer, even though the variability of the OFF band in type 20 made it difficult to distinguish type 20 and 22 cells, possibly causing some misclassification. Type 18 shows a sharp band at about 70% and a broader band touching the INL. Types 13 and 14 were difficult to distinguish, but 14 has a clearer gap to the INL and a less dense dendrite. Types 16 and 17 differ in lateral size. Some overlap between 16 and 15 cannot be completely ruled out but most type 15 cells are shorter and end mostly in a dense band. Types 19 and 21 differ in lateral size (21 and 42 may be the same type).

Next we considered cells (types 25, 28, 30, 32, 37, 39, 41, 47, 53 and 57) in which the branching pattern suggested wide fields, for example, because only few of their branches only adjacent to the INL. Many of these cells (types 25, 37, 39, 41, 47 and 53) show a sharp laminar in depth. Only type 25 ramifies close to the INL. Type 30 is more strongly branched than 28 and ramifies broadly in depth, unusual for wide field cells. Type 28, unlike type 30, has two branches leaving in opposite directions. Type 31 dendrites, uniquely among the cells reconstructed, go off into a narrow segment. Type 32 ramifies in the OFF ChAT band, but branches differ from those of the OFF SAC (type 33). Type 39 has only a single primary branch, whereas type 41, which stratifies at almost the same depth, has several. Note that types 37, 41/39 and 47 subdivide the space between the ChAT bands into three equal sublaminae.

The remaining amacrine cells are medium field cells (types 26, 27, 29, 33, 36, 38, 40, 42, 46, 48, 52 and 54-57), including the remaining ON SACs (types 33 and 51, see above). Types 34 (an interplexiform cell), 49 and 52 similarly branch all the way across the IPL. Type 49 has the very distinctive ‘waterfall’ anatomy and type 52 lacks the sharp band right outside the INL of type 34. Types 35 and 38 were distinguished by how far their dendrites reached towards the GCL. Types 48 and...
50 differ in primary dendrite shape and in plane size but may still be the same type. Type 45 has more primary dendrites than type 54.

To classify bipolar cells (types 58 71) we first tried to establish similarity to the types described previously. The correspondence was mostly obvious for RBCs (type 71) and ON CBCs (types 63 70) see the main text for CBCs (types 63 65) and XBC (type 66) but rather difficult for OFF CBCs (types 58 62).

First, all OFF bipolar cells were sorted using the seventy fifth percentile of the cumulative skeleton density in depth (starting at 0% then, the lower 58.2% (their prevalence; see Table 1 in ref. 28) of cells were placed in the CBC3A/3B/4 and the remainder into the CBC1/2 category. The former was then sorted by the twenty fifth percentile. Because this distribution was not clearly separable (consistent with the CBC4 width being smaller and more variable than drawn previously), we began to collect the CBC4 cells starting at the highest twenty fifth percentile numbers, adding cells consistent with the mosaic until the required prevalence was reached. The same procedure, now using the axonal coverage area, was used to separate CBC3A from B, reported to be larger than the twenty fifth percentile. A file (128 voxels) was used as the initial training data, which was gradually augmented by CBC3A (ref. 28), and CBC1/2 using the spread in depth of the axon (twentieth to eightieth of the volumes (each 517 voxels), which resulted in 0.6\% 2,000 400, 0.6; 2,000 800, 0.6; 2,000 1,600, 0.6; 2,000 1,600, 0.6; 5,000 2,000, 0.6; 10,000 3,000, 0.6; 20,000 4,000, 0.6; 25,000 5,000, 0.6; 30,000 6,000, 0.6.

This increased the average segment size to 2,443 voxels. Segments were then assigned to that skeleton that had the most nodes in the segment (only a small fraction contained nodes from more than one skeleton). All segments assigned to a skeleton comprise the volume reconstruction of the corresponding cell. The volume fraction erroneously assigned was estimated by summing the volume of all segments that contained multiple skeletons, weighted by the fraction of minority nodes in the segment and divided by the total volume of segments assigned to any skeleton.

Contact detection. To quantify contacts between segments, segment to segment overlap matrices were calculated between the original segmentation and versions shifted by one voxel, respectively, in the x, y and z directions. The resulting three collections of overlapping voxels were combined and classified and grouped into ‘contacts’ (Supplementary Data 8) using a dilation based proximity measure. The contact areas were calculated using the following weights (nm²) depending on the combination of direction sets they occurred in: 412.5 (x or y), 272.25 (z), 583.3631 (x and y), 494.2432 (x or y) and 2,643.7644 (x, y and z). This corrects for the anisotropy in voxel size and to some extent for the error introduced by the angle of the contact surface. For surfaces perpendicular to one of the principal axes, the face diagonals, or the space diagonal this estimate is exact.

Error estimation. To probe the frequency of missed contacts (false negatives) we selected 100 random locations on one skeleton (cell 17, gc36 51, W3) and searched for true contacts with an, according to the cell cell matrix, highly connected cell (cell 344, ac34 84). All 16 true contacts found were also found by the automated detection routine. To estimate the false positive rate we randomly selected 20 of the 7,217 contacts that the same gangleion cell made with other cells and visually inspected the corresponding locations in the raw data. In one case no actual contact existed (a piece of debris was erroneously attributed by the segmentation routine to cell 344).

Sizes for synaptic and non synaptic contacts. Synaptic and non synaptic contacts in the conventionally stained data set (563) were selected and their contact area determined in one of two ways. (1) Starting from a bipolar cell axon terminal, a synaptic ribbon was located (Fig. 3d), the two postsynaptic dyadic partners were found and their class determined, using the presence or absence of synaptic vesicles (found in amacrine but not ganglion cell dendrites). All three dyadic partners and, in addition, a nearby non synaptic contact were manually reconstructed using the KLEE software tool (M.H. et al., manuscript in preparation) in a region including all three contacts. The contact areas were determined as follows. Surface triangulations were generated for each volume reconstruction, then for each triangle it was determined whether there was another object within 144 nm above it, next the contact area with this object was calculated as the sum over all hits in that object weighted by the triangle areas. (2) All contacts with bipolar cells were reconstructed on several pieces of ganglion cell dendrite, quantified, and classified as synaptic when a ribbon was present and non synaptic otherwise. Classification, segmentation and contact detection were performed independently for each member of a set of overlapping cubes (257 voxels on a side), one cube for each interior data cube (128 voxels on a side). Each of those cubes overlays one data cube completely and 26 cubes partially. To avoid double counting, we counted a contact only when the largest part of the contact was inside the completely overlapped (central) data cube.
