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Double cones and the diverse connectivity of photoreceptors and bipolar cells in an avian retina

Avian double cone and bipolar cell connectivity

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Abstract

Double cones are the most common photoreceptor cell type in most avian retinas, but their precise functions remain a mystery. Among their suggested functions are luminance detection, polarized light detection, and light-dependent, radical-pair-based magnetoreception. To better understand the function of double cones, it will be crucial to know how they are connected to the neural network in the avian retina. Here we use serial sectioning, multi-beam scanning electron microscopy (ssmSEM) to investigate double cone anatomy and connectivity with a particular focus on their contacts to other photoreceptor and bipolar cells in the chicken retina. We found that double cones are highly connected with neighbouring double cones and with other photoreceptor cells through telodendria-to-terminal and telodendria-to-telodendria contacts. We also identified 15 bipolar cell types based on their axonal stratifications, photoreceptor contact pattern, soma position, and dendritic and axonal field mosaics. Thirteen of these 15 bipolar cell types contacted at least one or both members of the double cone. All bipolar cells were bi- or multistratified. We also identified surprising contacts between other cone types and between rods and cones. Our data indicate a much more complex connectivity network in the outer plexiform layer of the avian retina than originally expected.

Significance statement

Like in humans, vision is one of the most important senses for birds. Here, we present the first serial section multi-beam scanning electron microscopy dataset from any bird retina. We identified many previously undescribed rod-to-cone and cone-to-cone connections. Surprisingly, of the 15 bipolar cell types we identified, 11 received input from rods and 13 of 15 received at least part of their input from double cones. Therefore, double cones seem to play many different and important roles in avian retinal processing, and the neural network and thus information processing in the outer retina is much more complex than previously expected. These fundamental findings will be very important for several fields of science, including vertebrate vision, avian magnetoreception, and comparative neuroanatomy.

Introduction

The visual system of birds is one of the most complex among vertebrates and its importance for birds is reflected by the size of their eyes occupying up to 50% of the cranial volume (Waldvogel, 1990), their large number of retinal neurons (Seifert et al., 2020) and the presence of one or two foveas and/or a visual streak in the retina of many avian species (Wood, 1917). It is thus surprising that little is known about the anatomical ultrastructure and the connectivity of avian retinal cells.

Beside rods, birds have four types of single cones enabling tetrachromatic vision (Hart, 2001). Additionally, birds have double cones which often make up 40% of the cone population in the retina. Double cones are common among vertebrates except for eutherian mammals, elasmobranches and catfish (Walls, 1942; Ebrey and Koutalos, 2001). In contrast to some fish double cones, both members of bird double cones contain the same long wavelength sensitive opsin (LWS) (Hart, 2001). The principal member contains one oil droplet whereas the accessory member can have several oil droplets which can differ in size and number (Pedler and Boyle, 1969). In general, oil droplets act as long-pass filters and can have different absorption properties (Stavenga and Wilts, 2014).

The function of double cones is still debated (Seifert et al., 2020) and may differ between vertebrate classes. In fish, double cones have been implicated in colour vision due to different opsins in the individual double cone members (Ebrey and Koutalos, 2001). Additionally, they might be involved in polarisation vision since they form mosaics with neighbouring double cones being oriented 90° to each other (Horváth, 2014). Double cones in birds could play a role in movement detection, because the spectral sensitivity of motion-sensitive cells is comparable to the spectral sensitivity of birds' LWS pigments expressed in double cones and long wavelength single cones (Jones and Osorio, 2004; Osorio and Vorobyev, 2005). Furthermore, behavioural conditioning experiments link double cones to mediating fine pattern recognition (Lind and Kelber, 2011).

Additionally, double cones were suggested to be involved in light-dependent, radical-pair-based magnetoreception (Zapka et al., 2009; Hore and Mouritsen, 2016; Günther et al., 2018) which is

important for orientation and navigation (Wiltschko and Wiltschko, 1995; Kishkinev et al., 2015; Chernetsov et al., 2017; Mouritsen, 2018). Double cones of several bird species including chicken express cryptochrome 4 (Günther et al., 2018) which could be the primary magnetic sensory molecule as it binds the crucial FAD co-factor (Hore and Mouritsen, 2016; Zoltowski et al., 2019). Double cones could be suitable locations for magnetoreceptive molecules since light intensity and polarization information could be separated from the magnetic field information by having two sensors oriented differently in each member of double cones (Worster et al., 2017).

Fundamental anatomical insights into pigeon photoreceptors were provided by Mariani and Leure-du Pree (1978) showing e.g. that rods and double cones stratify in the first (outermost) stratum of the tri-stratified outer plexiform layer (OPL) which was also found in chicken (Tanabe et al., 2006). Furthermore, electron microscopic data from chick retina suggest a coupling of both members of a double cone through junction-like structures (Nishimura et al., 1981). However, a systematic structural analysis of double cones is missing. Two studies identified bipolar cell types in the chicken (Quesada et al., 1988) and pigeon retina (Mariani, 1987). Although these studies provide important knowledge about photoreceptors and bipolar cells in the avian retina, information on potential photoreceptor/photoreceptor and bipolar cell/photoreceptor connections are lacking.

Since these classic electron microscopic studies, electron microscopic techniques underwent a constant development (for review see Peddie and Collinson, 2014). By using a new development, namely serial sectioning multi-beam scanning electron microscopy (ssmSEM) (Eberle and Zeidler, 2018), the aim of the present study was to provide a detailed analysis of the double cones' anatomy and their connections to other photoreceptor and bipolar cell types. Furthermore, we reconstructed 74 complete bipolar cells through which an extended bipolar cell type classification emerged.

Materials and Methods

Animals

105 One-week old domestic chickens (*Gallus gallus domesticus*) were hatched in the teaching and research
106 station Frankenforst (Königswinter, Germany). All animal procedures were approved by the Animal
107 Care and Use Committees of the Niedersächsisches Landesamt für Verbraucherschutz und
108 Lebensmittelsicherheit (LAVES, Oldenburg, Germany) and the Landesamt für Natur, Umwelt und
109 Verbraucherschutz Nordrhein-Westfalen (LANUV, Recklinghausen, Germany). The age of an
110 experimental animal may have a crucial influence on the morphology and connectivity of the analyzed
111 cells. However, retinal cell type differentiation and synaptogenesis in the plexiform layers in chicken
112 already starts at an embryonic stage (Drenhaus et al., 2003, 2007), and major receptor types can
113 already be detected at E12 (Hering and Kröger, 1996), for review see (Mey and Thanos, 2000).
114 Therefore, the cell development in our one-week old chicken is completed and the synaptogenesis is
115 advanced, but it is unclear whether it is entirely completed.

116 **Sample preparation for electron microscopic recordings**

117 Birds were killed by decapitation and eyes were removed immediately. Lens apparatus and vitreous
118 body were removed, and eyecups were fixed in a 0.08 M cacodylate (Sigma Aldrich, St Louis, USA)
119 buffer (pH=7.4) containing 2.5% paraformaldehyde (Carl Roth, Karlsruhe, Germany) and 1.25%
120 glutaraldehyde (EMS Hatfield, USA) for 30 minutes at room temperature. Retinas were removed from
121 the eyecup and transferred into a 0.08 M cacodylate buffer (pH=7.4) two times for 15 minutes each.

122 Basic staining procedure was performed after Hua et al. 2015. Before embedding, the tissue was
123 dehydrated through a graded ethanol series (50%, 75%, 100%, 30 minutes each at 4 °C), followed by
124 washing three times in 100% anhydrous acetone (VWR, Radnor, USA) at room temperature for 30
125 minutes each. For epon infiltration, the tissue was first incubated in 1:2 mixture of anhydrous acetone
126 and Embed812 resin hard formulation (20 ml Embed812, 9 ml DDSA, 12 ml NMA and 0.72 ml DMP-30;
127 EMS) over night at room temperature followed by 8 h incubation in pure Embed812 resin at room
128 temperature. Retinas were cut into smaller pieces and position within the retina was mapped before

transferring the smaller tissue pieces into embedding moulds (Ted Pella) for polymerisation at 70 °C for 48 h.

Sample sectioning and data acquisition for electron microscopy

After polymerisation, a piece of the dorsal periphery in the left eye was pre-trimmed with a hand saw and afterwards trimmed to a block face of approximately 900 µm x 250 µm using a Leica ultramicrotome UC7. For the 3D reconstruction dataset, serial sectioning of 40 nm thick slices from a sample in the periphery of the left eye of one chicken was performed with a Diatome ultra ats diamond knife with a knife angle of 35° (Science Services, Germany). 271 sections of 900 x 250 µm were collected on a glow discharged silicon wafer (Active Bizz, Germany) and dried on a heating plate at 50 °C until the water was fully evaporated. The wafer was mounted with silver paint (Plano) on a multi Sem Universal holder Version 2 and stored in a heated vacuum chamber until further use. An overview image from the complete wafer was recorded with a Zeiss Axio Imager.A2 Vario. Individual slices were marked and tracked using Zen2 (blue edition) (Zeiss). The chicken sample was recorded with a 91-parallel-beam Zeiss MultiSEM 506, where the parallel beams are hexagonally arranged to decrease electron-optical aberrations (Eberle et al., 2015). Recordings were performed with a beam current of 591 pA, landing energy of 1.5 keV, 400 ns pixel dwell time per beam and a pixel size of 4 nm. A volume area of approximately 9 x 10^3 µm^3 was imaged, resulting in 11,985 hexagonally shaped multi-beam fields of view (mFOV), containing more than one million high-resolution images and about 1.36 x 10^{10} megapixels. Hexagonal mFOVs consisted of 91 rectangular, single-beam image tiles with a tile size of 3,800 x 3,292 pixels for each tile. Tile overlap was 500 nm and mFOV overlap was 8%. Single-beam images were stored as TIF-files accumulating 14.3 TB of storage space. To correct for scan distortion, a built-in algorithm was used. 2D alignment of all tiles per region was also performed by a built-in algorithm. One retinal slide consisting of several mFOVs is further on named region. In total, 271 sections were recorded.

For detailed 2D analysis of double cone morphological structures, 40 nm thick sections of nine chicken were cut with an ultra 35° diamond knife (Diatome) and transferred on carbon coated copper grids

with a hole size of 35/10 nm (S35/10, Quantifoil, Großlöbichau, Germany). Images were recorded with a Jeol JEM-2200FS at an energy of 200 keV with a TEM-CAM F416 cmos camera (TVIPS) at a magnification of 40,000x resulting in a pixel size of 0.322 nm.

Image processing and reconstruction of electron microscopic serial sections

Due to the tile overlap of 500 nm of the mFOVs, the overlapping part of the image was exposed twice to an electron beam which caused higher bleaching than in the rest of the image. To correct for these differences in brightness, a self-written script containing a bandpass filter was applied on every single tile. Image processing was performed using the plugin TrakEM2 from the Fiji package (Schindelin et al., 2012). All single tiles from one region were loaded into TrakEM2 using the provided text file with individual tile coordinates. To decrease the size of the dataset and increase processing speed, the dataset was divided into seven sub-volumes. For each sub-volume, a single image per slice was created. Single images were afterwards merged into one 3D dataset using the tif2mrc program from the imod package (Kremer et al. 1996). For 3D alignment of the individual slides in the sub-volumes we used the MIDAS program from imod. For the 3D volume reconstruction of one individual double cone, the alignment of a smaller sub-volume reaching from the pigment epithelium to the beginning of the inner nuclear layer (INL) and a size of 47.7 µm x 75.6 µm x 10.4 µm was refined using MIDAS. Due to memory limitation on the computer, we created a volume of 140 µm x 148 µm x 10.4 µm size reaching from the outer nuclear layer (OPL) to the ganglion cell layer (GCL) to reconstruct complete bipolar cells and photoreceptor cells. For all 3D volume reconstructions of individual cells, the 3dmod program from imod was used (see Fig. 1-1F for the two sub-volumes analyzed).

Analysis of photoreceptor cells and bipolar cells in a 3D volume

To calculate the ratio of the different photoreceptor cell types, photoreceptors of the complete data set were analyzed. Only photoreceptor cells which could be clearly identified as one specific type of photoreceptor went into the statistical analysis. Based on the stratification level in the OPL, long wavelength single cones (red cones) and middle wavelength single cones (green cones) could not be

distinguished and were sampled in one group. Likewise, short wavelength single cones (blue cones) and ultra-short wavelength single cones (violet cones) could not be distinguished and were also sampled in one group. To further analyse the structure and connectivity strength of the cells' terminals, we counted all the ribbon synapses present in twenty terminals of rods, single cones, and double cones. Afterwards, the mean and standard deviation was calculated. Telodendria or bipolar cell dendrites that reached into the terminal of a photoreceptor and ended there with contacting the terminal other than at a ribbon synapse were classified as "basal contact". Telodendria or bipolar cell dendrites that reached into the terminal of a photoreceptor and ended without making a contact were classified as "no clear contact". If a bipolar cell dendrite or photoreceptor telodendrion contacted a terminal from a photoreceptor but did not end there, it was also classified as "no clear contact". For evaluating the dendritic and axonal fields of the bipolar cells, we drew the convex hull for each axon terminal and dendritic field. Classification of bipolar cell types was performed blindly by four untrained researchers to avoid bias. Connectivity matrices were calculated based on the normalized number of basal or ribbon synapses, respectively, using MATLAB (Higham and Higham, 2016). Pre- and postsynaptic distributions from basal and ribbon contacts were calculated as followed: For the presynaptic distribution of basal contacts, we counted the basal contacts (Fig. 3-2 triangles) within the terminal of one photoreceptor cell type to a specific postsynaptic cell (Fig. 3-2A, e.g., bright blue for AC) and divided it by the number of all the detected basal contacts in the terminals (Fig. 3-2A, all triangles). The same procedure was performed for the presynaptic distribution of ribbon contacts, where we divided the number of ribbon contacts from one cell type by the number of all ribbon synapses identified within the terminal. For the postsynaptic distribution of synapses, we also counted all the basal and ribbon contacts that a postsynaptic cell type made within the terminal of a specific photoreceptor cell type (Fig. 3-2A, triangles or squares in bright blue for AC) and divided it by the total number of basal or ribbon synaptic contacts that photoreceptor cell type made (not shown in the figure).

Basal contacts were divided into three classes based on their distance to the nearest ribbon synaptic contact: triad associated, middle non-triad associated, and marginal non-triad associated (Fig. 4-1 to Fig. 4-15). Basal contacts were classified as middle non-triad associated if at least two dendrites separated this dendrites from the nearest ribbon synapse and as marginal non-triad associated if they contacted the terminal at the outer margin or at elongations of the terminal other than telodendria (see Tsukamoto and Omi, 2015).

The stratification profiles in Fig. 5 for each bipolar cell type were calculated as probability density function estimates, using the scatterhist function in MATLAB after calculating the mean cell volume of each bipolar cell type for each drawn contour. In Fig. 4-1 to Fig. 4-15 panel B the sum of each cell volume along the y-axis was calculated and overlayed with the total sum of all cells within the volume (black line).

Immunohistochemistry

For immunohistochemical analysis, eyecups from four one-week old chicken were prepared by cornea dissection followed by removal of the lens and vitreous body. Eyecups were fixed in 4% PFA/PBS for 30 min and afterwards washed three times for 10 min in PBS. Part of the retina was removed from the eyecup as stripes (width ~4 mm), reaching from the dorsal periphery to the centre tip of the pecten. The retinal stripes were afterwards embedded in 4% high-melting agarose. for vibratome sections, 100 µm thick vibratome sections were collected and triple staining using GNB3 antibody (CatNo. LS-B8347, Lifespan Biosciences, USA), diluted 1:200, PKC alpha clone MC5 (CatNo. K01107M, Biodesign International, USA), diluted 1:100, and Ctbp2 (CatNo. 193003, Synaptic Systems, Germany), diluted 1:5000 in PBS including 1% TritonX-100 and 3% normal donkey serum, was performed overnight at room temperature. At the next day, retina slices were washed four times 10 minutes in PBS and GNB3 visualization was performed using Alexa 488, Cy3 and Alexa 647-conjugated secondary antibodies (Dianova, Germany) 1:500, in PBS including 1% Triton and 3% normal donkey serum for two hours at room temperature. In addition to the secondary antibodies, we included DAPI (Sigma Aldrich, USA; 1:10.000) to stain cell nuclei. Retinal slices were again washed four times 10 minutes, mounted on

slides and covered with Aqua-Poly/Mount (Polysciences, Germany) and a coverslip. Confocal micrographs of fluorescent retinas were analyzed with a TCS SP8 confocal microscope (Leica Camera AG, Wetzlar, Germany) using the 405 nm and 488 nm lines and the PMT settings were chosen to avoid cross-talk between the different lines. Scanning was performed with the glycine immersion HC PL APO 63x / 1.30 GLYC CORR CS2 at a resolution of 2,048 x 2,048 pixels. Stacks were recorded with a thickness of 12 µm and final images were z-projections of the complete stack adjusted in brightness and contrast in ImageJ (NIH, Bethesda, MD, USA).

Results

Identification of different photoreceptor types

We used the novel ssmSEM technique to acquire a high-resolution 3D electron microscopic dataset from a 900 x 250 x 10.4 micrometre area in the dorsal periphery of the left retina of a one-week-old chicken to analyse the morphology of double cones in 3D and gain insights into their connections to bipolar cells and other photoreceptor cells. For a first estimation, we identified all the photoreceptor cell types in the complete dataset using already described morphological features, such as the presence of an oil droplet in all cones (Bowmaker, 2008), location of the cell bodies of photoreceptor cells in the outer nuclear layer (ONL), the ending of the synaptic terminals in the three strata of the OPL (Mariani and Leure-du Pree, 1978; Mariani, 1987), and glycogen accumulations in the inner segments of rods (hyperboloid) and the accessory member of the double cones (paraboloid) (Meyer and May, 1973). As the green and red single cones both have their synaptic terminals located in the second strata and the blue and violet single cones both have their synaptic terminals located in the third strata in the OPL (Mariani and Leure-du Pree, 1978), we were not able to differentiate between the two cell types of each pair and therefore grouped them together as green/red (G/R) and blue/violet cones (B/V). In total, 706 photoreceptor cells were counted. 228 of these were identified as double cones, 130 as combined green (medium-wavelength sensitive, MWS) or red (long-wavelength sensitive, LWS) single cones, 51 as blue (short-wavelength sensitive, SWS2) or violet (short-wavelength

sensitive, SWS1) single cones, and 244 as rods. We were able to identify 53 additional photoreceptors as single cones, but we could not characterise them further because essential morphological features were missing, e.g., the terminal within the OPL. In summary, we found 32.3% double cones, 34.6% rods and 33.1% single cones. Thus, 49.4% of all cones were double cones.

In the process of collecting serial sections, some slices were lost, leading to a ~160 nm gap (marked with a black line in Fig. 1A) in the 140 µm x 148 µm x 10.4 µm sub-volume. Despite this gap, we were still able to track most of the processes from photoreceptors as well as the dendrites and axons from bipolar cells (Fig. 1B, Fig. 1-1).

The terminals of the rods and both members of double cones form a mosaic (Fig. 1C, 1D) as do the terminals from the green/red cones (Fig. 1E, green). The terminals of the blue/violet cones seem to cluster together (Fig. 1E, magenta). The photoreceptor terminals from rods and double cones can be found in stratum 1 of the OPL whereas the terminals from G/R single cones are located in the second stratum and the terminals from B/V single cones in the third stratum (Fig. 1F).

Morphological characterisation of the double cone

With the high-resolution 3D reconstruction of a complete double cone, including all its major compartments, we reinvestigated the double cone anatomy in the chicken retina (Fig. 2A, sub-volume 1, Fig. 1-1F). We could identify multiple tiny oil droplets in the accessory member of the double cone and a single oil droplet in the principal member as indicated by Pedler and Boyle (1969). Due to the small size of the multiple oil droplets in the accessory member, they may have been overlooked in light microscopic studies (López-López et al. 2008) leading to different opinions on whether or not the accessory member of double cones has a fractionated oil droplet. The principal member has a mean number of 9.1 ± 3.2 (n=14, mean ± SD) processes, which spread radially but never exceed the first stratum of the OPL. In contrast, the accessory member extends several short processes towards the principal member and additionally 2.8 ± 0.7 (n=14) long processes, which pass underneath the terminal

of the principal member and can reach the third stratum of the OPL (Fig. 2A). Processes were only analyzed from double cone members where the complete terminal was in the volume.

For an even more detailed view on specific structures in the double cone, we used transmission electron microscopy. By analysing the highly stacked and ordered membranes in the outer segments of the double cone, we validated the tissue preservation (Fig. 2B) and were able to visualize the outer membrane invaginations (Fig. 2C). A closer look into the opposing membranes of the double cone members confirmed junction-like structures along the inner segments of the double cones (Fig. 2D), which may represent gap junctions as described by Nishimura and colleges (Nishimura et al., 1981). Additionally, we observed that only the accessory member of the double cone expressed a paraboloid which is a region of accumulated glycogen surrounded by a large density of endoplasmic reticuli (Cohen, 1972). However, we did not find any specialized structure that could have evolved specifically for the putative functions of chicken double cones in magnetoreception and/or polarized light vision.

Classification of photoreceptor contacts

Having confirmed the morphology of double cones in chicken, we investigated how double cones are connected to other photoreceptors and how the photoreceptors are connected to bipolar cell types. To characterize connecting cells, we first determined what is an actual contact and which type of contact cells make with each other. However, the typical morphology of ribbon synapses (Meller, 1964; Dowling and Boycott, 1966; Lagnado and Schmitz, 2015) and basal (flat) contacts (Dowling and Boycott, 1966; Haverkamp et al., 2000) in the photoreceptor terminals could still be identified and were used to classify the type of contact (for details, see method section). Ribbon synapses are located inside the photoreceptor terminal (Fig. 2E). Two lateral horizontal cell dendrites and one or two central bipolar cell dendrites invaginate the photoreceptor terminal at sites where ribbons are anchored (Dowling and Boycott, 1966). In general, ON bipolar cells usually make invaginating (central) contacts, OFF bipolar cells usually make basal (flat) contacts at the base of the photoreceptor terminal (Haverkamp et al., 2000). But there are several exceptions, where OFF bipolar cells occupy the central part of a ribbon synapse (fish: Saito et al., 1985 or ON bipolar cells make basal contacts Calkins et al., 1996). In the

turtle, the lateral or central positions could also be occupied by a process from another photoreceptor (Mariani and Lasansky, 1984).

The number of ribbon synapses

We first counted the number of ribbon synapses expressed in a subset of rod and cone terminals. Since the terminals of green and red cones as well as blue and violet cones terminate in the second and third strata of the OPL, respectively, the number of ribbons provided below is the average number for the respective two types combined. In contrast to rodent rods, which only express one ribbon per terminal (Cohen, 1960; Ladman, 1958), rods from chicken contained 3 ± 0.8 (n=20) ribbons per terminal. In double cones, the mean number of ribbons was larger in the principal member (18.5 ± 2.0, n=20) than in the smaller accessory member (8.7 ± 0.8, n=20). The terminals of single cones contained a similar number of ribbons (green/red single cones: 9.5 ± 1.4, n=20; blue/violet single cones: 8.9 ± 1.3, n=10). Ribbon synapses were only counted in complete terminals of rods and cones throughout the dataset.

Identification of photoreceptor/photoreceptor connections

Telodendria are present at the photoreceptor terminals of various vertebrate species (e.g. cats (Kolb, 1977), primates (O'Brien et al., 2012), zebrafish (Li et al., 2009; Noel and Allison, 2018)), and birds (Mariani and Leure-du Pree, 1978)). While telodendria were shown to be coupled by gap junctions in a number of species [e.g., ground squirrel (DeVries et al., 2002); primate (Hornstein et al., 2005); and zebrafish (Noel and Allison, 2018)], telodendria of turtle photoreceptors invaginate into neighboring terminals, making chemical contacts at the ribbon synapse (Ammermüller and Kolb, 1996; Mariani and Lasansky, 1984; Kolb and Jones, 1985; Owen, 1985).

The analysis of photoreceptor/photoreceptor contacts was performed in sub-volume 2 (140 µm x 148 µm x 10.4 µm Fig. 1-1F). By analysing tip-to-tip and tip-to-shaft telodendria contacts (for definition, see Fig. 3-1A, B), which most likely represent gap junctions, we found that tip-to-tip contacts only occurred between cells of the same cell type, predominantly between rods and in smaller numbers also between the principal members of the double cone and green/red single cones (Fig. 3-1C). Tip-to-

shaft telodendrial contacts were detected between cells from the same type but also between cells from different photoreceptor cell types with the highest numbers being observed between different single cone types (Fig. 3-1D). Although telodendria are abundant in the chicken OPL, contacts of telodendria-telodendria are rather restricted (18.4% of total number of detected contacts) and most of the photoreceptor telodendria make contact within the terminals of other photoreceptors.

However, as in the turtle, chicken photoreceptor telodendria frequently contacted other photoreceptor terminals via basal and ribbon contacts which looked similar to the basal and ribbon contacts between bipolar cells and photoreceptor cells described above. The accessory members of double cones are highly connected with their affiliated principal member by telodendrial basal and ribbon contacts in the terminal of the principal member (Fig. 3B, F-I). In contrast, the principal member is less connected, making only basal contacts with its affiliated accessory member and no ribbon contacts at all (Fig. 3B right, F-I). Principal members also made basal contacts to the terminal of other principal members (Fig. 3A, F-I). In contrast, accessory members from different double cones never made any contacts to each other (Fig. 3F-I).

Due to the length of the descending telodendria of the accessory members, we could not identify all contacts because the processes reached the end of the volume. Only in six AC cells we identified either basal or ribbon contacts directly to green/red cone terminals (Fig. 3C, F-I). In one case, we found a long telodendrion of an accessory member making a basal contact to a blue/violet cone (Fig. 3F, G).

Reconstructing the group of blue/violet cones, we discovered that these single cones not only have long telodendria at their terminals but also extend shorter telodendria into the first stratum of the OPL. However, some primary telodendria reached the end of the volume and therefore the area in the first stratum could not be completely reconstructed. Nonetheless in three of the cells, we found that these shorter telodendria made basal contacts at the terminal of the principal member of double cones (Fig. 3D, F, G). We found that B/V single cones do not contact each other but rather contact G/R single cones (Fig. 3F-I).

Almost all rods (90%) contacted the terminal of principal members of double cones with their telodendria via basal contacts (Fig. 3E, F, G). Although these rods received major input from the principal member, it is interesting to note that these rods were mostly presynaptic to other rods representing an asymmetry in signal transduction.

However, immunohistological stainings of connexins, the building blocks of gap junctions, indicated that gap junctions could also be present within the terminals of photoreceptors in the chicken retina (Kihara et al., 2009). Therefore, it could be possible that telodendria and photoreceptor terminals form not only chemical synapses but also gap junctions.

In summary, these analyses reveal a surprisingly complex network of connections among bird photoreceptors, suggesting that the bird retina is more similar to other sauropsida retinas such as the turtle (Mariani and Lasansky, 1984) than to the mammalian (Hornstein et al., 2005) or fish retina (Noel and Allison, 2018) in this regard. All individual photoreceptor contacts are listed in figures 3-3 to 3-6.

Identification of bipolar cell types

Since the connectivity between photoreceptors and bipolar cells is unknown for birds and because the number of previously anatomically described bipolar cell types in the bird retina seemed surprisingly low (11 in chicken, 8 in pigeon) (Mariani, 1987; Quesada et al., 1988), in sub-volume 2 (Fig. 1-1F), we reconstructed all bipolar cells that contacted at least one of the reconstructed photoreceptors. Bipolar cells which had their main dendritic and axonal fields inside the volume were considered as complete cells (n=74). These cells were used for identifying the connectivity between bipolar cells and photoreceptor cells and for defining bipolar cell types. Bipolar cells, of which the primary axon reached the end of the volume or major parts of the dendritic field were missing, were defined as partial bipolar cells (n=72) and were only further analyzed if they could be clearly assigned to one bipolar cell type (e.g., based on contacted photoreceptor types, fitting into the existing dendritic mosaic). Dendrites that contacted photoreceptors but were not connected to a soma were ignored. In order to identify the stratification level of the axons in the inner plexiform layer (IPL), we chose to divide the IPL in eight

381 strata based on an earlier study analysing the stratification of ganglion cells in chicken retina (Naito
382 and Chen, 2004).

383 With the dataset at hand, we not only analyzed the morphology (dendritic and axonal stratification,
384 soma position) of the bipolar cells, but also their contacts to the different photoreceptor types to
385 define bipolar cell types. In a final step, we double checked the different types by analysing the
386 dendritic and axonal field mosaics as described for bipolar cells from other species, such as mouse
387 (Wässle et al., 2009; Tsukamoto and Omi, 2017) and primates (Boycott and Wässle, 1991). In total, we
388 identified 15 types of bipolar cells in the chicken retina, sorted by the position of their axonal
389 stratification (Fig. 4 A). Detailed information on each bipolar cell type is given in Fig. 4-1 to Fig. 4-15. In
390 addition, we classified two fully reconstructed bipolar cells as “orphan” because they could not be
391 assigned to any other bipolar cell type and are also different from each other (Fig.4-16).

392 For all bipolar cells, we analyzed the type of contact they made to photoreceptor terminals (ribbon
393 versus basal contacts). We further categorized the basal contacts based on their distance to the
394 nearest ribbon synapse (Fig. 4-1F to Fig. 4-15F). Examples of this analysis are shown in Fig. 4B-G with
395 B2 as a potential ON bipolar cell type, B5a as a potential midget bipolar cell type and B7 as a potential
396 OFF bipolar cell type. We also analyzed whether bipolar cells contacted all the photoreceptors of a
397 specific type within their dendritic field and found that no bipolar cell type seemed to avoid a terminal
398 of a photoreceptor type it normally contacts (Fig.4-1G to 4-15G). We calculated the stratification
399 density probabilities of each individual bipolar cell type based on the calculated volume of the cells
400 along retina depth (Fig. 5) reflecting the overall stratification pattern in the OPL and IPL. To visualize
401 how much volume each bipolar cell contributes to the different strata in the IPL, we calculated the
402 mean volume of all cells from a bipolar cell type along IPL depth (Fig. 4-1B to Fig.4-15B, coloured lines)
403 and calculated the sum of all bipolar cells (Fig.4-1B to Fig.4-15B, black line) which enabled us to identify
404 the overall distribution of bipolar cells along IPL depth. We found that strata 3, 5 and 8 have the lowest
405 density of bipolar cell terminals. However, we cannot exclude that we are missing bipolar cell types
406 because our data set is limited in the z-direction.

In the OPL, we found some exclusive connections between bipolar cells and photoreceptors. Surprisingly, only one type (B9) exclusively contacts green/red single cones and one type (B10) exclusively contacts blue/violet cones. B10 bipolar cells may represent the “blue bipolar cell” known from mammalian species [e.g. mouse (Haverkamp et al., 2005), primate (Mariani, 1984; Ghosh et al., 1997) and ground squirrel (Li and DeVries, 2006)], but we would have had to be able to separate blue from violet single cones in the dataset to clearly identify single cone type specific bipolar cells. One type (B6) seems to contact almost exclusively the accessory member of double cones (Fig. 4A and Fig. 4-11). All other bipolar cell types (except for B9 and B10) make contacts to several photoreceptor cell types but receive most of their input from the principal member of double cones (Fig. 4-1 to 4-13). Interestingly rods make most of their ribbon synaptic contacts to B2 bipolar cells (Fig. 4-3). Additionally, 12 of the 15 identified bipolar cells receive input from rods.

Identification of the ON-OFF border in the IPL

We were able to identify 15 different bipolar cell types including their connectivity to photoreceptor cells. Since electrophysiological data from avian bipolar cells are completely lacking, we aimed to identify a putative border between the OFF and ON strata in the IPL using immunohistochemistry in order to analyse which of the identified cell types may represent ON or OFF bipolar cells. Although this border is well described for mammalian species, it remains unclear in birds since even the number of strata is not entirely resolved (Millar et al., 1987; Naito and Chen, 2004; Ritchey et al., 2010). We used antibodies against PKC alpha to label a subpopulation of putative ON bipolar cells (Ritchey et al., 2010) (Fig. 6 grey), GNB3 as a putative marker for all ON bipolar cells (Ritchey et al., 2010) (Fig. 6 green) and Ctbp2 to visualize all ribbon synapses in our tissue (Fig. 6 magenta). PKC positive cells were found to be positive for GNB3, indicating that these cells are indeed ON bipolar cells (Fig. 6A-F). Comparing the morphology and stratification levels of PKC positive bipolar cells with our 3D reconstructed cells, we find a high similarity with B9 and B10 bipolar cells, which are exclusively contacting single cones. The only difference between the PKC positive cells and the B9 or B10 bipolar cell types, respectively, is the stratification in the inner most stratum (S6/7) which is more excessive in the PKC cells than in our

433 reconstructions. Additionally, if the GNB3 marker indeed labels the ON strata of the avian IPL and we
434 assume eight strata, then the border between OFF and ON would be between S3 and S4 (Fig. 6E, F).
435 Comparing the GNB3 staining with Ctbp2 labelling, we found that synapses in the supposed OFF
436 stratum 1 are GNB3 positive which indicates that ON bipolar cells also stratify in the distal OFF layer.
437 Likewise, we found Ctbp2 positive but GNB3 negative areas between S4 and S5, S6 and S7 and in S7
438 and S8 indicating that OFF bipolar cells stratify in the ON strata of the IPL as well (Fig. 6 I, J). This
439 eliminates the clear separation between ON and OFF signals in the IPL. If we apply this stratification
440 pattern to our reconstructed bipolar cells and combine it with the type of contacts a bipolar cell makes
441 to the photoreceptor terminals, we suggest that bipolar cells belonging to our groups B1, B3, B7, and
442 B8 are putative OFF bipolar cells, whereas the groups of B2, to B6, B9 and B10 are putative ON bipolar
443 cell candidates. Clearly, electrophysiological studies are needed to corroborate these putative
444 assignments.

445 ## Discussion

446 We obtained the first 3D electron microscopic dataset for the avian retina using ssmSEM. We used this
447 dataset to 1) revisit the anatomy of double cones, 2) present insights into double cone connectivity to
448 other photoreceptors, and 3) provide the first classification of avian bipolar cell types that considers
449 photoreceptor to bipolar cell connectivity. Additionally, we found a surprisingly large and diverse
450 number of photoreceptor-photoreceptor connections, which, to our knowledge, were not previously
451 described in such diversity in any bird, and a surprising number of inputs from several different
452 photoreceptor cells to most of the individual bipolar cell types.

453 **Photoreceptor-photoreceptor connections**

454 Evidence from mammals and fish suggests that telodendria contact each other and form gap junctions
455 (DeVries et al., 2002; Noel and Allison, 2018), which are thought to have various functions. Cone-cone
456 coupling e.g. correlates common light-mediated input and reduces noise from individual
457 photoreceptors by averaging across the coupled network (DeVries et al., 2002; O'Brien et al., 2012),

458 thereby increasing the signal-to-noise ratio. Rod-rod coupling was shown to enhance contrast
459 detection in dim light, at the cost of reducing absolute sensitivity (Li et al., 2012). Here, we found that
460 predominantly rod photoreceptors form tip-to-tip and tip-to-shaft telodendrial contacts (Fig. 3-1).
461 Since we were not able to identify gap junctions in our ssmSEM data set, we can only speculate that
462 rod-rod electrical coupling is more abundant than cone-cone or rod-cone coupling in the chicken and
463 that they may serve similar functions as in other vertebrate classes.

464 In turtles, rods and cones also form chemical synapses between telodendria and photoreceptor
465 terminals (Mariani and Lasansky, 1984; Kolb and Jones, 1985; Owen, 1985). Functional experiments in
466 tiger salamander also suggest that rods and cones are electrically and chemically coupled (Attwell et
467 al., 1983). Here, we report, for the first time in birds, that also chicken photoreceptor cells make
468 telodendrial contacts with other photoreceptor terminals. These contacts mainly involved one or both
469 members of a double cone and other photoreceptor cell types but also occurred between green/red
470 single cones.

471 Our data suggest that rod-rod coupling in the chicken retina could primarily be mediated by electrical
472 synapses whereas cone-cone or rod-cone connections could be mediated by chemical synapses. In
473 turtles, sites of chemical synapses between red and green cones were hypothesised to lead to an
474 excitatory colour mixing which was found in electrophysiological recordings (Kolb and Jones 1985).
475 Since rods and double cones are likely to be involved in luminance detection rather than colour vision
476 (Lind et al., 2014), the chemical synapses between these two photoreceptor types could serve to
477 enlarge the operational range of the luminance channel and thereby enhance its sensitivity.

478 **Bipolar cell classification and connectivity to photoreceptor cells**

479 The bipolar cell classification provided here comprises 15 different types. Interestingly, we found that
480 the axonal stratification pattern and photoreceptor connectivity were the most defining factors for the
481 types: bipolar cells with the same axonal arbour contacted different photoreceptors. This is similar to
482 the zebrafish retina (Li et al., 2012) but in contrast to the mammalian retina where the same axonal

stratification is associated with the same photoreceptor connectivity (Euler et al., 2014). The presence or absence of a Landolt's club does not seem to be a defining feature because cells belonging to the same bipolar cell type, based on uniform dendritic and axonal mosaics, can possess, or lack this special protrusion (e.g., types 3a, 4c; Fig. 4-4 and 4-8). If the Landolt's club was a defining feature, we would expect the mosaics to show higher inhomogeneity and overlap within our defined bipolar cell types.

For the retina of a tetrachromatic animal (Hart, 2001), it seems very surprising that most bipolar cells make contacts to different photoreceptor cell types, thereby mixing colour channels. Furthermore, most bipolar cell types seem to get most of their input from the principal member of double cones (Fig. 4A; Fig. 4-1 to Fig.4-15). This may indicate that double cones are involved not only in luminance detection, but also in colour vision, presumably processed in parallel bipolar cell pathways. Bipolar cells in other animals contacting exclusively one type of single cone usually have wide dendritic fields [e.g. in rabbit (MacNeil and Gaul, 2008) and mouse (Haverkamp et al., 2005)], and these are the ones in our dataset which have reached the end of the volume more frequently than small-field bipolar cells. Therefore, bipolar cells exclusively contacting individual photoreceptor types, i.e., presumed colour-sensitive bipolar cells, may be underrepresented in our analysis which could explain the low number of bipolar cell types contacting the B/V single cones. Nevertheless, it is surprising that only one bipolar cell type contacted the B/V single cones whereas at least in zebrafish the UV single cones were found to be frequently contacted by a large number of bipolar cells (Li et al., 2012). Bipolar cell type B5a may represent a bird midget bipolar cell type. Primate midget bipolar cells are characterized by a very small dendritic and axonal field and they contact only one single cone in the central retina (Polyak, 1941; Puller et al., 2007). Therefore, midget bipolar cells mediate high-acuity vision (Euler et al., 2014). Since our dataset is from peripheral chicken retina it might be possible that this cell type receives single cone input in the area centralis. For other vertebrate retinas, it was proposed that bipolar cell types follow a so called "block wiring", meaning that they contact spectrally neighbouring photoreceptor cells (red and green) rather than leaving a spectral gap (red and blue) [for overview see (Baden and Osorio,

2019)]. Our data set also supports this hypothesis for the chicken retina, although we cannot separate all single cone types.

Recently, a detailed analysis on chicken retinal cell types was performed using single cell transcriptomics, and 22 different bipolar cells were identified (Yamagata et al., 2021), indicating that we are missing 7 bipolar cell types. One reason could be the limited z-depth of our dataset which potentially leads to a reduced number of wide-field bipolar cells. In addition, separating the R/G and B/V group of single cones could also lead to a regrouping of the bipolar cell types. Compared to previous anatomical studies, we were able to link all of the bipolar cell types identified in pigeon (Mariani, 1987) to our data. We also found similarities to the identified bipolar cells in chicken retina, but also identified new types (Quesada et al., 1988) (for detailed comparison see Fig.4-17).

In general, it is striking that all bipolar cell types are at least bistratified in the IPL whereas mammalian bipolar cells are mostly monostratified (Euler et al., 2014) and also fish have a few monostratified cells in addition to several multistratified types (Li et al., 2012). Since the border between the ON and OFF layer in the IPL is not resolved in the chicken, we used the GNB3 staining as a reference for ON bipolar cells (Ritchey et al., 2010) and Ctbp2 as ribbon synaptic marker. Combining the axonal stratification of the identified bipolar cells with the immunostaining and the type of contacts the cells made to the photoreceptor terminals, it is likely that OFF bipolar cells also stratify in the ON strata of the IPL as it was demonstrated in turtle (Ammermüller and Kolb, 1995) and zebrafish retina (Connaughton, 2001). Our Ctbp2 staining supports that hypothesis because we found ribbon synapses in GNB3-negative strata in the ON part of the IPL. Additionally, OFF bipolar cells can form ribbon synapses with photoreceptors (e.g. in fish (Saito et al., 1985; Sakai and Naka, 1983) and we cannot conclude that all bipolar cells that make basal contacts are OFF cells because, in primates, ON bipolar cells also form basal contacts with the photoreceptor terminal (Hopkins and Boycott, 1996). Thus, functional data are needed to unequivocally decide which bipolar cell types are ON and OFF cells.

Could the region in the retina change proportion of bipolar cell types or shape?

Our dataset was recorded in the dorsal area of the left eye of a one-week old chicken (*Gallus gallus domesticus*). The photoreceptor density and composition in the retina of birds and other animals change from the periphery to the centre and can, in some cases, be divided into special areas (for overview see (Seifert et al., 2020). Not only the ratio between the photoreceptor types change between periphery and centre, the total number of photoreceptor cells and ganglion cells also decrease from the centre to the periphery (Bueno et al., 2011). The increasing number of photoreceptor cells could lead to a higher density of bipolar cells with smaller dendritic fields in the central retina. Also, we cannot exclude that the central retina harbours bipolar cell types which have been missing from the more peripheral area analyzed here. Additionally, it was reported that the left eye is more involved in high spatial acuity tasks than the right eye (for review see Seifert et al. 2020), indicating that the retinal wiring may differ between both eyes.

Double cone anatomy

The detailed reconstruction of an individual double cone largely confirmed previous data: 1) accessory members show fragmented oil droplets, and 2) principal and accessory members are connected by junction-like structures. These structures may, at first glance, contradict the idea that light-dependent magnetoreception could be based on the differential signalling of avian double cones by expressing two sensors arranged in different orientations (Hore and Mouritsen, 2016; Worster et al., 2017). Electrically mixing the resulting signals already at the photoreceptor level seems counterintuitive. However, as we do not know under which light conditions (Yang and Wu, 1989) or time of the day (Ribelayga et al., 2008) the gap junction-like structures can pass signals, these gap junctions might be closed under the light conditions when magnetoreception is most relevant. Further research should investigate this issue.

Conclusion

Here, we present the first 3D reconstructed bird retina data set. We identified many previously undescribed photoreceptor-photoreceptor connections in the chicken retina which suggests

numerous interactions between rods and cones as well as between different cones types at the first stages of visual processing. We increased the number of morphologically known bipolar cell types from 11 to 17 and identified their complete connectivity to photoreceptor cells. Surprisingly, most bipolar cell types contact several different photoreceptor cell types. Astoundingly, 13 out of 15 identified bipolar cell types got at least part of their input from double cones. Therefore, our data suggest that the double cones play many different and important roles in avian retinal processing and that the neural network and thus information processing in the outer retina is in general much more complex than previously expected.

Author contributions

Experiment Design and Supervision, H.M., K.D., S.I., A.G., S.H., K.B.; Experiments, A.G., S.I., S.H.; Data Analysis, A.G., K.D., S.H., and H.M.; Writing, A.G., K.D., H.M, S.H., K.B.

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781

782

Figure 1. 3D reconstructed sub-volume used for bipolar and photoreceptor cell connectivity analysis. (A) 3D volume representation of the original EM data set highlighting the dimensions of the data set. **(B)** Model representation of all double cones (principal member in dark blue and accessory member in bright blue), rods (brown), green/red cones (green) and blue/violet single cones (magenta) as well as all complete bipolar cell grouped in the identified bipolar cell types indicated in different shades of yellow-orange-red-violet. **(C)** Bottom view of double cone (principal member in dark blue and accessory member in bright blue) terminals. **(D)** Bottom view of green/red cone (green) and blue/violet cone (magenta) terminals **(E)** Bottom view of rod terminals (brown). **(F)** Front view of all photoreceptor cell types within the volume with OPL lamina highlighted. Examples for the trackability of the data set are given in Figure 1-1 including an outline of a complete section with the highlighted sub-volumes. PR = principal member, AC = accessory member, G/R = green/red single cones, B/V = blue/violet single cones.

Figure 1-1. Trackability of cells through the gap between slice 72 and 73. (A) Schematic representation of a x/y slice from the sub-volume used for bipolar cell reconstruction highlighting positions for examples to check for trackability through the gap. **(B and C)** EM images from the OPL showing the slice before and after the lost slices for comparison. Large features can be used as landmarks in order to track smaller structures as well. **(D and E)** EM images representing example positions in the IPL to compare the slice before and after the gap. Most of the structures can be tracked through the gap; only thin, sideways moving structures were sometimes lost. **(F)** Example section of the dataset. Complete section used for photoreceptor cell types abundancy. Highlighted are the subvolumes for single double cone reconstruction (1) and bipolar cell and photoreceptor connectivity (2). ONL= outer nuclear layer, OPL= outer plexiform layer, INL= inner nuclear layer, IPL= inner plexiform layer, GCL= ganglion cell layer. Scale bar B-E: 2 µm, F: 100µm.

Figure 2. Double cone anatomy in the chicken retina. (A) 3D reconstruction of both members of a double cone, principal member (blue) and accessory member (pale orange) from the outer segments

809 containing densely packed disc membranes to the photoreceptor terminal where the signal transfer
810 occurs. The oil droplets in both members (green) are located in the inner segments directly at the
811 border to the outer segments. In the accessory member multiple small oil droplets or granules could
812 be found. The more than 200 mitochondria per cell (yellow) are densely packed in the ellipsoid part
813 of the inner segment. The golgi apparatus (red) is located in the myoid part of the inner segment
814 close to the nucleus (magenta). The accessory member of the double cone additionally contains a
815 paraboloid (dark orange) in the myoid part of the inner segment. **(B)** TEM image of the outer
816 segment of an accessory member from a double cone. Densely packed discs are visible as well as two
817 of multiple oil droplets and calycal processes on both sides of the outer segment indicated by the
818 arrows. **(C)** Magnified area from (B) reveals the typical invaginations formed in the outer segments of
819 cones (arrow). **(D)** TEM images of the outer membranes of both double cone members which form
820 junction-like structures (arrow) along the inner segments. **(E)** TEM image of a ribbon synapse in the
821 principal member of a double cone terminal. AC = accessory member, PR = principal member, HC =
822 horizontal cell dendrite, BC = invaginating bipolar cell dendrite. Scale bars: B = 2 µm, C and D = 100
823 nm, E = 200 nm.

824

825 **Figure 3. Connections from the double cone to other photoreceptors.** The two images on the left
826 show front and bottom view of two neighboring 3D reconstructed photoreceptor terminals. **(A)**
827 Contacts between the PRs of neighboring double cones (dark and bright blue). The EM images on the
828 right show that both PRs make basal contacts in the neighboring PR terminal (white arrows). **(B)**
829 Contacts between the accessory member (AC, turquoise) and principal member (PR, dark blue) of a
830 double cone. The EM image on the right shows that double cone members make basal contacts in
831 the other member's terminal, and that the AC additionally makes contacts through ribbon synapses
832 in the PR terminal (white arrows). **(C)** Contact between the AC (turquoise) and a G/R cone (green).
833 The two images on the left indicate that only the AC makes a contact to the G/R cone terminal but

not the other way around. The EM image on the right shows that the AC makes a ribbon synapse contact to the G/R cone terminal. **(D)** Contact between the PR of a double cone and a B/V cone. The two images on the left indicate that only the B/V cone makes contacts to the PR terminal whereas the PR does not seem to contact the B/V cone terminal. The EM image on the right shows that the B/V cone makes multiple basal contacts to the PR terminal. **(E)** Contacts between PR and rods. The EM images show that only the rod makes a basal contact with the PR terminal, but not the other way around. **(F)** Connectivity matrix with percental representations (see method section) of presynaptic basal contacts from PR (total # contacts=248), AC (total # contacts=26), rods (total # contacts=3), G/R (total # contacts=180) and B/V (total # contacts=12). **(G)** Connectivity matrix with percental representations of postsynaptic basal contacts from PR (total # contacts=100), AC (total # contacts=96), rods (total # contacts=79), G/R (total # contacts=151) and B/V (total # contacts=43). **(H)** Connectivity matrix with percental representations of presynaptic ribbon contacts from PR (total # contacts=45), AC (total # contacts=0), rods (total # contacts=0), G/R (total # contacts=35) and B/V (total # contacts=0)**. (I)** Connectivity matrix with percental representations of postsynaptic ribbon contacts from PR (total # contacts=0), AC (total # contacts=48), rods (total # contacts=0), G/R (total # contacts=31) and B/V (total # contacts=1). Tip-to-tip and tip-to-shaft photoreceptor contacts are shown in Figure 3-1 and the synaptic connectivity of a principal member terminal is shown in Figure 3-2. All photoreceptor cell contacts are shown in Figure 3-3 to Figure 3-6. G/R= green/red single cones, B/V= blue/violet single cones. All scale bars = 2 µm.

Figure 3-1. Telodendrial contacts between photoreceptor/photoreceptor within the chicken retina. (A) Example of a tip-to-tip contact between two photoreceptor telodendria. (B) Example of a tip-to-shaft contact between two photoreceptor telodendria. (C) Connectivity matrix representing the fractional number (see method section) of tip-to-tip contacts between telodendria from PR (total # contacts=6), AC (total # contacts=0), rods (total # contacts=60), G/R (total # contacts=2) and B/V (total # contacts=0). (D) Connectivity matrix representing the fractional number of tip-to-shaft

860 contacts between telodendria from PR (total # contacts=8), AC (total # contacts=0), rods (total #
861 contacts=24), G/R (total # contacts=20) and B/V (total # contacts=5). PR= Principal member of a
862 double cone, AC= accessory member of a double cone, G/R= green and red single cones, B/V= blue
863 and violet single cones.

864

865 **Figure 3-2. Synaptic connectivity of photoreceptors cell types. (A)** Schematic representation of a
866 principal member terminal showing the positions and types of each photoreceptor contact within the
867 terminal. Basal contacts represented by triangles; ribbon synapses represented by ovals. Length of
868 ovals represents size of ribbon synapse. **(B)** Schematic representation of principal member terminal
869 showing the positions and types of each bipolar cell contact within the terminal**. (C)** Front view of a
870 principal member terminal (blue) with exemplary basal and ribbon synaptic contacts (grey). All scale
871 bars = 2 µm. PR= principal member, AC= accessory member.

872

873 **Figure 3-3. Contacts of double cone members to other photoreceptor cells**. Principal and accessory
874 members from all double cones in the sub-volume are listed with all their different types of contacts
875 to other photoreceptor cells. Number of synapses are represented in the form (x/y) where x
876 represents the basal contacts and y the ribbon contacts. If displayed as (x) only basal contacts
877 occurred. The table also shows number of telodendria which reached the end of the volume or which
878 made no clear contacts meaning that the telodendria ended in the OPL without making any clear
879 contact to any cell.

880

881 **Figure 3-4. Contacts of rods to other photoreceptors**. All rods from the sub-volume are listed with all
882 their different types of contacts to other photoreceptor cells. Number of synapses are represented in
883 the form (x/y) where x represents the basal contacts and y the ribbon contacts. If displayed as (x)
884 only basal contacts occurred. The table also shows number of telodendria which reached the end of

885 the volume or which made no clear contacts meaning that the telodendria ended in the OPL without
886 making any clear contact to any cell. Rod 16 was excluded from the statistical evaluation because
887 only a very small part of the cell was in the volume.

888

889 **Figure 3-5. Contacts of G/R single cones to other photoreceptor cells**. All G/R cones in the sub-
890 volume are listed with all their different types of contacts to other photoreceptor cells. Number of
891 synapses are represented in the form (x/y) where x represents the basal contacts and y the ribbon
892 contacts. If displayed as (x) only basal contacts occurred. The table also shows number of telodendria
893 which reached the end of the volume or which made no clear contact meaning that the telodendria
894 ended in the OPL without making any clear contact to any cell.

895

896 **Figure 3-6. Contacts of B/V single cones to other photoreceptor cells.** All B/V single cones in the
897 sub-volume are listed with all their different types of contacts to other photoreceptor cells. Number
898 of synapses are represented in the form (x/y) where x represents the basal contacts and y the ribbon
899 contacts. If displayed as (x) only basal contacts occurred. The table also shows number of telodendria
900 which reached the end of the volume or which made no clear contact meaning that the telodendria
901 ended in the OPL without making any clear contact to any cell.

902

903 **Figure 4. Bipolar cell types in the chicken retina. (A)** Bipolar cell types are sorted based on their
904 axonal stratification level in the IPL. Below each bipolar cell type, the numbers of cells within this cell
905 type are shown, including their abundance within the bipolar cell class. Contacted photoreceptor
906 types are highlighted with respective color. Number of contacted photoreceptor cells and combined
907 mean number of basal and ribbon contacts per photoreceptor below. **(B-D)** Mean numbers of ribbon
908 and basal contacts per photoreceptor terminal for bipolar cell types B2, B5a and B7. Figures also
909 shown in Fig. 4-3, 4-9 and 4-12 for overall bipolar cell type summary. **(E-G)** Histograms showing the

910 averaged proportion of the three different classes of basal contacts (triad-associated, TA; marginal
911 and middle non-triad associated, NTA) of bipolar cell types B2, B5a and B7. Figures also shown in Fig.
912 4-3, 4-9 and 4-12 in the overall bipolar cell type summary. Analyses of each bipolar cell type are
913 represented in Figure 4-1 to 4-16 and a comparison between identified bipolar cells in this study and
914 other publications is shown in Figure 4-17. OPL = outer plexiform layer, INL = inner nuclear layer, IPL
915 = inner plexiform layer, PR = principal member, AC = accessory member, G/R= green/red single
916 cones; B/V = blue/violet single cones.

917

918 **Figure 4-1. Characterization of bipolar cell type B1a. (A)** 3D volume reconstruction of complete
919 bipolar cells from this type. Number of contacted photoreceptors and number of contacts per
920 photoreceptor terminal are indicated below each cell. Numbers of synapses are represented in the
921 form (x/y) where x represents the basal contacts and y the ribbon contacts. If displayed as (x), only
922 basal contacts occurred. **(B)** Mean cell density representation for B1a bipolar cells (yellow) based on
923 the volume of all cells from this bipolar cell type along the IPL depth. The sum of all mean bipolar cell
924 type densities in the IPL (black) indicates that strata 3, 5 and 8 are only weakly occupied by bipolar
925 cell axons. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. Front view of all
926 bipolar cells, which belong to one bipolar cell type, showing the overall distribution of the cells in the
927 data set. Dendrites are shown from a top view including dendritic fields, which are represented
928 through convex hull estimations to show dendritic field mosaic. Axonal fields are shown for each
929 stratum in the inner plexiform layer and are represented through convex hulls **(D)** Correlation
930 between missing branches and number of contacts/bipolar cell indicates that the number of contacts
931 does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal
932 contacts per photoreceptor type terminal. One way ANOVA including Tukey test reveals that the
933 most contacted terminal from the principal member of a double cone has significantly more contacts
934 than the second most contacted terminal. Therefore, B1a bipolar cells contact predominantly one
935 principal member of a double cone and make less contacts to other principal members. **(F)** Histogram

showing the averaged proportion of the three different classes of basal contacts for each photoreceptor cell type based on the classification by Tsukamoto and Omi (2015). **(G)** Distribution of different types of synapses at the dendrite of one B1a bipolar cell (marked with two asterisks in A). Basal contacts are represented as triangles and ribbon contacts as squares. Colors indicate type of contacted photoreceptor cell; photoreceptor terminals are also represented in the corresponding colors. B1a bipolar cells make most of their contacts to one central principal member of a double cone and in significantly lower numbers also contacts to other photoreceptor cell types. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-2. Characterization of bipolar cell type B1b. (A) 3D volume reconstruction of complete bipolar cells from this type. Same representation as in Fig.4-1. **(B)** Profile sum calculation for B1b bipolar cells (orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Due to a cell number of 2 it was not possible to perform any statistical analysis. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of one B1b bipolar cell (** in A). Dendrites (orange) make basal (triangle) contacts. Colors indicate type of photoreceptor cell that is contacted. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-3. Characterization of bipolar cell type B2. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B2 bipolar cells (orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Photoreceptor terminals are not significantly contacted with different numbers of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of one B2 bipolar cell (** in A). Dendrites (orange) make both basal (triangles) and ribbon (squares) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-4. Characterization of bipolar cell type B3a. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B3a bipolar cells (orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. One way ANOVA including Tukey test reveals that the most contacted terminal from the principal member of a double cone has significantly more contacts than the second most contacted terminal. Therefore, B3a bipolar cells contact predominantly one principal member of a double cone and make less contacts to other principal members. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of

B3a bipolar cells. Dendrites (orange) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-5. Characterization of bipolar cell type B3b. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B3b bipolar cells (orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates, that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. One way ANOVA including Tukey test reveals that the most contacted terminal from the principal member of a double cone has significantly more contacts than the second most contacted terminal. Therefore, B3b bipolar cells contact predominantly one principal member of a double cone and make less contacts to other principal members. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of one B3b bipolar cell (** in A). Dendrites (orange) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-6. Characterization of bipolar cell type B4a. (A) 3D volume reconstruction of complete bipolar cells from this type. **(B)** Profile sum calculation for B4a bipolar cells (red) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell

types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does show a higher variation with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Photoreceptor terminals are not significantly different in their number of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of one B4a bipolar cell. Dendrites (red) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-7. Characterization of bipolar cell type B4b. (A) 3D volume reconstruction of complete bipolar cells from this type. **(B)** Profile sum calculation for B4b bipolar cells (dark red) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. One way ANOVA including Tukey test reveals that the most contacted terminal from the principal member of a double cone has significantly more contacts than the second most contacted terminal. Therefore, B4b bipolar cells contact predominantly one principal member of a double cone and make less contacts to other principal members **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of one B4b bipolar cell. Dendrites (dark red) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-8. Characterization of bipolar cell type B4c. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B4c bipolar cells (red) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. One way ANOVA including Tukey test reveals that the most contacted terminal from the principal member of a double cone has significantly more contacts than the second most contacted terminal. Therefore, B4c bipolar cells contact predominantly one principal member of a double cone and make less contacts to other principal members **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of B4c bipolar cells. Dendrites (red) make basal (triangles) and ribbon (squares) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 μm.

Figure 4-9. Characterization of bipolar cell type B5a. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B5a bipolar cells (dark orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts may decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. One way

ANOVA including Tukey test reveals that the most contacted terminal from the principal member of a double cone has significantly more contacts than the second most contacted terminal. Therefore, B5a bipolar cells contact predominantly one principal member of a double cone and make less contacts to other principal members. This cell type is a good candidate for a midget bipolar cell homolog **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of B5a bipolar cells. Dendrites (dark orange) make basal (triangles) and ribbon (squares) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-10. Characterization of bipolar cell type B5b. (A) 3D volume reconstruction of complete bipolar cells from this type. **(B)** Profile sum calculation for B5b bipolar cells (yellow) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Low number of cells didn't allow further statistical tests. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B5b bipolar cell. Dendrites (yellow) make basal (triangles) and ribbon (squares) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-11. Characterization of bipolar cell type B6. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B6 bipolar cells (dark orange) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Photoreceptor terminals are not significantly different in their number of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B6 bipolar cell. Dendrites (orange) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-12. Characterization of bipolar cell type B7. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B7 bipolar cells (light pink) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Photoreceptor terminals are not significantly different in their number of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B7 bipolar cell. Dendrites (light pink) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer,

PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-13. Characterization of bipolar cell type B8. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B8 bipolar cells (pink) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Photoreceptor terminals are not significantly different in their number of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B8 bipolar cell. Dendrites (pink) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-14. Characterization of bipolar cell type B9. (A) 3D volume reconstruction of complete bipolar cells from this type. Partial bipolar cells that could be assigned to this bipolar cell type are displayed as well. **(B)** Profile sum calculation for B9 bipolar cells (dark pink) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does not decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type.

Photoreceptor terminals are not significantly different in their number of contacts. **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B9 bipolar cell. Dendrites (dark pink) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-15. Characterization of bipolar cell type B10. (A) 3D volume reconstruction of complete bipolar cells from this type. **(B)** Profile sum calculation for B10 bipolar cells (dark red) and profile sum of all bipolar cell types (black) in the IPL. **(C)** Dendritic and axonal field mosaics of identified bipolar cell types. **(D)** Correlation between missing branches and number of contacts/bipolar cell indicates that the number of contacts does decrease with an increase of missing branches. **(E)** Mean numbers of ribbon and basal contacts per photoreceptor terminal from each photoreceptor cell type. Not enough cells to do further analysis **(F)** Histogram showing the averaged proportion of the three different classes of basal contacts. **(G)** Distribution of different types of synapses at the dendrite of a B10 bipolar cell. Dendrites (dark red) make basal (triangles) contacts. OPL = outer plexiform layer, INL = inner nuclear layer, IPL= inner plexiform layer, PR = Principal member of a double cone, BC = bipolar cell, AC = accessory member of a double cone, G/R = green and red single cones, B/V = blue and violet single cones, TA = triad associated, NTA = non triad associated. Scale bar: G= 5 µm.

Figure 4-16. Orphan bipolar cells. Two bipolar cells which do not map with any of the other types. Stratification pattern in the IPL is similar but the position of the soma in the INL differs and one cell contacts all single cone types and the accessory member of the double cone whereas the other cell only makes one contact to one G/R single cone.

Figure 4-17. Comparison of bipolar cell types described in different publications. (A) Identified bipolar cells in the pigeon retina (Mariani 1987) reorganised to fit our identified bipolar cell types. We could assign all the identified bipolar cells from Mariani (1987) to our bipolar cells. **(B)** Overview of our identified bipolar cells from Fig. 4. **(C)** Stratification examples from the chicken retina (adapted from Yamagata et al. 2021, with permission) showing six out of 22 identified bipolar cells with their potential stratification in the IPL. We could not find any monostratified cells in our dataset. **(D)** Identified bipolar cells from the chicken retina (Quesada et al. 1988). Several of the described bipolar cells look similar to our bipolar cells, however, some of the wide-field bipolar cells are missing in our dataset.

Figure 5. Stratification level of the individual bipolar cell types in the OPL and IPL. (A-C) Bipolar cell stratification profile in the OPL represented by a probability density function estimate for bipolar cell types B1a-B3b, B4a-B5b, and B6-B10, respectively. Density functions are calculated based on the volume of the cells along OPL depth. Dendrites increase in volume with increasing distance to the connected terminals. Therefore, the OPL density estimate is slightly shifted towards the INL and the peak density estimate does not represent area of synaptic contacts. **(D-F)** Bipolar cell stratification profile in the IPL represented by a probability density function estimate for bipolar cell types B1a-B3b, B4a-B5b, and B6-B10, respectively. OPL = outer plexiform layer, IPL= inner plexiform layer.

Figure 6. IPL stratification in the chicken retina. Vertical vibratome sections of the chicken retina were labelled with PKC (grey; A-D, F, and I), Ctbp2 (magenta; B-C, G-I), GNB3 (green; C, E-F, H-I) and Dapi (blue; C, F, I). PKC labelled bipolar cell axon terminals are also GNB3 positive in the IPL indicating that these bipolar cells (high similarity with B9 and B10 in Fig. 4) are ON bipolar cells (D-F). Double labelling with Ctbp2 and GNB3 reveal ON bipolar axon terminals in the presumed OFF layer and OFF

1194 axon terminals in the presumed ON layer of the IPL (G-J). Complete stack is available in Figure 6-1.

1195 Scale bar in C applies for A-C, scale bar in F for D-J, all scale bars are 20 µm.

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1197 **Figure 6-1 Confocal image stack with GNB3 (green) and Ctbp2 (magenta) staining within the IPL**.

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