Neurons of the primate retina: A qualitative and quantitative analysis

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Abstract

Parallel processing of visual information begins at the first synapse in the retina, where input from photoreceptors is transmitted to 12 types of bipolar cell. Bipolar cells are interneurons that propagate visual signals to at least 17 types of ganglion cell, which are the output neurons of the retina. In this way the so-called vertical pathways are formed that deliver different sensory signals to the brain. This thesis comprises a detailed map of the cell types that contribute to parallel processing in primate retina. Knowledge of the morphology and spatial distribution of retinal cells is pivotal in understanding the way the visual scene is processed, and more broadly in understanding mechanisms of neuronal wiring.

Chapter 1 introduces the structure of the primate retina and describes the morphology of retinal cells and their contribution to visual processing.

Chapter 2 provides a survey of the ganglion cell types in marmoset retina. Ganglion cells were transfected with a plasmid for the expression of a synaptic marker, postsynaptic density 95, conjugated to green fluorescent protein. After transfection the retinas were processed with immunohistochemical markers for bipolar and/or amacrine cells to determine ganglion cell stratification. At least seventeen morphological types of ganglion cell were identified. The contribution of widefield ganglion cells to peripheral vision is much greater than their contribution to foveal vision, whereas the fovea is dominated by midget and parasol cells. Outside the fovea the ganglion cell diversity in marmoset retina is likely as great as that reported for non-primate retinas.

In Chapter 3, particle-mediated gene transfection was applied to post mortem human retina. Human retinas maintained their morphology and immunohistochemical properties for at least three-days in culture. Based on dendritic field size and stratification at least eleven morphological types of retinal ganglion cell were distinguished. This study demonstrates that gene transfection can be used to target cells in the human retina, with the potential to study their connectivity and structural changes in diseases.

Chapter 4 provides a quantitative analysis of the major cell populations in the inner nuclear layer of normal human retina. Immunohistochemical markers were applied to vertical sections in order to label and quantify horizontal, bipolar,
amacrine and Müller cells across the retina. In addition, cone photoreceptors and ganglion cells were counted. With the exception of the fovea, which is dominated by midget bipolar cells, the proportion of different cell populations in the inner nuclear layer is comparable across all eccentricities and comparable to non-human primates and other mammals. Analysis of the midget pathway (high acuity and colour vision), revealed that the ratio of cone photoreceptors to recoverin labelled OFF midget bipolar cells does not change between central and peripheral retina, suggesting that the acuity of the cone array is delivered to the inner retina in the peripheral as well as in the foveal retina. The density of the diffuse cone bipolar cell types DB3a and DB3b which provide input to the parasol pathway (contrast and motion detection) was less than half that of midget bipolar cells at any given eccentricity. The cone to cone bipolar cell ratio was constant across the retina suggesting that convergence and divergence do not change with eccentricity.

In conclusion, the data provided in this thesis will serve as a reference for the interpretation of abnormalities in disease, and the informed targeting of treatments in human retinas.
Statement of originality

This statement is to certify that:

i. To the best of my knowledge, this thesis comprises my original work toward the PhD except where indicated in the Authorship Attribution Statement;

ii. Due acknowledgement has been made in the text to all other material used and external assistance received;

iii. This thesis has not been submitted for any other degree.

Rania Masri
29/07/2019
Authorship Attribution Statement

The experiments in Chapter Two of this thesis were initiated during my Honours candidature and were completed during the first year of my PhD. Retinas analyzed in Chapter Two were provided by Prof. Amane Koizumi and immunohistochemically labelled under the supervision and guidance of Dr. Kumiko Percival. The process of particle-mediated gene transfection described in Chapter Three was carried out in collaboration with Dr. Sammy C.S. Lee. One of the preparations presented in Chapter Four (#14064) was sectioned and partly analyzed by Dr. Siva Purushothuman.

Some of the results described in the present thesis have appeared in the following publications and abstracts:

Publications

- Chapter Two comprises the following publication:


  My individual contribution to the above publication included the following: study concept and design, acquisition of data, analysis and interpretation of data, drafting and critical revision of the manuscript.

- Chapter Three comprises the following publication:


  My individual contribution to the above publication included the following: study concept and design, acquisition of data, analysis and interpretation of data, drafting and critical revision of the manuscript.

Published Abstracts


Non-published abstracts


During the course of my PhD, I also contributed to the following publications and abstracts, which involved experiments not described by this thesis:

Publications:


Published Abstracts:


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29/07/2019
As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Ulrike Grünert
29/07/2019
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BT</td>
<td>broad thorny</td>
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<tr>
<td>CaBP</td>
<td>calbindin</td>
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<td>CD15</td>
<td>carbohydrate epitope 15</td>
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<td>choline acetyl transferase</td>
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<td>CtBP2</td>
<td>c-terminal binding protein 2</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole dihydrochloride</td>
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<tr>
<td>DB</td>
<td>diffuse bipolar</td>
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<tr>
<td>DIC</td>
<td>differential interference contrast</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>FMB</td>
<td>flat midget bipolar</td>
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<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
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<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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<td>GCL</td>
<td>ganglion cell layer</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HFL</td>
<td>henle fibre layer</td>
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<td>IMB</td>
<td>invaginating midget bipolar</td>
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<td>INL</td>
<td>inner nuclear layer</td>
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<tr>
<td>IPL</td>
<td>inner plexiform layer</td>
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<tr>
<td>LBS</td>
<td>large bistratified</td>
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<td>LGN</td>
<td>lateral geniculate nucleus</td>
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<td>NFL</td>
<td>nerve fibre layer</td>
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<td>NT</td>
<td>narrow thorny</td>
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<td>ONL</td>
<td>outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>outer plexiform layer</td>
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<tr>
<td>PB</td>
<td>phosphate buffer</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PSD95</td>
<td>postsynaptic density 95</td>
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<td>RBPMS</td>
<td>RNA-binding protein with multiple splicing</td>
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<td>recursive bistratified</td>
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<tr>
<td>RPE</td>
<td>retinal pigment epithelium</td>
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<tr>
<td>S-, M-, L-</td>
<td>short, medium, long wavelength sensitive</td>
</tr>
<tr>
<td>SBS</td>
<td>small bistratified</td>
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<td>SM</td>
<td>smooth monostratified</td>
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CHAPTER ONE
1. Introduction

1.1 Introduction to the primate retina

The retina is a layer of nerve tissue that lines the back of the eye. Light that enters the eye is focused by the cornea and lens onto the retina which carries the sensory neurons (photoreceptors). Photoreceptors convert light to an electrical signal that subsequently modifies neurotransmitter release. There are four types of photoreceptor in the primate retina; rods specialised for scotopic (night) vision, as well as short (S), long (L), and medium (M) wavelength sensitive cones which are active under photopic (daylight) conditions. There are also intrinsically photosensitive melanopsin-containing cells which are involved in regulating circadian rhythms. Sensory information is carried from photoreceptors to retinal ganglion cells in parallel vertical pathways, each of which delivers a different message to the brain about the visual world (Wässle, 2004; Field and Chichilnisky, 2007; Masland, 2012). There are roughly 30 different areas in the brain devoted to the processing of visual sensory information that is received through direct or indirect input from the retina (Felleman and Van Essen, 1991). Knowledge of retinal neurons and their connectivity is pivotal in understanding visual processing, and more broadly in understanding mechanisms of neuronal wiring. In the living eye, the structure of the retina can be resolved to the level of the retinal layers through non-invasive methods such as optical coherence tomography (Chen et al., 2006; Curcio et al., 2011; Yoshioka et al., 2019). This is a valuable diagnostic tool for diseases that affect the eye and the central nervous system.

This thesis aims to provide a map of the cells that make up the primate (including human) retina, in particular the density and distribution of different cell populations across the retina, as well as detailed characterization their connections. The research presented here is derived from human and non-human primate retinas. Primates are the only mammals that contain a fovea, which is a region of the retina specialized for high acuity vision (see section 1.1.2). In the chapter 2, I employ the New World marmoset (*Callithrix jacchus*) as a non-human primate model for the human visual system. The marmoset visual system bares many similarities to that of Old World monkeys and humans (Troilo et al., 1993; Solomon and Rosa, 2014). Figure
1.1 shows images of a marmoset (A) and a human (B) retina, revealing comparable macroscopic features such as the yellow pigment of the macula. The data presented in chapters 3 and 4 are obtained from *post mortem* human retinas. Since humans and monkeys have evolved independently for over 1.5 million years, it is important to establish the potential specializations that may distinguish human and non-human primates.

The following sections of this chapter will provide an overview of the cell types in the retina and outline current knowledge on their connectivity and function in visual processing.

**Figure 1.1** Comparison of a marmoset and a human retina. Micrographs of retinas from an adult male marmoset and a 47 year old female human donor. The retinas were dissected out of the eye cup and flattened onto a dish using relieving cuts. The yellow pigment of the macula is visible, indicating the location of the fovea in both retinas. The hole in the centre of the retinas represents the site of the optic nerve head. S: superior, I: inferior, T: temporal, N: nasal. Scale bar = 5 mm. (*Image courtesy Ulrike Grünert*).
1.1.1 Structure of the retina

The basic structure of the retina can be appreciated in Figure 1.2, which is a schematic representation of a vertical section. There are five neuronal cell types that are organized in vertical and horizontal laminations; photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. The retina also contains three types of glial cell; astrocytes, microglia, and Müller cells. The neurons of the retina are neatly packed in a multilayered organisation, which can be separated into nuclear layers containing the cell bodies, and plexiform layers containing the cell dendrites and synapses. The photoreceptors are positioned sclerad so that the outer segments of rod and cone photoreceptors are interacting with the retinal pigment epithelium, which provides trophic and structural support. The cell bodies of the photoreceptors form the outer nuclear layer (ONL), and their axon terminals form the outer plexiform layer (OPL). The OPL is where the photoreceptors transmit signals to the dendrites of bipolar cells and the processes of horizontal cells. The somas of bipolar cells, horizontal cells, amacrine cells, and Müller cells form the inner nuclear layer (INL). The axons of bipolar and amacrine cells and the dendrites of ganglion cells stratify in the inner plexiform layer (IPL). The bipolar axon terminals contact the dendritic arbour of ganglion cells in the inner plexiform layer. Different types of bipolar cell stratify at different levels of the IPL, resulting in the anatomical derivation of five strata (S1-S5) within this layer (Cajal, 1972). The cell bodies of horizontal cells are located at the border of the inner nuclear layer with the outer plexiform layer and the somas of amacrine cells are located at the border of the inner nuclear layer with the inner plexiform layer (Fig 1.2). The ganglion cell layer (GCL) houses the somas of the ganglion cells and displaced amacrine cells. The nerve fibre layer of the retina consists of ganglion cell axons which exit the retina at the optic disc and form the optic nerve. Signals transmitted to the ganglion cells from the outer retina are delivered to the brain through the optic nerve.
Figure 1.2 Schematic drawing of the laminar organisation of the retina. The retinal layers are indicated on the right and the cell types are indicated on the left of the schematic. Rod and cone photoreceptors occupy the outer (scleral) layer and their cell bodies form the outer nuclear layer. The somas of horizontal, amacrine and bipolar cells are located in the inner nuclear layer. The cell bodies of Müller cell glia are also found in the inner nuclear layer and their processes span the entire thickness of the retina. Ganglion cells form the innermost (vitreal) layer and their axons come together in the nerve fibre layer to form the optic nerve, through which visual signals are transmitted out of the eye to the brain.
1.1.2 The fovea

The fovea is a specialised region of primate retina which is located ~3-4 mm temporal to the optic disc in human and ~2 mm temporal in marmoset (Fig 1.1) (Troilo et al., 1993; Drasdo et al., 2007). Light passes through the layers of the retina to reach the photoreceptors, as shown in Figure 1.2. In the fovea, the inner layers are shifted laterally and there is a direct path to the photoreceptors (Fig 1.3). At the centre of the fovea, photoreceptors are very densely packed (Curcio et al., 1991) in order to allow high acuity spatial sampling of the visual world. The very centre of the human fovea lacks rod photoreceptors and short-wavelength cones (S-cones). The rod free area is roughly 350 to 750 µm wide (Polyak, 1941; Curcio et al., 1991; Strettoi et al., 2018) and extends beyond the S-cone free area. The absence of rods from the centre of the fovea is not surprising, given that rods are specialised for high sensitivity under low light conditions and are not involved in the transmission of high acuity signals. The reason for the absence of S-cones from the foveas of humans and macaques is less clear, and in the marmoset retina S-cones are present at the foveal centre (Martin and Grünert, 1999). Long and medium wavelength cones are at their peak density at the foveal centre, and these cones mediate high spatial resolution by maintaining one-to-one connections with downstream neurons. Consequently, the cells connecting to this dense network of cones are also densely packed near the fovea, but are displaced from their receptive field at the foveal centre. The axons of cone photoreceptors (Henle fibres) radiate from cone somas to the cone pedicles, forming a layer of obliquely orientated fibres travelling in the direction of downstream neurons displaced from the foveal centre (Figure 1.3). The degree of lateral displacement is measured in the human fovea and results are presented in Chapter 4 to determine the anatomical convergence of input within vertical pathways.

The structure and composition of the fovea is different from the rest of the retina, and it is for this reason that eccentricity (distance from the foveal centre) is an important consideration when studying the primate retina. Cell density decreases away from the fovea, and the dendritic and axonal fields of retinal cells become larger (Watanabe and Rodieck, 1989; Dacey and Petersen, 1992; Rodieck and Watanabe, 1993; Ghosh et al., 1996). Most notable are changes in ganglion cell dendritic fields (Fig.1.4). Recent studies have shown that the specialised function of the fovea is
facilitated by differences in the physiology of retinal circuits within the fovea compared to the periphery (Sinha et al. 2019).

Figure 1.3 Vertical view through a human fovea. Micrograph of a vertical section through a 36 year old female donor retina. The foveal slope extends from either side of the centre of the fovea (foveola). The foveola consists primarily of the outer layers carrying the photoreceptors. The Henle fibre layer (HFL) shows horizontally orientated fibres radiating from cone photoreceptor somas at the foveola to the downstream neurons in laterally displaced retinal layers. The inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL) are displaced away from the foveola and gradually increase in thickness along the foveal slope. ELM: external limiting membrane, ONL: outer nuclear layer. Scale bar = 50 µm.

Figure 1.4 The dendritic field size of ganglion cells increases with distance from the fovea. Drawings of midget ganglion cells in marmoset retina. The numbers next to each cell indicate the distance from the foveal centre (eccentricity). Arrows point to ganglion cell axons. The dendritic field size increases with increasing eccentricity. Scale bar = 50 µm. (Ghosh et al., 1996).
1.1.3 Synapses in the retina

Retinal neurons transmit information via electrical and chemical synapses. Cone photoreceptors form invaginating and flat/basal contacts types of contacts in the outer plexiform layer. Each cone pedicle contains between 20 and 50 synaptic invaginations. Each synaptic invagination usually contains one presynaptic ribbon and three postsynaptic processes (two horizontal dendrites and one or two bipolar cell dendrites) (Boycott and Dowling, 1969; Chun et al., 1996). In addition, cone bipolar cells make flat contacts at the base of the pedicle. Horizontal cells are coupled to each other by gap junctions, which allows these cells wide lateral reach across the retina.

In the inner plexiform layer, bipolar cell axons form ribbon synapses. At these synapses, presynaptic ribbons are located ~ 200 nm above the plasma membrane (Sterling and Matthews 2005) and are involved in tethering synaptic vesicles and moving them to the release site at the base of the ribbon (Zenisek and Matthews, 2000; Migdale et al., 2003). C-terminal binding protein 2 (CtBP2) is a transcriptional repressor that forms part of a major protein unique to the synaptic ribbon (Schmitz et al., 2000). Most bipolar terminals are characterised by varicosities along their length, which are assumed to be sites of synaptic contact (Grüner and Martin, 1991; Jacoby and Marshak, 2000; Zenisek and Matthews, 2000). For most cone bipolar cells, each synaptic ribbon apposes a ganglion cell and an amacrine cell process (Figure 1.5). Amacrine cells make conventional synapses with bipolar cells, ganglion cells, and each other. The bipolar synapse onto ganglion cells is excitatory, and so it is characterised by thick material at the postsynaptic density opposed to the presynaptic ribbon of the bipolar cell (Dowling and Boycott, 1966; Raviola and Raviola, 1982; Jacoby et al., 1996; Grüner et al., 2002). PSD-95 (postsynaptic density protein-95) aggregates at the postsynaptic sites of the ganglion cell and is presumed to be associated with the clustering of a subset of ionotropic glutamate receptors (Koulen et al., 1998).

More recently, the method of biolistic transfection has been applied to retinal tissue to detect synaptic input onto retinal ganglion cells. First developed in rabbit retina, the authors used a gene gun to propel microcarriers (bullets) coated with an expression plasmid for the postsynaptic density protein PSD95 conjugated to green fluorescent protein into retinal tissue (Koizumi et al., 2007). This is followed by a three-day culture period to allow for the expression of the transfected protein by the target
cells. PSD95 was used to target the postsynaptic sites of retinal ganglion cells (Jakobs et al., 2008). Particle-mediated gene transfection has been applied to rodent (Moritoh et al., 2010; Morgan et al., 2011), and marmoset (Moritoh et al., 2013; Percival et al., 2014; Masri et al., 2016) retina to analyze bipolar to ganglion cell connectivity. In Chapter 3 of this thesis this method is applied to post mortem human retinas.

**Figure 1.5** Schematic drawing of synapses in the inner plexiform layer. Bipolar axons form ribbon synapses onto two postsynaptic profiles. The bipolar terminal is shown in magenta with varicosities containing multiple synaptic ribbons. The bipolar terminal (BP) contacts ganglion cell dendrites (GC, green) and amacrine cell processes (AC, blue). Amacrine cells form conventional synapses onto bipolar, ganglion, or other amacrine cells. The inset shows the region outlined in white at the bipolar ribbon synapse. (Modified from Wässle, 2004).
1.2 Cell types in the retina and their role in visual processing

1.2.1 Amino acid signatures and molecular markers

Retinal neurons can be categorized by their expression of biochemical markers. A detailed description of the amino acid signatures that distinguish different retinal cells in the primate was carried out by Kalloniatis and colleagues (1996). The key amino acid signatures in the primate retina are glutamate, glycine, gamma-aminobutyric acid (GABA) and taurine. GABA and glycine are fast inhibitory neurotransmitters that dominate retinal processing. Broadly, photoreceptors express glutamate, as do cone bipolar cells and most ganglion cells. Amacrine cells express glycine, GABA, or both, and Müller cells are taurine-rich. Horizontal cells express GABA, but to a lesser extent compared to amacrine cells.

Peng et al. (2019) carried out a near complete molecular taxonomy of primate retinal neurons using sophisticated RNA sequencing. Peng and colleagues generated high-quality cell profiles in order to identify molecular signatures specific to subpopulations of retinal neurons. Consistent with the work of Kalloniatis and colleagues, the method of molecular sequencing showed that GABAergic and glycinergic neurons can be further separated based on variation in their expression of biochemical signatures. The study by Peng et al. produced an extensive database of these signatures that can be used to isolate or cluster cell populations. Interestingly, the authors also observed eccentricity-related differences in GABAergic expression in amacrine cells and phototransduction proteins in photoreceptors.

1.2.2 Photoreceptors

Photoreceptors are the principal sensory neuron of the retina. The outer segments of photoreceptors contain a protein called opsin which is embedded in a stack of membranes. In rods, the membrane consists of intracellular disks which are not connected to the surface membrane whereas in cones the photosensitive membrane is continuous with the surface membrane. Opsin is bound to 11-cis retinal and absorbs photons of light. Due to the high light sensitivity of rod opsins
(rhodopsin), rods are specialised for vision under dim light, also known as scotopic vision (Baylor et al., 1984; Kraft et al., 1993). Cone opsins are less sensitive than rods to light (Baylor, 1987). This means they are elemental in photopic vision which occurs under well-lit conditions, when rods are saturated (Baylor et al., 1984). Under mesopic (moderately lit) conditions, both types of photoreceptor are responsive (Schneeweis and Schnapf, 1995; Hornstein et al., 2005).

Photoreceptors convey changes in illumination through signalling cascades initiated by the absorption of photons of light by the light sensitive molecule retinol. This leads to conformational changes in photoreceptor opsins which causes the closure of cyclic guanosine monophosphate gated channels in the membranes of the outer segments. The resultant hyperpolarisation of the photoreceptor reduces the release of glutamate at the photoreceptor terminal. Under dim light levels the outer segment channels remain open and release sodium predominantly, leading to the depolarisation of the photoreceptor which in turn facilitates the steady release of glutamate from the presynaptic terminal (Baylor, 1996). At the photoreceptor synapse, the glutamate receptors located at the tip of the bipolar dendrites shape the signal that is delivered to the inner retina as a result of changes in illumination.

Photopic (daylight) vision allows the perception of colour through different types of cones carrying opsin proteins that vary in their peak wavelength sensitivity across the visible spectrum. Cones with peak wavelength sensitivity in the short range of the visible spectrum, near spectral blue, are called S-cones (or blue cones). Similarly, M-cones are medium wavelength sensitive cones near spectral green and L-cones are long-wavelength sensitive cones near spectral red. The peak wavelength sensitivities of S-, M- and L-cones in human are 430, 531 and 561 nm, respectively (Schnapf et al., 1987). Perception of colour is determined by the ratio of the quantum catch of these cones in response to reflected wavelengths of light.

The S-cones can be distinguished from other cones by subtle differences in their morphology (Ahnelt et al., 1987; Curcio et al., 1991), molecular signatures (Peng et al., 2019), and by immunohistochemical labelling with antibodies directed against S-cone opsin (Szel et al., 1988; Curcio et al., 1991; Martin and Grünert, 1999). Understanding the relative proportion of different photoreceptor subtypes,
as well as the relative convergence of photoreceptors to downstream retinal neurons, provides an insight to the signals that are transmitted to the inner retina.

1.2.3 Neurons of the vertical ‘through’ pathway

Bipolar cell types

There are multiple subtypes of bipolar cell in the retina of primates and other mammals (Boycott and Wässle, 1991; MacNeil et al., 2004; Wässle et al., 2009). In primate retina, the cone driven bipolar cells comprise two types of midget (flat and invaginating), seven types of diffuse (DB), and one type of blue cone bipolar cell (Fig. 1.6). In addition, a large-field cone bipolar cell type named “giant bipolar” has been described (Kolb et al., 1992; Joo et al., 2011; Tsukamoto and Omi, 2016).

Bipolar cell types were first distinguished by their morphology in Golgi labelled preparations (Polyak, 1941; Boycott and Dowling, 1969; Cajal, 1972; Boycott and Wässle, 1991; Kolb et al., 1992), and later the different morphological types were further characterised by expression of specific marker proteins (Dizhoor et al., 1991; Grünert and Martin, 1991; Kouyama and Marshak, 1992; Milam et al., 1993; Grünert et al., 1994; Wässle et al., 1994; Jacoby et al., 2000; Chan et al., 2001b; Haverkamp et al., 2003; Puthussery et al., 2011). Consistently, recent studies using single-cell RNA sequencing molecularly characterised primate bipolar cells into twelve types (Peng et al. 2019). Morphological differences between bipolar cells can be seen in Figure 1.6. These differences include the shape of the dendritic tree in the outer plexiform layer, the position of the soma in the inner nuclear layer, and the shape and position of the axon terminals in the inner plexiform layer. In the outer plexiform layer, diffuse bipolar cells can be differentiated from midget bipolar cells by the number of cone contacts. Diffuse bipolar cells contact between 5-10 cones (Boycott and Wässle, 1991) whereas midget bipolar cells contact between 1-3 (Kolb and Dekorver, 1991). Midget bipolar cells therefore have smaller dendritic bouquets at the same eccentricities compared to the diffuse bipolar cells. In the inner plexiform layer, where bipolar cell terminals contact ganglion cell dendrites, the axons of bipolar cells terminate in distinct strata (Fig. 1.6). Reconstructions of bipolar cells in macaque retina using electron microscopy (Calkins et al., 1994; Calkins et al.,
1998; Calkins and Sterling, 2007) (Tsukamoto and Omi, 2015, 2016) confirmed the morphological types described previously.

Figure 1.6 shows the immunohistochemical markers that can be used to identify bipolar subtypes in human and marmoset retina. Milam et al. (1993) first showed in human retina that antibodies against recoverin are expressed by flat midget bipolar cells, which is also true in macaque retina (Milam et al., 1993; Wässle et al., 1994). In marmoset retina, antibodies against CD15 are used to label flat midget bipolar cells and DB6 cells (Chan et al., 2001b) whereas in human retina CD15 is expressed by DB3b cells (Haverkamp et al., 2003; Putthussery et al., 2013). Across human, macaque and marmoset retina, rod bipolar cells and DB4 cells can be labelled with antibodies against Protein kinase C alpha (PKC$\alpha$) (Grüner and Martin, 1991; Kolb et al., 1993; Chan et al., 2001b; Haverkamp et al., 2003) and DB3a cells can be labelled with antibodies against calbindin (CaBP) (Martin and Grüner, 1992; Grüner et al., 1994; Luo et al., 1999; Haverkamp et al., 2003). Glutamate transporter 1 (Glt1) is also consistently expressed across primates in subtypes of OFF bipolar cells (FMB, DB2 and DB3b) (Grüner et al., 1994; Jacoby et al., 2000; Haverkamp et al., 2003; Weltzien et al., 2015). ON bipolar cells can be labelled with antibodies against the transcription factor Islet-1 (Haverkamp et al., 2003) or the G protein alpha (Go$\alpha$) (Haverkamp et al., 2003; de Souza et al., 2012). In human and marmoset retina, antibodies against the hyperpolarisation activated cyclic nucleotide gated potassium channel 1 (HCN1) are expressed in DB3a, DB3b and DB4 cells (Putthussery et al., 2013). Haverkamp and colleagues (2003) provide a detailed comparison of the immunohistochemical markers of bipolar subtypes in human and macaque retina. The macromolecular markers applicable to bipolar cells in marmoset retina were most recently described by Weltzien et al. (2015), and in human retina by deSouza et al. (2016). Overall, the morphology of bipolar cell types appears to be consistent across primates however the expression of macromolecular markers shows some variation.
Figure 1.6 Bipolar cell types in primate retina. The cells are arranged according to the level of axon terminal stratification in the inner plexiform layer. The bipolar cell types are named in bold above each cell. The layers of the retina are indicated on the right and the five strata within the inner plexiform layer (S1-S5) are indicated on the left side. The dotted line represents the border between the ON and the OFF sublamina. Known immunohistochemical markers for each bipolar cell type are indicated for marmoset (above) and human (below) retina. DB: diffuse bipolar, FMB: flat midget bipolar, IMB: invaginating midget bipolar, BB: blue cone bipolar, RB: rod bipolar. OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. CD15: carbohydrate epitope 15, CaBP: calbindin, CCK: cholecystokinin, Glt1: glutamate transporter 1, Goα: G-protein alpha, HCN1: hyperpolarisation activated cyclic nucleotide gated potassium channel 1, Islet1: LIM-homeodomain transcription factor Islet-1, PKCα: protein kinase C alpha, Rec: recoverin. (Modified from Boycott & Wässle, 1999; Haverkamp et al. 2003).
**Bipolar cell function**

Bipolar cells transmit signals from photoreceptors to the inner retina. At this level in the vertical pathway, the twelve bipolar types are already forming parallel information channels to be propagated through the visual system. For example, bipolar cells can be broadly categorised into ON or OFF cells based on the polarity of their responses to light. An increase in illumination causes a decrease in the release of glutamate at the photoreceptor synapse. In the postsynaptic dendrites of ON and OFF bipolar cells, different types of glutamate receptors cause a range of responses to changes in illumination. Glutamate receptors can be broadly subdivided into two main types; metabotropic and ionotropic receptors. Metabotropic receptors (mGluR6) are located in the dendritic tips of ON bipolar cells and lead to hyperpolarisation of ON cells in response to light increments (Vardi, 1998; Vardi et al., 2000). In contrast, ionotropic glutamate receptors are depolarised in response to light increments and hyperpolarised during light decrements, when there is an increase in glutamate release at the photoreceptor synapse (Massey, 1990). OFF bipolar cells express different subtypes of ionotropic glutamate receptors on their dendrites called AMPA (\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors (Haverkamp et al., 2001b, a; Puthussery et al., 2014) which contribute to variation in physiological properties of these bipolar cells (DeVries, 2000). ON and OFF bipolar cells can also be distinguished anatomically based on their stratification in the inner plexiform layer. Figure 1.6 shows ON and OFF subdivisions of the inner plexiform layer, where bipolar cells stratifying in the inner half of the inner plexiform layer are ON cells while the cells in the outer half are OFF cells (Famiglietti and Kolb, 1976; Nelson et al., 1978; Schiller, 1992). Electrophysiological recordings from bipolar cells show that ON and OFF bipolar cells can be further separated into those with transient (high frequency) and sustained (low frequency) responses to light (Awatramani and Slaughter, 2000; DeVries, 2000).

The connectivity and distribution of many bipolar subtypes can provide a clear indication of their function in visual processing. For example, blue cone bipolar cells selectively contact S-cones and so are assumed to carry the chromatic blue-ON signal to the inner retina (Kouyama and Marshak, 1992; Calkins, 2001). Kolb and Dekorver (1991) showed in Golgi preparations of human
retina that midget bipolar cells near the foveal centre contact a single cone photoreceptor. It is widely believed that this feature of the midget circuitry evolved for high spatial resolution in primates (Kolb and Dekorver, 1991; Calkins et al., 1994; Martin, 1998). As a consequence of this one-to-one connectivity, midget bipolar cells establish a private line of transmission for chromatic signals from medium and long wavelength cones to the output neurons of the retina (Fig. 1.7). Thus, in central retina, the midget system serves high spatial acuity and red/green colour vision. In keeping with this hypothesis, the density of midget bipolar cells is highest near the fovea and decreases towards peripheral retina (Wässle et al., 1994; Weltzien et al., 2015).

Diffuse bipolar cells indiscriminately contact multiple cones, and so much of their function is inferred with respect to stratification and connectivity in the inner plexiform layer as opposed to cone-type specificity. As such, diffuse bipolar cells are not involved in the transmission of chromatic signals to the inner retina. Electrophysiological recordings of diffuse bipolar types have assisted the characterisation of the role of these cells in processing visual stimuli (Puthussery et al., 2013; Puthussery et al., 2014). The OFF diffuse cell DB3 is the most well studied diffuse bipolar cell type. Puthussery and colleagues (2013 and 2014) showed that the DB3 cell can be separated into two distinct types; DB3a cells which exhibit sustained, and DB3b cells which exhibit transient responses to glutamate. The authors suggest that this is mediated by the different subunits of kainate receptors expressed by DB3a and DB3b cells.

DB3a cells stratify at the same level as OFF parasol ganglion cells, and the two cells have demonstrated robust synaptic connectivity in macaque and marmoset retina (Jacoby and Marshak, 2000; Jacoby et al., 2000; Tsukamoto and Omi, 2015; Masri et al., 2016). A schematic of the parasol pathway is shown in Figure 1.7. The DB3a cell drives the parasol pathway and contributes to the detection of motion and contrast in primate vision (Puthussery et al., 2013; Puthussery et al., 2014). In Chapter 4 the DB3a and DB3b subtypes are identified and quantified in human retina.
Figure 1.7 Bipolar cells in the midget (acuity) and parasol (motion detection) pathways. The schematic on the left shows one-to-one connectivity between cone photoreceptors and midget bipolar cells and this is characteristically found in foveal retina. The schematic on the right represents circuitry for the parasol pathway. Parasol ganglion cells are receiving input from diffuse bipolar cells which contact multiple cone photoreceptors.
Chapter One
Introduction | 18

Ganglion cell types

Ganglion cells are the output neurons of the retina. They receive signals from photoreceptors via bipolar cells and amacrine cells, and transmit information directly to visual nuclei in the brain. Ganglion cells have been categorized based on their central projections, morphology, and electrophysiological properties. Polyak (1941) initially described midget, parasol and “shrub” ganglion cells in Golgi-labelled preparations. Shrub cells were later classified as small bistratified ganglion cells. Midget, parasol and small bistratified cells make up most of the ganglion cell population, and so are the most studied in primate retina. Using retrograde labelling, Perry and colleagues (1984) revealed the projections of these cells to different layers of the lateral geniculate nucleus in the dorsal thalamus. Watanabe and Rodieck (1989) and Rodieck and Watanabe (1993) combined retrograde tracers from multiple visual nuclei in the brain with intracellular injection of labelled somas in the retina to reveal the ganglion cell dendritic tree. In this way, the researchers were able to characterise the morphological features and projections of different ganglion cell types. Concurrently, Dacey and Peterson described the morphology of midget and parasol ganglion cells in human retina using intracellular injection in vitro (Dacey and Petersen, 1992), and later applied the same method to identify non-midget, non-parasol ganglion cells (Peterson and Dacey, 1999, 2000). In 2003, Dacey and co-workers. developed a method called ‘photofilling’, where the morphology of cells retrogradely labelled using rhodamine dextran is revealed when the somas are exposed to light, causing the dextrans to diffuse through the dendrites. This technique revealed previously unidentified cell types in macaque retina and led to the classification of 17 morphological types of ganglion cell (Fig 1.8). The same method was used to identify ganglion cells projecting to the koniocellular layers of the lateral geniculate nucleus in marmoset (Szmajda et al., 2008; Percival et al., 2011, 2013). Another method to identify ganglion cells in retina is particle mediated gene transfection (see Section 1.1.3 above). In the aforementioned studies the target protein was located at the excitatory post synaptic sites on ganglion cell dendrites. One advantage of this method is that a large number of cells can be labelled directly by shooting plasmid-coated microcarriers into retinal tissue. Thus, this method allows the detection of a variety of different ganglion cell types regardless of their projections. This method was used to survey different morphological types of ganglion cell in...
marmoset retina in Chapter 2 and was also applied to human retina in Chapter 3. Recent work by Peng and colleagues (2019) used molecular RNA sequencing to find 18 subtypes of ganglion cell, most of which were present both in central and peripheral retina. This confirmed the conclusions drawn in Chapter 2 in marmoset and in Dacey’s previous work in macaque.

Non-midget, non-parasol, non-small bistratified ganglion cells are collectively referred to as widefield ganglion cells based on the extent of their dendritic fields, which are ‘wider’ compared to the midget, parasol and small bistratified ganglion cells (Polyak, 1941). Widefield ganglion cells make up less than 20% of all ganglion cells (Dacey, 2004). At least 13 types of widefield ganglion cell have been distinguished based on their morphology and stratification in the inner plexiform layer (Ghosh et al., 1996; Peterson and Dacey, 1999, 2000; Dacey et al., 2003; Dacey, 2004; Yamada et al., 2005). Among these cells are monostratified (smooth monostratified, large sparse, giant sparse, recursive, narrow thorny), bistratified (recursive and large bistratified), and broadly stratified (broad thorny) ganglion cells (Fig. 1.8). The use of molecular RNA sequencing has allowed the identification of molecular fingerprints which can be used to target particular ganglion cell types. For example, Dhande et al. (2019) showed that the transcription factor Satb2 is specific to direction selective cells in the mouse and to a large sparse cell type in the primate.

Each ganglion cell type tiles the entire retinal surface to ensure efficient sampling of the visual scene (Wässle et al., 1981; Dacey, 1993b). The cell bodies are spaced in a regular mosaic and their processes cover the areas between each cell body. The degree of overlap between the dendritic arbors of two adjacent ganglion cells of the same type is measured by the coverage factor. The coverage factor is the product of ganglion cell density and dendritic field size divided by retinal area (Mcllwain, 1986; Masland, 2012). A coverage factor of one indicates perfectly tiled dendrites with no overlap and no gaps. Most ganglion cell types have coverage factors close to one (Masland, 2012), meaning that every point in the visual scene is simultaneously reported to the brain by at least 17 independent cells, each transmitting a different aspect of the stimulus.
**Figure 1.8** Ganglion cell types in macaque retina. Schematic representation of ganglion cell types identified after retrograde tracer injections into the lateral geniculate nucleus and subsequent photofilling. Ganglion cells are either monostratified in the ON or OFF sublamina of the inner plexiform layer (IPL), bistratified, or broadly stratified in the middle of the IPL. The disks above the schematic depict dendritic field size of the cells in peripheral retina. Different shapes and densities of the dendritic tree can also observed in the drawings of ganglion cells within the disks. *(Modified from Dacey, 2004).*
**Ganglion cell function**

The ON/OFF dichotomy of bipolar subtypes is propagated through the inner plexiform layer to yield ON and OFF postsynaptic responses in ganglion cells. Midget and parasol ganglion cells have concentric receptive fields with an ON centre and an antagonistic OFF surround, or vice versa (Croner and Kaplan, 1995). ON centre ganglion cells are excited by light increments while OFF centre ganglion cells are excited by light decrements in the centre of their receptive field. The midget ganglion cell receives chromatic input to its receptive field centre and spectrally opponent input to its surround (Lee et al., 1998; Martin et al., 2001; Diller et al., 2004). The output of midget ganglion cells is important for encoding high resolution vision as well as red/green colour vision.

Input to parasol ganglion cells is achromatic, since they predominantly contact diffuse bipolar cells which transmit signals from multiple cones with varied wavelength sensitives. Parasol ganglion cells have larger receptive fields and higher contrast sensitivity compared to midget ganglion cells (Croner and Kaplan, 1995). In addition, midget ganglion cells show sustained responses to maintained stimuli while parasol ganglion cells respond transiently to stimulus onset or offset (Levitt et al., 2001; Solomon et al., 2006; Field et al., 2007; Crook et al., 2008b). These properties implicate parasol ganglion cells in the detection of luminance changes in low contrast stimuli, and in the perception of fast flicker and motion.

Small bistratified ganglion cells have two dendritic tiers in the inner plexiform layer; one in the ON and one in the OFF sublamina. The ON tier receives input from blue cone bipolar cells while input to the OFF tier is less clearly understood. Small bistratified ganglion cells exhibit sustained excitatory responses to blue light, presumed to arise from the blue ON, yellow OFF dichotomy between the two dendritic tiers (Dacey and Lee, 1994; Field et al., 2007). It was previously thought that yellow OFF signals to the outer tier arise from input from OFF diffuse bipolar cells (Calkins et al., 1998). However recent studies have conferred that the yellow OFF signal arises from negative feedback from horizontal and amacrine cells (Field et al., 2007). Although the mechanisms have not been fully elucidated, the small bistratified ganglion cell is known to contribute to encoding colour perception in the blue ON/yellow OFF channels (Crook et al., 2009).
Electrophysiological recordings of some widefield ganglion cells have provided insight to their role in vision. For example, giant sparse ganglion cells have been shown to be melanopsin expressing ganglion cells in macaque retina (Dacey et al., 2005). These cells are intrinsically photosensitive and project to the lateral geniculate nucleus, the olivary pretectal nucleus and the suprachiasmatic nucleus, and are likely involved with regulating circadian rhythms and other non-image forming functions such as the control of pupil size (Hannibal et al., 2002; Hattar et al., 2002; Dacey et al., 2005). Recordings have also been made from smooth monostratified ganglion cells which have similar contrast and temporal sensitivity to parasol ganglion cells, and receive diffuse achromatic input, but project primarily to the superior colliculus whereas parasol ganglion cells project to the lateral geniculate nucleus (Petrusca et al., 2007; Crook et al., 2008). Puller et al. (2015) recorded from broad thorny ganglion cells and suggested from their responses to stationary and drifting stimuli that broad thorny cells are involved in the detection of retinal image slip during smooth pursuit of moving objects in visual space. The physiological function of most widefield ganglion cell types is still a growing area of research in primates because the low density of these cells makes them difficult to target and characterise. For some widefield ganglion cells, their function has been inferred based on their connectivity in the visual pathway. The diffuse bipolar cell type 6 (DB6) was shown to contact narrow thorny ganglion cells preferentially. Narrow thorny cells project to layer K1 of the lateral geniculate nucleus (Percival et al., 2014). Cells in this part of the lateral geniculate nucleus have been shown to play a role in motion sensitivity (Solomon et al., 1999; Puller et al., 2015; Eiber et al., 2018), and some bypass the visual cortex and project directly to the motion selective temporal area (Sincich et al., 2004).

Different types of widefield ganglion cells are likely to carry information about the visual world to different nuclei in the brain. It is therefore important to establish a method to effectively target and identify these cells. In this thesis, the method of particle-mediated gene transfection is applied to label ganglion cells in human and marmoset retinas. One of the aims was to establish whether the ganglion cell types identified in macaque are comparable in marmoset retina. Consistency amongst ganglion cell types in primates would imply that visual information is delivered to the brain in the same kind of information channels, and
thus the visual system of primates would integrate information in a similar way in order to create perception.

1.2.4 Neurons of the lateral pathway

**Horizontal cells**

Horizontal cells provide inhibitory feedback to rod and cone photoreceptors in order to hone the signal that is delivered to the inner retina. Horizontal cells sample cones across a broad area and therefore have a large receptive field on account of their laterally extending processes as well as the chemical synapses between neighbouring cells. When a central cone is excited the neighbouring cones are also activated, horizontal cells are subsequently hyperpolarized and are able to sum the light signals from cones across their receptive field and subtract this from the local signal. This creates feedback inhibition which reduces the light response of a maximally excited cone by the illumination of neighbouring cones. (Dacey et al., 1996; Dacey et al., 2000; Masland, 2001; Verweij et al., 2002). The feedback process in most vertebrates studied has been attributed to horizontal cell release of GABA, which is one of the main inhibitory neurotransmitters in the retina and has also been localised to horizontal cells in primates (Grüntert and Wässle, 1990; Kalloniatis et al., 1996). It has also been suggested that horizontal cells mediate inhibitory feedback via the induction of an outward current of calcium ions from the cone photoreceptors (Verweij et al., 1996; Verweij et al., 2003; Thoreson and Dacey, 2019). Horizontal cells are hyperpolarized by light (Dacheux and Raviola, 1990) and as calcium ions flow through cation-permeable channels in the horizontal cell membrane, the extracellular space becomes more negative (Kamermans et al., 2001). The hyperpolarisation of horizontal cells thus triggers pH-dependent voltage changes which result in the depolarisation of the cone photoreceptors (Hirasawa and Kaneko, 2003; Davenport et al., 2008; Warren et al., 2016).

There is general agreement that there are two types of horizontal cell in most mammals. In primate retina, they are named H1 and H2 horizontal cells and they differ in their connections. H1 cells make strong contacts with L and M cones, but avoid S-cones whereas H2 cells make strong connections with S-cones and make sparse contacts with M- and L-cones (Ahnelt and Kolb, 1994; Goodchild et al., 1996a; Chan and Grüntert, 1998). Consistently, S-cone responses are not
detected in recordings from H1 cells, which are most strongly activated by excitation of M- and L-cones (Dacey et al., 1996), and H2 cells respond strongly to S-cone modulation and to a lesser extent, to L- and M-cone excitation. Despite this seemingly preferential cone input, horizontal cells do not exhibit spectrally opponent responses i.e. they are excited by all wavelengths non-selectively. The function of horizontal cells in visual processing is more involved with centre-surround inhibition than with colour opponency (Dacey, 1996; Dacey et al., 1996).

**Amacrine cells**

Amacrine cells are inhibitory interneurons which are found mostly in the inner nuclear layer but can be displaced to the ganglion cell layer. Amacrine cells usually express either GABA or glycine as their neurotransmitters (Wässle and Boycott, 1991). GABAergic amacrine cells have large dendritic field diameters but are narrowly stratified in the inner plexiform layer. Glycinergic amacrine cells have small dendritic field diameters and are broadly stratified across the ON and OFF strata of the inner plexiform layer. Superimposed onto the GABA/glycine classification are at least 26 morphological types of amacrine cell in the primate retina (Mariani, 1990; Kolb et al., 1992), which can be further distinguished based on dendritic field size, stratification, connectivity in the inner plexiform layer, and their expression of immunohistochemical markers and other neurotransmitters.

For amacrine cells with known functions, it is apparent that each amacrine cell does a specific job in shaping ganglion cell responses. For example, starburst amacrine cells make excitatory synapses onto ganglion cells that are sensitive to moving stimuli and shape the direction selective response of these ganglion cells (He and Masland, 1997; Yoshida et al., 2001; Vaney et al., 2012). Starburst amacrine cells express both GABA and acetylcholine as neurotransmitters (Rodieck and Marshak, 1992). In primate retina, starburst amacrine cells can be labelled with antibodies against acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) (Rodieck and Marshak, 1992).

AII cells are the most frequent amacrine cell type in all mammals studied. They are glycinergic amacrine cells which integrate signals from the inner and outer sublaminae of the inner plexiform layer (Strettoi et al., 1992; Wässle et al., 1995). Under scotopic conditions, signals from rod photoreceptors are transmitted via rod bipolar cells to AII amacrine cells, which deliver the rod signal to ON and OFF
cone bipolar cells since rod photoreceptors do not possess a direct line of connectivity to retinal ganglion cells.

Many amacrine cell types can be distinguished in primate retina using molecular markers, and this has greatly facilitated the characterisation of different amacrine cell types in recent years (Majumdar et al., 2008; Klump et al., 2009; Neumann and Haverkamp, 2013; Weltzien et al., 2014; Marshak et al., 2015).

1.2.5 Glia

There are three types of glial cell in the retina: microglia which are found across various retinal layers, astrocytes which are located primarily in the nerve fibre layer, and Müller cells which span the entire thickness of the retina. The glial network provides mechanical, trophic and immune support to the neuroretina.

Müller cells are the principal glial cell in the primate retina, and the only glial cell present at the primate fovea (Provis et al., 2000; Bringmann et al., 2018). Müller cells have honeycomb-like processes surrounding the cone soma at the external limiting membrane, and extend to the outer plexiform layer by following the course of photoreceptor fibres. The cell body of Müller cells is situated in the inner nuclear layer between the somas of amacrine and bipolar cells. The endfeet of Müller cells form part of the internal limiting membrane. Müller cells provide support to retinal neurons through the formation of a functional column within which photoreceptors and interneurons surround a single Müller cell (Bringmann et al., 2018). Müller cells have a range of functions concerned with maintaining the healthy and homeostatic function of retinal neurons (Reichenbach and Robinson, 1995). Thus, the morphology, density, and protein expression of Müller cells are important indications of a healthy retina (Bringmann et al., 2006). Müller cells have also been ascribed a role in the processing of light in the retina; the shape of Müller cells, their refractive index, and their orientation along the passage of light have been shown to contribute to minimising scatter and guiding light with minimal intensity loss towards the photoreceptors (Franze et al., 2007).
1.3 Aims of this thesis

The broad aim of this thesis was to elucidate, through quantitative and qualitative analyses, the different cell types that make up the retina, and study their morphology and distribution across the retina. Specifically, this thesis addresses the following questions:

Chapter 2:
- How many ganglion cell types can be identified in the marmoset retina and how are they different or similar those identified previously in macaque retina?
- How does the proportion of ganglion cell types change across the retina?

Chapter 3:
- Do post-mortem human retinas maintain structural integrity and immunohistochemical expression after three days of organotypic culture?
- Can particle-mediated gene transfection followed by organotypic culture be successfully applied to human retinas to label ganglion cells in vitro?

Chapter 4:
- How does the distribution of bipolar cells in midget and parasol pathways vary across the retina?
- What is the numerical convergence of cone photoreceptors to bipolar cells in the midget and parasol pathway in human retina?
- What is the proportion of amacrine, horizontal, bipolar, and Müller cells in the inner nuclear layer of the human retina and does this vary with eccentricity?
CHAPTER TWO
Survey of retinal ganglion cell morphology in marmoset

2.1 Abstract

In primate retina, the midget, parasol, and small bistratified cell populations form the large majority of ganglion cells. In addition, there is a variety of low-density wide-field ganglion cell types, which are less well characterized. Here retinal ganglion cells in the common marmoset, *Callithrix jacchus*, are studied using particle-mediated gene-transfer. Ganglion cells were transfected with an expression plasmid for the postsynaptic density 95-green fluorescent protein. The retinas were processed with established immunohistochemical markers for bipolar and/or amacrine cells in order to determine ganglion cell dendritic stratification. In total, over 500 ganglion cells were classified based on their dendritic field size, morphology and stratification in the inner plexiform layer. Over 17 types were distinguished, including midget, parasol, broad thorny, small bistratified, large bistratified, recursive bistratified, recursive monostratified, narrow thorny, smooth monostratified, large sparse, giant sparse (melanopsin) ganglion cells, and a group that may contain several as-yet uncharacterized types. Assuming each characterized type forms a hexagonal mosaic, the midget and parasol cells account for over 80% of all ganglion cells in central retina but only ~50% of cells in peripheral (>2 mm) retina. We conclude that the fovea is dominated by midget and parasol cells. Outside the fovea, the ganglion cell diversity in marmoset is likely as great as that reported for non-primate retinas. Taken together, the ganglion cell types in marmoset retina resemble those described previously in macaque retina with respect to morphology, stratification and change in proportion across the retina.
2.2 Introduction

Different areas in the brain play different roles in visual processing and receive functional input from different retinal ganglion cell types. Retrograde labeling studies of the macaque visual system demonstrated that the parvocellular layers of the dorsal lateral geniculate nucleus (LGN) receive their major input from midget (or Pβ) ganglion cells whereas the magnocellular layers receive their major input from parasol (or Pα) cells (Leventhal et al., 1981; Perry and Cowey, 1981; Perry et al., 1984), reviewed by Rodieck (Rodieck et al., 1985; Rodieck, 1988). A third well-characterized ganglion cell type is the small bistratified (blue-ON/yellow-OFF) cell (Rodieck, 1991; Dacey and Lee, 1994), which preferentially targets the koniocellular layers of the LGN (Martin et al., 1997; Szmajda et al., 2008; Roy et al., 2009).

Additional ganglion cell types project to the superior colliculus, the pretectum (Perry and Cowey, 1984; Rodieck, 1991; Rodieck and Watanabe, 1993; Dacey et al., 2003), and the suprachiasmatic nucleus (Hannibal et al., 2014). Some or all of these cells also project to the LGN (Rodieck and Watanabe, 1993; Dacey, 2004), where they preferentially target the koniocellular layers (Szmajda et al., 2008; Percival et al., 2011, 2013, 2014). Irrespective of their projections, these ganglion cells have dendritic fields larger than those of midget, parasol and small bistratified cells and thus are collectively referred to as wide-field cells (Peterson and Dacey, 1999, 2000; Yamada et al., 2005). Wide-field ganglion cells are considered to be low-density cell types because midget, parasol and small bistratified ganglion cells are thought to account for the large majority of retinal ganglion cells in primate (Polyak, 1941; Perry et al., 1984; Silveira and Perry, 1991; Dacey, 1993a, b; Ghosh et al., 1996; Yamada et al., 1996, 1998). Knowledge about the number and proportion of wide-field types across the retina, however, is still incomplete.

The total number of ganglion cells varies significantly between primate and other mammalian species. Macaque monkeys and humans have between 1 and 1.5 million ganglion cells (Curcio and Allen, 1990; Sandell and Peters, 2001), cats have between 120,000 and 150,000 cells (Stone, 1978; Illing and Wässle, 1981); rabbits have 380,000 cells (Vaney, 1980); rats have between 113,000 and 117,000 cells (Forrester and Peters, 1967; Potts et al., 1982; Perry et al., 1983) and mice have about 50,000
ganglion cells (Dräger and Olsen, 1981; Jeon et al., 1998). If one assumes that the large majority of retinal ganglion cells in primates are midget and parasol cells, the remaining number of ganglion cells in primate retina could be as high as 200,000 ganglion cells (Wässle, 2004). Thus, the number of wide-field cells in primate retina is roughly in the range of the total number of retinal ganglion cells in other mammalian species.

Studies of cat (O'Brien et al., 2002; Berson, 2008) and rabbit (Roska and Werblin, 2001; Rockhill et al., 2002) retina identified respectively, ten and twelve morphologically and physiologically distinct ganglion cell types. In primates, at least 17 morphological types of ganglion cell were distinguished (Polyak, 1941; Watanabe and Rodieck, 1989; Kolb et al., 1992; Peterson and Dacey, 1999, 2000; Dacey et al., 2003; Dacey, 2004; Yamada et al., 2005) but only a few of these have been characterized physiologically (Dacey and Lee, 1994; Dacey et al., 2003; Crook et al., 2008a, b; Puller et al., 2015). Comparable numbers of ganglion cell types were originally thought to be present in mouse retina, as reviewed by (Masland, 2011). Recent studies, however, have suggested that there exist at least 30 morphological (Helmstaedter et al., 2013; Dunn and Wong, 2014; Sanes and Masland, 2015) types of retinal ganglion cells in mouse, many of which can be genetically defined (Sümbül et al., 2014). The number of 30 ganglion cell types in mouse retina is thought to account for more than 95% of the ganglion cells (Sanes and Masland, 2015) but Baden and colleagues identified 32 functional types and suggest that the number may increase to as many as 40 types (Baden et al., 2016).

The question whether the morphological types identified in primate retina account for the total number of ganglion cells has only been addressed for macaque (Dacey, 2004; Dacey et al., 2010). Dacey and colleagues estimated for one eccentricity in peripheral retina, that the 17 ganglion cell types they analyzed account for 85% of all macaque ganglion cells leaving an undefined number of low-density types to be classified.

In the present study, ganglion cell types in the marmoset retina were labeled using a relatively unselective (nominally random) method, that is, particle-mediated gene transfer. The cells were classified using dendritic field size, eccentricity, dendritic
tree morphology and stratification. The density of the cell types was estimated and compared to the total ganglion cell density across the retina.

2.3 Methods

2.3.1 Animals

We obtained three retinas from one male (2 retinas) and one female (1 retina) adult marmoset. The marmosets were purchased at CLEA Japan Inc. and maintained in the National Institute for Basic Biology Bioresource Centre under the guidelines of the National Institute for Physiological Sciences (NIPS) code of practice for the care and use of animals. Procedures were approved by the institutional ethics committee of NIPS (No. 11A172) and the National Institutes of Natural Sciences (NINS; Nos. 08B005 and 10B001). The animals were anaesthetized with medotomidine (0.01 mg/kg, i.m.; DomitorOrion) and ketamine (10 mg/kg; Ketalar, Daiichi Sankyo Espha). All animals were sacrificed with an overdose of 80-150 mg/kg sodium pentobarbitone.

2.3.2 Labeling of ganglion cells by particle-mediated gene transfer

Details of the procedures have been described previously (Koizumi et al., 2007; Moritoh et al., 2010; Percival et al., 2014; Masri et al., 2016). Retinas were dissected in Ames’ medium, cut into quadrants and mounted ganglion cell side upward onto Millicell Millipore tissue culture plate inserts. The vitreous was removed with fine forceps. A Helios gene gun system (Bio-Rad) was used to propel gold microcarriers coated with the cytomegalovirus (CMV) PSD95-GFP fusion plasmid (gift from Dr. Masaki Fukata, NIPS) into the tissue. The ratio of DNA to the gold microcarriers (mean diameter 1.6 µm) was 1.5 µg plasmid/1 mg gold. The bullets were delivered at 110 psi at a distance of 5 mm from the retina so that cells in the ganglion cell layer would express PSD95-GFP. Each retinal quadrant was then cultured for three days in more than 26 ml Ames’ medium containing 0.192% sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.292 mg/ml L-glutamine (Gibco, Grand Island, NY), supplemented with 10% horse serum (Sigma- Aldrich, St. Louis, MO). Cultured retinas
were fixed in 4% PFA in phosphate-buffered saline (PBS), pH 7.4, for 1 hour at 4°C and kept on the tissue culture insert for further processing.

2.3.3 Antibody characterization

The antibodies used in the present study are summarized in Table 2.1. The mouse and rabbit antibodies against the calcium binding protein D-28k (CaBP) were characterized by the manufacturer via Western blot analysis. Both antibodies yield a single band at 28 kDa when characterized in brain homogenates from various species, including macaque monkey.

The antibody against CD15 was derived from hybridoma cell lines (MMA, ATCC HB78) and reacts with the 3-fucosyl-N-acetyl-lactosamine antigen (Andressen and Mai, 1997).

The antibody against choline acetyl transferase (ChAT) has been characterized by the manufacturer (Millipore, Temecula, CA) in Western blots of mouse brain lysates; it stains a single band of 68–70 kDa.

The antibody against the C-terminal binding protein 2 (CtBP2) has been characterized by Western blots of mouse retina (Schmitz et al., 2000). It recognizes a 110-kDa and a 120-kDa band (representing the RIBEYE B-domain) and a 50-kDa band (CtBP1) (tom Dieck et al., 2005).
Table 2.1. Antibodies

<table>
<thead>
<tr>
<th>Antibody name (cell type labeled)</th>
<th>Immunogen</th>
<th>Source, catalogue number; RRID</th>
<th>Antibody type</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaBP (DB3a)</td>
<td>Recombinant rat calbindin D-28k</td>
<td>Swant, Marly, Switzerland, CB38, lot: 5.5; RRID: AB_10000340</td>
<td>Rabbit polyclonal</td>
<td>1:20,000</td>
</tr>
<tr>
<td>CaBP (DB3a)</td>
<td>CaBP-D-28kD purified from chicken gut. Hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunised mouse</td>
<td>Swant, Marly, Switzerland, 300, lot: 07(F); RRID: AB_10000347</td>
<td>Mouse monoclonal</td>
<td>1:10,000</td>
</tr>
<tr>
<td>CD15 (FMB, DB6)</td>
<td>Membrane preparation of the human histiocytic cell line U-937</td>
<td>Gift from J.K. Mai, University of Düsseldorf, Germany; RRID: AB_2314137</td>
<td>Mouse monoclonal</td>
<td>1:6</td>
</tr>
<tr>
<td>ChAT (starburst amacrine)</td>
<td>Purified human placental choline acetyltransferase enzyme</td>
<td>Millipore, Billerica, MA, AB144P, lot: NG1780580; RRID: AB_2079751</td>
<td>Goat polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>CtBP2 (synaptic ribbons)</td>
<td>Amino acids 361-445 of mouse C-terminal binding protein 2</td>
<td>BD Biosciences, Heidelberg, Germany, 612044; RRID: AB_399431</td>
<td>Mouse monoclonal</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>
2.3.4 Immunohistochemistry

Retinas were processed free floating without removing the filter membrane. The tissue was preincubated for 1 hour at room temperature, in PBS containing 5% bovine serum albumin (BSA, Sigma, St. Louis) and 0.5% Triton X-100 (BDH Chemicals, Kilsyth, Australia). This was followed by incubation in a mixture of two primary antibodies diluted in PBS containing 0.5% Triton X-100 in PBS and 0.05% NaN₃ and 1% bovine serum albumin for one week at 4°C. Eleven retinal quadrants were used: six quadrants were processed with antibodies against CaBP and CD15, two quadrants were processed with antibodies against CaBP and CtBP2, one quadrant was processed with antibodies against CD15 and ChAT, one quadrant was processed with antibodies against ChAT and protein kinase C, and one quadrant was processed with antibodies against CD15 and GluR4. In the two latter quadrants only the antibody staining for ChAT and CD15 was of sufficient quality to be analyzed. After rinses, retinal pieces were incubated overnight at 4°C in a mixture of secondary antibodies (made in donkey, coupled to Alexa 594 (1:500) or Alexa 647 (1:250), Jackson Immunoresearch, West Grove, PA) diluted in PBS containing 0.5% Triton X-100 and 1% BSA. Preparations were mounted onto polysine slides (Menzel-Gläser, Braunschweig, Germany) and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA).

2.3.5 Microscopy

Stacks of images (7 to 28 optical slices) of individual ganglion cells were taken from the ganglion cell layer to the inner nuclear layer using a confocal microscope (Zeiss LSM700) equipped with 488, 555, and 639 lasers at 1-µm increments using a 20 × objective (Plan Apochromat, 20 × / 0.8, No. 420650-9901) at a resolution of 2,048 pixels. The brightness and contrast of the images were adjusted in Zen (Zeiss), Adobe Photoshop CS6, or Imaris (Bitplane, Zurich, Switzerland) software. The eccentricity relative to the fovea for each cell was calculated from a low-power montage of the quadrant(s) for each retina.
2.3.6 Classification of ganglion cell types

Ganglion cells were reconstructed in 3D from confocal image stacks using the filament tracer in Imaris software (Bitplane, Zurich, Switzerland), or traced in 2D from collapsed image stacks in Adobe Illustrator CS6. Dendritic field size was measured from collapsed image stacks of individual ganglion cells in Fiji (Schindelin et al., 2012). The outermost dendrites of the ganglion cell were connected to form a convex polygon, and the average of the minimum and maximum diameter of the polygon was taken as the dendritic field diameter (Szmajda et al., 2008). Ganglion cells were classified using morphological criteria, such as branch density, soma location with respect to the dendritic field, dendritic stratification in the inner plexiform layer, dendritic field size with respect to eccentricity, and the presence of an axon. For small and large bistratified cells the number of branch points and the number of thorns were determined for regions of interest (62.5 x 62.5 µm) in the middle of the dendritic field using Imaris Software.

The stratification of the ganglion cell dendrites in the inner plexiform layer was determined qualitatively by going through the stacks of images and determining the location of the dendrites with respect to immunohistochemical labeling and/or neighboring midget and parasol cells. For a small sample of cells, we measured the stratification in regions of interest (400 x 200 µm). Orthogonal projections were created using Zen software and the position of the dendrites of ganglion cells and the processes of bipolar and amacrine cells were measured. The percentage stratification of the ganglion cell in the inner plexiform layer was determined relative to the known stratification of the bipolar and amacrine cell processes, with 0% indicating the border with the inner nuclear layer and 100% indicating the border with the ganglion cell layer.

2.3.7 Density and proportion of ganglion cells

We estimated the local spatial density of each identified ganglion cell population by calculating, for each labeled cell, the equivalent population density $D$. For a hexagonal matrix this value is given by $D = \frac{1}{\left(\frac{a^2}{2} \sqrt{3}\right)}$, where $D$ is cell density (cells / mm$^2$) and $a$ is the distance between a neighboring cell centroid. For a non-overlapping hexagonal matrix of circular elements, $a$ is the same as the element diameter $d$. For
overlapping matrices, the value is $a = d/l$, where $l = 1$ indicates no overlap and $l = 2$ indicates 50% overlap (a matrix where the edge of each circle touches the neighboring centroids). The relationship between dendritic field overlap and coverage is approximated by a power function $y = ax^b + c$, where $y$ is dendritic field overlap expressed as percentage, $a = -2,210$, $b = -0.01357$, and $c = 2,210$ (Szmajda et al., 2005). Thus, a matrix with $l = 2$ (overlap 50%) has a coverage factor ~5.). We set overlap values for each population using our own measures of dendritic overlap and $l$ or by converting values of coverage taken from the literature.

2.3.8 Definition

The term “central retina” refers to the first 10 degrees of visual angle (1.5 mm in marmoset) and the term “peripheral retina” refers to eccentricities above 1.5 mm (Wilder et al., 1996; Percival et al., 2013). No correction for shrinkage was applied because paraformaldehyde fixation was assumed to produce minimal shrinkage and the tissue was mounted using a water based mounting medium (Vectashield, Vector).
2.4 Results

2.4.1 Ganglion cell analysis

As has been reported in previous studies of various mammalian species including marmoset (Koizumi et al., 2007; Jakobs et al., 2008; Morgan et al., 2008; Moritoh et al., 2013), particle mediated gene-transfer enables labeling of a large number of ganglion cells (Figure 2.1). The images of labeled cells show brightly labeled puncta distributed along the dendritic tree (e.g. Figures 2.6C, F; 2.7E, H; 2.8F, H, J, 2.9D). These puncta represent postsynaptic densities (Jakobs et al., 2008; Bleckert et al., 2013; Percival et al., 2014). Here, we analyzed a total of 574 ganglion cells. The large majority of cells was derived from two temporal pieces from two different animals. In order to classify the ganglion cells, we first measured the dendritic field size and the eccentricity and then considered other morphological criteria, such as dendritic branch density and dendritic stratification.

Figure 2.1. PSD95-GFP expression in ganglion cells of marmoset retina. A: Confocal micrograph of the temporal quadrant of a retina transfected with postsynaptic density 95 (PSD-95) plasmid. B: Drawing of the quadrant shown in A. Each dot marks a labeled ganglion cell that was classified. X indicates the location of the fovea. Scale bar = 1 mm.
The morphological types we found are largely comparable to those observed in previous studies of marmoset, using a variety of methods including intracellular injections (Ghosh et al., 1996), retrograde tracer labeling from the LGN together with photofilling (Szmajda et al., 2005, 2008; Percival et al., 2009, 2011; Abbott et al., 2012; Percival et al., 2013), and AAV-mediated expression (Ivanova et al., 2010).

In peripheral retina, the dendritic stratification was analyzed by comparing it with the stratification of CD15 immunoreactive OFF midget bipolar and DB6 cells (Chan et al., 2001b), calbindin immunoreactive DB3a cells (Weltzien et al., 2015; Masri et al., 2016) and/or choline acetyltransferase immunoreactive starburst amacrine cells (Moritoh et al., 2013) (Figure 2.2). In marmoset retina OFF midget bipolar cells stratify in stratum S1 close to the inner nuclear layer at about 5% to 15% depth of the inner plexiform layer and DB6 bipolar cells stratify in S5 at 88% to 96% depth (Jusuf et al., 2004). Calbindin positive DB3a cells stratify in S2 at 15% to 30% depth (Ghosh et al., 1997); and the processes of choline acetyltransferase (ChAT) positive (starburst) amacrine cells occupy two bands in the inner plexiform layer located at 18% and 78% (S2 and S4) of the inner plexiform layer (Moritoh et al., 2013). The outer starburst band is usually more weakly labeled (Rodieck and Marshak, 1992; Majumdar et al., 2008; Moritoh et al., 2013), thus only the inner ChAT band was used in the analysis of the whole mount preparations. In central retina antibody penetration was insufficient and thus dendritic stratification was determined by going through the stack of images and estimating the borders of the inner plexiform layer.
Figure 2.2. Immunohistochemical labeling of bipolar and amacrine cell terminals can be used to define the strata of the inner plexiform layer in marmoset retina. Confocal images of immunolabeled vertical vibratome sections through marmoset retina. Sections shown in A-C are from about 1 mm eccentricity. A: CD15 immunoreactivity is expressed by two bipolar cell types: OFF midget bipolar and DB6 cells. B: Calbindin (CaBP) immunoreactivity is expressed by DB3a cells. C: Starburst amacrine cells are immunoreactive to choline acetyl transferase (ChAT). The processes close to the GCL are more strongly labeled than the outer processes (arrows). D: Section from 6 mm eccentricity, note the difference in the thickness of the IPL. Calbindin immunoreactivity is found in cone photoreceptors, DB3a cells and some cells in the GCL. The INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar shown in D = 25 µm, applies to all panels.
At all eccentricities a variety of ganglion cell types was found (Figure 2.3) with a morphology that matched previous descriptions in Old World (Dacey et al., 2003; Yamada et al., 2005) and New World monkeys (Ghosh et al., 1996; Yamada et al., 1996). Consistent with these previous studies, the dendritic field size of midget and parasol cells increases with the distance from the fovea and is smaller than that of the other ganglion cell types at all eccentricities (Figure 2.4A).
Figure 2.3. Morphology of ganglion cell types identified using particle mediated gene transfer. Eccentricity in mm is indicated below each cell. The outer dendrites of bistratified cells are shown in red. Scale bar = 100 µm.
Figure 2.4. Dendritic field size increases with eccentricity. A: Scatterplot showing the dendritic field diameter as a function of retinal eccentricity. B: The dendritic field size of midget and parasol cells is compared to two types of wide-field cells. C: The dendritic field size of midget cells is compared to three types of wide-field cells. D: The dendritic field size of midget cells is compared to four types of wide-field cells.
2.4.2 Midget and parasol ganglion cells

Midget ganglion cells (Figure 2.5A, C, D, F, G) usually have a single primary dendrite that branches densely into a small dendritic tree. The soma is usually positioned away from the centre of the dendritic field. The dendrites stratify either close to the inner nuclear layer (outer midget cells) or close to the ganglion cell layer (inner midget cells). The dendrites of the outer cells co-stratify with the axon terminals of CD15 labeled OFF midget bipolar cells (Jusuf et al., 2006a, b) but mostly above the DB3a cells. The dendrites of the inner midget cells stratify mostly immediately above the axon terminals of DB6 bipolar cells with some dendrites intermingled with DB6 axon terminals.

Parasol ganglion cells (Figure 2.5A, D, F, G) have two to four primary dendrites emerging radially from the soma and forming a dense, regularly branched dendritic tree with the soma located in the centre of the dendritic field. The dendrites of the outer parasol ganglion cells stratify at the level of the calbindin labeled DB3a cells (Jacoby et al., 2000; Masri et al., 2016). The inner parasol ganglion cells stratify well above the DB6 cells towards the middle of the inner plexiform layer. For macaque retina it has been reported that ON parasol cells co-stratify with starburst amacrine cells (Jacoby et al., 1996). Whether the same co-stratification exists in marmoset could not be determined because the retinal pieces processed for ChAT immunoreactivity did not contain any ON parasol cells.

The dendritic field size of midget and parasol ganglion cells was in the range of our previous measurements (Ghosh et al., 1996; Szmajda et al., 2005, 2008; Abbott et al., 2012), although there seemed to be a tendency of a slightly larger average dendritic field size of midget cells in the present study.

Of the midget ganglion cell population in peripheral retina 23% (35/152) were inner and 77% (117/152) were outer stratifying cells. Of the parasol ganglion cell population in peripheral retina 29% (27/ 94) were inner stratifying 71% (67/94) were outer stratifying cells. The non-midget, non-parasol population comprised at least fifteen ganglion cell types. Using the terminology from Dacey (Dacey et al., 2003; Dacey, 2004) these included narrow thorny, recursive monostratified, smooth monostratified, sparse, broad thorny, and various types of bistratified cells (Figure 2.3).
The morphology and stratification of non-midget, non-parasol ganglion cells are described below. We will first describe cells in peripheral retina (beyond 1.5 mm eccentricity) and then discuss the cells in central retina in a separate paragraph at the end of the results section.

2.4.3 Ganglion cells in peripheral retina

In peripheral retina (eccentricities beyond 1.5 mm), 261 non-midget, non-parasol cells were analyzed. As shown in Figure 2.4A, and consistent with previous findings, midget cells consistently had the smallest dendritic fields followed by parasol cells (Ghosh et al., 1996; Gomes et al., 2005; Szmajda et al., 2005) and small bistratified cells (Ghosh et al., 1997). The wide-field cell types all had larger dendritic field sizes than parasol cells. Similar to findings in macaque retina (Dacey et al., 2003; Dacey, 2004), the dendritic field diameter of broad thorny cells in marmoset retina was comparable to that of narrow thorny, large bistratified, large sparse and recursive mono- and bistratified cells, whereas the giant sparse ganglion cells had on average the largest dendritic field. These latter cells are presumed to be the melanopsin-expressing ganglion cells (Jusuf et al., 2007).
Figure 2.5A-F. Ganglion cells at about 4 mm eccentricity. (See next page for legend).
Figure 2.5A-F. Ganglion cells at about 4 mm eccentricity. The images are photomontages from collapsed confocal image stacks. The eccentricity in mm is given in the upper right hand corner of each panel. BT, broad thorny; SBS, small bistratified; RBS, recursive bistratified; LBS, large bistratified; NT, narrow thorny. G: Same montage as shown in F. The colored symbols indicate the cell type. Scale bar in F = 50 µm applies to all.
Small bistratified and large bistratified ganglion cells

The main difference between the small and large bistratified cell types is that the dendritic field of large bistratified cells is sparser and larger (Figures 2.3, 2.4C, 2.5B,C, D; 2.6A, B). In addition, the large bistratified cells have fewer hooks and thorns in comparison to the small bistratified cells. For 10 small bistratified and 12 large bistratified cells located between 2.3 and 11.7 mm eccentricity, the number of branch points and the number of thorns was calculated for an area in the middle of the dendritic field. On average the number of branch points per 100 µm dendritic length was 2.6 (± 0.5 S.D.) for small bistratified cells and 1.8 (± 0.4 S.D.) for large bistratified cells. The average number of dendritic thorns per µm dendritic length was 4.2 (± 0.7 S.D.) for small bistratified cells and 2.2 (± 1.1 S.D.) for large bistratified cells. Both the difference in the number of branch points and the difference in the number of thorns were statistically significant (p=0.05, paired Wilcoxon nonparametric rank sum test).

The inner dendrites of the small bistratified (n = 19) and large bistratified (n = 17) cells in our sample stratify at or above the level of DB6 axons and the smaller outer dendritic tier stratifies near or above the level of DB3a axons (Figure 2.6). The dendritic field size of small bistratified cells is comparable to that of parasol cells, whereas the dendritic field size of large bistratified cells is in the range of the other wide-field cells (Figure 2.4A, C).
Figure 2.6. Small and large bistratified cells. **A**: Line drawing of a small bistratified cell (SBS) **B**: Line drawing of a large bistratified (LBS) cell. The inner tier is drawn in black and the outer tier in red. The eccentricities in mm are indicated in the upper right hand corner for each cell. **C-H**: Single confocal planes from the stack of images taken of the LBS cell shown in **B**. The inner (z03) and outer (z06) dendrites of the LBS cell are shown in green; the CD15-labeled DB6 axon terminals (**D**) and calbindin labeled DB3a axon terminals (**G**), respectively are shown in magenta. The merged images show that the inner dendrites of the LBS cell stratify in the same plane as the DB6 axons and the outer dendrites stratify in the same plane as the DB3a axon terminals. Scale bar in **B** = 50 µm, applies to **A** and **B**. Scale bar in **H** = 20 µm applies to **C-H**.
Broad thorny ganglion cells

Broad thorny cells have fine densely branching dendrites bearing numerous fine thorns (Figures 2.3; 2.5A, E, F, G; 2.7C), which stratify broadly in the middle of the inner plexiform layer (Percival et al., 2011). A few of the outermost dendrites reach above the level of the calbindin labeled axon terminals of DB3a cells (Figure 2.7D, E), whereas the innermost dendrites are found mostly above the axon terminals of CD15 labeled DB6 cells at the level of the ON-ChAT band (Figure 2.7H-M).

In total 116 broad thorny cells were classified in peripheral retina, thus broad thorny cells made up nearly half of the wide-field cells in our sample. Their dendritic field size ranged from 170 µm at 2 mm to 470 µm at 10 mm eccentricity (Figure 2.4A, D). These values are comparable to previous results from intracellular injection and retrograde labeling (Ghosh et al., 1996; Szmajda et al., 2008; Percival et al., 2011). Broad thorny cells in macaque retina had a dendritic field size of up to 600 µm (Rodieck and Watanabe, 1993; Dacey et al., 2003; Puller et al., 2015); the difference in dendritic field size between the two species may be attributable to the larger eye size of macaques.

Narrow thorny ganglion cells

Narrow thorny cells (n = 36) resemble broad thorny cells in that they have fine dendrites that carry numerous thorns but narrow thorny cells are more sparsely branched than broad thorny cells are (Figures 2.5E, 2.7A, B). Two types of narrow thorny cells were found, inner stratifying (n = 18) and outer stratifying (n = 17) cells. Inner narrow thorny cells stratify close to the ganglion cell layer, where they make contact with DB6 cells (Percival et al., 2014). Outer narrow thorny cells stratify mostly just above the level of the calbindin labeled DB3a cells (Figure 2.7F, G). The dendritic field diameter of narrow thorny cells ranged from 190 µm at about 2 mm to over 300 µm at about 7 mm and thus is within the same range as that of broad thorny cells (Figure 2.4D).
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Figure 2.7. Broad thorny and narrow thorny cells. (See next page for legend).
**Figure 2.7.** Broad thorny and narrow thorny cells. **A - C:** Collapsed stacks of confocal images showing narrow thorny (A, B) and broad thorny (C) cells in whole-mount view. Broad thorny cells have a denser dendritic tree than narrow thorny cells. The parasol outer cell in B has a smaller dendritic field. The eccentricity in mm is indicated in the upper right corner for each cell. **D-M:** High-resolution confocal images of dendrites of thorny ganglion cells. Single confocal planes are shown. **D, E:** The calbindin (CaBP) labeled DB3a axon terminals (magenta) co-stratify with the outer dendrites of a broad thorny cell (BT, green). **F, G:** The DB3a axon terminals (magenta) are located below the dendrites of a narrow thorny (NT, green). **H - M:** The innermost dendrites of a broad thorny cell (BT, green) co-stratify with the inner band of ChAT labeled starburst amacrine cell processes (blue) but are mostly located above the CD15-labeled DB6 terminals (red). Scale bar in C = 50 µm applies to A-C. Scale bar in G = 10 µm applies to D-M.
**Recursive ganglion cells**

Recursive cells are characterized by fine recursive dendrites lacking dendritic thorns (Figure 2.8A-D). We distinguished recursive monostratified and recursive bistratified ganglion cells. **Recursive bistratified** cells (n = 27) have dendritic field diameters that are comparable to those of large bistratified cells (Figure 2.4C). The inner dendrites of recursive bistratified cells are found above the axons of DB6 and above the dendrites of inner midget ganglion cells. Four of the recursive bistratified cells were found in a retinal piece that was processed for ChAT immunoreactivity. The inner dendrites of these cells are intermingled with the processes of inner stratifying ChAT positive amacrine cells but did not show any significant co-fasciculation with the ChAT cells (Figure 2.8F, G). Most of the outer dendrites of recursive bistratified ganglion cells are located slightly above the level of the DB3a cells, which is consistent with previous findings in marmoset retina (Moritoh et al., 2013; Masri et al., 2016). The stratification thus is different from that of recursive bistratified cells in macaque where these cells stratify closer to the middle of the inner plexiform layer, that is, between the inner and outer parasol cells (Dacey, 2004).

Four **recursive monostratified** cells were found in far peripheral retina. They had dendritic field diameters (260 to 370 µm) in the lower range of the wide-field ganglion cells (Figure 2.4B). The dendrites of recursive monostratified cells were located near or above the inner ChAT band (Figure 2.8J, K). Some of the dendrites of recursive monostratified cells co-stratified with DB6 axon terminals (Figure 2.8H, I) suggesting that they may get some input from DB6 cells.

**Smooth monostratified ganglion cells**

Five cells with a morphology similar to parasol cells but a larger dendritic field were classified as **smooth monostratified** cells (Crook et al., 2008a). These cells have relatively straight dendrites, which branch regularly and show fewer dendritic branchlets compared to parasol cells, hence the name smooth (Figures 2.3, 2.9A-C). Only outer stratifying cells were observed in our sample and these cells co-stratified with calbindin labeled DB3a cells (Figure 2.9 D-F). The dendritic field diameter of smooth monostratified cells ranged between 250 and 340 µm (Figure 2.4B).
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**Figure 2.8.** Recursive cells. (See next page for legend).
Figure 2.8. Recursive cells. A-D: Collapsed images of a stack of confocal images showing recursive bistratified (A, B) and recursive monostratified (C, D) cells. The recursive cells are distinguished by the curvature of their smooth dendrites. The eccentricity in mm is indicated in the upper right corner for each cell. E: Line drawing of a recursive bistratified cell showing the inner dendritic tier in black and the outer tier in red. The eccentricity is noted above the cell drawing. F-K: High-power images of single confocal planes showing ganglion cell dendrites in green and bipolar or amacrine cell processes in magenta. F, G: The inner dendrites of a recursive bistratified cell (RBS, green) stratifies slightly above the inner ChAT-band (magenta). H, I: recursive monostratified (RM, green) cell shows some overlap with a CD15-labeled DB6 axon terminals (magenta), but little co-stratification with the inner ChAT-band. Scale bar in D = 50 µm applies to A-D. Scale bar in E = 50 µm. Scale bar in G = 10 µm applies to F-K.
Figure 2.9. Smooth monostratified cells. (See next page for legend).
Figure 2.9. Smooth monostratified cells. **A-C:** Collapsed stacks of confocal images showing smooth monostratified (SM) cells together with parasol and midget cells. Eccentricity in mm is indicated in the upper right hand corner for each cell. **D-F:** Single confocal planes showing dendrites of a smooth monostratified cell (green) that costratify and make potential contacts with calbindin labeled DB3a axon terminals (magenta). Scale bar in A= 50 µm applies to A-C. Scale bar in F = 20 µm applies to D-F.
Sparse ganglion cells

Sparse ganglion cells have a sparse dendritic tree with straight dendrites, which lack hooks or thorns. Sparse cells were subdivided into large sparse and giant sparse cells (Figure 2.3). In total six large sparse cells were encountered; five of which were located in peripheral retina and had dendrites stratifying close to the ganglion cell layer at the level of the DB6 axons. This stratification is consistent with a previous study (Percival et al., 2011). The dendritic field diameter was determined for three peripheral cells and ranged between 240 and 333 µm (Figure 2.4D). The only large sparse cell that was labeled in central retina (at 0.8 mm) had a dendritic field size of 104.2 µm and stratified in the OFF sublamina.

Giant sparse cells (Figure 2.3 and Figure 2.10C) show morphology, dendritic field size and stratification that match previous descriptions of melanopsin-containing cells (Jusuf et al., 2007). The dendritic field diameters of giant sparse cells ranged between 441 and 533 µm (Figure 2.4D). Five inner stratifying and three outer stratifying giant sparse cells were labeled. The dendrites of the inner cells stratify near the ganglion cell layer at the level of the DB6 axons (Grünter et al., 2011); the dendrites of the outer cells stratify close to the inner nuclear layer.
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Figure 2.10. Rare ganglion cell types. (See next page for legend).
Figure 2.10. Rare ganglion cell types. Collapsed images of stacks of confocal images. Eccentricity in mm is shown in upper right hand corner for each cell. A: Two unclassified cells are shown. Both cells stratify in stratum 1, close to the inner nuclear layer. B: Cell classified as narrow thorny cell with an unusually large dendritic field. C: Giant sparse (presumed melanopsin-containing cell) together with an outer midget ganglion cell is shown. D: Cell classified as recursive bistratified with an unusually large dendritic field. Scale bar in D = 50 µm applies to A-D.
Miscellaneous ganglion cells

Some cells had an unusually large dendritic field (Figures 2.4A; 2.10B, D), in the range or even exceeding that of giant sparse (melanopsin-containing) cells (Figure 2.10C). Unusually large cells were encountered in peripheral and central retina and included broad and narrow thorny, recursive monostratified, large bistratified and recursive bistratified cells.

In total, 31 ganglion cells were not classified because they did not fit any of our criteria and/or their dendrites were intermingled with other cells and/or the quality of the staining was insufficient. Figure 2.10A shows two examples of unclassified cells from about 3 mm eccentricity. Both cells stratified close to the inner nuclear layer, had relatively large dendritic fields (267 and 404 µm) and showed some resemblance to the ganglion cells in the S-group of Rodieck and Watanabe (1993).

Eight cells had an asymmetric dendritic field (Figure 2.11) stratifying in the outer region of the inner plexiform layer. These cells were collectively named asymmetric cells but most likely do not represent a homogenous group. Some cells had morphology resembling the jam-B cells described in mouse retina (Kim et al., 2008) and the G3 cell in rabbit retina (Hoshi and Mills, 2009). The orientation of the dendritic field of the cells, however, was variable; four cells had dendritic fields pointing away from the fovea at a right angle (e.g., Figure 2.11A, B, C); two cells had a dendritic tree pointing towards the fovea and two cells had a dendritic field pointing away from the fovea (2.11D). Two of the asymmetric cells (Figure 2.11C, D) had somas that were immunoreactive for calbindin (Figure 2.11H-M) but the other asymmetric cells were calbindin negative (e.g., Figure 2.11E-G). Thus calbindin immunoreactivity cannot be used as an unequivocal marker in identifying these cells.
Figure 2.11. Asymmetric cells. A-D: Collapsed images of stacks of confocal images are shown. The arrows point to the location of the fovea, the arrowheads indicate the axon. Eccentricity in mm is shown in upper right hand corner for each cell. E - G: Soma of the asymmetric cell shown in B. This cell is not calbindin (CaBP, magenta) positive. H - J: Soma of the asymmetric cell shown in C. This cell is CaBP positive. K - M: Soma of the asymmetric cell shown in D. This cell is CaBP positive. Scale bar in C = 50 µm, applies to A-D; scale bar in M = 20 µm, applies to E-M.
2.4.4 Central ganglion cells

Ganglion cells encountered in central retina (n=67) resembled their peripheral counterparts but usually had much smaller dendritic fields (Figure 2.4). Due to poor antibody penetration, the immunolabeling in central retina was weak and stratification of the cells could not be determined. In total 30 midget ganglion cells, 16 parasol ganglion cells, six broad thorny, six small bistratified, three large bistratified, two narrow thorny, one recursive bistratified, one recursive monostratified, and one large sparse cell were identified, while one cell remained unclassified. Examples of central cells are shown in Figure 2.12.

For each cell type we compared its proportion within the population of labeled ganglion cell types in peripheral and central retina (Figure 2.13), and found that at eccentricities below 1.5 mm midget (45%) and parasol (24%) cells made up the majority of labeled cells, whereas small bistratified cells (8%) and broad thorny cells (7%) were the most commonly labeled wide-field ganglion cell type. In peripheral retina, the proportion of labeled midget and parasol cells decreased, while the proportion of wide-field cells increased with broad thorny cells being the most frequently labeled wide-field cell type. It should be noted that our sample of labeled ganglion cells is likely biased against midget and parasol ganglion cells because we initially focused on wide-field ganglion cell types when scanning the tissue. The relatively high proportion of broad thorny ganglion cells in the peripheral retina will be discussed below.
**Figure 2.12.** Ganglion cell types in central retina. **A-C:** Collapsed images of stacks of confocal images taken from central retina. Eccentricity in mm is shown in upper right hand corner for each cell. **D:** Line drawings of ganglion cell types found in central retina. Eccentricity is indicated below each cell. Axons, where visible, were drawn in light grey, the outer dendritic tier of bistratified cells is shown in red. Scale bar in A = 50 µm applies to A-C. Scale bar in D = 50 µm.
Figure 2.13. The proportion of wide-field cells increases with distance from the fovea. Bar graph showing the proportion of midget, parasol, small bistratified (SBS), broad thorny and the remaining wide-field ganglion cell types classified from particle-mediated gene transfer in the present study. Eccentricity ranges are indicated.
2.5 Discussion

In the present study we identified at least 17 morphological types of ganglion cell in marmoset retina using particle mediated gene transfer. The cells matched the morphology of wide-field ganglion types in Old World and New World primates identified by other methods, including Golgi-impregnation (Polyak, 1941; Boycott and Dowling, 1969; Kolb et al., 1992), intracellular injection (Ghosh et al., 1996; Yamada et al., 1996; Peterson and Dacey, 1999, 2000; Yamada et al., 2005), Dil labeling (Yamada et al., 1998), and retrograde labeling (Watanabe and Rodieck, 1989; Rodieck and Watanabe, 1993; Dacey et al., 2003; Dacey, 2004; Crook et al., 2008a, b; Szmajda et al., 2008). The homology of ganglion cells within different species (Berson, 2008; Sanes and Masland, 2015) and the different nomenclature within different studies of primate retina (Yamada et al., 2005) have been discussed previously and thus will not be dealt with here.

2.5.1 Ganglion cell stratification

Particle-mediated gene-transfer has the advantage that it allows simultaneous labeling of large numbers of ganglion cells including low-density cell types (Jakobs et al., 2008; Morgan et al., 2008; Moritoh et al., 2010, 2013). An important criterion in distinguishing ganglion cell types is the stratification in the inner plexiform layer, because this determines the synaptic input to these cells (Boycott and Wässle, 1991). Here we analyzed the ganglion cell stratification by comparing it with that of well characterized bipolar (diffuse bipolar types DB3a and DB6 and flat midget bipolar) and amacrine (the inner and outer ChAT) populations. Our measurement precision is limited by a number of factors. The inner plexiform layer in peripheral retina is only about 15 µm thick (Figure 2.2D) and the borders of the inner plexiform layer are hard to define in the absence of multiple criteria. Future studies could include image stacks using DAPI and/or Nomarski optics to address this problem. Furthermore, the quantitative stratification measurements were taken only in specific regions of the dendritic tree and only for a small sample of cells in well-labeled preparations. More measurements will be needed to give a more accurate reflection of the ganglion cell stratification but this was beyond the scope of the present study.
2.5.2 Seventeen morphological ganglion cell types were distinguished

Despite the limitations outlined above, we are confident that the summary diagram shown in Figure 2.14, is a good representation of the stratification of ganglion cells types in marmoset retina. Monostratifying cell types (Figure 2.14A) included inner and outer pairs of midget, parasol, narrow thorny, smooth monostratified, large and giant sparse cells and one type of recursive monostratifying (inner) cell. Bistratified or broadly stratified (presumed ON/OFF) ganglion cells had dendrites in both the inner and the outer sublamina (Figure 2.14B).

One of the cell types found in previous studies of macaque (Dacey, 2004; Crook et al., 2008a) i.e., inner smooth monostratified (named large radiate in Yamada et al., 2005) was not detected in the present study and neither inner nor outer smooth monostratified cells were reported in other studies of marmoset retina (Szmajda et al., 2008; Ivanova et al., 2010; Percival et al., 2011; Moritoh et al., 2013). We do not think, however, that this ganglion cell type is absent from marmoset retina, because marmoset and macaque otherwise do not show fundamental differences in their retinal wiring. Thus, both inner and outer smooth monostratified cells are included in our diagram. Inner smooth monostratified cells were assumed to co-stratify with inner parasol cells (Crook et al., 2008a).

In summary, our findings suggest that the ganglion cell types in marmoset retina are comparable to those found in other primates such as macaque (Dacey, 2004; Yamada et al., 2005) and human (Kolb et al., 1992; Peterson and Dacey, 1999, 2000).
Figure 2.14. Summary diagram showing ganglion cell types and their stratification in marmoset retina. The stratification of most of the ganglion cell types was determined by comparing to the stratification of immunohistochemically labeled bipolar and amacrine cells except for the following cells: ON parasol cells (Ghosh et al., 1996); inner tier of small bistratified cells (Ghosh et al., 1997); outer large sparse cells (Ivanova et al., 2010); inner smooth monostratified cells (Crook et al., 2008).
2.5.3 Do seventeen types of ganglion cell account for all types?

The number of retinal ganglion cell types in the present study is in the range of the number of types reported for other primates, including macaque and human (Kolb et al., 1992; Dacey et al., 2003; Dacey, 2004; Yamada et al., 2005). As outlined in the introduction, in mouse at least 32 retinal ganglion cell types have been found and this number has been suggested to increase to 40 types (Baden et al., 2016).

The question arises whether the 17 morphological types of ganglion cell types detected in the present study account for all ganglion cell types in the marmoset retina. In order to address this question, we took the dendritic field size of each cell we had identified and calculated the density for each cell across the retina assuming a coverage factor of 1.0 for midget cells and a coverage of 1.7 for all other cell types. The reader should note that density estimates derived this way should be treated with caution, because they are based on individual size and overlap properties of small cell numbers. In absence of specific population markers for most widefield types, such calculations nevertheless can provide the best available "ballpark" estimate of ganglion cell populations in marmoset retina. We allowed for four types of recursive bistratified cells (representing four types of ON-OFF direction selective cells, see discussion below), and three types of recursive monostratified (ON-direction selective) cells. In order to maximize the cell sample across eccentricity we pooled data from the present study with data from our previous studies (Szmajda et al., 2008; Percival et al., 2013). The equivalent packing density was calculated from dendritic field diameter and coverage for each cell, then for each population the cell density was estimated using a two-stage exponential fit: \( y = a \cdot e^{bx} + c \cdot e^{dx} \), where \( y \) is cell density (cells / mm\(^2\)), \( x \) is eccentricity (mm), \( e \) is the natural exponent, and \( a, b, c, d \) are coefficient values. Coefficients were estimated using a nonlinear least square algorithm (Matlab cftool). The total ganglion cell density and proportions across eccentricity were then estimated (Figure 2.15). On comparing our total estimate (Figure 2.15A, asterisk symbols) we see good agreement with total ganglion cell counts (Figure 2.15A, broken line) from our previous study of marmoset retina (Wilder et al., 1996). In central retina the total density of the cell types in our sample accounts for 84%; but in peripheral retina it accounts for ~60% of the total ganglion cell density we previously estimated. Thus our
results raise the possibility that some ganglion cell types are not yet characterized. Similar calculations and conclusions were made for one eccentricity each in rabbit (Rockhill et al., 2002) and macaque (Dacey et al., 2010) retinas.

Using these data we also estimated the proportion of wide-field cells across the retina with respect to midget, parasol, and small bistratified cells (Figure 2.15B). The figure shows that the proportion of midget ganglion cells decreases from about 80% in central retina, to about 15% in peripheral retina, whereas the proportion of parasol cells remains relatively constant at around 10%. Similarly, the proportion of midget ganglion cells was estimated to decrease from 95% in central to 45% in peripheral retina of human (Dacey, 1993b); and in peripheral retina of macaque a proportion of 50% midget and 16% parasol cells was calculated (Dacey, 2004). In the present study, the proportion of small bistratified cells rises slightly from less than 2% in central retina to ~8% in peripheral retina. These data are consistent with data from macaque retina, where the small bistratified cell has been estimated to make up a proportion between 1% in central retina and 6 to 10% in peripheral retina (Dacey, 1993a). Thus, in general the proportions of the three major ganglion cell types in marmoset and macaque retinas are comparable.

In the present study, the proportion of wide-field cells increased steadily with the distance from the fovea and by 7.5 mm eccentricity the wide-field cells outnumber midget cells and thus make up the majority of all ganglion cells. This change in proportion is largely due to the fact that the dendritic field size and thus the density of midget ganglion cells changes more rapidly than in other ganglion cell types (Figure 2.4).

What then are the missing ganglion cell types? As outlined in the result section we found single examples of well-labeled cells, which did not fit any criterion, so we cannot rule out the possibility that some low-density types were missed. Furthermore, we did not use any sophisticated measures as have been applied to differentiate ganglion cell types in mouse retina (Sümbül et al., 2014). Applying more quantitative stratification and/or branch density measurements may reveal that some of ganglion cell types described here can be further subdivided.
2.5.4 What is the total number of wide-field ganglion cells?

As outlined in the introduction it has been suggested that the total number of wide-field ganglion cells in primate retina is more than an entire cat retina (Wässle, 2004). We estimated the total number of retinal ganglion cells in marmoset retina from the product of local cell density (Figure 2.15A) and retinal area, in 1 mm eccentricity increments. This calculation yields a total of ~1 million ganglion cells with midget cells making up the large majority (700,000 cells) of this total. The central retina (up to 1.5 mm eccentricity) contains approximately 700,000 ganglion cells, 100,000 of which are wide-field cells. For peripheral retina (beyond 1.5 mm) we calculate a total of 300,000 ganglion cells with a total of 100,000 wide-field ganglion cells. Thus, marmoset retina is estimated to contain a total of ~200,000 wide-field cells, exceeding the total number of ganglion cells in a mouse retina by a factor of four. As noted above, however, it is important to remember that these estimates are based on sample populations, not on specific markers that label entire cell populations. Measurements made using these more accurate methods will be needed to verify our calculations.
Chapter Two

Survey of retinal ganglion cell morphology in marmoset | 75

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Figure 2.15. Estimates of ganglion cell densities and proportion across marmoset retina. (See next page for legend).
Figure 2.15. Estimates of ganglion cell densities and proportion across marmoset retina. A: Estimated packing density of ganglion cells in marmoset temporal, inferior, and superior retina. Lines show two-stage exponential fit functions derived from dendritic field diameter and overlap for each population as described in the text. Fit coefficients (a, b, c, d): midget: 5.962 x 10^4, -2.310, 1.829 x 10^3, -0.316; parasol: 5.397 x 10^3, -1.757, 303.27, -0.1509; small bistratified: 1.119 x 10^3, -0.7989, 15.234, -0.0774; broad thorny: 0.809 x 10^3, -1.518, 54.363, -0.146; other wide-field: 0.6705 x 10^3, -1.1058, 21.532, 9.345 x 10^-6. Raw data are shown for broad thorny cells. Asterisks show total ganglion cell estimate from sum of the individual curves. Broken line shows total ganglion cell estimate from Wilder et al. (1996). B: Proportion of total ganglion cells. Here, broad thorny cells are incorporated in the wide-field cell counts.
2.5.5 Brain projection of wide-field ganglion cells

In the present study, broad thorny cells (n=122) form a large proportion of the labeled cells in peripheral retina. In our previous studies, where ganglion cells were labeled via retrograde tracer injections into the koniocellular layers of the LGN, broad thorny cells were only rarely encountered (Szmayda et al., 2008; Percival et al., 2011, 2013). Similarly, recursive cells were only encountered in one of our tracer injection studies (Percival et al., 2013) but in the present study, these cells were encountered relatively frequently. This result may imply that the major projection of broad thorny and recursive cells is not to the LGN but to other brain areas. Consistently, studies in macaque have shown that retinal ganglion cells with a variety of morphologies resembling broad thorny, narrow thorny and recursive cells project to the superior colliculus (Rodieck and Watanabe, 1993; Dacey et al., 2003). Equivalent studies are missing for marmoset.

The retrograde tracer injections and gene gunning methods likely have different sorts of bias resulting in different proportions of labeled cells (Kong et al., 2005). The nature of any bias in gene gunning is unclear, but most certainly it is not related to soma size as midget ganglion cells (n=182) have the smallest soma size (Ghosh et al., 1996) yet comprise the most frequently identified cell type in the present study. In favor of the bias hypothesis is the fact that small bistratified, inner midget and inner parasol cells were underrepresented whereas broad thorny cells, outer midget and outer parasol cells were overrepresented in our sample (compare Figures 2.13 and 2.15A). Similarly, Moritoh et al. (2010) using the same method as in the present study, found relatively high numbers of broad thorny and recursive cells but low numbers of small bistratified cells.

The relative high proportion of wide-field cells estimated for peripheral retina (Figure 2.15B) is in contrast to studies in macaque that estimated that non-midget, non-parasol cells make up between 20% (Perry and Cowey, 1984; Perry et al., 1984) and 26% (Dacey, 2004) of the total ganglion cell population in peripheral retina. This discrepancy could represent differences between macaque and marmoset retina. More studies, involving tracer injections from the superior colliculus and other potential targets of retinal ganglion cells are needed to address this question. Whether the wide-
field ganglion cells in primate retina show the same diversity in their central projections as has recently been reported for ganglion cells in non-primates (Dhande and Huberman, 2014; Robles et al., 2014; Gauvain and Murphy, 2015; Sanes and Masland, 2015) will likewise need to be studied in the future.

2.5.6 Functional role and connectivity of wide-field ganglion cells

The functional role of most wide-field ganglion cells is still unknown and to date only two studies have carried out electrophysiological recordings from ganglion cells in marmoset retina (Protti et al., 2014; Huang and Protti, 2016). In these studies, Protti and colleagues recorded from different types of ganglion cells and concluded that marmoset ganglion cells, much like those in other mammals, receive bipolar and amacrine inputs that are modulated by lateral inhibition originating in the inner plexiform layer. Our finding that all ganglion cell types express PSD95-GFP across their dendritic tree is consistent with the physiological findings.

The physiological properties of specific wide-field ganglion cell types were summarized previously (Dacey, 2004; Yamada et al., 2005). More recently, smooth monostratified cells were characterized as Y-like cells because of similarities in their response properties to parasol cells (Crook et al., 2008a, b). Our finding that DB3a cells stratify at the same level as smooth monostratified cells together with our previous findings showing that DB3a cells provide only 30% of their output to OFF parasol cells (Masri et al., 2016) is consistent with the suggestion that OFF smooth monostratified cells receive input from the same bipolar type(s) as OFF parasol cells. Similarly, ON smooth monostratified cells, like ON parasol cells are likely to receive input from DB4 cells (Puthussery et al., 2013) and/or DB5 cells (Boycott and Wässle, 1991).

Recursive monostratified cells have been suggested to be the correlate of ON-direction selective and recursive bistratified cells have been suggested to be the correlate of ON-OFF direction selective cells (Dacey, 2004) but electrophysiological evidence demonstrating direction-selective ganglion cells in any primate retina is still missing. In the current study only four out of 24 recursive bistratified cells were located in retinal pieces that were processed with antibodies to ChAT. These four cells stratified in the same region as starburst cells but did not co-fasciculate with them,
suggesting that these cells are probably not direction selective (Vaney et al., 2012). On the other hand, previous studies of macaque (Yamada et al., 2005) and marmoset (Moritoh et al., 2013) retinas found a low number of cells that co-stratified with ChAT cells. Thus it is likely that a subpopulation of the cells classified as recursive bistrafied cells found in the present study are in fact direction selective cells, but further studies are needed to verify this speculation.

Broad thorny cells stratify broadly in the middle of the inner plexiform layer (Ghosh et al., 1996; Szmajda et al., 2008; Moritoh et al., 2013) suggesting that they receive input from multiple types of ON and OFF bipolar cells (Percival et al., 2011; Masri et al., 2016). Similar cells have been described in macaque retina (Dacey et al., 2003) and based on their similarity to cells found in rabbit retina, broad thorny cells have been suggested as candidates for local edge detectors (Berson, 2008). Recent electrophysiological recordings from these cells in macaque retina, however, found that broad thorny cells have transient ON-OFF responses (Puller et al., 2015), whereas local edge detectors in rabbit retina have sustained responses (van Wyk et al., 2006). Thus, Puller et al. (2015) suggested that broad thorny cells are involved in visual guidance of pursuit movements (Puller et al., 2015).

2.6 Conclusions

Our main conclusions are as follows. Consistent with our previous studies, we found that wide-field cells (that is, non-midget, non-parasol pathways) do contribute to foveal vision (see discussion in Percival et al., 2013). However, here we found that the wide-field cells make a much smaller contribution to foveal vision than their contribution to peripheral vision, and this result is consistent with results from human and macaque retina showing the fovea is dominated by midget and parasol cells (Dacey, 1993b; Dacey, 2004). In respect of the high morphological diversity and numerical dominance of wide-field cells, the peripheral retina of primates resembles the entire retina of non-primate mammals (rodents and carnivores) studied so far.
CHAPTER THREE
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3.1 Abstract

Purpose: Particle-mediated gene transfer has been used in animal models to study the morphology and connectivity of retinal ganglion cells. The aim of this chapter was to apply this method to transfec ganglion cells in post-mortem human retina.

Methods: Post-mortem human eyes from male and female donors aged 40 to 76 years old were obtained within 15 hours after death. In addition, two marmoset retinas were obtained immediately after death. Ganglion cells were transfected with an expression plasmid for the postsynaptic density 95 protein conjugated to green or yellow fluorescent protein. Retinas were cultured for three days, fixed and then processed with immunohistochemical markers to reveal their stratification in the inner plexiform layer.

Results: The retinas maintained their morphology and immunohistochemical properties for at least three days in culture. Bipolar and ganglion cell morphology was comparable to that observed in non-cultured tissue. The quality of transfected cells in human retina was similar to that in freshly enucleated marmoset eyes. Based on dendritic field size and stratification at least eleven morphological types of retinal ganglion cell were distinguished.

Conclusions: Particle-mediated gene transfer allows efficient targeting of retinal ganglion cells in cultured post-mortem human retina.

Translational Relevance: The translational value of this methodology lies in the provision of an in vitro platform to study structural and connectivity changes in human eye diseases that affect the integrity and organization of cells in the retina.
3.2 Introduction

The organotypic culture of mammalian retina has provided an efficient model for the study of retinal biology (Pinzón-Duarte et al., 2000; Pérez-León et al., 2003; Koizumi et al., 2007; Binley et al., 2016), disease therapy (Ruscher et al., 2007), and pathophysiology (Manabe et al., 2002; White et al., 2015; Johnson et al., 2016; Valdés et al., 2016). Retinal explants can be targeted with gene promoters and viral vectors, and have the advantage over single cell cultures that neural networks are preserved.

In the past decade, the development of organotypic human retinal cultures has provided the opportunity to record from and manipulate intact human retina. Human ex vivo retinas have been transfected with adeno-associated viruses for application in gene therapy (Fradot et al., 2010) and optogenetic studies (Busskamp et al., 2010; Sengupta et al., 2016). Additionally, human retinal explants have been used to test the effects of neurotoxic insult on ganglion cells (Niyadurupola et al., 2011) and to study retinal degeneration patterns (Fernandez-Bueno et al., 2012). In recent studies, organotypic culture has been combined with the use of biolistics in order to introduce transgenes to retinal neurons. This method has been applied to murine (Kerschensteiner et al., 2009; Moritoh et al., 2010), rabbit (Koizumi et al., 2007), and marmoset monkey (Moritoh et al., 2013; Percival et al., 2014; Masri et al., 2016, 2017) retinas but to date has only been applied to fetal human retinal tissue (Zhang et al., 2018). The aim of the present study was to use particle mediated gene transfer in post-mortem human retina in order to enable studies of retinal ganglion cells and their potential connectivity. Particle mediated gene transfection provides a reliable way of manipulating transgenic expression in cultured human retinal tissue in vitro within days. Here we combined biolistic labeling with immunohistochemistry using markers that would be useful in the analysis of potential ganglion cell contacts in the inner plexiform layer.
3.3 Methods

3.3.1 Post-mortem donor tissue

Post-mortem human eyes from adult donors with no known history of eye disease were obtained from the Lions NSW Eye Bank (Sydney Eye Hospital) with consent and ethical approval from The University of Sydney Human Research Ethics Committee (HREC# 2012/2833); the protocols adhered to the Declaration of Helsinki. Table 3.1A and B summarizes the information on donor eyes. Eyes were placed in Hibernate-A-Medium (Gibco, Thermo Fisher Scientific, North Ryde, NSW) or CO₂ Independent Medium (Gibco) at the Eye Bank. Subsequently, posterior eye cups were immersed for 30 minutes in oxygenated Ames’ medium (Sigma Aldrich, St Louis, MO) containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.292 mg/ml L-glutamine (Gibco), supplemented with 0.5% normal horse serum (Antibodies Australia, Clayton, VIC). The eye cups were gently squeezed outside from the optic nerve downwards to detach the vitreous humor. Retinas were dissected in supplemented Ames’ medium in sterile conditions and cut into 4 to 6 pieces (Fig. 3.1). Each retinal piece was halved into a central and peripheral portion (maximum 10 mm x 15 mm) and cultured separately. The retinal pigment epithelium was retained in two experiments (Table 3.1A) for co-culture with the retina (Kaempf et al., 2008; Di Lauro et al., 2016).

Non-cultured retinas used for comparison were initially fixed in the eye cup prior (n=1) or after (n=3) vitreous removal in 2 or 4% paraformaldehyde (PFA, Table 3.1B) in 0.1 M phosphate buffer (PB), pH 7.4, rinsed in PB and then dissected.

Pieces from cultured and non-cultured retinas intended for immunohistochemistry were immersed in 30% sucrose in 0.1 M PB, frozen in liquid nitrogen, and kept at -80°C until use.
### Table 3.1. Donor retinas.

**A. Retinas processed for organotypic culture and particle mediated gene transfection.**

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*The medium was Hibernate-A for #15155; and CO₂ Independent Medium for the other retinas; all retinas were placed in Ames’ medium for at least 30 minutes before culture. Times are shown to the nearest hour.

**B. Non-cultured retinas.**

<table>
<thead>
<tr>
<th>ID</th>
<th>Eye</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Death to Enucleation (hours)</th>
<th>Enucleation to Medium* (hours)</th>
<th>Time in Medium (hours)</th>
<th>Death to Fixation (hours)</th>
<th>Time in Fixative (hours)</th>
<th>Concentration of Fixative (% PFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13587</td>
<td>R</td>
<td>F</td>
<td>44</td>
<td>2.3</td>
<td>N/A</td>
<td>N/A</td>
<td>4.3</td>
<td>12</td>
<td>2%</td>
</tr>
<tr>
<td>13699</td>
<td>R</td>
<td>M</td>
<td>56</td>
<td>2</td>
<td>N/A</td>
<td>N/A</td>
<td>9.2</td>
<td>21.17</td>
<td>2%</td>
</tr>
<tr>
<td>15022</td>
<td>R</td>
<td>M</td>
<td>69</td>
<td>9.2</td>
<td>4.5</td>
<td>14</td>
<td>33</td>
<td>1</td>
<td>4%</td>
</tr>
<tr>
<td>15054</td>
<td>R</td>
<td>F</td>
<td>76</td>
<td>8.5</td>
<td>2</td>
<td>11.5</td>
<td>29.5</td>
<td>1</td>
<td>4%</td>
</tr>
</tbody>
</table>

*The medium used was Hibernate-A. Retinas #15022 and #15054 were also immersed in carboxygenated Ames’ medium for 1 hour before fixation. Times are shown to the nearest hour. PFA: Paraformaldehyde
3.3.2 Marmoset tissue

Two retinas were obtained from one male adult marmoset (*Callithrix jacchus*, aged 1.8 years) were used. The animal was obtained from the Australian National Health and Medical Research Council’s (NHMRC) combined breeding facility. All procedures were carried out according to the provisions of the NHMRC Code of Practice for the Care and Use of Animals, were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee of The University of Sydney (AEC #558).

The marmoset was sedated with an intramuscular injection of 12 mg/kg Alfaxan (Jurox, Rutherford, NSW, Australia) and 3mg/kg diazepam (Roche, NSW, Australia) and then euthanized with an overdose of sodium pentobarbitone (Letho-barb, Virbac, Milperra, NSW, Australia). The animal was transcardially perfused using 500 ml chilled sucrose-based solution consisting of 205 mM sucrose, 2.5 mM KCl, 26 mM NaHCO$_3$, 1.2 mM NaH$_2$PO$_4$.H$_2$O, 0.1 mM CaCl$_2$, 5 mM MgCl$_2$. 6H$_2$O, and 10 mM D-glucose (Pietersen et al., 2014). The eyes were enucleated, dropped into 3% povidone-iodine solution in phosphate buffered saline (PBS), rinsed with sterile PBS, and kept on ice in freshly made Ames’ medium for a maximum of 45 minutes. The eyes were then immersed in oxygenated Ames’ medium (95%O$_2$ / 5%CO$_2$) for thirty minutes. The anterior portion of the eye cup was removed, and the eye was gently squeezed from the optic nerve downwards to remove the vitreous humor. The retina was dissected out of the eye cup and cut into four quadrants.

3.3.3 Particle-mediated gene transfer and organotypic culture

The protocol for organotypic culture and gene transfection is a modification of a previously published protocol (Koizumi et al., 2007; Moritoh et al., 2013). Each retinal piece was flattened ganglion cell side up onto a 0.4 µm pore size Millicell cell culture insert (30 mm diameter, Millipore, Temecula, CA). Excess medium was removed using filter paper in order to enable the retina to adhere to the membrane. A Helios gene gun system (Bio-Rad, Hercules, CA) was used to propel gold microcarriers into the tissue. The gold microcarriers (1.6 µm diameter) were coated with a cytomegalovirus (CMV) postsynaptic density 95 - green fluorescent protein (PSD95-GFP) fusion plasmid in a ratio of 1.5 µg plasmid/1 mg gold (gift from Prof. Masaki Fukata, National
Institute for Physiological Science, Osaka, Japan). Plasmids conjugated to yellow fluorescent protein (PSD95-YFP, gift from Prof. Rachel Wong, University of Washington, Seattle, WA) were used in one case (#15233). Bullets used within two months of preparation yielded optimal expression of the protein. The bullets were delivered at 100 psi. Retinas were shot through the filter of Transwell membrane cell culture inserts (3 µm pore size membrane, 12 mm insert, Corning, Sigma Aldrich, St Louis, MO) at a distance of ~ 50 mm. Initially, a modified gene gun barrel was used for retina #15155 at 40 mm distance from the tissue (O’brien and Lummis, 2006). However, we found the original gun barrel to be less damaging to the retina and to provide a better distribution of the bullets. The Millicell inserts were fitted onto custom printed filter stands and placed in a tissue culture dish (100 x 20 mm, Falcon, Corning, NY, USA). The retinal pigment epithelium (if retained) was positioned at the base of the tissue culture dish (Di Lauro et al., 2016). Dishes were filled with supplemented Ames’ medium so that the photoreceptor side was in contact with the medium and the ganglion cell side was exposed to the atmosphere (Koizumi et al., 2007). The entire chamber was placed in an incubator (37°C/ 5%CO₂; SANYO, Osaka, Japan, MCO-18M) and the retina cultured for 67 – 89 hours (Table 3.1A). The medium was replaced daily.

After culture, the retinal pieces were removed from the incubator and the medium was replaced with 4% PFA in 0.1M PB. Pieces were fixed for one hour. After rinses in 0.1 M PB, the pieces were processed for immunohistochemistry attached to the Millicell filter membrane, unless otherwise indicated.

### 3.3.4 Immunohistochemistry

Pieces from one cultured retina (#15233) were peeled off the filter membrane and used to cut vertical cryostat sections (Leica CM 3050 S) at a thickness of 14 µm. Sections were pre-incubated for 30 minutes in 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc. PA, USA) in PBS containing 0.5% Triton X-100 (BDH Chemicals, Kilsyth, Australia). Primary antibodies (see Table 3.2) were diluted in 0.5% Triton X-100 in PBS and 0.05% NaN₃ with 3% NDS and applied overnight. Secondary antibodies were donkey anti-mouse, anti-rabbit, or anti-goat immunoglobulins (IgG) coupled to Alexa 594 or Alexa 488 (Jackson ImmunoResearch Laboratories Inc., PA, USA). The sections were coverslipped using Mowiol (Hoechst
Australia Ltd., VIC) containing 10% DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma Aldrich, Milwaukee, WI).

Other pieces from cultured (#15155; #15233; #15512) and non-cultured retinas (Table 3.1B) were processed free floating. The tissue was processed as described above except that the incubation time in primary antibodies was at 4°C for 4-7 days and that 10μg/ml DAPI (1:1,000, 4’,6-diamidino-2-phenylindole dihydrochloride, D9542, Sigma) was added to the secondary antibody diluent. Some retinal pieces were incubated in antibodies against green fluorescent protein in order to enhance GFP labeling. The tissue was flat mounted onto poly-lysine coated microscope slides and coverslipped with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA).

3.3.5 Microscopy

Tissue was imaged using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) or a confocal scanning microscope (Zeiss LSM700) equipped with 405, 488, 555, and 635 nm lasers at a resolution of 2,048 x 2,048 pixels. Stacks of images were obtained at 0.5 – 1 µm increments using a 20 × objective (Plan Apochromat no. 420650-9901) or a 40 × water immersion objective (Plan Apochromat no. 421767-9970). The brightness and contrast of the images was adjusted in Zen Blue software (Zeiss) software or Adobe Photoshop CS6 (San Jose, CA).

3.3.6 Analysis

The dendritic field size of ganglion cells was measured from stacks of images using Fiji (Schindelin et al., 2012) as described in the previous Chapter. The eccentricity of ganglion cells was calculated from low-power montages of the retinal pieces. Stratification of ganglion cell dendrites was determined using orthogonal projections in Zen Blue, relative to the DAPI nuclear staining at the borders of the inner plexiform layer and/or to the stratification of calbindin labeled diffuse bipolar DB3a cells (Haverkamp et al., 2003; Puthussery et al., 2013), recoverin labeled flat midget bipolar cells (Milam et al., 1993), and ChAT labeled starburst amacrine cells (Rodieck and Marshak, 1992). Some retinal ganglion cells were reconstructed using the filament tracer in Imaris (Bitplane, Zurich, Switzerland) software.
<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Immunogen</th>
<th>Source, catalogue number, RRID</th>
<th>Antibody type</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin (CaBP)</td>
<td>Recombinant rat calbindin D-28k</td>
<td>Swant, CB38, lot: 5.5 RRID: AB_10000340</td>
<td>Rabbit polyclonal</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Calretinin (CaR)</td>
<td>Produced against recombinant human calretinin</td>
<td>Swant, 7699/4, lot: 1§.1; RRID: AB_10000321</td>
<td>Goat polyclonal</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Choline acetyl transferase (ChAT)</td>
<td>Purified human placental choline acetyltransferase enzyme</td>
<td>Millipore, AB144P, lot: NG1780580; RRID: AB_2079751</td>
<td>Goat polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>C-terminal binding protein (CtBP2)</td>
<td>Amino acids 361-445 of mouse C-terminal binding protein 2</td>
<td>BD Biosciences, 612044; RRID: AB_399431</td>
<td>Mouse monoclonal</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Green fluorescent protein isolated directly from the jellyfish Aequorea victoria.</td>
<td>ThermoFisher Scientific, A-6455, lot: 1692915; RRID: AB_221570</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
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<tr>
<td>Green fluorescent protein</td>
<td>Derived from the jellyfish Aequorea victoria. Immunogen is a fusion protein corresponding to full length amino acid sequence 246</td>
<td>Rockland Immunochemicals, 600101215, RRID: AB_218182</td>
<td>Goat polyclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>Glutamine Synthetase</td>
<td>Human Glutamine Synthetase amino acids 1-373, recognizes band of 45 kDa</td>
<td>BD Transduction Laboratories, 610518; RRID: AB_2313767</td>
<td>Mouse monoclonal</td>
<td>1:6,000</td>
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<tr>
<td>Islet-1</td>
<td>C-terminal portion of rat Islet-1</td>
<td>Developmental Studies Hybridoma Bank, 39.3F7; RRIDAB_1157901</td>
<td>Mouse monoclonal</td>
<td>1:250</td>
</tr>
<tr>
<td>Melanopsin</td>
<td>N-terminal peptide of human melanopsin protein (Liao et al. 2017)</td>
<td>Dr. King-Wai Yau, Johns Hopkins University School of Medicine, Baltimore, MD</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>RNA-Binding Protein with Multiple Splicing (RBPMS)</td>
<td>Recombinant protein encompassing a sequence within the centre region of human RBPMS</td>
<td>Gene Tex, Sapphire Bioscience, GTX118619, lot: 40415; RRID: AB_10720427</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
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<tr>
<td>Recoverin</td>
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<td>Millipore, AB5585, lot: LV1480447; RRID: AB_2253622</td>
<td>Rabbit polyclonal</td>
<td>1:10,000</td>
</tr>
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</table>
3.4 Results

3.4.1 Preservation of gross morphology and retinal architecture

Retinas to be used for organotypic culture (Fig. 3.1) were checked to be in good condition after dissection, in particular we checked macroscopically that the photoreceptor layer was intact and did not peel off. Retinas co-cultured with the retinal pigment epithelium adhered more strongly to the filter membrane and were thus better preserved but this was not investigated systematically.

The main aim of the present study was to establish biolistic transfection in post-mortem human retina but in order to check whether the transfected and cultured retinal tissue appeared qualitatively normal we compared non-cultured and cultured retinas. A more quantitative analysis was not carried out as it would require comparison of tissue at the exact same eccentricity, the same age, and the same post-mortem delay. This was beyond the scope of the present study.

**Figure 3.1.** Micrograph of a freshly dissected human retina. The dissection was performed in Ames’ medium and relieving cuts were made to flatten the retina. The macula (yellow) and surrounding blood vessels are visible. 1 mm grid paper pictured for scale.
Vertical cryostat sections or retinal flat mounts were processed with established immunohistochemical markers for neurons and Müller cells (Milam et al., 1993; Haverkamp et al., 2003; de Souza et al., 2016; Lee et al., 2016). Figure 3.2A-E shows images of vertical sections through a cultured retina processed with these markers. The retinal layers are intact but the inner and outer segments of the photoreceptors were damaged when the tissue pieces were peeled off the filter membrane. The appearance of glutamine synthetase labeled Müller cells (Fig. 3.2A), recoverin labeled flat midget bipolar cells (Fig. 3.2B,C), Islet-1 positive ON bipolar cells (Fig. 3.2B), and calretinin labeled amacrine cells (Fig. 3.2D) resembles previously published data of normal human retina (Milam et al., 1993; Haverkamp et al., 2003; de Souza et al., 2016; Lee et al., 2016). The dendritic terminals of PKC labeled rod bipolar cells are clearly visible in Figure 3.2D but rod bipolar somas and axons were only weakly labeled. Figure 3.2E shows a vertical section labeled with antibodies against the ganglion cell marker RBPMS (Kwong et al., 2010; Rodriguez et al., 2014). Consistent with previous studies, expression of RBPMS was restricted to the somas of ganglion cells (see also Fig. 3.6A).

Flat mount preparations of cultured (Fig. 3.2F,G) and non-cultured retinas (Fig. 3.2H,I) showed comparable expression of the Müller cell marker glutamine synthetase. Similarly, the morphology of bipolar and amacrine cells resembled that shown in previous studies of human retina (Rodieck and Marshak, 1992; Haverkamp et al., 2003). Figure 3.3 shows examples of calbindin and recoverin labeled bipolar somas and axon terminals in non-cultured (Fig. 3.3A,B,E,F) and cultured (Fig. 3.3C,D,G,H) retinas, as well as ChAT expression in ON starburst somas and processes of non-cultured (Fig. 3.3I, J) and cultured (Fig. 3.3K,L) retinas. Taken together, our results show that cells in post-mortem human retina maintained their morphology and immunohistochemical properties after three days of organotypic culture.
Figure 3.2. Organotypically cultured human retinas maintain their structure and immunohistochemical properties. (A) to (E) Conventional fluorescence micrographs (A, B) and confocal images (C,D,E) of vertical cryostat sections through cultured human retina. (A) Immunoreactivity for glutamine synthetase is present in Müller cell somas, end-feet and processes. (B, C) Recoverin immunoreactivity (magenta) is present in photoreceptors and OFF midget bipolar cells, ON bipolar cell somas express Islet (green). (D) Calretinin is localized to amacrine cells in the inner nuclear layer, and PKC\(\alpha\) is localized to rod bipolar cells whose dendrites are more strongly labeled. (E) RNA-binding protein with multiple splicing (RBPMs) is restricted to ganglion cells. Nomarski optics reveals the retinal layers (ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer). (F-I) Single confocal images of retinal flat mounts. Single confocal planes from a stack of images showing expression of glutamine synthetase by Müller cell bodies in cultured (F) and non-cultured flat mount retina (H). The images in G and I are orthogonal projections of images stacks from the preparations shown in F and H, respectively, with glutamine synthetase shown in red and DAPI-labeled nuclei shown in blue. Scale bar shown in E = 20 µm, applies to A-E; scale bar shown in H = 20 µm (applies to F-I).
Figure 3.3. Comparison of bipolar and amacrine markers in cultured and non-cultured retinal flat mount preparations of human retina. (A-D) Confocal images of calbindin (CaBP) expressing bipolar cells. A shows the somas and B shows the axon terminals in non-cultured retina, C shows the somas and D the axon terminals in cultured retina. (E-H) Confocal images of recoverin expressing flat midget bipolar cells. E shows the somas and F shows the axon terminals in non-cultured retina, G shows the somas and H shows the axon terminals in cultured retina. (I-L) Choline acetyl transferase (ChAT) immunoreactive displaced amacrine cells and their processes in non-cultured (I,L) and cultured (K,L) retina. Scale bar = 20 µm in A (applies to all).
3.4.2 Particle-mediated gene transfection of retinal ganglion cells

In both, marmoset and human retina a variety of ganglion cell types were labeled using particle-mediated gene transfection (see below). Transfected cells are characterized by brightly labeled puncta distributed along the dendritic tree. The quality of the PSD95-GFP labeling in marmoset retina was superior to that found in human retina where the puncta were more irregular with respect to their size and distribution on the dendrites (see below). Nonetheless our results show that ganglion cells in post-mortem human retina can express PSD95-GFP.

Figure 3.4 demonstrates the punctate appearance of PSD95-GFP labeling on the dendrites of ganglion cells in marmoset (Fig.3.4A, inset) and human retina (Fig. 3.4B). These puncta are thought to be the postsynaptic sites to bipolar input (Jakobs et al., 2008; Bleckert et al., 2014; Masri et al., 2016). The piece of human retina was also processed with antibodies against the C-terminal binding protein 2 (CtBP2) (Schmitz et al., 2000), a marker for pre-synaptic ribbons (Fig. 3.4C) and the merged image shows that some of the PSD95-GFP puncta are located close to CtBP2 immunoreactive puncta (arrows in Fig. 3.4D) consistent with the presence of synaptic connections. Similar results have been obtained in other species (Jakobs et al., 2008; Bleckert et al., 2014; Masri et al., 2016) but will need to be analyzed more quantitatively in human retina.

The key factors for successful particle-mediated gene transfection in post-mortem human retina were that 1) the retinas were processed within 15 hours of death, 2) the vitreous was completely removed, and 3) the explants were cultured for three to four days. When the time delay between death and immersion in Ames’ medium exceeded 15 hours (n=11 retinas), no particle-mediated labelling was observed. These retinas are not included in Table 3.1A.
Figure 3.4. Expression of PSD95-GFP in retinal ganglion cells. (A) Marmoset retina: Photomontage of collapsed stacks of confocal images of ganglion cells labeled using particle-mediated gene transfection. Numbers indicate the distance from the fovea in mm for each cell. The inset represents a region of interest from the dendritic tree of the broad thorny ganglion cell shown on the left, showing the punctate expression of PSD95-GFP. (B-D) Human retina: Confocal images of the dendrites of a biolistic transfected ganglion cell processed with antibodies against the pre-synaptic ribbon protein CtBP2. Arrows point to PSD95-GFP puncta (green) associated with ganglion cell dendrites opposed to presynaptic ribbons (CtBP2, magenta). Scale bar = 50 µm in (A), 5 µm in B (Applies to inset in A and B-D).
Figure 3.5 compares the expression of PSD95-GFP in midget ganglion cells of marmoset (Fig. 3.5A,B) and human retina (Fig. 3.5C). Due to their small dendritic field size, midget ganglion cells were more prone to over-expression of PSD95-GFP along their dendrites (Masri et al., 2017). Further experiments are required to discern whether shorter incubation times reduce over-expression of PSD-95 puncta along ganglion cell dendrites.

The distribution of the PSD95-GFP puncta along the dendrites of ganglion cells with larger dendritic fields is shown for a recursive bistratified cell in marmoset retina (Fig. 3.5D) and a parasol cell in human retina (Fig. 3.5E). As pointed out above, the PSD95-GFP puncta on the dendrites of ganglion cells in marmoset have a more uniform size and a more regular distribution.

In order to demonstrate that the ganglion cell layer in transfected and cultured retinas remains intact, some retinal pieces were processed with antibodies against RBPMS. Figure 3.6A shows a micrograph of such a retina and demonstrates that RBPMS labeling is present in cells with relatively large somas (presumed ganglion cells), whereas unlabeled cells are thought to be displaced amacrine, glial and endothelial cells.

Figure 3.6B shows a transfected ganglion cell with a very large sparse dendritic field (880 µm diameter) in human retina. This cell stratified mostly close to the ganglion cell layer but also had some dendrites close to the inner nuclear layer. Based on its large dendritic field size, we classified this cell as giant sparse cell (Rodieck and Watanabe, 1993). Giant sparse cells are thought to be equivalent to melanopsin-expressing (intrinsically photosensitive) ganglion cells which can be identified with antibodies against melanopsin (Dacey et al., 2005; Nasir-Ahmad et al., 2017). Here we applied these antibodies to cultured (Fig. 3.6C) and non-cultured (Fig. 3.6D) human retinas. In both cases, the typical morphology of melanopsin-expressing ganglion cells can be distinguished, demonstrating that the morphology of intrinsically photosensitive ganglion cells is conserved in cultured retinas. Double labeling experiments would be required to confirm that transfect giant sparse cells like the one shown in Figure 3.6B are indeed melanopsin expressing cells.
Figure 3.5. Expression of PSD95-GFP in ganglion cells labeled using particle-mediated gene transfection in marmoset (A,B,D) and human (C, E) retinas. The numbers indicate the eccentricities of the cells in mm. (A) Fluorescence micrograph of a midget ganglion cell, imaged at the level of the inner plexiform layer. The same ganglion cell is shown in (B) together with differential interference contrast optics (DIC). (C) Fluorescence micrograph of midget ganglion cells in human retina, shown at the level of the dendrites. (D) Confocal projection of the dendritic tree of a recursive bistratified cell in marmoset retina. (E) Confocal projection of a parasol ganglion cell in human retina. Scale bar = 50 µm in C (applies to all).
Figure 3.6. Human retina: ganglion cell labeling in cultured and non-cultured retinas. (A) Confocal image of a flat mounted cultured human retina showing expression of RNA-binding protein with multiple splicing (RBPMS, green). The focus is on the ganglion cell layer. DAPI labeled nuclei are shown in blue. (B) Maximum intensity projection of a giant sparse ganglion cell labeled using particle-mediated gene transfection. (C) Maximum intensity projection of a melanopsin-expressing ganglion cell in cultured retina. (D) Maximum intensity projection of a melanopsin-expressing ganglion cell in non-cultured retina. Scale bar in A = 20 µm, scale bar = 100 µm in D (applies to B-D).
3.4.3 Classification of ganglion cell types labeled by particle-mediated gene transfection

In total 126 transfected ganglion cells were analyzed in human retina. Examples of these cells are shown in Figures 3.5 – 3.8. Please note that the micrographs of the cells are maximum intensity projections from confocal stacks resulting in a less punctate appearance of the dendritic tree. The cells were derived from seven retinal pieces at eccentricities between 6 mm and 18 mm. Labelling near central retina was limited because the thick nerve fibre layer obstructed the passage of the bullets. Ganglion cells were classified based on the presence of an axon, dendritic field size relative to eccentricity, the morphology of their dendritic tree and the stratification of their dendrites in the inner plexiform layer (Rodieck et al., 1985; Dacey and Petersen, 1992; Dacey, 1993b, a; Peterson and Dacey, 1999, 2000; Dacey et al., 2003; Nasir-Ahmad et al., 2017).
Figure 3.7. Human retina: confocal micrographs of ganglion cell types identified using particle-mediated gene transfection. (See preceding page for legend).
Figure 3.7. Human retina: confocal micrographs of ganglion cell types identified using particle-mediated gene transfection. The numbers indicate the eccentricities of the cells in mm. The insets in A, E, and J show the stratification of the cells. Blue indicates DAPI labelling, the ganglion cell layer is located at the lower edge of the inset. (A) Collapsed stack of images of a smooth monostratified (SM) ganglion cell. (B-C) Single confocal planes showing the dendrites of the SM cell (green) co-stratifying with calbindin-labeled bipolar axon terminals (magenta). (E) Collapsed stack of images of a small bistratified ganglion cell. (F-H) Single confocal planes showing the dendrites of the SBS cell (green) with the calbindin-labeled bipolar axon terminals (magenta). (I) Collapsed stack of a large sparse ganglion cell. (J) Collapsed stack of a large bistratified (LBS) ganglion cell. A midget cell can also be seen in the lower left corner of the image (arrow). Eccentricity in mm is indicated in the upper left-hand corner for each cell. Scale bar = 10 µm in B (applies to B-D), 20 µm in F (applies to F-H), 50 µm in J (applies to A, E, I, J).
In total, 93 cells were classified as midget (73%), six cells were classified as parasol (5%) and eight cells were classified as small bistratified (6%). The remaining ganglion cells were widefield ganglion cell types (16%). The proportions of labelled cell types could be skewed by some bias in gene-gun labelling, which is addressed in the discussion of this chapter. Figure 3.7 shows examples of labeled cells, Figure 3.8 shows reconstructions of ganglion cell types identified in this study, and Figure 3.9 shows their dendritic field diameters with respect to eccentricity.

Midget ganglion cells have the smallest dendritic field size at all locations, a single primary dendrite, and a small soma that is positioned away from the dense dendritic tree (Figs. 3.5A-C, 3.8). Parasol cells have a large soma and thick primary dendrites (Fig. 3.5E, 3.8). Although parasol ganglion cells in our samples exhibited typical characteristics and dendritic field diameter, the dendritic tree was weakly labeled, asymmetrical in some cases, and sparser than expected. Small bistratified cells have a sparse bistratified dendritic tree and a dendritic field size comparable to parasol cells. Examples of small bistratified cells are shown in Fig. 3.7E and 3.8. They have a comparatively large inner dendritic tree and a small outer dendritic tree. The outer dendritic tree stratifies slightly sclerad relative to the terminals of calbindin labeled DB3a cells (Fig. 3.7F-H) in stratum 3 and the inner dendritic tree stratifies in stratum 5 of the inner plexiform layer.

Widefield ganglion cells included smooth monostratified, large bistratified, narrow thorny, sparse and giant sparse ganglion cells. Smooth monostratified cells (n=2) have smooth, straight dendrites that radiate from the soma in the centre of the dendritic field (Fig. 3.7A, 3.8). Their dendrites stratified in the OFF sublamina at the same level as DB3a axon terminals (Fig. 3.7B-D). Large sparse cells (n=3) had dendritic trees that were consistently smaller than giant sparse cells but stratified at similar levels of the inner plexiform layer. An example of an inner stratifying large sparse cell is shown in Figure 3.7I. Large bistratified cells (n=7, Fig. 3.7J) stratified at the same level as the small bistratified cells in strata 3 and 5 of the inner plexiform layer (inset in Fig. 3.7J). In addition to the giant sparse cell shown above (Fig. 3.4B), we found five other cells with this morphology and dendritic field size (Fig. 3.8 and 3.9), four cells stratified close to the inner nuclear and two cells stratified close to the ganglion cell layer.
Figure 3.8. Human retina: reconstructions of ganglion cell types identified using particle-mediated gene transfection. The numbers indicate the distance from the fovea in mm. Arrows point to axons, the outer dendrites of bistratified cells are shown in red. Scale bar = 100 μm.
Figure 3.9. Human retina: Scatter plot showing dendritic field diameter as a function of retinal eccentricity for ganglion cells labeled using particle-mediated gene transfection.
3.5 Discussion

The main finding of this study is that particle-mediated gene transfection can be used to label ganglion cells in organotypic cultured post-mortem human retina. We also show that transfected retinal tissue can be processed with bipolar markers. This will enable future studies of the synaptic input to labeled ganglion cells (Masri et al., 2016).

The morphology of transfected ganglion cells from freshly enucleated marmoset retinas was similar to the human retinas with less than 15 hours post-mortem delay. However, there were discrepancies with respect to the quality of the expression of PSD95-GFP. These differences between marmoset and human retina are most likely due to the differences in the preparation and treatment of the eyes and retina prior to biolistic transfection and organotypic culture. Marmoset eyes were perfused with a chilled sucrose-based solution, enucleated and then placed immediately into Ames’ medium, while human eyes were stored in CO₂ Independent medium for up to 9 hours prior to being placed into Ames’ medium. A reduced time window between death and gene transfection is likely to improve results in human retinal explants.

Ganglion cells identified in this study largely resembled those identified previously in humans using a variety of other methods (Rodieck et al., 1985; Kolb et al., 1992; Peterson and Dacey, 1999, 2000; Nasir-Ahmad et al., 2017). Similarly, previous studies in animals have shown that retinal ganglion cells labeled by particle-mediated gene transfection (Jakobs et al., 2008; Moritoh et al., 2013; Masri et al., 2017) have comparable morphology to that detected with other methods (Perry and Cowey, 1984; Rodieck and Watanabe, 1993; Ghosh et al., 1996; Rockhill et al., 2002; Dacey et al., 2003; Yamada et al., 2005; Szmajda et al., 2008). However, we found differences in the proportions of cells labeled in human retina compared to previous studies. Parasol ganglion cells were found in much lower proportions than expected and had sparser dendritic trees than previously reported (Rodieck et al., 1985; Dacey and Petersen, 1992). In macaque monkey, cells with large axon diameters have been found to be more susceptible to glaucoma (Glovinsky et al., 1991). Whether our findings in human suggest that parasol cells are more susceptible to post-mortem changes needs further investigation.

As discussed previously (Kong et al., 2005; Masri et al., 2017) biolistic labelling is probably biased but it is unlikely that it is biased towards a particular soma or dendritic
field size. Labelling by particle-mediated gene transfection depends on the particles effectively reaching the cell nucleus and on the ability of the cell to transcribe and express the transgene. In marmoset retina, thorny ganglion cells comprised a large proportion of the transfected cells (Moritoh et al., 2013; Masri et al., 2017) but these cells are near absent in the human samples. It is possible that the dense dendritic tree of thorny ganglion cells is less likely to express PSD95-GFP in post-mortem cultures but this needs further investigation. Another interesting result is that we found six giant sparse cells (presumed melanopsin expressing cells) although these cells make up less than 1% of the retinal ganglion cell population (Liao et al., 2016; Nasir-Ahmad et al., 2017). Previous studies have shown that melanopsin-expressing ganglion cells are more resistant to neurodegeneration compared to other ganglion cell types after optic nerve injury (La Morgia et al., 2010; de Sevilla Müller et al., 2014). Thus, is it possible that melanopsin-expressing cells are more easily transfected than other ganglion cell types. Alternatively, the transfected cells classified as giant sparse could include a yet to be described ganglion cell type.

Our study shows that expression of ganglion and glial cell markers is comparable in cultured and non-cultured retinas. These markers are important indicators of the preservation of retinal architecture (Johnson and Martin, 2008; Niyadurupola et al., 2011; Di Lauro et al., 2016) since protein expression in these cell types is altered in response to injury (Bringmann et al., 2006; Pattamatta et al., 2016). In future, it would also be of interest to assess reactive gliosis in cultured human retinas over time using antibodies against glial fibrillary acidic protein. Previous studies have shown that degeneration of the retina becomes evident after a culture period longer than three days (Jakobs et al., 2008; Fernandez-Bueno et al., 2012; Müller et al., 2017). Other studies have cultured human retinas for several weeks to achieve transduction of adeno associated viral vectors (Busskamp et al., 2010; Fradot et al., 2010), but the viability of ganglion cells in these retinas was not systematically assessed.

The present study followed a protocol first applied to primate retina by Moritoh and colleagues (Moritoh et al., 2013) who used Ames’ medium supplemented with normal horse serum and antibiotics (Koizumi et al., 2007; Moritoh et al., 2013). Ames’ medium is known to facilitate the survival of retinal neurons for up to six days for electrophysiological recordings (Ames and Nesbett, 1981; Koizumi et al., 2007). Previous studies in murine culture systems excluded serum from the culture media
because much of its composition is unknown (Romijn et al., 1988; Caffe et al., 2002; Valdés et al., 2016). Studies on rat retina determined that serum-free defined culture media containing B27/N2 supplements extended explant viability by at least one week (Johnson and Martin, 2008) but in human retina the survival of neurons decreased after two days in serum-free medium (Niyadurupola et al., 2011). It is unclear whether the use of serum had any adverse or positive effect on our samples of human retina, but serum was also included in the culture of marmoset retina with excellent results.

The applications of biolistic transfer have been demonstrated previously in mouse (Morgan et al., 2011), rabbit (Rockhill et al., 2002; Koizumi et al., 2007; Jakobs et al., 2008), and marmoset (Moritoh et al., 2013; Percival et al., 2014; Masri et al., 2016) retinas. The feasibility of using this method in human retina allows for a broader use of post-mortem donor tissue. Particle-mediated gene transfer is relatively rapid, requires little maintenance, and provides a system within which multiple parameters can be controlled and easily manipulated. Biolistic labelling can be carried out using combinations of plasmids targeting different genes in the same preparation. Ultimately, the system described here holds potential for clinical translation in allowing genetic manipulation, electrophysiological recording, and evaluation of changes in specific cell types in human retinal tissue.
CHAPTER FOUR
4. Quantitative analysis of the major cell populations in the human retina.

4.1 Abstract

A map of the inner human retina is necessary to identify target areas for treatment in retinal diseases, and to support current diagnostic tools. The current study measured the spatial density of neurons in the inner nuclear layer including horizontal, bipolar, amacrine, and Müller cells. In particular, this study focused on bipolar cells involved with high acuity and colour vision (midget bipolar) and diffuse bipolar cells involved in motion detection (DB3a,b). Data were obtained from vertical Vibratome sections across the horizontal meridian of six post-mortem human donor retinas (aged 30-60 years). Sections were labelled using cell-type specific immunohistochemical markers and imaged using high-resolution, multi-channel fluorescence microscopy. Cells were counted across the length and depth of multiple sections to obtain population densities. In the midget (acuity) pathway, the numerical convergence between cones and midget bipolar cells is 1:1 up to at least 10 mm eccentricity. Diffuse bipolar cells in the parasol (motion detection) pathway make up less than half the density of midget bipolar cells at any given eccentricity. In order to determine the numerical convergence of cones onto ganglion cells near the fovea, the lateral displacement was measured from cone somas to the dendritic bouquets of bipolar cells and subsequently from bipolar cells to ganglion cell dendrites. The ratio of ganglion cells to cones at the fovea was 2:1. The density of H1 and H2 horizontal cells, GABAergic and glycinergic amacrine cells, and Müller cells was also determined across the retina. These cells exist in consistent proportions across the human retina. In the inner nuclear layer, bipolar cells make up the majority of cells, followed by amacrine, Müller, and horizontal cells.
4.2 Introduction

Knowledge of the structure of human retina is important for understanding normal visual function and to assist in the diagnosis and treatment of diseases that cause blindness. In the inner nuclear layer of the retina, there exists a diversity of neurons which form parallel pathways to different ganglion cell types (Boycott and Wässle, 1999; Dacey, 2000; Masland, 2001). Horizontal cells and amacrine cells form inhibitory (lateral) pathways, which serve to modulate visual input from photoreceptors. Bipolar cells are part of the vertical (through) pathway, where signals from rod and cone photoreceptors are transmitted to at least 17 types of ganglion cell (Kolb et al., 1992; Rodieck and Watanabe, 1993; Ghosh et al., 1996; Dacey, 2004; Masri et al., 2017). The best studied pathways in primates are the midget and parasol pathways (Rodieck et al., 1985; Watanabe and Rodieck, 1989; Kolb and Dekorver, 1991; Dacey and Petersen, 1992; Wässle et al., 1994).

The midget pathway is involved in high acuity vision and red/green color vision (Lee et al., 2010), and this is mediated by the one-to-one connectivity between cones, midget bipolar, and midget ganglion cells (Kolb and Dekorver, 1991; Calkins et al., 1994) which dominate the fovea (Perry et al., 1984; Dacey, 1993b). There are two bipolar cell types that transmit signals from the cone photoreceptors in the midget pathway; flat midget bipolar cells (or OFF midget bipolar cells) which contact OFF centre midget ganglion cells, and invaginating midget bipolar cells (or ON midget bipolar cells) which contact ON centre midget ganglion cells (Kolb, 1970; Kolb and Dekorver, 1991). Studies comparing the spatial density of neurons in macaque retina have indicated that the one-to-one ratio between cones and midget bipolar cells extends outside the fovea (Wässle et al., 1994). The first aim of this study is to compare the relative proportions of cells in the midget pathway in central and peripheral retina by accounting for the total lateral displacement of neurons near the fovea. In doing so, I hope to determine whether one-to-one connectivity extends outside the fovea in human retina, which would imply that the chromatic acuity of the cone array is preserved in the periphery.

The parasol pathway is involved in motion detection (Merigan et al., 1991; Croner and Kaplan, 1995; Crook et al., 2008b) and receives cone input via diffuse bipolar (DB) cells which contact multiple cone photoreceptors. Six to eight types of diffuse bipolar cell types were identified in primate retina using Golgi stained preparations.
(Boycott and Wässle, 1991; Kolb et al., 1992; Joo et al., 2011), electron microscopic reconstructions (Tsukamoto and Omi, 2015, 2016), and specific patterns of immunoreactivity (Grünert et al., 1994; Kalloniatis et al., 1996; Chan et al., 2001b; Haverkamp et al., 2003; Puthussery et al., 2011).

The connectivity of ON and OFF parasol cells has been studied in macaque and marmoset retinas. These studies found that ON parasol ganglion cells receive input from DB4 and DB5 cells (Puthussery et al., 2013; Tsukamoto and Omi 2016) and OFF parasol cells receive input from DB3a and DB3b cells (Jacoby and Marshak, 2000; Jacoby et al., 2000; Calkins and Sterling, 2007; Puthussery et al., 2013; Tsukamoto and Omi, 2015; Masri et al., 2016; Tsukamoto and Omi, 2016). DB3a and DB3b cells express the same voltage dependent channels at the axon terminals, but express different voltage dependent ion channels at the axon initial segments, and different glutamate receptors on their dendrites (Puthussery et al., 2013; Puthussery et al., 2014). These differences contribute to variation in physiological response properties between DB3a and DB3b cells, for example DB3a cells exhibit sustained whereas DB3b cells exhibit transient responses to glutamate. DB3a and DB3b cells also differ in their expression of molecular markers in human, macaque and marmoset retinas (Haverkamp et al., 2003; Puthussery et al., 2013; de Souza et al., 2016).

While the distribution and density of DB3a cells has been investigated in macaque (Martin and Grünert, 1992; Grünert et al., 1994) and marmoset (Luo et al., 1999; Chan et al., 2001b; Weltzien et al., 2015) neither DB3a nor DB3b have been studied systematically in human retina. The second aim of this study was to quantify the density of DB3a and DB3b cells in human retina and compare the distribution of diffuse bipolar cells in the parasol pathway to midget bipolar cells in the midget pathway.

In marmoset and macaque retina, it has been shown that bipolar cells make up the majority of cells in the inner nuclear layer, and that midget bipolar cells outnumber other bipolar types (Martin and Grünert, 1992; Grünert et al., 1994; Chan et al., 2001b; Ahmad et al., 2003; Weltzien et al., 2015). In human retina, quantitative data on cells in the inner nuclear layer is scarce. The third aim of this study is to reveal the spatial density and immunoreactivity of cells in the inner nuclear layer of the human retina. At present, bipolar cells, horizontal cells, amacrine cells, and Müller cell glia can be identified with immunohistochemical markers (Milam et al., 1993; Haverkamp et al., 2003; de Souza et al., 2016). I have applied these markers to post-mortem samples
of human retina in order to map the cell populations that make up the inner nuclear layer and to compare their distribution across the retina.

### 4.3 Methods

**Tissue collection and preparation**

*Post-mortem* human eyes from donors with no known history of eye disease were obtained from the Lions NSW Eye Bank (Sydney Eye Hospital) with consent and ethical approval from The University of Sydney Human Research Ethics Committee (HREC# 2012/2833). Information about the donor eyes is summarized in Table 4.1. Only retinas from donors under 60 years of age with a *post-mortem* delay shorter than 10 hours from death to fixation were used. Eyes were received with the cornea removed and the eye cup immersed in 2% paraformaldehyde (PFA). In one case (ID#15415), eyes were first placed into CO₂ Independent Medium and subsequently fixed in 2% PFA. Upon receipt, eyes were rinsed in 0.1M phosphate buffer (PB) and the retina was then dissected out of the eye cup. The vitreous was carefully removed off the retinal surface and relieving cuts were made to flatten the retina. Retinas were immersed in 30% sucrose overnight, then snap frozen in separate parts using liquid nitrogen and stored at -80°C until use. Retinal pieces measuring 3-5 mm in width and 4-5 mm in length were prepared from defined eccentricities along the nasal-temporal axis (1mm nasal to 15mm temporal). The retinal pieces were embedded in 3% low melting Agarose (Sea Plaque Agarose, Rockland, ME) dissolved in phosphate buffered saline (PBS) and sectioned vertically at a thickness of 100 µm using a Vibratome (VT 1200 S, Leica Microsystems, Nußloch, Germany). Sections were processed immediately or kept in 0.1M PB containing 0.05% NaN₃ for up to 4 weeks until use.
Table 4.1. Donor retinas

<table>
<thead>
<tr>
<th>ID</th>
<th>Eye</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Time to Enucleation (hours)</th>
<th>Time to Fixation (hours)</th>
<th>Time in Fixative* (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13587</td>
<td>Right</td>
<td>F</td>
<td>44</td>
<td>2</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>13699</td>
<td>Right</td>
<td>M</td>
<td>56</td>
<td>2</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>14064</td>
<td>Right</td>
<td>F</td>
<td>44</td>
<td>1</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>14458</td>
<td>Left</td>
<td>F</td>
<td>31</td>
<td>3</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>15415**</td>
<td>Left</td>
<td>F</td>
<td>54</td>
<td>3</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>15649</td>
<td>Left</td>
<td>F</td>
<td>36</td>
<td>0.5</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

*All retinas were fixed in 2% paraformaldehyde.
**The eye was placed into CO$_2$ Independent Medium for 1 hour before fixation.
Times are shown to the nearest hour.
**Immunohistochemistry**

Sections were pre-incubated in 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc., PA, USA) and 0.5% Triton X-100 (BDH Chemicals, Kilsyth, Australia) in PBS for 1 hour or overnight. The antibodies used in this study are summarized in Table 4.2. Sections were incubated with primary antibodies diluted in PBS containing 0.5% Triton X-100, 0.05% NaN₃, and 3% NDS. Secondary antibodies (made in donkey) coupled to Alexa 594, Alexa 488 or Alexa 647 (Jackson ImmunoResearch Laboratories Inc., PA, USA) were diluted in PBS containing 0.5% Triton X-100 and 3% NDS. DAPI (1:1,000, 4’,6-diamidino-2-phenylindole dihydrochloride, D9542, Sigma) was usually added to the secondary antibody diluent. Sections were incubated in primary antibody for 4 - 7 days and in secondary antibody for 14 - 24 hours. During incubation periods, sections were kept at room temperature on a shaker during the day and in the fridge at 4°C overnight. Adhesive spacers (20 mm diameter, 0.12 mm depth; ThermoFisher Scientific) were fitted onto poly-lysine coated microscope slides. The tissue was rinsed, mounted within the wells of the adhesive spacers, then coverslipped using Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA).

**Microscopy**

Images were obtained using a confocal scanning microscope (Zeiss LSM700) equipped with 405, 408, 555, and 635 nm lasers. Entire vibratome sections (at least 3 mm in length and a maximum of 100 µm in thickness) were imaged in tiled stacks which were subsequently stitched together using Zeiss ZEN Black software. Images were taken at a resolution of 2,048 x 2,048 or 1,024 x 1,024 pixels and a step size of 0.87-1 µm for each optical section. Stacks of images were obtained using 20 × air objective (Plan Apochromat no. 420650-9901) and some regions were imaged at higher magnification using 40 × water immersion objective (Plan Apochromat no. 421767-9970). The contrast and brightness of the images were adjusted using Zeiss Zen Blue, Adobe Photoshop, or Imaris (Bitplane, Zurich, Switzerland) software.
# Table 4.2. Antibodies

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Immunogen</th>
<th>Source, catalogue number, RRID</th>
<th>Antibody type</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calbindin (CaBP)</td>
<td>CaBP-D-28kD purified from chicken gut. Hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunised mouse</td>
<td>Swant, 300, lot: 07(F); RRID: AB_10000347</td>
<td>Mouse monoclonal</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Calbindin (CaBP)</td>
<td>Recombinant rat calbindin D-28kD</td>
<td>Swant, CB38, lot: 5.5 RRID: AB_10000340</td>
<td>Rabbit polyclonal</td>
<td>1:20,000</td>
</tr>
<tr>
<td>CD15</td>
<td>U-937 histiocytic cell line, purified from tissue culture supernatant or ascites by affinity chromatography</td>
<td>BD Biosciences, 559045; RRID: AB_3067776</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Cone Arrestin (7G6)</td>
<td>Developed using macaque light-adapted retinal extract as immunogen. Gives single band of 44-46 kDa on Western blots of human retina.</td>
<td>Gift from Prof. Peter Macleish, Morehouse School of Medicine, Atlanta, Georgia, USA.</td>
<td>Mouse monoclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>Glutamic Acid Decarboxylase 65 (GAD-6)</td>
<td>Affinity purified from rat brain, recognizes single band of 64kDa</td>
<td>Deposited to the Developmental Studies Hybridoma Bank by Gottlieb, D.I. (Product GAD-6); RRID: AB_528264</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Glutamic Acid Decarboxylase 67 (GAD67)</td>
<td>Recombinant GAD67 protein, clone 1G10.2. Reacts with 67kDa isofrom of Glutamate Decarboxylase</td>
<td>Millipore, MAB5406, lot: 2283338; RRID: AB_2278725</td>
<td>Mouse monoclonal</td>
<td>1:8,000</td>
</tr>
<tr>
<td>Glycine Transporter (Glyt1)</td>
<td>Raised against a synthetic peptide corresponding to the carboxyl terminal region of Glyt1. A single band is detected in Western blots.</td>
<td>Gift from Prof. David Pow, ImmunoSolution, QLD, Australia, IG1122; RRID: AB_2314597</td>
<td>Rabbit polyclonal</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Glutamine Synthetase</td>
<td>Human Glutamine Synthetase amino acids 1-373, recognizes band of 45 kDa</td>
<td>BD Transduction Laboratories, 610518; RRID: AB_2313767</td>
<td>Mouse monoclonal</td>
<td>1:6,000</td>
</tr>
<tr>
<td>Islet-1</td>
<td>C-terminal portion of rat islet-1</td>
<td>Developmental Studies Hybridoma Bank, 39.3F7; RRID: AB_1157901</td>
<td>Mouse monoclonal</td>
<td>1:250</td>
</tr>
<tr>
<td>Parvalbumin (PV)</td>
<td>Purified from carp muscles. Stains the 39Ca-binding spot of parvalbumin</td>
<td>Swant, 235, lot: 10-11 (F); RRID: AB_10000343</td>
<td>Mouse monoclonal</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Parvalbumin (PV 25)</td>
<td>Produced against rat muscle parvalbumin</td>
<td>Swant, PV 25, lot: 5.10; RRID: AB_10000344</td>
<td>Rabbit polyclonal</td>
<td>1:10,000</td>
</tr>
<tr>
<td>RNA-Binding Protein with Multiple Splicing (RBPMS)</td>
<td>Synthetic peptide corresponding to amino acid residues from the N-terminal region of the rat RBPMS sequence, recognizes the 24kDa RBPMS protein</td>
<td>Lubio, PhosphoSolutions, 1832-RBPMS, lot: NB9160; RRID: AB_2492226</td>
<td>Guinea Pig polyclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>RNA-Binding Protein with Multiple Splicing (RBPMS)</td>
<td>Recombinant protein encompassing a sequence within the centre region of human RBPMS</td>
<td>Gene Tex, Sapphire Bioscience, GTX118619, lot: 40415; RRID: AB_10720427</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>Recoverin</td>
<td>Recombinant human recoverin, recognizes single band of 26 kDa</td>
<td>Millipore, AB5585, lot: LV1480447; RRID: AB_2253622</td>
<td>Rabbit polyclonal</td>
<td>1:10,000</td>
</tr>
<tr>
<td>S-Opsin</td>
<td>Raised against a peptide mapping at the N-terminus of the opsin protein encoded by OPN1SW of human origin.</td>
<td>Santa Cruz, sc-14363, lot: A2813; RRID: 21246551</td>
<td>Goat polyclonal</td>
<td>1:100</td>
</tr>
</tbody>
</table>
Analysis

The density of cells was determined from tiled stacks of images using ZEN Blue software. Cells were counted from volumetric reconstructions of the Vibratome sections; the length of the section was divided into bins of 100 µm width and cells were counted within each bin across a minimum depth of 10 µm in the z-plane. Cells were counted when the following criteria applied: the morphology was comparable to previous descriptions of that cell type, the pattern of expression was consistent with cells of the same classification within the same preparation, and the stratification of the cell soma and/or dendrites in the retina was as expected for that cell type. Areal densities (cells/mm²) were calculated for each bin. The eccentricity was calculated by taking into account the triangulated distance from the fovea. Average densities for each cell population were determined from different samples at various increments along the horizontal meridian. A correction factor was applied for depth readings taken using the air objective to account for the refractive index of the Vectashield mounting medium (1.45). No correction for shrinkage was applied because paraformaldehyde fixation was assumed to produce minimal shrinkage and the tissue was mounted using a water based mounting medium (Vectashield, Vector).

Density calculations were fit to a curve as a function of eccentricity for each cell population. Curves were fit in Matlab V9.2 (Mathworks, Natick NJ) with three-stage difference-of-exponentials functions, and these were adjusted across all eccentricities from 0 to 15 mm along the temporal horizontal axis. It is for this reason that some resolution at the foveal centre is sacrificed, and the fit is anchored at 0. A precise representation of the fovea can be achieved if the foveal data is graphed separately, for which the present study would require additional data due to the limitation of the number of sections available through the fovea. Optimal fit parameters are given in Table 4.3. Cumulative density across the horizontal meridian was determined by accounting for the increase in containing volume with eccentricity. This required circular integration of spatial densities within annuli of defined eccentricity ranges radiating from the foveal centre in a “bullseye” pattern.
The following formula was applied to calculate the number of cells within each annulus:
\[
Density \left( \frac{\text{cells}}{\text{mm}^2} \right) \times \text{Annulus Area (mm}^2) \\
\text{Annulus Area} = (\pi \times r_o^2) - (\pi \times r_i^2)
\]

Where \( r_o \) represents the radius of the outer circle and \( r_i \) represents the radius of the inner circle.

**Table 4.3. Best fit parameters for pooled data**

<table>
<thead>
<tr>
<th></th>
<th>( c_1 )</th>
<th>( \lambda_1 )</th>
<th>( c_2 )</th>
<th>( \lambda_2 )</th>
<th>( c_3 )</th>
<th>( \lambda_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cones</td>
<td>3.673e+05</td>
<td>-7.828e</td>
<td>-2e+05</td>
<td>-2.000e+03</td>
<td>2.034e+04</td>
<td>-2.164e-01</td>
</tr>
<tr>
<td>S-Cones</td>
<td>5.048e+03</td>
<td>-3.014e</td>
<td>-1.1+04</td>
<td>-7.869e</td>
<td>1.455e+03</td>
<td>-1.370e-01</td>
</tr>
<tr>
<td>Ganglion cells</td>
<td>5.717e+05</td>
<td>-1.031e</td>
<td>-6e+05</td>
<td>-1.261e</td>
<td>-5.527e+01</td>
<td>-9.658e+01</td>
</tr>
<tr>
<td>Flat Midget Bipolar cells</td>
<td>4.208e+05</td>
<td>-1.225e</td>
<td>-4.551e+05</td>
<td>-1.387e</td>
<td>1.230e+04</td>
<td>-6.986e-02</td>
</tr>
<tr>
<td>ON Bipolar cells</td>
<td>1.788e+05</td>
<td>-4.755e-01</td>
<td>-1.836e+05</td>
<td>-5.618e-01</td>
<td>8.894e+03</td>
<td>-1.832e-02</td>
</tr>
<tr>
<td>DB3a cells</td>
<td>4.275e+03</td>
<td>-1.949e-01</td>
<td>-9.614e+03</td>
<td>-5.831e</td>
<td>3.336e+02</td>
<td>5.811e-03</td>
</tr>
<tr>
<td>DB3b cells</td>
<td>7.061e+03</td>
<td>-1.511e-01</td>
<td>-7.869e+03</td>
<td>-3.120e</td>
<td>-3.653e+03</td>
<td>-3.347e-01</td>
</tr>
<tr>
<td>Horizontal cells</td>
<td>2.051e+04</td>
<td>-3.850e-01</td>
<td>-4.246e+04</td>
<td>-4.196e</td>
<td>2.672e+03</td>
<td>-1.097e-02</td>
</tr>
<tr>
<td>H1 cells</td>
<td>1.659e+04</td>
<td>-3.334e-01</td>
<td>-4.227e+06</td>
<td>-1.633e+01</td>
<td>1.280e+03</td>
<td>4.952e-02</td>
</tr>
<tr>
<td>H2 cells</td>
<td>5.131e+03</td>
<td>-2.197e-01</td>
<td>-5.440e+03</td>
<td>-6.706e-01</td>
<td>-8.006e-01</td>
<td>-5.171e+01</td>
</tr>
<tr>
<td>Glycinergic amacrine cells</td>
<td>1.614e+04</td>
<td>-1.26e-01</td>
<td>-3.944e+05</td>
<td>-5.923e</td>
<td>-6.498e+02</td>
<td>-5.675e+01</td>
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<tr>
<td>GABAergic amacrine cells</td>
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<td>-1.001e</td>
<td>-1.354e+05</td>
<td>-1.113e</td>
<td>7.605e+03</td>
<td>-6.347e-02</td>
</tr>
<tr>
<td>Müller cells</td>
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<td>-1.845e</td>
<td>-2.369e+05</td>
<td>-2.362e</td>
<td>1.379e+04</td>
<td>-2.815e-02</td>
</tr>
</tbody>
</table>
4.4 Results

The data presented in this study were obtained from one male and five female human donor retinas aged of 30 and 60 years (Table 4.1). Although we did not specifically address the question of age-related differences in the distribution of retinal neurons, we observed that the variation between retinas in our sample range was largely independent of age. All six retinas exhibited morphology similar to that observed macroscopically in Figure 4.1A; i.e. they were free from any visible pathology, with the macula lutea distinguished by the yellow pigment which facilitates the demarcation of the location of the fovea.

Figure 4.1B-E shows confocal images of a vertical section through the fovea. The foveal pit indicated by the arrow in Figure 4.1B has a diameter of approximately 1 mm and is deepest at the foveola where photoreceptor density is highest and inner retinal layers are displaced. The fovea is ~ 1.6 mm in diameter in human retina (Polyak, 1941; Hendrickson, 2005; Strettoi et al., 2018) and contains all retinal layers displaced from the foveola forming the thick walls of the foveal pit. Central retina comprises the foveola, fovea, parafovea and perifovea (Polyak, 1941; Hendrickson, 2005). We use the term central retina to refer to eccentricities (up to 3.0 mm, first 10° of visual angle) where the ganglion cell layer is more than one cell thick, roughly equivalent to the diameter of the macula lutea (Boycott et al., 1987; Hendrickson, 2005; Bringmann et al., 2018). We refer to eccentricities between 3.0 mm and 6 mm as mid-peripheral retina and eccentricities beyond 6 mm as far peripheral retina (Martin and Grünert, 1992; Goodchild et al., 1996b). Vertical sections through the retina were obtained from retinal pieces (3-4 mm wide) at eccentricities between 1 mm nasal to 15 mm temporal.

Multiple immunohistochemical markers were used to compare different populations of neurons across eccentricities in the same preparation. The section shown in Figure 4.1B was incubated with DAPI nuclear stain (Fig 4.1C) to label the nuclei of all cells, antibodies against S-opsin to label S-cones (Fig 4.1D), and antibodies against RBPMS to label ganglion cell somas (Fig 4.1E). The section was imaged across its entire length and depth at high resolution in order to distinguish retinal layers and to quantify individual cells as shown in Figure 4.1F-I. Sections used in this study were generally of excellent quality, however in every preparation there
were small areas where accurate quantification of retinal cells was not feasible due to distortion of retinal layers or poor immunohistochemical staining.

Figure 4.1. Processing of post-mortem human donor retina. A: Photomontage of a human retina dissected out of the eye cup. Relieving cuts are made to flatten the retina and to preserve the nasal-temporal axis of the fovea. The yellow pigment of the macula is visible. B-E: Confocal images of a vertical section cut through the fovea. B: Nomarski optics are used to reveal the retina layers, the arrow points to the foveal pit. The section was incubated in DAPI nuclear stain (C), and stained with antibodies against S-Opsin (D) and RBPMS (E) to label S-Cones and ganglion cells respectively. F: A region of interest from the retina in B shown at higher magnification to reveal the vertical layers at roughly 1.5 mm eccentricity. The same region is shown in panels G-I, DAPI stain is shown in G, S-Opsin in H, and RBPMS in I. ONL: outer nuclear layer, HFL: Henle fibre layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar in A = 5 mm, Notches in B are 500 µm apart, Scale bar in I = 50 µm (applies to F-I).
4.4.1. NEURONS IN VERTICAL PATHWAYS

4.4.1.1 Bipolar cells in the midget pathway

Flat midget bipolar cells in macaque and human retina can be identified with antibodies against recoverin (Milam et al., 1993; Wässle et al., 1994; Haverkamp et al., 2003). Here we applied antibodies against recoverin to vibratome sections in order to quantify flat midget bipolar cells across the human retina.

Figure 4.2A-D shows that recoverin is expressed in the primary dendrite, the soma, and the axon terminal of bipolar cells. The broadly stratifying axonal terminals are located in outer half of the in the inner plexiform layer. This morphology is consistent with descriptions of flat midget bipolar cells (Boycott and Wässle, 1991; Kolb et al., 1992; Milam et al., 1993). Thus we assumed that all bipolar cells counted using antibodies against recoverin were flat midget bipolar cells.

At all eccentricities, recoverin is expressed by rod and cone photoreceptors and a large number of bipolar cells which are located in the middle of the inner nuclear layer (Fig 4.2A-F). The expression of recoverin in the inner nuclear layer varied between preparations. In some preparations, recoverin-labelled somas can be seen near the border with the inner plexiform layer (e.g. Fig 4.2F). Due to the position of these cells, they are unlikely to be bipolar cells and thus were excluded when quantifying flat midget bipolar cells. In the case of preparation #15415, recoverin immunoreactivity was present in photoreceptors but near absent in bipolar cells, except for some labelling near the fovea. This preparation was not included in the density counts for midget bipolar cells. The expression of all other immunohistochemical markers was normal in this preparation (e.g. see Figure 4.7, 4.11, 4.14).

There is no specific marker for invaginating (ON) midget bipolar cells in human, however antibodies against the transcription factor Islet-1 can be used to label the entire population of ON bipolar cells (Haverkamp et al., 2003; de Souza et al., 2016). Consistently, we found expression of Islet-1 in the nuclei of bipolar cells in the inner nuclear layer (Fig 4.2G-H). Antibodies against Islet-1 and recoverin were used in the same preparation as shown in Figure 4.2E-J. As expected, we found no overlap between the two markers. The intensity of expression of Islet-1 differed between cell
somas in the inner nuclear layer, but further experiments are required to discern whether the difference in expression pertains to different ON bipolar cell types.

In order to quantify ON bipolar cells, we counted Islet-1 positive nuclei in the inner nuclear layer, excluding rare nuclei that were found at the border of the outer plexiform layer (arrows in Fig 4.2G-H). These cells presumably are horizontal cells which also expressed calbindin and/or parvalbumin in double labelled preparations (not shown).

Figure 4.3 shows the spatial density of recoverin labelled flat midget bipolar cells (Fig 4.3A,B) and Islet-1 labelled ON bipolar cells (Fig 4.3C,D). The density of flat midget bipolar cells peaks at roughly 1 mm at an average of 22,000 cells/mm² (range 19,700 to 31,800 cells/mm²) and decreases to 6,300 cells/mm² at 9 mm eccentricity.

The average density of ON bipolar neurons peaks at roughly 2 mm eccentricity with 18,000 cells/mm² (range 19,300 to 28,000 cells/mm²) and gradually decreases across the retina to 7,000 cells/mm² by 13 mm eccentricity. The curve of pooled densities of Islet-1 positive neurons has a relatively flat shape at the peak because invaginating midget bipolar cells and rod bipolar cells, which are presumed to make up the majority of the population, peak at separate eccentricities between 1 and 3 mm.

The average density of flat midget bipolar cells is higher than that of all labelled ON bipolar neurons between 0.4 and 1.8 mm temporal to the foveal centre. Data from individual preparations where both recoverin and Islet-1 labelled bipolar cells were quantified (Fig 4.3 A,C) show that the density of flat midget bipolar cells exceeds that of ON bipolar cells in two of these preparations (#13587 and #13699), and in the remaining two (#14064 and #15649) the density of flat midget bipolar cells is equal to or lower than the density of ON bipolar cells. Beyond 2 mm eccentricity, the density of Islet-1 ON bipolar neurons is equivalent to or higher (on average 20-30% higher) than flat midget bipolar neurons across all preparations.
Figure 4.2. Flat midget bipolar cells and ON bipolar cells. A-D: Fluorescent image with Nomarski optics showing recoverin expressed by flat midget bipolar cells in the inner nuclear layer at various eccentricities (indicated in the upper right corners) from preparation #13587. The inner nuclear layer becomes thinner and the flat midget bipolar cells become sparser with increasing eccentricity. E-J: regions of interest from preparation #15649 stained with antibodies against recoverin (green) to label OFF (flat) midget bipolar cells and islet-1 (magenta) to label ON bipolar cell nuclei. Eccentricity is indicated in panel E for E,G,I and in panel F for F,H,J. Merged images I and J show that there is no colocalisation between recoverin and islet-1. OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer. Scale bar in D = 50 µm (applies to A-D), 20 µm in J (applies to E-J).
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Chapter Four

Figure 4.3. Spatial density of flat midget bipolar cells and ON bipolar cells. A: Spatial density of flat midget bipolar cells quantified using antibodies against recoverin, plotted against eccentricity across three preparations. Each preparation is allocated a unique symbol and each point on the graph represents the density (y-axis) of the target cell type within a counting window at a particular eccentricity (x-axis). B: Pooled data from the three preparations in C, fit to a curve to illustrate population density of flat midget bipolar cells across the retina. C: Spatial density of ON bipolar cells quantified across six preparations labelled with antibodies against Islet-1. Density is plotted against eccentricity. D: Pooled data across the six preparations in A, fit to an exponential curve to illustrate the population density of ON bipolar cells across the human retina. Flat midget bipolar cells peak closer to the fovea compared to ON bipolar cells. Fit parameters are given in Table 4.3.
4.4.1.2 Bipolar cells in the parasol pathway

We aimed to identify and quantify diffuse bipolar cell types involved with OFF parasol cells in human retina. Figure 4.4A shows a vertical section labelled with antibodies against calbindin. As shown previously calbindin is expressed by the DB3a cell (Milam et al., 1993; Haverkamp et al., 2003). The axon terminals of DB3a cells form a distinct band in stratum 2 of the inner plexiform layer (Fig 4.4A,B and E). Two other bands can be distinguished in the inner plexiform layer using antibodies against calbindin, one in the centre and one at the border with the ganglion cell layer (stratum 5). These bands are presumably formed by amacrine and ganglion cell processes.

Consistent with previous findings sections labelled with antibodies against CD15 (Fig 4.4C-E) revealed a single bipolar cell type, the DB3b cell which stratifies in stratum 2 of the inner plexiform layer (Haverkamp et al., 2003; Puthussery et al., 2013; Tsukamoto and Omi, 2015). Antibodies against CD15 also revealed weak expression in the ganglion cell layer and diffuse expression across the inner plexiform layer (Figure 4.4E). In preparations double labelled with CD15 and calbindin, we found no colocalization between the two antibodies in the inner nuclear layer, indicating that two distinct bipolar cell types are labelled (Fig 4.4D-E).

We quantified the spatial density of DB3a and DB3b cells in six preparations (Fig 4.5) and found that DB3a and DB3b cells follow a very similar pattern of distribution. The first DB3a cell nuclei were counted as close as 100 µm from the foveal centre. Both cell types have a peak density at around 1 mm eccentricity. The average peak density of DB3a cells is 3,900 cells/mm² (range 4,000 and 6,900 cells/mm²). The average peak density of DB3b cells is close to 3,500 cells/mm² (range of 3,000 to 7,000 cells/mm²). Both DB3a and DB3b cells decrease in density towards peripheral retina to less than 1,500 cells/mm² by 10 mm eccentricity. Our results show that DB3a and DB3b cells exist in equal proportions across the human retina.

Compared to recoverin-labelled flat midget bipolar cells, the density of DB3a and DB3b cells is consistently lower across the retina. Furthermore, when the densities of DB3a and DB3b cells are added, the flat midget bipolar cells still outnumber the diffuse bipolar cells. In fact, the ratio of flat midget bipolar cells compared to the sum of the densities of DB3a and DB3b cells is close to 3:1 across
the human retina. The higher proportion of flat midget bipolar cells supports low convergence between cones and midget bipolar cells which will be quantified in the following sections. In addition, it also reflects the higher proportion of midget ganglion cells compared to parasol ganglion cells which receive input from DB3a and DB3b cells.
Figure 4.4. Diffuse bipolar cells DB3a and DB3b can be distinguished in human retina. (See next page for legend).
Figure 4.4. Diffuse bipolar cells DB3a and DB3b can be distinguished in human retina. 

A: Vibratome section 2 mm temporal from preparation #13587 stained with antibodies against calbindin, imaged with Nomarski optics to reveal retinal layers. Calbindin expression can be observed in cone photoreceptors, horizontal cells, amacrine cells, and a population of bipolar cells known as DB3a cells (arrowheads). 

B-D: Region of interest from a section labelled with antibodies against calbindin (green) antibodies against CD15 (magenta). 

B: Bipolar cells labelled with antibodies against calbindin are a homogenous population of DB3a cells with distinct dendrites meeting cell bodies in the centre of the INL and axon terminals stratifying in S2 of the IPL. 

C-D: The population of bipolar neurons immunoreactive to CD15 has a similar morphology to DB3a cells and terminate in the same stratum in the IPL, but are a distinct population of bipolar cells called DB3b cells. 

E: Overview image of a vibratome section 2 mm temporal to the fovea labelled with antibodies against calbindin (green) and CD15 (magenta). 


Scale bar = 100 µm in A (applies to A,E), 25 µm in D (applies to B-D).
Figure 4.5. Spatial density of diffuse bipolar cells in human retina. **A**: Density of DB3a cells quantified using antibodies against calbindin in six preparations. **B**: Data from **A** fit to an exponential curve to illustrate the spatial density of DB3a cells across temporal retina. **C**: The density of DB3b cells plotted against eccentricity for five preparations. DB3b cells were identified with antibodies against CD15. **D**: Pooled data across these five preparations fit to an exponential curve to show the population density of DB3a cells up to 15 mm temporal to the fovea. DB3a and DB3b cells have comparable densities across the retina.
4.4.1.3 Identification and quantitative analysis of cone photoreceptors

Cone photoreceptors were labelled using monoclonal antibodies (7G6) against cone arrestin (Wikler et al., 1997; Zhang et al., 2003). Cone arrestin is expressed in the entire cone including the outer and inner segments, the axon (Henle fibre) and the pedicles of all cone types. This pattern of expression is consistent across all eccentricities (Fig 4.6A-E). Figure 4.6 also shows that the cone size increases and the density decreases with eccentricity. In two preparations (#13587 and #15415), we used antibodies against calbindin to quantify cones in mid-peripheral and far peripheral retina. It has been shown that the calcium binding protein calbindin is expressed by medium and long wavelength but not short wavelength cone photoreceptors in human retina (Chiquet et al., 2002). Thus these counts will not include S-cones. Since S-cones make up less than 7% of the cone population outside of central retina (Curcio et al., 1991), cone densities measured in this study using antibodies against calbindin are slightly underestimated.

Short-wavelength cones (S-cones) were identified with a commercially available anti-blue opsin protein which was localized most strongly at the outer and inner segments but was also found in the Henle fibres and pedicles. Figure 4.6F shows the distribution of S-cones at the fovea. As expected, S-cones are sparse to absent at the foveola but comparatively dense in the surrounding fovea (Curcio et al., 1991).

The Henle fibres formed by the axons of cone photoreceptors can be observed in Figure 4.6A-D and Figure 4.6F, indicating the lateral displacement of the postsynaptic targets of the cone photoreceptors in the inner nuclear layer. Figure 4.6A-E shows that the Henle fibres gradually decrease in length with eccentricity (measurements of the lateral displacement are provided below).

Previous studies have measured the topography and density of cone photoreceptors in human retina from whole mount preparations (Curcio et al., 1990). Here, we determined the cone density from vertical sections at the level of the cone inner segments (arrow, Figure 4.6E). At the foveola and surrounding fovea, we used a 40 x water immersion objective to obtain stacks of images in order to better distinguish individual cones. The peak cone density for two preparations at the foveola was 150,000 cells/mm² (Fig 4.6G,H). This is lower than the average peak density of
199,000 cells/mm² reported by Curcio et al. (1990), although our recorded value lies within the range of individual variability in their study. Overall, the pattern of cone distribution across the retina was consistent with previous reports (Osterberg, 1935; Polyak, 1941; Curcio et al., 1990); cone density declines steeply after the peak, reaching an average of 15,000 cells/mm² within 1 mm of the foveal centre. The cone density continues to decline gradually in mid-peripheral retina and drops to ~3,500 cells/mm² beyond 12 mm eccentricity (Fig 4.6G,H).

S-cones were counted at the level of the inner segments (#13587 #13699 #14064) and the somas (#15415 and #15649) (Fig 4.6I). Consistent with previous reports (Curcio et al., 1991), average S-cone density peaks at 0.36 mm eccentricity temporal with a density of 2,400 cells/mm² (range 2,100 to 2,880 cells/mm²) (Fig 4.6J). The variability in density between preparations as well as the irregular distribution of the S-cones across the retina is evident in the relatively broad cloud of data points representing S-cone densities in different preparations in Figure 4.6I. The location of the peak also varied slightly between preparations, ranging from 0.23 mm to 0.32 mm. The S-cone free zone was measured across the temporal axis in one preparation (#15415), it had a diameter of 70 µm, with the first S-cones appearing as close as 30 µm temporal (Fig 4.6F, inset). Consistent with previous reports in human (Curcio et al., 1991) and non-human primates (Marc and Sperling, 1977), S-cones made up 6% of cones at their average peak density, and this proportion gradually increased to about 8-10% beyond 1 mm eccentricity.

Taken together, our results show that the density of cone photoreceptors can be quantified with reasonable accuracy using immunolabelled vertical sections through the retina. This validates our method for the quantification of neurons which have not been measured previously in the inner nuclear layer of human retina, and allows the comparison of cone density with postreceptoral neurons within the same preparation.
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Figure 4.6. Vertical vibratome sections were used to quantify the density of cone photoreceptors. (See next page for legend).
**Figure 4.6.** Vertical vibratome sections were used to quantify the density of cone photoreceptors. **A-E:** Regions of interest from vibratome sections stained with antibodies against cone arrestin to reveal all cone photoreceptors in preparation #14064. Eccentricities are indicated in the upper right corners of the images. The diameter of the cone inner segments increases with eccentricity as cones become less tightly packed. The arrow in **E** points to the anatomical level at which cones were counted across the retina. **F:** Vertical section through the fovea showing expression of S-Opsin by S-Cone photoreceptors. S-Cones are near-absent at the fovea. The Henle fibres of the S-Cones can be observed extending across the Henle fibre layer. The inset in **F** is roughly 115 µm wide and reveals the blue-free zone at the foveal centre. Density of cones photoreceptors (**G**) and S-cones (**I**) is plotted against eccentricity from up to six preparations and the pooled data across preparations is shown in **H** for cones and in **J** for S-cones. ONL: outer nuclear layer, HFL: Henle fibre layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 50 µm in **E** (applies to A-E), 100 µm in **F**.
4.4.1.4 Identification and quantitative analysis of ganglion cells

Ganglion cells were labelled with antibodies against RBPMS, a specific ganglion cell marker (Rodriguez et al., 2014). RBPMS was localized to the somas of all ganglion cells (Fig 4.7A-E) as well as some primary dendrites. Displaced amacrine cells do not express RBPMS (Rodriguez et al., 2014). Consistent with previous reports, (Polyak, 1941; Curcio and Allen, 1990; Grünert et al., 1993) sparse ganglion cell nuclei were observed on the foveal floor (Fig 4.1E). DAPI labelled nuclei which did not colocalize with RBPMS were also seen at the centre of the fovea (Fig 4.1C), indicating that in addition to ganglion cells other cell types may also be localized to the foveal pit. The ganglion cell layer is deepest at 1 mm eccentricity (Fig 4.7B, 6 cells deep in the preparation shown) and is ~1 cell deep by 5 mm eccentricity.

The ganglion cell density was determined for five preparations in central retina and two preparations in mid-peripheral and far peripheral retina. The values are comparable with previous reports (Curcio and Allen, 1990). The peak ganglion cell density is located at around 1 mm eccentricity at an average of 34,000 cells/mm² (Fig 4.7K), and varied between individuals (range 28,000 – 50,000 cells/mm², Fig 4.7J). Ganglion cell density decreases at a steep rate within the central 3 mm temporal to the fovea, and then declines more gradually until it reaches 300 cells/mm² at 12 mm eccentricity.

As shown in Figure 4.7H the calcium binding protein parvalbumin is expressed in cells in the ganglion cell layer (Wässle et al., 2000; de Souza et al., 2016; Kántor et al., 2016). Thus in some preparations antibodies against parvalbumin were used to determine the ganglion cell density.

In order to determine whether parvalbumin positive cells include all ganglion cells, vertical sections were double labelled with antibodies against parvalbumin (Fig 4.7G) and antibodies against RBPMS (Fig 4.7H). The results showed that most but not all retinal ganglion cells express parvalbumin (Fig 4.7G-I). This finding was quantified in two preparations (#13587 and #15649) where on average, 92% of ganglion cells were labelled for parvalbumin. The proportion varied between 80-100% for individual regions and was not dependent on eccentricity. Conversely, some cells in the ganglion cell layer were parvalbumin positive but RBPMS negative (not shown), indicating that parvalbumin expressing cells include displaced amacrine cells.
Parvalbumin positive displaced amacrine cells had small somas, were usually located close to the border with the inner nuclear layer and were omitted from our counts of parvalbumin positive ganglion cells. Therefore, although samples where ganglion cell density was determined using antibodies against parvalbumin (#13587) are slight underestimates, the results are comparable with preparations where the ganglion cell density was determined using antibodies against RBPMS (Figure 4.7J). Ganglion cells were always counted in conjunction with the DAPI nuclear stain in order to avoid counting the same cell twice.

In summary, the ganglion cell density across the human retina determined here from vertical sections is in good agreement with previous studies (Curcio and Allen, 1990) using whole mount preparations. Measurements of cone photoreceptor and ganglion cell density are useful in estimations of convergence of input within vertical pathways in the retina.
Figure 4.7. Identification of retinal ganglion cells and quantification of their spatial density. (See next page for legend).
Figure 4.7. Identification of retinal ganglion cells and quantification of their spatial density. **A-E:** Regions of interest from vertical sections of the preparation #15415 at different eccentricities stained with antibodies against RBPMS to reveal ganglion cell somas. The ganglion cell layer is thickest at 1 mm eccentricity. The ganglion cell layer is reduced to a single row of ganglion cells by 5 mm eccentricity. Displaced ganglion cells can be observed in the inner nuclear layer. **F-I:** Parvalbumin is expressed by most (but not all) ganglion cell somas. Confocal images of a region of interest from a vertical section at 1.2 mm eccentricity labelled with antibodies against parvalbumin (magenta) and antibodies against RBPMS (green). **F:** Nomarski optics reveal the vertical layers of the retina. **G:** RBPMS labelled ganglion cell somas. **H:** Parvalbumin-labelled cells in the ganglion cell layer: **I:** The merged image shows that most ganglion cells are double labelled for RBPMS and parvalbumin but there is a small population of ganglion cells that do not express parvalbumin (arrows). **J, K:** Ganglion cell density is shown relative to eccentricity. Data from five preparations is shown in **J** and average density across all preparations is shown in **K.** INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 50 µm in A (applies to A-E), 20 µm in F (applies to F-I).
4.4.1.5 Receptoral and postreceptoral displacement

Cone photoreceptors are densely packed at the centre of the fovea and the remaining layers of the retina are shifted to allow a direct path of light to the photoreceptors. This results in lateral displacement of downstream neurons at two main stages; firstly due to the Henle fibres radiating from cones at the foveal centre to contact bipolar cells (receptoral), and secondly due to the oblique path of the bipolar cells through the inner nuclear layer which further offsets contacts with retinal ganglion cells (postreceptoral) (Schein, 1988). Knowledge of the total lateral displacement allows accurate estimations of the number of cones to bipolar cells to ganglion cells in the foveal region. We measured the displacement of the inner retinal neurons at both stages in vertical visual pathways.

Receptoral displacement (Henle fibre length) was determined as the distance between the centre of the cone soma and the centre of the cone pedicle (Boycott et al., 1987; Sjöstrand et al., 1999). The procurement of confocal image stacks through the length and depth of foveal sections allowed us to follow the Henle fibres of individual cone photoreceptors to the pedicle (Fig 4.8A-B). In Figure 4.8C, the eccentricity of the cone photoreceptor soma is plotted against the horizontal distance to the cone pedicle. We measured the receptoral displacement from cone somas as close as 125 µm from the foveal centre. Consistent with previous reports in monkey (Perry and Cowey, 1988; Schein, 1988; Bringmann et al., 2018) and human (Polyak, 1941; Sjöstrand et al., 1999) retina, we found short Henle fibres (230 µm) closer to the foveal centre, and long fibres (300 – 400 µm) at cone soma eccentricities between 0.5 – 1 mm temporal. The Henle fibre length systematically decreased with eccentricity (Fig 4.8A-C). By about 6 mm eccentricity, cone photoreceptor somas are vertically aligned with their pedicles (Fig 4.6E).

Postreceptoral displacement was measured as the distance from the midline of the dendritic tree (presumed location of the cone pedicle) of a bipolar cell to the midline of the bipolar axon terminal. Measurements were obtained from recoverin labelled flat midget bipolar cells, calbindin labelled DB3a cells and CD15 labelled DB3b cells all of which showed comparable displacement. Measurements were obtained from bipolar cell dendritic trees located at eccentricities ranging from 0.24 – 8.6 mm temporal. The largest postreceptoral displacement occurs between 0.3 and 0.8 mm temporal to the
foveal centre with an average length of 55 µm (Fig 4.8D). Individual bipolar axon terminals were displaced by a maximum of 70 µm. Similar measurements of postreceptoral displacement were reported by Sjöstrand et al. (1999) in human retina and by Perry and Cowey (1968) in macaque retina. From about 2.5 mm eccentricity there is little to no appreciable displacement of bipolar axon terminals.

The average receptoral and postreceptoral displacements were added in Figure 4.8E in order to demonstrate the total lateral displacement between cones and bipolar axon terminals. The peak total displacement was found 0.5 mm from the foveal centre at 389 µm. Most of the total displacement is accounted for by the length of the Henle fibres (receptoral displacement). At the peak, postreceptoral displacement contributes a maximum of 15% to the total displacement.

Existing data places the total displacement in human retina in the range of 370 µm (Sjöstrand et al., 1999) to 526 µm (Drasdo et al., 2007). In keeping with previous studies, peak total lateral displacement of 389 µm is a reasonable estimation of the displacement in the human fovea. This data enables quantification of numerical convergence in the fovea where cones are most significantly displaced from their postreceptoral targets.
Figure 4.8. Total lateral displacement in human retina. (See next page for legend).
Figure 4.8. Total lateral displacement in human retina. Images in A and B are taken at 1 mm and 3 mm eccentricity respectively, from a vertical section through the fovea labelled with antibodies against cone arrestin. The lateral distance of the cone pedicle from the soma is plotted as receptoral displacement on the y-axis in C. The eccentricity of the soma is plotted on the x-axis. D: Post-receptoral displacement is plotted against eccentricity for 3 preparations. The displacement was measured from the centre of the dendritic bouquet to the centre of the axon terminal of calbindin, CD15, and recoverin labelled bipolar cells. On the x-axis, the distance of the centre of the dendritic bouquet from the fovea is plotted as the assumed eccentricity of the cone pedicle. E: Total lateral displacement calculated from average receptoral and postreceptoral displacement across preparations which are also shown. The data was fit with a three-stage difference-of-exponentials function. ONL: outer nuclear layer, HFL: Henle fibre layer, OPL: outer plexiform layer. Scale bar = 50 µm in B (applies to A-B).
4.4.1.6 Convergence and divergence within vertical pathways

There is converging evidence from primate retina that one-to-one connectivity between cones and midget bipolar cells extends beyond the fovea (Milam et al., 1993; Wässle et al., 1994; Kolb and Marshak, 2003). We directly compared the spatial density of cone photoreceptors and flat midget bipolar cells within two preparations (#13587 and #14064). Figure 4.9 shows a micrograph of a vibratome section processed with antibodies against cone arrestin (Fig 4.9A) and antibodies against recoverin (Fi. 4.9B). In regions of interest from sample points in central (Fig 4.9C), mid-peripheral (Fig 4.9D) and far peripheral (Fig 4.9E) retina, we observed flat midget bipolar cells with a single apical dendrite contacting a single cone. Flat midget bipolar cells contacting multiple cones are observed at higher eccentricities, but this was not quantified systematically. We compared the spatial densities of cones and flat midget bipolar cells from 1 mm to 11 mm temporal for retinas #13587 (Fig 4.9F) and #14064 (Fig 4.9G). The ratio of cones to flat midget bipolar cells is close to 1:1 up to at least 10 mm eccentricity in both preparations.
Figure 4.9. Numerical convergence in the midget pathway. A, B: Vertical vibratome section 300 µm superior to the fovea stained with antibodies against cone arrestin and recoverin. C-E: Single confocal images from sections stained with cone arrestin and recoverin to illustrate connectivity between cone photoreceptors and flat midget bipolar cells at various eccentricities. Insets in each image show that single-headed flat midget bipolar cells can be identified up to at least 10 mm eccentricity. Eccentricity is indicated in the lower left corner. F-G: Cell density is plotted against eccentricity for cone photoreceptors and flat midget bipolar cells in preparation #13587 (F) and #14064 (G). The curves in F and G represent the average density of cone photoreceptors across all preparations. In both preparations, the ratio of cones to flat midget bipolar cells is 1:1 in peripheral retina. Scale bar = 200 µm in B (applies to A,B), 50 µm in E (applies to C-E).
In order to accurately determine numerical convergence within central retina, cumulative numbers of cones, midget bipolar cells, DB3a and ganglion cells were compared while accounting for lateral displacement. Figure 4.10A (left panel) shows the average density for cones up to 2 mm eccentricity. This data was then used to calculate the cumulative number of cones at the same eccentricities (right panel). The same calculations are shown in Figure 4.10B for flat midget bipolar cells. The cumulative numbers of midget bipolar cells are shifted to the left in order to account for receptoral displacement due to Henle fibres. These results are demonstrated by the red dotted line in the right panel in Figure 4.10B. By 2 mm eccentricity, the number of cones is roughly 240,000. The ratio of cones to flat midget bipolar cells is 1:1 by 0.4 mm. At 0.5 mm the ratio increases to 1.2 cones per midget bipolar cell. Between 0.5 mm and 2 mm, the average number of flat midget bipolar cells exceeds that of cones and the ratio of cones to midget bipolar cells drops to 0.8:1. The density of midget bipolar cells also slightly exceeds that of cones when data is compared within individual preparations. We suspect the latter result is likely due to an overestimation of flat midget bipolar cells in central retina (see Section 4.5). Overall, after accounting for receptoral displacement due to Henle fibres, the number of cones to flat midget bipolar cells in central retina lies close to a ratio of a single cone for every flat midget bipolar cell.

For DB3a cells, the total number of cells at 1 mm is ~22,200 (Fig 4.10C), which translates to roughly 4 cones converging onto a single DB3a cell. This is in agreement with the bipolar to cone ratio measured in macaque retina in DB3 cells (Boycott and Wässle, 1991). The ratio of cones to DB3a cells is more than four times that of bipolar cells in the midget pathway, and is fairly consistent across eccentricities. A similar pattern of convergence is to be expected for DB3b cells across the retina, although this was not directly measured in this study.

The majority of ganglion cells in primate fovea are midget ganglion cells (Leventhal et al., 1981; Perry et al., 1984; Rodieck et al., 1985; Dacey, 1993b; Masri et al., 2017) and most publications attest to the prevalence of two midget ganglion cells (one ON and one OFF) for every cone (Schein, 1988; Watanabe and Rodieck, 1989; Curcio and Allen, 1990; Kolb and Marshak, 2003). The graph on the right in
Figure 4.10D shows the total number of ganglion cells at 1 mm eccentricity is roughly 181,500 cells. The number of ganglion cells is almost double the number of cones at 1 mm eccentricity. In this case the numbers have been shifted to account for the total lateral displacement. The ratio of 1 cone for every 2 ganglion cells is consistent up to 2 mm eccentricity. This is in agreement with the one-to-one pathway described for midget bipolar and midget ganglion cells in central retina. In peripheral retina, although flat (OFF) midget bipolar cells appear to maintain a one-to-one ratio with cone photoreceptors, multiple midget bipolar cells then converge onto a single ganglion cell (Dacey and Petersen, 1992; Dacey, 1993b; Kolb and Marshak, 2003).

In summary, the midget pathway maintains one-to-one connectivity with cone photoreceptors up to at least 10 mm eccentricity. In the parasol pathway, multiple cone photoreceptors converge onto a single DB3a cell.
Figure 4.10. Cumulative densities of cells in the midget and parasol pathways of the retina, accounting for displacement in the fovea. The panels on the left represent average densities for cones (A), flat midget bipolar cells (B), diffuse bipolar cells (C), and ganglion cells (D) from 0 to 2 mm eccentricity. The panels on the right show the cumulative counts for each aforementioned cell population across the same eccentricity. The red dotted lines represent the cumulative numbers for each cell type adjusted for receptoral (B and C) and total lateral displacement (D).
4.4.2 COMPOSITION OF THE INNER NUCLEAR LAYER

4.4.2.1 Identification and quantitative analysis of horizontal cells

Horizontal cells in the human retina can be labelled with antibodies against the calcium binding proteins parvalbumin and calbindin (Haverkamp et al., 2003; de Souza et al., 2016). Consistently, in parvalbumin labelled preparations, we observed very dense expression in the horizontal cell layer, while expression in the ganglion cell layer was much weaker (Fig 4.11A-D). Parvalbumin positive horizontal cells were observed as close as 100 µm from the foveal centre (Fig 4.11A). Parvalbumin is expressed in the cell body and dendritic processes of the horizontal cells (Fig 4.11A-E).

In accordance with previous studies in monkey retina, we assumed parvalbumin was expressed by both H1 and H2 cells while calbindin was only expressed by H2 cells (Röhrenbeck et al., 1989; Wässle et al., 2000). Presumed H2 cells had a more rounded, smaller cell body compared to the H1 cells. Expression of parvalbumin was comparable between H1 and H2 cells. Expression of calbindin was localised to the cytoplasm and the fine dendrites of H2 cells (Fig 4.11F).

In parvalbumin-labelled preparations, we also observed displaced horizontal cells which have been described previously in macaque retina (Wässle et al., 2000). These cells had somas in the ganglion cell layer but did not have an axon descending into the nerve fibre layer, and their somas showed noticeably stronger expression of parvalbumin immunoreactivity compared to ganglion cells (Fig 4.11H). Displaced horizontal cells had “interplexiform processes” (Wässle et al., 2000) which branched in the inner plexiform layer and extended through the inner nuclear layer to meet the processes of other horizontal cells (arrow in Fig 4.11H).

We quantified the spatial density of H1 and H2 cells across the retina. Figure 4.12A shows the densities of all parvalbumin immunoreactive H1 and H2 horizontal cells from six preparations. The pooled data is shown in Figure 4.12B. Horizontal cell density peaks at an average of 16,200 cells/mm² at roughly 0.75 mm temporal to the foveal centre. The peak varies between individuals in the narrow range of 17,000 to 20,000 cells/mm². Horizontal cell density declines steeply within central retina and then plateaus but remains in excess of 2,500 cells/mm² at 10 mm eccentricity. The spatial distribution of H1 and H2 cells is compared in Figure 4.12C-D.
Consistent with previous findings in macaque (Wässle et al., 1989; Wässle et al., 2000), the density of H1 cells is higher than that of H2 cells at all eccentricities. The two cell types peak at disparate eccentricities; the H1 cells close to 0.6 mm and the H2 cells at 2.5 mm temporal. The density of H1 cells peaks at an average of 15,000 cells/mm$^2$ and drops to roughly 2,500 cells/mm$^2$ by 12 mm eccentricity. The density of H2 cells declines steadily across the retina from a peak density of 2,000 cells/mm$^2$ to less than 300 cells/mm$^2$ at our most peripheral sample points. The peak density of H2 cells between individuals was in the range of 2,000 – 4,000 cells/mm$^2$ within 1 and 2.5 mm eccentricity. Given the differential distribution of H1 and H2 cells, we expect the ratio of the two cell types to vary across the retina. At the peak density of H1 cells the ratio of H1 to H2 cells is 15:1, and at 2.5 mm where the H2 cell density is at its peak the ratio is 4.4:1. At 4 mm and 7 mm, the ratio is 3.4:1 and 3.3:1 respectively. The distribution of H1 and H2 cells in human retina thus follows a similar trend to that described in macaque (Wässle et al., 1989; Wässle et al., 2000).

In addition, in all preparations double labelled with parvalbumin and calbindin antibodies, we observed a rare presumed horizontal cell that expressed calbindin but not parvalbumin (arrow in Fig 4.11G). The distribution of these cells was irregular across the retina, as they were sometimes absent within counting windows wider than 200 µm. Even at the horizontal cell peak, its spatial density was less than or equal to 1000 cells/mm$^2$ (total count = 35 cells from 6 vertical sections). The irregular distribution of these cells suggests they do not represent a separate population of horizontal cells.
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Figure 4.11. Immunohistochemical labelling of horizontal cells in vertical sections through retina #15415. (See next page for legend).
**Figure 4.11.** Immunohistochemical labelling of horizontal cells in vertical sections through retina #15415. 

A: Vertical section 100 µm superior to the fovea labelled with antibodies against parvalbumin (green) and imaged with Nomarski optics to reveal the retina layers. Horizontal cells labelled in the inner nuclear layer can be observed close to the foveal pit. 

B: The same section as shown in A at an eccentricity of 1 mm where horizontal cells are densely packed and ganglion cells span multiple layers. Panels C and D show regions of interest from vibratome sections stained with antibodies against parvalbumin at 2 mm and 3.5 mm, respectively. 

E-G: Images from a section double labelled with antibodies against parvalbumin (E) and antibodies against calbindin (F). G: Merged image showing horizontal cells expressing parvalbumin alone (H1 cells) and horizontal cells expressing both parvalbumin and calbindin (H2 cells, arrowheads). A horizontal cell expressing calbindin alone can also be observed (arrow). 

H: Collapsed image from a stack of confocal images across 15 z-slices showing the morphology of a displaced horizontal cell immunoreactive to parvalbumin at 8 mm eccentricity. ONL: outer nuclear layer, HFL: Henle fibre layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 50 µm in A (applies to A,B), 20 µm in E (applies to C-G), 20 µm in H.
Figure 4.12. Spatial density of horizontal cell populations in six preparations of human retina. A: Density of horizontal cells labelled using antibodies against parvalbumin (H1 and H2) from six preparations plotted against eccentricity. B: Data from A fit to an exponential curve showing the average distribution of horizontal cells across the retina. C-D: H1 and H2 populations could be quantified in three preparations (#13587, #14064, #15415) which were double labelled for parvalbumin and calbindin. The average density across the retina for H1 cells is shown in C and for H2 cells is shown in D.
4.4.2.2 Identification and quantitative analysis of amacrine cells

Amacrine cells are the most diverse neurones in the primate retina, with an excess of 20 types identified in human (Kolb et al., 1992). Amacrine cells can be classified more broadly into two groups based on their expression of inhibitory neurotransmitters; GABA or glycine (Massey and Redburn, 1987; Wässle and Boycott, 1991). In our preparations, we used antibodies against glycine transporter 1 (GlyT1) to distinguish glycinergic amacrine cells and antibodies against the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) to distinguish GABAergic amacrine cells (Massey and Redburn, 1987; Kalloniatis et al., 1996; de Souza et al., 2016). Amacrine cell somas were located in one or two rows close to the border of the inner nuclear layer with the inner plexiform layer. Using antibodies against GlyT1 we found strong expression in the cell membranes and dendritic processes of amacrine cells (Fig 4.13A). Using antibodies against GAD, we observed GABAergic amacrine cells in the inner nuclear layer (Fig 4.13B) and displaced amacrine cells in the ganglion cell layer (not shown). GAD immunoreactivity is observed in the cell membrane and dendritic processes of amacrine cells. GABAergic amacrine cells tend to have larger dendritic trees, which is likely to contribute to the denser expression of GAD in the inner plexiform layer compared to GlyT1 (Figure 4.13A,B). In contrast to GABAergic amacrine cells, glycinergic amacrine cells were not present in the ganglion cell layer (Figure 4.13A). Displaced amacrine cells were not quantified in this study.

We quantified the spatial density of amacrine cells across two preparations (#13587 and #14064). We found GABAergic and glycinergic amacrine cells as close as 100 µm temporal to the foveal centre. Both glycinergic and GABAergic amacrine cell types followed a similar pattern of distribution; the peak density lies between 1-2 mm (closer to 1 mm for glycinergic and closer to 2 mm for GABAergic amacrine cells) and the density gradually declines within the central 5 mm and steadies thereafter (Fig 4.13C-D). On average, the density of glycinergic amacrine cells is higher than that of GABAergic cells across all eccentricities (Fig 4.13E). The peak density of glycinergic amacrine cells is 13,550 cells/mm² compared to 8,500 cells/mm² for GABAergic amacrine cells. The ratio of glycinergic amacrine cells to GABAergic amacrine cells is 1.6:1 at their respective peak densities and 1:1 outside central retina.
Figure 4.13. Immunohistochemical labeling and spatial density of glycinergic and GABAergic amacrine cells. **A-B:** Fluorescent images from vibratome sections taken at eccentricities between 2 and 3 mm using Nomarski optics to reveal retinal layers. **A:** Glycinergic amacrine cells express glycine transporter 1 (GlyT1). **B:** GABAergic amacrine cells are labelled with antibodies against glutamic acid decarboxylase (GAD-6). The spatial density of glycinergic amacrine cells (**C**) and GABAergic amacrine cells (**D**) was quantified in two preparations. **E:** Exponential fits for GlyT1 positive glycinergic and GAD positive GABAergic amacrine cells up to 15 mm eccentricity. HFL: Henle fibre layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 50 µm in B (applies to A,B).
4.4.2.3 Identification and quantitative analysis of Müller cells

Müller cells make up the large majority of glial cells in the retina (Bringmann et al., 2006). We used antibodies against glutamine synthetase to identify Müller cells (de Souza et al., 2016). Glutamine synthetase is localised to the processes of Müller cells, and is strongly expressed in the fibres and end feet (Fig 4.14A-D). Glutamine synthetase immunoreactivity in the inner nuclear layer appears to vary slightly with eccentricity; it is observed in the cytoplasm in central retina (Fig 4.14A-B) and then more strongly in the cell membrane in midperipheral and peripheral retina (Fig 4.14C-D). The morphology of Müller cells observed in our preparations was consistent with previous descriptions (Bringmann et al., 2006; de Souza et al., 2016). The fibres form honeycomb-like processes at the level of the external limiting membrane where they ensheath photoreceptor cell bodies. The cell bodies are located sclerad to the amacrine cell layer and have a characteristic polygonal shape. The broad cone-shaped end feet of Müller cells form part of the internal limiting membrane.

The spatial density of Müller cells was determined across the temporal retinal axis. We counted Müller cells in combination with DAPI labelling and differential interference contrast to improve accuracy in identifying the polygonal cell bodies. The peak density of Müller cells was 24,000 cells/mm\(^2\) at roughly 0.8 mm from the foveal centre (Fig 4.14E-F). Although our sample of preparations stained with glutamine synthetase that were of acceptable quality for counts was limited, it appears as though there may be considerable variation in peak densities of Müller cells between individuals. In the far periphery, the density declines to ~10,000 cells/mm\(^2\) by 9 mm eccentricity. Comparable densities have been reported in human (Bringmann et al., 2018) and macaque (Martin and Grünert, 1992) retina.
Figure 4.14. Immunohistochemical labeling and spatial density of Müller cells across the retina. **A-D:** Fluorescent images of vertical vibratome sections labelled with antibodies against glutamine synthetase to reveal the morphology of Müller cells at various eccentricities (indicated in the lower left corners). **E:** Spatial density of Müller cells quantified from four preparations. **F:** Population fit for the density of Müller cells in human retina across the four samples shown in E. ONL: outer nuclear layer, HFL: Henle fibre layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar = 20 µm in D (applies to A-D).
4.4.2.4 Cell populations in the inner nuclear layer

We estimated the relative composition of the inner nuclear layer at specific eccentricities across the retina. Flat midget bipolar, ON bipolar, DB3a, horizontal cells, amacrine and Müller cells were counted in two preparations (#13587 and #14064) at 1 mm, 2 mm, 3 mm, 5 mm and 9 mm temporal to the foveal centre in regions 100 µm wide and 20 µm deep. Rod bipolar counts in the same preparations were obtained from Dr. Sammy Lee (Lee et al., 2016) and DAPI nuclear stain was used to estimate the total number of cells in the inner nuclear layer. The proportion of OFF diffuse bipolar cells for which direct counts could not be obtained (DB1, DB2, DB3b) was estimated by subtracting the total number of neurons in a counting window (DAPI counts) from the sum of all other cells at the same eccentricity.

The proportions of bipolar, horizontal, amacrine and Müller cells are shown at different eccentricity points for one preparation in Figure 4.15A. Results were comparable in the other preparation (not shown). The relative proportion of different cell types in the inner retina did not vary considerably across eccentricities. The proportion of bipolar cells ranges from 47-58% of all cells in the inner retina from 1 mm to 9 mm temporal. Amacrine cells make up 13-23% of the total cell count. We noticed a slight trend in the proportion of horizontal cells which decreased from 13% near central retina to 9% in the periphery. The opposite was true for Müller cells, which gradually increased in proportion with eccentricity from 16% in central retina to 25% in the periphery. No such trends were observed for bipolar cells and amacrine cells across the retina. Thus bipolar cells make up most of the cells in the inner nuclear layer (~50%), followed by amacrine, Müller, and horizontal cells.

The relative proportions of bipolar cell populations are broken down for two preparations at 1 mm, 2 mm and 3 mm in Figure 4.15B. On average, OFF bipolar cells exist in higher proportions compared to ON bipolar cells. The proportion of rod bipolar cells is lowest at 1 mm eccentricity and increases by more than twofold at 2 mm eccentricity. The proportion of ON cone bipolar neurons was obtained by subtracting rod bipolar counts from counts of Islet-1 cells and ranges from 20-30% of all bipolar neurons. Flat midget bipolar cells make up roughly a quarter of bipolar neurons while DB3a cells make up less than 10% of all bipolar cells up to 3 mm eccentricity. In far peripheral retina (9 mm eccentricity, not shown), we found the proportions of bipolar
cells to be largely consistent with the proportions at 3 mm eccentricity: rod bipolar cells made up 24%, ON cone bipolar cells made up 15%, flat midget bipolar cells made up 28%, DB3a cells 4% and the remaining OFF diffuse cone bipolar cells made up 29% of all bipolar cells in the inner nuclear layer.

Thus, the relative fractions of neuronal cell types and Müller cells in the inner nuclear layer are consistent between preparations and between primate species, despite absolute variation in density. Between bipolar subtypes, there are notable eccentricity related differences in proportional distribution across the inner nuclear layer.
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Chapter Four

Quantitative analysis of the major cell populations in human retina

Figure 4.15. Proportion of neurons in the inner nuclear layer. (See next page for legend).
Figure 4.15. Proportion of neurons in the inner nuclear layer. The top panel shows the proportions of different cell populations in the inner nuclear layer across five eccentricities for one preparation (#13587). The total number under each graph represents the total density of cells (in cells/mm²) sampled within each eccentricity point, and was obtained by counting DAPI-labelled cell nuclei in the inner nuclear layer. The bottom two panels show the proportions of bipolar cell populations in the inner nuclear layer for two preparations (Top row #13587, Bottom row #14064) at three eccentricities.
4.5 Discussion

This study has compiled the first comprehensive view of cell populations in the inner nuclear layer of human retina. The main findings of this study include 1) measurements of numerical convergence in the midget and parasol pathways showing one-to-one connectivity between cones and midget bipolar cells and divergent input between cones and diffuse bipolar cells, and 2) a comparison of the distribution of neurons and glia in the inner nuclear layer of the human retina.

4.5.1 Strengths and limitations of the quantification of cell populations using immunohistochemically labelled vertical sections.

In this study multiple neuronal populations have been successfully quantified within the same preparation. Immunohistochemical markers were used in order to target and directly quantify retinal cells. This reduces error and ambiguity in cell counts, as bipolar subtypes can be reliably distinguished from one another based on their molecular signatures. Another example that has been a source of error in previous quantitative studies is the inclusion of displaced amacrine cells in quantification of retinal ganglion cells, which can be overcome with the use of cell type specific antibodies. Vertical sections were imaged across 3-5 mm eccentricity in one continuous stack, which allowed a reliable view of the qualitative and quantitative changes across temporal retina. Counts of retinal cells were obtained from up to six preparations. Not all cell types were counted in all six preparations and at all eccentricities due to a number of limitations including areas of tissue damage from processing or areas of poor staining quality, as well as limits to the number of antibodies that can be applied to each section due to the fluorescent tags available and cross reactivity between antiseras. One of the main sources of error in the quantification of spatial density across the retina is the misidentification of the foveal centre (Packer et al., 1989; Curcio et al., 1990). Vertical sections in this study could be imaged at high resolution across 0.1 mm depth and 3 mm in length, which ensured that the entire foveal region could be visualised in one confocal stack of images. In this way, the area where the foveal pit was deepest could be located, and morphological clues were subsequently used to identify the position of the foveal centre. This process minimises error in the identification of the foveal centre in vertical sections.
4.5.2 Comparison of neurons in vertical pathways in the retinas of human and non-human primates

**Cone photoreceptors and retinal ganglion cells**

Previous studies by Curcio and coworkers (Curcio and Allen, 1990; Curcio et al., 1990; Curcio et al., 1991) have used Nomarski optics and a 100 × objective in flat-mounted preparations to count cone photoreceptor and ganglion cell density across human retina. We distinguished cone photoreceptors and ganglion cells by immunohistochemical markers in vertical sections imaged using a confocal microscope under a 20 x or 40 x (cone counts at the foveal centre) objective. Despite the methodological differences, our results are largely in agreement with Curcio’s data. The cone density peaked at an average of 150,000 cells/mm², S-cones at 2,400 cells/mm², and ganglion cells at 34,000 cells/mm², compared to a range of 190,000 cells/mm², ~1,900 cells/mm², and 35,000 cells/mm² reported by Curcio for cones, S-cones and ganglion cells respectively. At the foveal centre, an error of 100 µm could reduce the peak cone density by half (Packer et al., 1989), and so it is possible that a narrower counting window at higher magnification would result in higher peak density counts. Variation between preparations was evident most strongly amongst S-cone counts (the low density of S-cones makes for noisier data within a narrow counting window), and in ganglion cell density. Similar to this study, Curcio and Allen (1990) reported a two-fold variation in ganglion cell density and added that individual differences reflect a large variation in the total number of ganglion cells serving central vision. Yoshioka et al. (2017) reported age related thinning of the ganglion cell layer which was most pronounced past 60 years of age, however Tong et al. (2019) suggested these changes occur gradually from late 30s onwards. As stated above we did not observe any age-related changes in cell density. For example, preparations from older donors (#15415 and #13699) showed comparable ganglion cell densities to the retinas from younger donors (Fig 4.7J). A much larger sample controlled for age-related differences would better indicate potential variation in densities with age. Peak cone density at the foveal centre was derived from only two preparations which demonstrated comparable density (150,667 cells/mm² and 141,935 cells/mm²). Outside of the foveal centre, the variation between different preparations was greatest for cones between 0.4 and 4 mm eccentricity, and fairly stable thereafter (Curcio et
al., 1990). A similar degree of variation (~twofold) was also reported for macaque retinas (Wikler et al., 1990), signifying that it is more likely a feature of primate retina rather than an artifact of differences in post mortem treatment or differences in age between preparations. Cone density in macaque is similar to human across the retina (Packer et al., 1989; Wässle et al., 1990; Wikler et al., 1990; Martin and Grünert, 1992; Wässle et al., 1994). The New World monkey, the marmoset Callithrix jacchus, demonstrates comparable cone density with human at the peak but higher density outside the fovea, where the marmoset retina is cone-dominated (Wilder et al., 1996). Psychophysical studies combined with anatomical investigations in the same individuals are required to ascertain whether variation in density of cone photoreceptors and/or retinal ganglion cells reflects variability in visual performance between individuals. Recent studies have used optical coherence tomography to correlate changes to visual field sensitivity with age-related reduction in ganglion cell layer thickness (Tong et al., 2019).

**Midget bipolar cells**

In keeping with previous studies (Milam et al., 1993; Wässle et al., 1994; Haverkamp et al., 2003) we found that recoverin is localized to flat midget bipolar cells. In our study, expression of recoverin appeared to be restricted to one morphological type, (Fig 4.2A-F). However, we cannot dismiss the possibility that recoverin is expressed in another bipolar cell type as has been suggest by Milam et al. (1993). For example, recoverin immunoreactivity was less distinctly uniform in central retina (Fig 4.2A compared to D), and this could be a reflection of the expression of recoverin in more than one bipolar cell type which would lead to the overestimation of flat midget bipolar cell density near the fovea.

In one preparation from the current study (#15415), recoverin expression in bipolar cells was near absent. There is no other evidence of clinical pathology in retina #15415, and the retina was fixed within five hours after death (see Table 4.1) which is comparable to the other retinas used here. The cause of death was cardiac arrest, and the associated changes in calcium levels could have contributed to the variability in bipolar cell immunoreactivity to recoverin, which is involved in the regulation of calcium dependent processes (Dizhoor et al., 1991). As indicated previously, this retina was not included in the quantification of midget bipolar cells.
The average peak density of flat midget bipolar cells was 22,000 cells/mm² in 4 preparations, which coincides with the peak density estimated in macaque retina (Wässle et al., 1994). Outside of the fovea, the density of midget bipolar cells dropped to roughly 5,000 cells/mm² which is comparable to values reported in macaque, but higher than that of marmoset retinas (Weltzien et al., 2015) because one-to-one connectivity in the midget pathway of marmosets is restricted to the fovea (Chan et al., 2001b; Telkes et al., 2008).

ON bipolar cells were quantified using antibodies against Islet-1. Previous studies in primates including human have shown that Islet-1 is a marker for ON bipolar cells including rod bipolar cells and DB4 cells (Haverkamp et al., 2003; Puthussery et al., 2014; de Souza et al., 2016). Our pooled data shows that on average flat (OFF) midget bipolar cells outnumber Islet-1 ON bipolar cells between 0.4 and 1.8 mm eccentricity. This presentation is not consistent when individual preparations are compared i.e. two preparations show higher densities for Islet-1 ON bipolar cells, one shows equivalent densities, and one shows lower densities when compared to flat midget bipolar cells (Fig 4.3A,C).

It is generally accepted that in the fovea ON and OFF midget bipolar cells exist in roughly equal proportions (Kolb and Dekorver, 1991; Dacey, 1993b; Ahmad et al., 2003). Thus, it is surprising to find that in some preparations, a single bipolar cell type, the flat midget bipolar cell, outnumbers all ON bipolar cells at the reported eccentricities. This implies that OFF midget bipolar cells exist in higher density compared to ON midget bipolar cells at the fovea. In macaque fovea, Ahmad and colleagues reported a slight (5%) difference between ON and OFF midget bipolar cells, reasoned due to OFF midget bipolar cell contacts with S-cones (Herr et al., 2003; Klug et al., 2003; Field et al., 2010; Dacey et al., 2017) while ON midget bipolar cells contacted only L and M cones. Interestingly, in our study, OFF midget bipolar cells outnumber ON bipolar cells in the eccentricity range where S-cones are at highest density. ON/OFF asymmetry has been reported in the midget and parasol system outside of central retina, where ON-centre midget and parasol ganglion cell dendritic fields were 30-50% larger than their OFF-centre counterparts (Dacey and Petersen, 1992; Dacey, 1993b). A consequence of this is that ON-centre cells would exhibit lower spatial resolution. Evidence from psychophysics and electrophysiological studies supports variation in the receptive fields of ON and OFF centre cells (Zemon
et al., 1988; Tyler et al., 1992; Chichilnisky and Kalmar, 2002). Further evidence and more detailed analysis of the foveal region is required to ascertain whether the relatively high density of OFF midget bipolar cells compared to ON cone bipolar cells in the fovea can be justified by ON/OFF asymmetry.

Another explanation for the relatively high density of flat midget bipolar cells in the fovea compared to ON cone bipolar cells is the combined effect of underestimated ON bipolar numbers and overestimated flat midget bipolar numbers near the foveal centre (see above). In some preparations, ON bipolar numbers are likely underestimated due to variation in the intensity of the expression of Islet-1 in bipolar cell nuclei. This is supported by the data which shows a greater difference between flat midget bipolar cells and ON cone bipolar cells in preparation #13587 (Fig 4.3A,C) where expression of Islet-1 was comparatively weak (not shown). Furthermore, Haverkamp and colleagues (2003) indicated that at least one ON bipolar type may not express Islet-1. This could also contribute to the underestimation of ON cone bipolar cells in the present study. The peak density (18,000 cells/mm²) of Islet-1 positive bipolar cells near the fovea (Fig 4.6A) is comparable to the peak of recoverin-labelled flat midget bipolar cells suggesting that Islet-1 labeling includes ON midget bipolar cells, but further studies are required to determine whether other ON cone bipolar cells express this transcription factor.

Taken together, it is likely that the high proportion of flat midget bipolar cells compared to ON cone bipolar cells in some preparations is shaped predominantly by a number of methodological factors, but could also carry the prospect of functional asymmetry in the ON/OFF pathways.

**Diffuse Bipolar Cells**

Electrophysiological studies have suggested that DB3a and DB3b bipolar cells provide input to OFF parasol ganglion cells in macaque retina (Puthussery et al., 2013; Puthussery et al., 2014). This is supported by anatomical evidence in macaque and marmoset retina, which also demonstrates that the majority of the input is derived from DB3a cells (Jacoby and Marshak, 2000; Jacoby et al., 2000; Tsukamoto and Omi, 2015; Masri et al., 2016). The specific role of DB3b cell input to parasol ganglion cells remains unclear, but it has been suggested that DB3b cells make some direct contacts
with and thus may play a role in stimulating parasol ganglion cells (Tsukamoto and Omi, 2014, 2015).

The spatial density of calbindin labelled DB3a cells has been studied across the retinas of marmoset (Weltzien et al., 2015), macaque (Martin and Grünert, 1992) and human retinas (this study). In all species the density of calbindin labelled DB3a cells ranges between 4,400 and 6,000 cells/mm$^2$ close to 1 mm eccentricity. It should be noted that in macaque but not in human retina, calbindin is also weakly expressed by DB6 cells (Haverkamp et al., 2003; Peng et al., 2019). However, the quantitative studies in macaque (Martin and Grünert, 1992; Grünert et al., 1994) and marmoset (Weltzien et al., 2015) like the present study only counted DB3a cells. The consistent density between species is likely a reflection of consistencies in the parasol pathway between species (Rodieck et al., 1985; Lee et al., 1990; Rodieck and Watanabe, 1993; Croner and Kaplan, 1995; Yeh et al., 1995; Ghosh et al., 1996; Kremers et al., 1997; Jacoby and Marshak, 2000; Masri et al., 2016).

DB4 cells provide major inputs to ON parasol ganglion cells (Jacoby et al., 1996; Puthussery et al., 2013; Puthussery et al., 2014; Tsukamoto and Omi, 2016). In this study the distribution of DB4 cells in human retina was not quantified but given the similarities in the parasol pathway between human, macaque and marmoset retina, the density of DB4 cells in non-human primate species can be compared with human data. Similar to DB3a cells, the density of DB4 cells peaks at 1 mm at 6,600 cells/mm$^2$ and drops to 1,000 cells/mm$^2$ in far peripheral retina (Grünert et al., 1994). Ahmad et al. (2003) reported that OFF diffuse bipolar cells make up more than twice the density of ON diffuse bipolar cells in a patch of macaque retina close to the foveal centre. In part, this could be explained by differences in distribution with eccentricity, and/or because there are more OFF diffuse (DB1, DB2, DB3a, DB3b) than ON diffuse (DB4, DB5, DB6) bipolar cell types. Previous studies in human retina have also reported ON/OFF asymmetry in the parasol pathway (Dacey and Petersen, 1992) however we were unable to observe this phenomenon in the absence of cell-type specific molecular markers for ON diffuse bipolar cells.

Consistent with previous studies this study shows that DB3b cells in human retina express CD15 (Haverkamp et al., 2003; de Souza et al., 2016). In contrast in marmoset retina CD15 is expressed by flat midget and DB6 cells (Chan et al., 2001b) and in macaque retina CD15 is expressed only by DB6 cells (Chan et al., 2001a).
According to Tsukamoto et al. (2014), DB3a and DB3b cell bodies are located at different levels in the inner nuclear layer however this was not discernable in our preparations (see Figure 4.4). There is no pre-existing data on the density of DB3b cells. Our results show that they exist in equal proportions to DB3a cells across the human retina. Given that Tsukamoto et al. (2014) reported that in macaque retina DB3a cells have larger axonal arbors compared to DB3b cells, it is possible that the two cell types have different coverage factors, but the axonal arbors of the two cell types in human was not determined here. DB3b cells are the only bipolar cell type in primate retina to make some direct contacts with rod photoreceptors (Tsukamoto and Omi, 2014), they are likely part of an alternate input to parasol ganglion cells that is active under mesopic and scotopic light conditions.

**Convergence and divergence within vertical pathways**

The total lateral displacement was measured in order to accurately calculate convergence within vertical pathways in the human fovea. Total displacement was calculated from measurements of receptoral and postreceptoral displacement. In addition, there is minor displacement in the ganglion cell layer due to the offset of the dendritic tree of ganglion cells from their somas (Rowe and Dreher, 1982; Perry and Cowey, 1988) however we did not measure this in the current study. Sjöstrand et al. (1999) extrapolated that displacement in the ganglion cell layer accounts for less than 1% of total lateral displacement. Few studies have directly measured displacement in human retina (Sjöstrand et al., 1999; Drasdo et al., 2007; Bringmann et al., 2018). In accordance with these studies, peak total lateral displacement in human retina measured here (389 µm) falls within the range of 370 µm (Sjöstrand et al., 1999) to 526 µm (Drasdo et al., 2007) reported previously. Drasdo et al. (2007) largely attribute this range to meridional differences, that is Sjöstrand et al. (1999) measured Henle fibres in the vertical meridian whereas Drasdo et al. (2007) measured Henle fibres in the horizontal meridian. No meridional differences, however, were found in macaque retinas (Perry and Cowey, 1988; Schein, 1988). Measurements in the present study were obtained from the horizontal meridian of four retinas and the measurements reported by Drasdo et al. (2007) are from six retinas but the authors excluded one retina with receptoral displacement lower than 400 µm because it was anomalous to their sample. It is possible that Drasdo et al. overestimated their correction for
shrinkage resulting in higher estimates of lateral displacement than previously reported in human retina. Bringmann et al. (2018) reported a total displacement of 400 µm in the horizontal meridian, which is consistent with results reported in this study (389 µm). Drasdo et al. (2007) also reported measurements of maximum postreceptoral displacement which were 50% higher than reported here. The differences in the total lateral displacement measured by Drasdo and colleagues and the present study might be due to the methodological differences outlined above.

In this study, measurements of postreceptoral displacement were obtained from immunohistochemically labelled bipolar cells in vertical sections, which offers little room for methodological error. In the same way, confocal images of large retinal sections (> 2 mm length) immunohistochemically labelled to reveal photoreceptor morphology significantly reduced ambiguity in following individual Henle fibres. Despite this, there are various challenges and limitations to achieving accurate measurements of total displacement, including the need for perfectly vertical vibratome sections, the introduction of distortions to the tissue (by flattening the naturally curved retina), and potentially misidentifying the foveal centre. Given consistency with recent reports in the human fovea (Bringmann et al., 2018), we are satisfied that our procedures were optimized for measurements of lateral displacement along the horizontal meridian.

Measurements of total lateral displacement were used to calculate numerical convergence in the fovea, where the midget system predominates. Cone photoreceptors and flat midget bipolar cells exist in a one-to-one ratio up to at least 10 mm eccentricity. This implies the preservation of the chromatic acuity of the cone array in the inner retina. Wässle et al. (1994) reported similar results in macaque retina. Electrophysiological recordings from macaque retina (Martin et al., 2001; Solomon et al., 2005) and psychophysical studies in human (Abramov et al., 1991; Mullen and Frederick, 2002) have shown that chromatic selectivity can be detected in the retinal periphery. Color vision is well preserved along the blue/yellow axis however the quality of the red/green opponent signals is reduced in the periphery (Mullen and Frederick, 2002). This is likely explained by the convergence at the level of the ganglion cells in the midget pathway. Ganglion cells outside the fovea are receiving input from multiple flat midget bipolar cells which hinders the distinction between L and M cone signals. Due to the sparse distribution of cone photoreceptors, and the increase in receptive
fields of midget ganglion cells, chromatic signals are best detected in the periphery at low spatial frequencies (in response to large stimuli). The exact mechanism for chromatic selectivity in the retinal periphery is yet unclear. However it is possible that the cone photoreceptors might form clusters which cause the predominance of a spectral signal within the ganglion cell receptive field (Mollon and Bowmaker, 1992; Kolb and Marshak, 2003), or that subtle differences in the synaptic connectivity between midget bipolar cells and midget ganglion cells enhance functional segregation between M and L cone inputs (Jusuf et al., 2006a; Field et al., 2010).

Various estimates of the sampling density of ganglion cells in the fovea have been proposed for human (Curcio and Allen, 1990; Sjöstrand et al., 1999; Drasdo et al., 2007) and non-human primate retinas (Perry and Cowey, 1988; Schein, 1988; Wässle et al., 1990; Goodchild et al., 1996b; Wilder et al., 1996; Ahmad et al., 2003), in the range of 2-4 ganglion cells for every cone. A ratio of 3-4 ganglion cells per cone (Wässle et al., 1990; Sjöstrand et al., 1999) implies that ON and OFF midget ganglion cells make up at best two thirds (i.e. 66%) of the ganglion cell population in the fovea, which is inconsistent with previous reports in primate retina (Leventhal et al., 1981; Perry et al., 1984; Rodieck et al., 1985; Dacey, 1993b; Masri et al., 2017). In this study the ratio of ganglion cells to cones was 2:1 in the fovea. This allows for one-to-one connectivity between all OFF midget bipolar cells and OFF midget ganglion cells in the fovea. Kolb and Marshak (2003) showed in human retina that ON midget bipolar cells in the fovea and parafovea do not all exhibit strict 1:1 connectivity. This is consistent with a ganglion cell to cone ratio close to 2:1 as it allows for one-to-one connectivity in the OFF midget pathway, a slightly lower ganglion cell to bipolar cell ratio in the ON midget pathway, and also accounts for a low density of non-midget ganglion cells close to the fovea.

In the parasol pathway, multiple cones converge onto a single diffuse bipolar cell. Parasol ganglion cells have consistently larger dendritic fields than midget ganglion cells and exist in lower proportions across the retina (Dacey and Petersen, 1992). In this study, we showed that the density of DB3a and DB3b bipolar cells, both signalling to parasol ganglion cells, was three times lower than the density of flat midget bipolar cells. The ratio of cones to diffuse bipolar cells was roughly 4:1 across the retina. In contrast to the midget pathway, the parasol pathway does not require high spatial resolution, but instead relies in part on a large receptive field and
specializations of diffuse bipolar cell ion channels to respond to high temporal frequency stimuli, and to detect motion and flicker in visual space.

4.5.3 Composition of the inner nuclear layer

**Horizontal cells in human and non-human primates**

This study is the first to quantify neurons serving the lateral visual pathway in human retina. We distinguished two horizontal cell types; the H1 cells (parvalbumin) and H2 cells (parvalbumin and calbindin). Horizontal cells provide feedback inhibition onto rod and cone photoreceptors in order to enhance the signal where the photoreceptors are maximally excited by the stimulus. H1 cells make preferential contacts with L and M cones whereas H2 cells preferentially contact S-cones (Ahnelt and Kolb, 1994; Goodchild et al., 1996a; Chan and Grünert, 1998). Consistently, H1 cells made up the majority of the horizontal cells (80-90%) in the human retina whereas H2 cells peaked at an average of 2,000 cells/mm², which coincides with the peak density of S-cones.

In parvalbumin-labelled preparations, we observed horizontal cells in the inner nuclear layer with processes descending into the inner plexiform layer, as well as displaced horizontal cells in the ganglion cell layer with processes ascending into the outer plexiform layer. The interplexiform processes appear to be a general feature of horizontal cell types, however the functional significance of the interplexiform horizontal cells remains unclear.

**Amacrine cells in human and non-human primates**

Amacrine cells are inhibitory interneurons which function to integrate signals in the inner retina in order to shape ganglion cell responses. GABA and glycine containing amacrine cells account for almost all amacrine cells in the inner nuclear layer. Previous studies of human and non-human primates have reported a small proportion (between 5 and 15%) of amacrine cells lacking immunohistochemical expression of both GABA and glycine in mid to far peripheral retina (Davanger et al., 1991; Koontz et al., 1993; Kalloniatis et al., 1996; Weltzien et al., 2015). The present study does not account for these cells, nor does it account for amacrine cells which express both GABA and glycine (3-4%) (Davanger et al., 1991), because amacrine cell populations were quantified separately.
Consistent with previous studies in human and non-human primates, the density of glycinergic amacrine cells was on average higher than that of GABAergic amacrine cells (Davanger et al., 1991; Koontz et al., 1993; Weltzien et al., 2015). The ratio of glycinergic to GABAergic amacrine cells at their respective peak densities (1.6:1 between 1-2 mm eccentricity) is higher than reported previously for central retina (~1.2:1) in marmoset (Weltzien et al., 2015) and macaque (Koontz et al., 1993). Outside of the area of peak density, GABAergic and glycinergic amacrine cells exist in equal proportions in human retina. It is possible that GABAergic amacrine cells were underestimated in our sample due to weaker immunohistochemical staining compared to the glycinergic cells. The effect of this underestimation could be exacerbated in central retina where GABAergic amacrine cells are more densely packed (Davanger et al., 1991; Koontz et al., 1993). Peng et al. (2019), found that GABAergic expression was on average lower in central retina compared to the periphery using molecular sequencing, which could also suggest that several GABAergic amacrine cell types are not found in the fovea.

**Distribution of cells in the inner nuclear layer across the primate retina**

The relative proportion of cells in the inner retina did not vary considerably across eccentricities, and this is in agreement with similar reports in marmoset (Weltzien et al., 2015), macaque (Martin and Grünert, 1992) and other mammalian retina (Strettoi and Masland, 1995; Jeon et al., 1998). The main exception was the proportion of rod bipolar cells, which is lowest at 1 mm eccentricity and increases by more than twofold at 2 mm eccentricity. Martin and Grünert (1992) reported similar changes in rod bipolar density in macaque, which contrasts with the cone dominated marmoset where the proportion of rod bipolar cells is basically unchanging across the retina (Weltzien et al., 2015). We found that on average, OFF bipolar cells exist in higher proportions compared to ON bipolar cells which is consistent with previous findings in foveal macaque retina (Ahmad et al., 2003). Despite the differences in absolute density between species, flat midget bipolar cells make up the largest fraction of all bipolar cell types in human (Fig 4.15) and marmoset (Weltzien et al., 2015) retina. Glycinergic and GABAergic amacrine cells each make up 9% of cells in the inner nuclear layer on average which is in agreement with previous estimations in human retina (Davanger et al., 1991). Müller cells made up an average of 20% of the total cell
population, which is slightly higher than reported in marmoset (Weltzien et al., 2015) and consistent with that reported macaque (Martin and Grünert, 1992), where Müller cell density was shown to closely match the density of amacrine cells across the retina. The proportional distribution of neurons in the inner nuclear layer appears to be relatively consistent between primates.

4.5.4 Relevance to the diagnosis and treatment of retinal diseases

In this study we have mapped neurons in the major visual pathways in the human retina and quantified the distribution of other neurons and Müller cells that make up the inner nuclear layer. These data were previously absent in the literature. Knowledge of the spatial density of neurons across the retina can provide important clues to the way vision is processed (Curcio et al., 1990; Wikler et al., 1990; Martin and Grünert, 1992; Dacey, 1993b; Wässle et al., 1994; Wässle et al., 1995; Wilder et al., 1996; Telkes et al., 2008). Furthermore, this data may serve as a reference for the interpretation of abnormalities in disease, and the informed targeting of treatments. The electroretinogram (ERG) measures the electrical activity of cells in the retina in response to light, and abnormal signals are an early sign of disease (Holder, 2001; Heckenlively et al., 2006). Our quantitative data on the density and proportional distribution of different neurons across the retina will aid in understanding the relative contribution of different retinal cell types to normal ERG signals.

Different retinal diseases attack different cell populations; for example, in age related macular degeneration (AMD) and retinitis pigmentosa, rod and cone photoreceptors degenerate but many downstream neurons survive (Stone et al., 1992; Santos et al., 1997; Medeiros and Curcio, 2001). Our data helps guide the delivery of treatments such as injectable viral vectors (Bainbridge et al., 2008) and micro-stimulation electrodes (Chow et al., 2004; Palanker et al., 2005; Zrenner et al., 2010) with improved precision to take advantage of the surviving neurons at the optimal location. Both the morphology and density of Müller cells is an important indicator of a pathological retina (Bringmann et al., 2006). Knowledge of the distribution of specific cell types such as Müller cells in healthy retinas provides a baseline for measurement of cell apoptosis and proliferation in diseased retinas.
Finally, qualitative descriptions of the expression of cell markers in neurons and glia in normal human retina also contribute to the characterisation of abnormal morphology and protein expression in retinal disease (Strettoi and Pignatelli, 2000; Strettoi et al., 2002; Bringmann et al., 2006; de Souza et al., 2016). In our study, we provide valuable insight into the morphology of neurons and their immunohistochemical expression across the retina, since pathology can be more or less prominent at different eccentricities depending on the disease.
CHAPTER

FIVE
5. CONCLUSION.

The studies described in this thesis sought to elucidate through quantitative and qualitative analyses the different cell types that make up the retina, their morphology and distribution across the retina.

Our survey of retinal ganglion cells in marmoset retina provides a near complete view of the output neurons in primate. The method of particle-mediated gene transfection ensured a nominally random way to obtain a large sample of ganglion cells in the retina regardless of their anatomical projections. The study could be furthered by investigating the connectivity of widefield ganglion cell types using quantitative three-dimensional analysis of the postsynaptic densities of ganglion cells and presynaptic bipolar terminals (Percival et al., 2014; Masri et al., 2016). Future studies could investigate the spatial density and distribution of widefield ganglion cells in primate retina using molecular markers (Chandra et al., 2017) which would enable analysis of entire populations of ganglion cell subtypes. Currently, there are limited molecular markers that target specific ganglion cell types in primate retina (Dacey et al., 2005; Rousso et al., 2016).

The application of particle-mediated gene transfection to human retina was an important technical development that has not been demonstrated previously. We describe various challenges in dealing with post-mortem human retinas, of which lengthy post-mortem delays was most trying. The ability to genetically manipulate human organotypic cultures in vitro opens new doors for testing genetic therapies, and the analysis of changes to the structure and connectivity of retinal cells under disease conditions. Further improvements could be targeted at optimizing the expression of postsynaptic density PSD95 by way of systematically testing the ideal time in culture and the most effective culture media for the preservation of synaptic integrity. Future studies could address the quantification of synaptic connectivity and the comparison of neuronal wiring in the retinas of human and non-human primates. Particle-mediated gene transfection provides a platform through which labelled cells can be targeted for electrophysiological recordings (Koizumi et al., 2007; Jakobs et al., 2008). This bridges a fundamental challenge in the understanding of ganglion cell function, which is the ability to target low density widefield ganglion cell types and measure their response to visual stimuli.
The data provided in Chapter 4 provides a reference for the interpretation of abnormalities in disease, and the informed targeting of treatment in human retinas. The study demonstrated that immunohistochemically labelled vertical vibratome sections across the horizontal meridian can be used to analyze the density of neurons across the retina, the numerical convergence of cells in vertical pathways through the retina, and the proportional distribution of neuronal cell types within retinal layers. Due to the variability between individual preparations, the study could be improved by increasing the number of preparations for statistical comparison, and by the application of more specific antibodies for different bipolar subtypes as they become available. For example, a specific marker for ON midget bipolar cells would aid in addressing questions on ON/OFF asymmetry in the fovea, and the comparative density of OFF and ON midget bipolar cells. Furthermore, it would be clinically useful to apply the same methodology to diseased retinas (i.e. in cases of diabetic retinopathy or age-related macular degeneration) to directly compare the spatial density of neurons and their proportional distribution with results from normal retina. Measurements of retinal layer thickness are often obtained from patients using optical coherence tomography in order to diagnose retinal atrophy and monitor disease progression. Future studies could obtain measurements of retinal layer thickness from histological sections of post-mortem human retinas in order to inform and validate data from optical coherence tomography. Finally, the data provided in Chapter 4 would be enhanced by extending the analysis to the nasal horizontal axis of the same preparations to provide a complete, clinically relevant picture of the human retina with data on naso-temporal asymmetries.
References


REFERENCES


REFERENCES


Tsukamoto Y, Omi N (2014) Some OFF bipolar cell types make contact with both rods and cones in macaque and mouse retinas. Front Neuroanat 8:105.


