

THE UNIVERSITY OF MELBOURNE
Department of Biomedical Engineering

NATIONAL VISION RESEARCH INSTITUTE

Adjusting the Parameters of Electrical Stimulation of Retinal Ganglion Cells to Reduce Neural Adaptation and Improve Efficacy of Retinal Prostheses

Author:

Artemio SOTO-BRECEDA
ORCID: 0000-0002-7162-2590
Student ID: 663743

Supervisors:

Prof. Anthony BURKITT
Dr. Tatiana KAMENEVA
Prof. Michael IBBOTSON
Dr. Hamish MEFFIN

Submitted in total fulfilment of the
requirements of the degree of
DOCTOR OF PHILOSOPHY

August, 2018

Abstract

Retinal prostheses aim to provide visual percepts through electrical stimulation of the retina to blind people affected by diseases caused by photoreceptor degeneration. Two challenges presented by current devices are a lack of selectivity in the activation of retinal ganglion cells (RGCs) and neural adaptation in the retina, which is believed to be the cause of fading—an effect where artificially produced percepts disappear over a short period of time, despite continuous stimulation of the retina. We aim to (1) understand the neural adaptation generated in RGCs during electrical stimulation, (2) obtain the preferred stimulation parameters (waveform) of each morphological class of RGCs and (3) use the preferred waveform of each morphological class to selectively activate different neurons.

RGCs have been classified by morphology into 4 main groups: A, B, C and D. We performed an spike-triggered covariance (STC) analysis on the responses of 44 RGCs to extracellular electrical white noise and 43 RGCs to intracellular white noise. We then recovered their temporal electrical receptive fields (tERF), or waveform. A number of RGCs were stimulated with all the previously recovered waveforms to test the efficacy of each waveform on each other. The waveform recovered from the responses to intracellular stimulation have shown that RGCs can be classified into their respective morphological types by using a K-means clustering algorithm. Extracellular stimulation did not result in waveforms with a clear correlation between clusters and morphological classes. Cells from B and D morphological types had lower thresholds when stimulated with the waveform recovered from cells in the same morphological class. A-RGCs on the contrary, did not seem to share the same temporal features in their waveform with other A-type neurons. Further studies involving a larger data set might determine whether the waveform could preferentially stimulate cells from a specific morphological class.

Current visual prostheses use electrical pulses with fixed frequencies and amplitudes modulated over hundreds of milliseconds to stimulate the retina. However, in nature, neuronal spiking occurs with stochastic timing, hence the information received naturally from other neurons by RGCs is irregularly timed. We used a single epiretinal electrode to stimulate and compare rat RGC responses to stimulus trains of biphasic pulses delivered at regular and random inter-pulse intervals (IPI), the latter taken from an exponential distribution. Our observations suggest that stimulation with random IPIs result in lower adaptation

rates than stimulation with constant IPIs at frequencies of 50 Hz and 200 Hz. We also found a high proportion of lower amplitude action potentials, or spikelets. The spikelets were more prominent at high stimulation frequencies (50 Hz and 200 Hz) and were less susceptible to adaptation, but it was not clear if they propagated along the axon. Using random IPI stimulation in retinal prostheses reduces the decay of RGCs and this could potentially reduce fading of electrically induced visual perception.

Declaration

I, Artemio Soto-Breceda, hereby declare that this Thesis comprises only my original work towards the PhD except where indicated in the Preface. Due acknowledgment has been made in the text to all other material used.

This Thesis is fewer than 100,000 words, exclusive of tables, figures, bibliography and appendices.

Signature:

Preface

Chapter 2 comprises entirely the following paper, published by the Journal of Neural Engineering on August 3, 2018 as a result of part of the work towards the completion of this PhD Thesis:

- Soto-Breceda, A., Kameneva, T., Meffin, H., Maturana, M. I., and Ibbotson, M. R. (2018). Irregularly timed electrical pulses reduce adaptation of retinal ganglion cells. *Journal of Neural Engineering*, 15(056017)

I acknowledge the contributions of Tatiana Kameneva, Hamish Meffin, Matias Maturana and Michael Ibbotson in the completion of the work cited above.

The data used in the intracellular stimulation sections in Chapter 4 and Chapter 5 was collected by Stephanie Kirkby (50 %) and Maximilian Slattery (50 %) within the in vitro lab facilities of the National Vision Research Institute during their Honours degrees.

This degree was funded by:

- CSIRO-Data61, Australia, Scholarship No. 663743.
- Consejo Nacional de Ciencia y Tecnología (CONACYT), México, Scholarship No. 399077.
- Australian Research Council Centre of Excellence for Integrative Brain Function (CE140100007).
- Melbourne International Fee Remission Scholarship (MIFRS), The University of Melbourne, Australia, 663743.

Acknowledgements

To my grandfather Miguel Ángel 'El Abuelo', who sowed the seed of this dream, to someday contributing into restoring vision to people whom, like him, endured retinitis pigmentosa through their life.

Many thanks to my parents Teresita and Roberto, and my sister Paula who have always supported me and encouraged me to work towards bigger goals. To Paula for being always a moral example and a loving friend.

I would also like to acknowledge my beloved aunt Arcelia Breceda, for always believing in me and who would have done anything in her power to help me and support me through my life. It is in great part thanks to her that I have had the opportunity and the desire to study and live in Australia.

To everyone in the NVRI (Ali, Scott, Jason, Wei, Molis and Maryam), thank you for your company during this years and for your invaluable collaborations and suggestions, and most importantly, thank you for the much needed coffee breaks. Special thanks to Matias for being an example of success and hard work, and for teaching me everything I needed to learn and more. To Steph and Max for working hard and keeping me company during hard times.

I am infinitely grateful to Tania, Hamish, Tony and Michael for all their support and for their help in revising my manuscripts as many times as I needed. Thanks for Michael for always being concerned about my success, and to Tania for worrying about my progress and for accepting to be my supervisor in the first place. My supervisors made me feel at home in The University and in the NVRI.

A special mention to Ludovico Einaudi who kept me company and inspired me during the long hours of thesis writing and experimental work since day one of these four years of PhD.

Finally, I would like to thank Marcela, who has never let me down and has been the greatest support during this journey, nothing would have been possible without her. Thank you for teaching me more about life than any University could ever have.

Contents

1	Introduction	1
1.1	The eye	2
1.1.1	How vision works	3
1.2	Retina	6
1.2.1	Retinal ganglion cells	9
1.3	Retinal Degeneration	12
1.4	Visual prostheses	14
1.4.1	Prosthetic retinas	14
1.4.2	Image fading	18
1.5	Thesis Outline	21
2	Irregularly timed electrical pulses reduce adaptation of retinal ganglion cells	24
2.1	Introduction	25
2.2	Methods	27
2.2.1	Animal preparation	27
2.2.2	Whole cell recordings	28

2.2.3	Light response	29
2.2.4	Blocking action potentials	30
2.2.5	Electrical stimulation	30
2.2.6	Data analysis	32
2.3	Results	33
2.4	Discussion and Conclusion	46
3	Morphological analysis	53
3.1	Introduction	53
3.1.1	Neurons in the retina	53
3.1.2	Cell recording	58
3.2	Methods	59
3.2.1	Light response	59
3.2.2	Morphological classification	60
3.3	Results	63
3.3.1	Class A	65
3.3.2	Class B	66
3.3.3	Class C	67
3.3.4	Class D	68
3.3.5	Polemic ON-OFF cells	68
3.4	Conclusions	71
4	Receptive fields	74

4.1	Methods	76
4.1.1	Gaussian white noise stimulation	76
4.1.2	Spike-Triggered Covariance	80
4.1.3	Waveform analysis	80
4.2	Results	84
4.2.1	Intracellular stimulation	84
4.2.2	Extracellular stimulation	90
4.2.3	Influence of latency on the temporal receptive field	93
4.3	Discussion and conclusion	99
5	Selectivity	102
5.1	Methods	103
5.1.1	Data analysis	105
5.2	Results	106
5.2.1	Extracellular stimulation	106
5.2.2	Intracellular stimulation	118
5.3	Discussion and conclusions	120
6	Conclusions	125
	Bibliography	128
	Appendices	142
A	Example stimulus trains from Chapter 2	144

B Efficacy of response per class	146
C Efficacy of response per cluster	152

List of Figures

1.1	Structure of the human eye	2
1.2	Central visual pathways	4
1.3	Centre-surround receptive fields	5
1.4	Modular organization of the visual cortex (hypercolumns)	6
1.5	Main types of retinal neurons	7
1.6	Retinal network pathways	9
1.7	Retinal layers and electrode implantation sites	15
1.8	Retinal prosthesis schematic	17
2.1	Experimental set-up	29
2.2	Membrane potential of example RGCs in response to full-field light stimulation	30
2.3	Amplitude of stimulation and artifact removal	31
2.4	Example trains of constant IPI and random IPI stimulation	34
2.5	Raster plot	35
2.6	Average spike rate of the response to constant IPI and random IPI stimulation	36
2.7	Decay of the spike rate during stimulation	37
2.8	Adaptation	39

2.9 Cathodic first stimulation	40
2.10 Spikelets	42
2.11 Rate of small amplitude action potentials	43
2.12 Results with both spikes and spikelets	44
2.13 Sodium channel blocker	45
3.1 Stratification of RGCs	54
3.2 Activation of RGCs by bipolar cells	55
3.3 Amacrine cells pathways	55
3.4 Morphological types of RGCs	57
3.5 Whole cell recording	59
3.6 Analysis of the morphology of RGCs	61
3.7 Stratification analysis of an example RGC	62
3.8 3D reconstruction of an A cell	65
3.9 3D reconstruction of a B cell	66
3.10 3D reconstruction of a C cell	67
3.11 3D reconstruction of a D cell	68
3.12 Reconstruction of a false ON-OFF RGC	69
3.13 Reconstruction of a second apparent ON-OFF RGC	70
3.14 Stratification analysis of apparent ON-OFF RGC	71
4.1 Spike-triggered covariance	75
4.2 Nonlinearity of model with a single filter	75

4.3 Stimulation and resting times	76
4.4 Stimulation protocol and STC analysis	79
4.5 Significance test	81
4.6 Example temporal electrical receptive field (waveform)	81
4.7 Eigenvector analysis to the covariance matrix of receptive fields	82
4.8 K-means clustering parameters	83
4.9 Waveform from example recording superimposed with a spike	84
4.10 Distribution of intracellularly stimulated RGCs	85
4.11 Intracellular clusters	86
4.12 Intracellular clusters (second part)	87
4.13 Intracellular clusters (third part)	88
4.14 Distribution and effectivity of intracellular stimuli	90
4.15 Distribution of RGC clusters	92
4.16 Extracellular clusters	93
4.17 Extracellular clusters - 3D figure	94
4.18 Extracellular clusters as functions of latency	95
4.19 Latency of the response to extracellular stimulation	96
4.20 Lower and upper amplitude stimulation thresholds	97
4.21 Distribution and effectivity of extracellular stimuli	98
5.1 Waveforms ensemble	104
5.2 Extracellular stimulation train	105
5.3 Sigmoid fit to the efficacy of response to waveforms	106

5.4	Example recording of the response to stimulus with train of waveforms	107
5.5	ERFs of each morphological class	108
5.6	Efficacy of response to own waveform (3 example cells)	109
5.7	Scatter plot by morphological class	110
5.8	Threshold of cells per morphological class	111
5.9	Mean efficacy per morphological class	112
5.10	ERFs of each waveform cluster	114
5.11	Efficacy of response to own waveform by cluster (3 example cells)	115
5.12	Mean efficacy per waveform cluster	115
5.13	Scatter plot by waveform cluster	116
5.14	Mean threshold of all the RGCs by waveform cluster	116
5.15	Mean efficacy per morphological class (intracellular stimulation)	119
5.16	Mean efficacy per waveform cluster (intracellular stimulation)	120
A.1	Example sequences of biphasic pulses with constant and random IPI. . . .	145
B.1	Efficacy response of each cell in class A, part 1 of 2	147
B.2	Efficacy response of each cell in class A, part 2 of 2	148
B.3	Efficacy response of each cell in class B, part 1 of 2	149
B.4	Efficacy response of each cell in class B, part 2 of 2	150
B.5	Efficacy response of each cell in class D	151
C.1	Efficacy response of each cell in Cluster 2	153
C.2	Efficacy response of each cell in Cluster 3	154

C.3 Efficacy response of each cell in Cluster 4	155
---	-----

Chapter 1

Introduction

Retinal prostheses aim to provide visual percepts to blind people affected by diseases caused by photoreceptor degeneration. Visual prostheses typically use electrical pulses to stimulate retinal ganglion cells (RGC), one of the many types of neurons in the retina. A critical objective of this research is to improve perceptual outcomes for patients with retinal prostheses. To achieve this ultimate goal, two projects oriented to optimize the electrical stimulation of the retina have been designed. The first of them seeks to understand the phenomenon of image fading on the retina and to find suitable stimulation procedures that reduce such phenomena. The temporal characteristics of RGC spiking during visual stimulation are observed to accurately follow a Poisson process. Under the hypothesis that mimicking a Poisson process with electrical stimuli could improve the efficacy of RGC responses, we studied the response of RGCs to irregularly timed bi-phasic pulses, delivered according to a Poisson process, and compared it to pulses delivered at regular intervals. The Poisson process significantly improved the efficacy of stimulation. These findings could help bionic eye users to continue perceiving a fixed image for longer than a few seconds, thereby improving the efficacy of visual prostheses and getting closer to restoring normal visual functions. There are more than 20 different types of RGCs, with each type conveying different visual information. The second project explores a technique where the retina is stimulated with white noise, with the intention of optimizing the electrical stimuli to elicit responses in specific RGC types. The analysis of the responses to white noise stimulation involves using a spike-triggered covariance analysis, which determines the patterns of stimulation that elicit a response pattern specific to each cell.

This introductory chapter presents a literature review of vision processing and the structure of the retina. It also discusses common causes of blindness, particularly diseases that cause retinal degeneration. A discussion follows on prosthetic vision, as used to surpass blindness caused by retinal degeneration. The review of bionic vision discusses its advantages and disadvantages, as well as the issues that need to be overcome to improve current devices.

1.1 The eye

Three distinct layers form the human eye. The support of the eyeball is provided by the outermost layer, consisting of the sclera (white) and cornea (clear), which share a border called the limbus. The second layer of the eye is the central vascular layer, which consists of the iris, the ciliary body, and the choroid. The choroid adjoins the inner most layer of the eye, formed by the retina (Kels et al., 2015). The retina is the main focus of this project, since it is responsible for transducing light into ionic currents. It will be discussed more deeply in further sections. Figure 1.1 presents a schematic of the structure of the eyeball and shows the main parts of the eye.

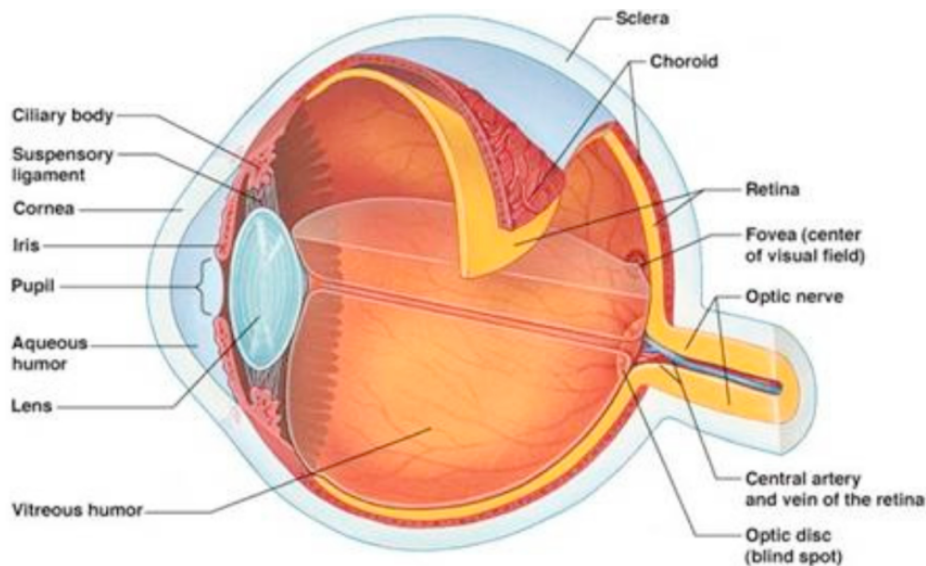


Figure 1.1: Structure of the human eye (Cholkar et al., 2012).

1.1.1 How vision works

The process of vision starts when light travels through the cornea, which is a clear transparent tissue and acts as a window for the light to enter the eye. The incoming light continues its path through the anterior chamber and the pupil, the latter being an aperture equivalent to the diaphragm or shutter of a camera. The light subsequently crosses the lens and the vitreous humour to finally finish its journey in the retina, where it is captured and transformed into neural impulses, undergoes processing by the neurons in the retina and is then transmitted to the brain (Kels et al., 2015). In order for the rays of light to be received adequately, the eye requires, first, to have a rigid spherical shape, given by the sclera. The sclera also provides an outer layer that can tolerate the high pressures exerted by the vitreous humour in the posterior chamber. Second, for light to reach the retina the cornea and the lens must focus the image appropriately. After pre-processing the signals transduced from the incoming light, the mammalian retina transmits most of the visual information to a highly organized cluster of cells in the thalamus, called the lateral geniculate nucleus (LGN), through the optic nerve (Figure 1.2). The LGN then sends signals to a large area of the brain dedicated to vision, the visual cortex (Dowling and Dowling, 2016c). Figure 1.2 shows a simple drawing of the central visual pathways, from the eye to the primary visual area (V1) in the visual cortex.

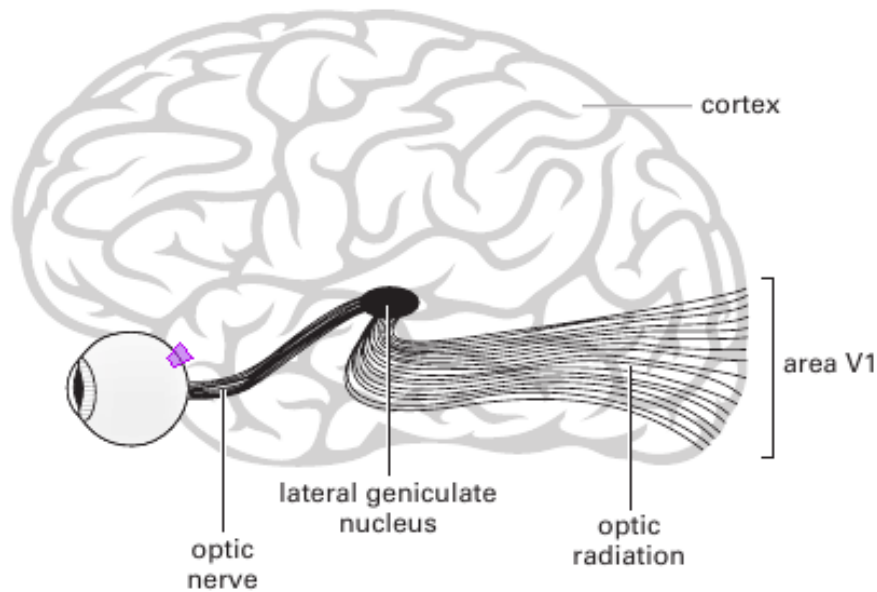


Figure 1.2: The central visual pathways. Light projects onto the retina at the back of the eye, where it is processed and transmitted through the optic nerve into the lateral geniculate nucleus (LGN). The visual information is then sent to the primary visual cortex (V1) in the brain. Figure taken from Dowling and Dowling (2016c).

Lateral geniculate nucleus

In primates, most of the signals transmitted from the retina are projected to the LGN. The LGN also gets substantial feedback information from the visual cortical areas and from the reticular formation. The latter contributes to the modulation of the arousal states of the brain and to the level of attention. This is important to the visual process because attention affects the responses of cortical neurons, i.e. the visual information may be modified by the LGN before going to the brain (Dowling and Dowling, 2016a).

LGN neurons have a centre-surround receptive field organization, which is illustrated in Figure 1.3. According to Alonso and Chen (2009), the receptive field of a neuron is "a portion of sensory space that can elicit neuronal responses when stimulated". In terms of visual neurons, either in the retina, LGN, or visual cortex, the visual receptive field can be defined as the area in visual space where a light stimulus can elicit responses from the cell (Alonso and Chen, 2009; Suematsu et al., 2013). There is an emphasis here on the *visual* receptive field, because a similar concept called *electrical* receptive field will be introduced in Chapter 4.

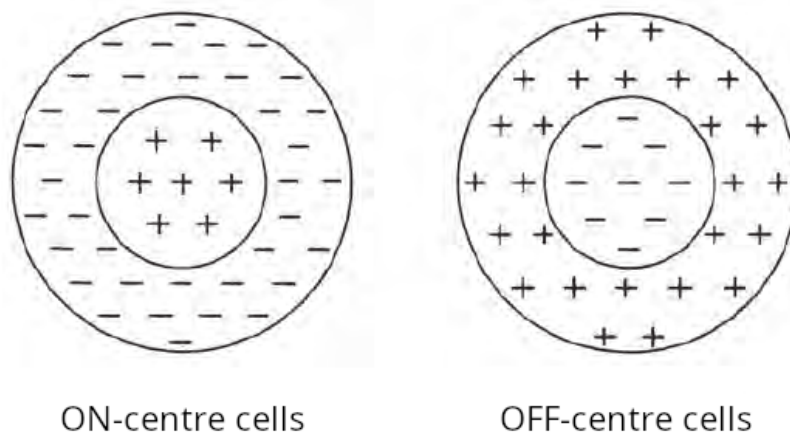


Figure 1.3: Centre-surround receptive fields in the LGN. An ON-centre neuron (left) responds to an increase of brightness in the centre of its receptive field, and to a decrease of brightness in the surrounding of its receptive field. An OFF-centre neuron (right) generates a spike in response to a decrease of brightness in the periphery of its receptive field and to a decrease of brightness in the centre of its receptive field. Figure adapted from Dowling and Dowling (2016a).

Visual Cortex

The primary visual cortex, or V1, consists of 6 layers, with layer 4 being the primary strata that receives signals from the LGN. Other areas in the cortex and thalamus also provide inputs to the visual cortex (Rosenfield et al., 2009). The neurons in V1 have much more complicated receptive fields than the retina or the LGN. V1 cells are selectively activated not only by the absence and presence of light, but they also respond to features like the shape, orientation, movement and direction of the stimulus (Dowling and Dowling, 2016a).

It has been proposed that the visual cortex is organized into hypercolumns as illustrated in Figure 1.4, which contain circuits to analyse a particular segment of visual space (Dowling and Dowling, 2016a). Although the path of most of the information in the cortex is across layers (vertical), there are also lateral neural connections (horizontal), which result in information being integrated over many millimetres of cortex. This means that a neuron receiving stimulation in one hypercolumn can affect the activity of other neurons in a different hypercolumn.

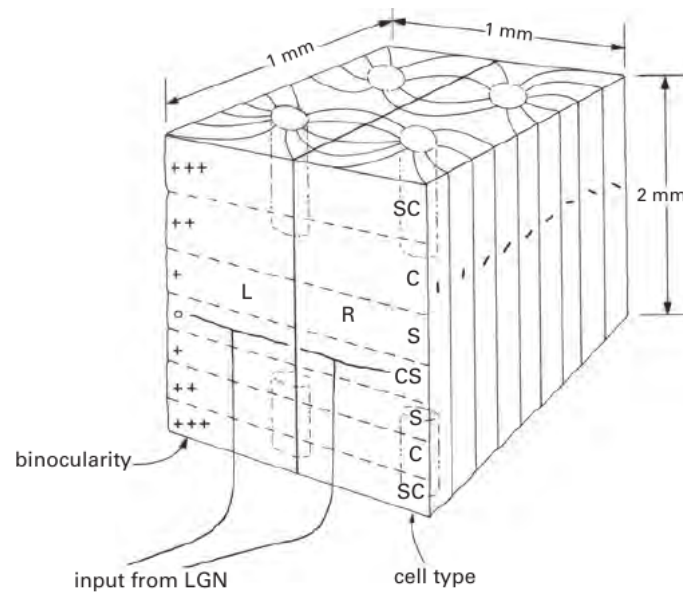


Figure 1.4: Modular organization of the visual cortex. A hypercolumn contains all the cells required to analyse a section of the visual space, all types of simple and complex cells, and all possible orientation selective cells. Figure taken from Dowling and Dowling (2016a)

1.2 Retina

The mammalian retina contains about 55 morphological types of neurons, including different kinds of photoreceptor cells (Masland, 2001). Its main function is to receive, process and transmit visual signals to the brain (Lin and Peng, 2013). Refer to Figure 1.5 for an illustration of the different cell types in the retina. Masland (2001) lists the different cell types present in the mammalian retina, which are classified as photoreceptors (cones and rods), horizontal cells, bipolar cells, amacrine cells and RGC, and these types are almost uniformly distributed horizontally across the retina, with the exception of horizontal cells. In addition, horizontal cells have much smaller density compared to the other retinal neurons (Masland, 2011). Figure 1.5 shows a diagram of the neural network in the retina, illustrating the synaptic interrelations between cells. The circuitry in the retina is evidently less complex than that in the visual cortex; it is also much more accessible and easier to study (Dowling and Dowling, 2016a). Hence, dissection and *in vitro* study of a small, healthy piece of retinal tissue is simpler than with the visual cortex. In addition, the layers of the retina are easier to segregate, identifying a RGC from a bipolar cell, for example, is mostly just a matter of determining how deep in the retina one is looking.

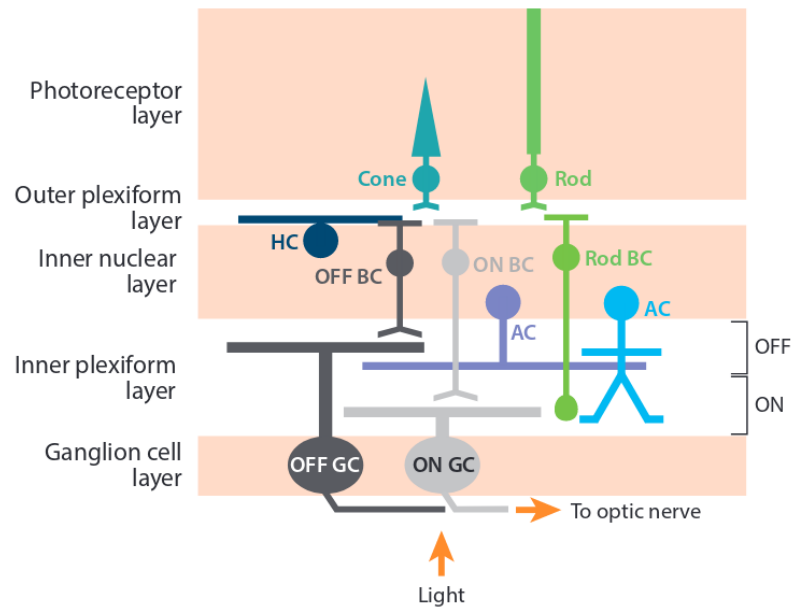


Figure 1.5: Synaptic interrelations of the main types of neuron in the retina. Photoreceptor cells (Cone and Rod), horizontal cell (HC), bipolar cells (BC), amacrine cells (AC) and retinal ganglion cells (GC). Figure taken from Demb and Singer (2015).

Photoreceptors

At the outer layer of the retina, alongside the pigment epithelium, lies a layer of light-sensitive neurons called photoreceptors (Dowling and Dowling, 2016b). These cells are elongated and have an outer-segment region specialized for the absorption of light, and an inner-segment, where the molecules and proteins necessary for phototransduction are manufactured. Then there is the nucleus of the cell and finally the synaptic terminals which transmit the visual information to the other layers of the retina. There are two types of "image forming" photoreceptors in the vertebrate retina: rods and cones. Rods are mainly responsible for peripheral and dim-light vision; they have high density in the area surrounding the fovea, which is the central part of the retina, but are virtually non-existent in the centre of the fovea. In contrast, cones have a very high density at zero eccentricity (the centre of the fovea) and low density elsewhere in the retina. Cones are dedicated to high acuity vision tasks and colour vision. Different types of cones contain different pigments, thus making them sensitive to different wavelengths of light. Humans have 3 different cone photoreceptors, which allow them to be maximally sensitive to red light, blue light, or green light. Conversely, most mammals have only 2 types of cones, hence they have dichromatic

vision. Moreover, foveal organization is only present in primate retina, not in rats (Masland, 2001, 2012).

When a neuron is stimulated, the ionic channels in its membrane open. Thus allowing positive charged ions into the neuron and causing it to depolarize, or increase its membrane potential. Surprisingly, when photoreceptors are stimulated with light, they hyperpolarize, i.e. their membrane potential decreases, and the synaptic activity of the cells becomes less active. This can be interpreted as photoreceptors being stimulated by darkness, and deactivated by light (Dowling and Dowling, 2016b).

Inner nuclear layer

The next layer of cell bodies in the retina corresponds to the inner nuclear layer, which is where the soma of bipolar cells, amacrine cells, and horizontal cells are found. Figure 1.6 illustrates the retinal network, where the bipolar cells sample the mosaic of cone photoreceptors. Diffuse bipolar cells receive input from all the cones within their reach, while blue bipolar cells receive input selectively from blue cones (short wavelength), preserving the chromatic purity of the responses of the blue bipolar cells (Figure 1.6a). The mammalian retinal network compares the output of short wavelength cones to that of long wavelength cones, which leads to colour vision. Different types of bipolar cells transmit different components of the cone output, for instance, increments in light intensity depolarize ON bipolar cells, while decrements in light intensity depolarize OFF bipolar cells (Demb and Singer, 2015). When talking about bipolar cells, there is no mention of 'activation', because bipolar cells do not elicit action potentials, instead, they show a variation in the membrane potential. In Figure 1.6b, all bipolar cells in contact with an individual cone (red arrow) receive information from just this cone. In this case, the functional type of the bipolar cells in red is the same, but the same cone would also transmit information to 10 other types of bipolar cells.

As mentioned previously, each type of bipolar cell transmits components of the cone output, and synapses onto different types of RGCs. Each RGC then receives a particular version of the visual input, causing the beginnings of ganglion cell diversity. In Figure 1.6c, the ganglion cell receiving information from the blue bipolar cell becomes a blue-sensitive RGC. However, information from bipolar cells is not the only input onto RGCs; amacrine

cells receive a direct excitatory input from bipolar cells, and make inhibitory synaptic inputs back to the bipolar cell axons, forming a classic feedback system. Amacrine cells also synapse onto each other and onto RGCs (Figure 1.6d). The action of amacrine cells causes the highly selective final response of the RGCs, meaning that each type of RGC responds to a particular kind of stimulus (direction of movement, low or high contrast, rapid movement, etc.) (Masland, 2011).

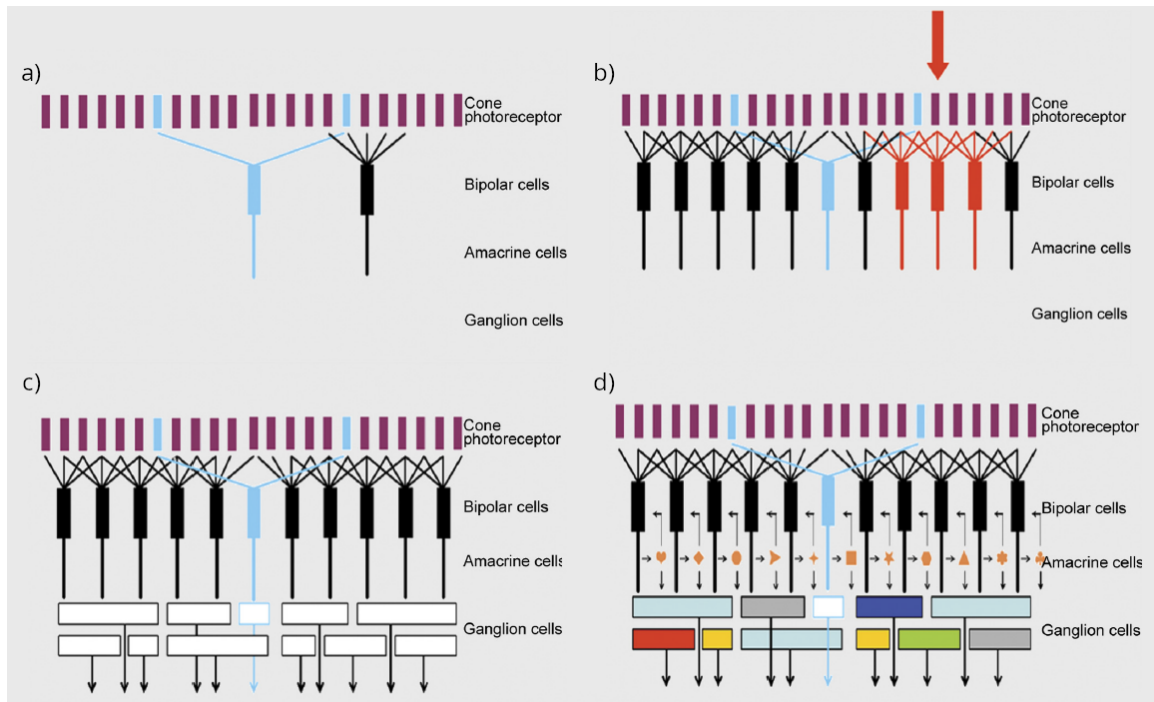


Figure 1.6: Retinal network pathways. **(a)** Cone photoreceptors sampled by a diffuse bipolar cell (black) and a blue bipolar cell (blue), **(b)** activation of multiple bipolar cells as a result of the stimulation of a single cone photoreceptor, **(c)** synapse of a blue bipolar cell on a blue-sensitive RGC, **(d)** diversity in RGCs from the action of amacrine cells. Figure adapted from Masland (2011).

1.2.1 Retinal ganglion cells

The RGCs are located in the inner layer of the retina (bottom row in Figures 1.5 and 1.6), and are responsible for transmitting visual information to the brain through the optic nerve, which is formed by the axons of the RGCs. Some RGCs receive their input from bipolar, and some from amacrine cells. RGCs also have a sub-classification, with there being at least 20 different types of RGCs in most mammalian retinas, including primates, which means that there are about 20 different messages about an image constantly being

transmitted to the higher visual pathways (Baden et al., 2016; Bae et al., 2018; Dowling and Dowling, 2016c; Masland, 2012; Wässle, 2004). Masland (2001) emphasizes the similarities of the cell types from different mammalian retinas; for example, most mammals (including rat, mouse and cat) have brisk-transient α ganglion cells and brisk-sustained β ganglion cells.

When a RGC is stimulated by another cell, the normally polarized membrane potential depolarizes, causing a sudden peak of voltage from -60 mV (resting potential) to around 20 mV. This process is called depolarization, and the peak is called an action potential, or spike. The spikes are transmitted through the synaptic connections in order for the other cells in the retina to register that light has been received (Dowling and Dowling, 2016b).

Like the neurons in the LGN, RGCs have centre-surrounding receptive fields, and they have been discovered to have elliptical receptive fields, which could account for a small proportion of cells having orientation tuning (Rodieck and Stone, 1965; Suematsu et al., 2013). The receptive fields of the RGCs near the fovea are smaller, or more focused than those in the visual periphery (Alonso and Chen, 2009). They are also arranged in a mosaic fashion and overlap between cells, which, according to Devries and Baylor (1997) prevents aliasing and provides improved sensitivity to the cell population. The type of light response of RGCs depends on the depth of their dendritic stratification. RGCs that arborize closer to the bipolar cells are OFF RGCs, and are activated by a transition from lights on to lights off. Conversely, RGCs whose dendrites stratify closer to the RGC layer, are ON RGCs, and respond to transition from lights off to lights on (Bae et al., 2018; Sanes and Masland, 2015). The diversity of light response in RGCs is introduced with more depth in Chapter 3. The existence of two different light sensitivity channels contributes to a fast perception of shape, colour, flicker and stereo image, but the two main reasons for the need of ON and OFF channels are to facilitate contrast sensitivity and for a rapid detection of brightness increment or reduction (Schiller et al., 1986).

RGCs can be classified morphologically by studying their soma size and dendritic field morphology. Although previous classifications have used this criteria, Huxlin and Goodchild (1997) used Dil (dye) labelling and intracellular Neurobiotin injection, which allowed them to stain the whole cell. They classified rat RGCs into three main groups: A, for RGCs presenting large somata and dendritic fields; B, for those whose somata and dendritic field size is small; and C, for small-to-medium somata and medium-to-large dendritic fields.

Table 1.1: Morphological characteristics of RGC classes.

Class	Soma diameter	DF diameter
A	large	large
B	small	small
C	small-to-medium	medium-to-large
D	varies*	varies*

* All bistratified RGCs belong to class D.

Each main group has subgroups depending on the shape of the soma and their level of stratification in the inner plexiform layer (inner or outer).

Similarly to Huxlin and Goodchild (1997), Sun et al. (2002b) used Dil-coated particles to stain and classify RGCs in three main groups: A, B and C, but made a more extended analysis with a larger sample, adding one main group (D) and several subtypes to the already existing ones. Table 1.1 summarizes the characteristics of RGCs in the 4 different groups.

Wong et al. (2012) studied 125 different RGCs from Long-Evans rats, classifying them by their morphology into the previously described groups, and then by their physiological characteristics. To analyse physiological features, whole cell current-clamp recordings were performed and a comparison of the passive membrane properties of every cell type was made. Resting membrane potential, input resistance, time constant, maximum firing rate, steady-state firing rate, frequency adaptation index, spike width and hyperpolarization-evoked sag potentials were compared in their study.

Recently, Tribble et al. (2014) proposed a novel method of morphological classification for RGCs based on the proximal dendritic features, such as primary dendrite thickness and asymmetry in the proximal dendritic tree. The proposed method was able to classify a small sample of RGCs in their groups A, B, C and D. Classification into subgroups cannot be achieved with this method. An important aspect of the method is the ability to classify degenerated cells since the parameters used in the analysis are resistant to early degeneration (Tribble et al., 2014).

Sun et al. (2002a) made a morphological classification of mouse RGCs and found the same 4 main groups as in the rat. A different morphological classification is proposed by Coombs et al. (2006), who found fourteen different classes of RGCs and designated

each class to a cluster, from M1 to M14 (M for mouse), where the first 10 clusters were for monostratified cells and the other 4 for bistratified. Their classification was based on morphological measurements of soma and dendritic field size, total dendrite length, number of dendritic branches, and other characteristics. Hu et al. (2013) used 5 of those groups (M1 to M5) to perform a physiological analysis of mouse RGCs and found interesting differences in spike threshold and sag amplitude between groups.

1.3 Retinal Degeneration

Degeneration of retinal photoreceptors, as occurs in diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), is the major cause of adult blindness in developed countries (Wright et al., 2010). RP refers to a set of hereditary retinal diseases that cause blindness due to a progressive loss of rod and cone photoreceptor cells (Gregory-Evans et al., 2013). Although RP presents a variety of symptoms for different patients, many of them fall into a classic pattern of loss of night vision in adolescence, loss of mid-peripheral visual field in young adulthood, tunnel vision in adulthood and, finally, loss of central vision by the age of 60 (Hartong et al., 2006). Retinal degeneration presents progressive loss of rod and cone photoreceptor cells. Commonly, loss of rod photoreceptor cells occurs in earlier stages of the disease, thus the first symptoms include low night vision and a progressively narrower visual field; generally, rod loss exceeds the loss of cones. As the disease advances, cone photoreceptor cells also start dying, causing the loss of visual acuity and general vision. Many amacrine cells, bipolar cells and horizontal cells also degenerate in later stages of retinal degeneration (Dowling and Dowling, 2016b; Hartong et al., 2006; Noell, 1963). Likewise, a form of AMD called Nonexudative AMD presents as gradual vision loss, as well as retinal pigmentary changes, drusen, and geographic atrophy of the retinal pigment epithelium. Exudative AMD presents as choroidal neovascularization, which causes rapid vision loss (Medeiros and Curcio, 2001).

Although RP and AMD cause irreversible loss of photoreceptors, it has been shown that other cell layers in the retina remain, if not intact, then with a large proportion of healthy cells. Medeiros and Curcio (2001) studied 21 patients with AMD and compared the number of surviving RGCs to that of photoreceptor cells. They found no loss of RGCs in one kind of AMD (nonexudative AMD) while in eyes with exudative AMD the remaining RGCs are

sufficient to electrical stimulation, thus making it possible to transmit a response through the optic nerve. Mazzoni et al. (2008) also found survival of RGCs in *rd* mice (*rd* = retinal degeneration) and, more importantly, the RGCs maintain their electrical functionality even long after the loss of photoreceptors.

The work from Hartong et al. (2006) involved an extensive review of RP. They studied the genes related to the inheritance of the disease. At the time Hartong et al. (2006) published their work, there were 45 identified genes for RP, which were found to be related only to 60% of cases. Wright et al. (2010) consider RP and AMD as the two main forms of photoreceptor degeneration. They also list the different genetic factors that contribute to RP and AMD. A subsequent study relating the survival of RGCs to photoreceptor degeneration was carried out by Lin and Peng (2013). Their paper investigated the morphological changes in RGCs in *rd1* mice (ages of 1 to 18 months) and reported no significant changes in the morphological characteristics of α ganglion cells. The density of such cells did not show any significant differences between early and advanced stages of retinal degeneration.

Pupil responses to light in mice and humans who lack functional rod and cone photoreceptors have been observed for a long time. The assumption that this light perception was due to survival of rods and cones has continued until recently. Lucas et al. (2001) disassociated the photosensitivity with residual rods and cones in *rd/rd cl* mice. Subsequently, Zaidi et al. (2007) found the same behaviour in rodless coneless human subjects and identified a new photoreceptor in the ganglion cell layer, therefore calling it a photoreceptor RGC (pRGC). In a related publication, Zaidi (2009) discussed the role of pRGC in visual perception and conscious sight. The previously mentioned work from Lin and Peng (2013) also analysed intrinsically photosensitive RGCs (ipRGC) and showed that they have similar response properties to those of the α RGC. Intrinsically photosensitive or photoresponsive RGCs express the photopigment melanopsin, hence are also known as melanopsin-containing RGCs (mRGCs) (Hattar et al., 2002; Schmidt et al., 2011). There are at least 6 types of ipRGCs and they are functionally and anatomically diverse, their role is mainly to provide non-image forming visual functions, like pupillary reflex and induction and regulation of sleep (Schmidt et al., 2011).

1.4 Visual prostheses

There is currently no clinically available treatment capable of reversing RP, but various teams around the world have been working to develop a prosthetic eye that could eventually provide useful vision to blind patients. The first visual prostheses to use a multielectrode array was implanted in 1967 on the visual cortex of a patient (Brindley and Lewin, 1968). The implant could achieve visual percepts. With new technology, the limitations of implanting electrodes on the cortex have slowly been overtaken and several groups are still trying to develop cortical prostheses. Stimulation of the optic nerve targets the axons of the RGCs, the problem with these devices is that the percepts resulting from stimulation with optic nerve implants are irregular in shape and visual location, hence they require a complex algorithm to provide a meaningful image (Lasker, 2014). Another possible approach is to directly stimulate the LGN (Panetsos et al., 2011).

1.4.1 Prosthetic retinas

The prosthetic vision devices that have been shown to partially restore functional vision to patients of retinal degeneration are the retinal prostheses. These devices seek to replace the function of the photoreceptors and bypass the retinal networks. They make use of the surviving RGCs and stimulate them to cause information to be transmitted through the optic nerve to the LGN and higher vision processing centres of the brain. Depending on the location of the stimulation electrodes in the retina, the prosthetic retinas can be divided into epiretinal, subretinal or suprachoroidal. See Figure 1.7 for the different possible locations of a retinal prosthesis.

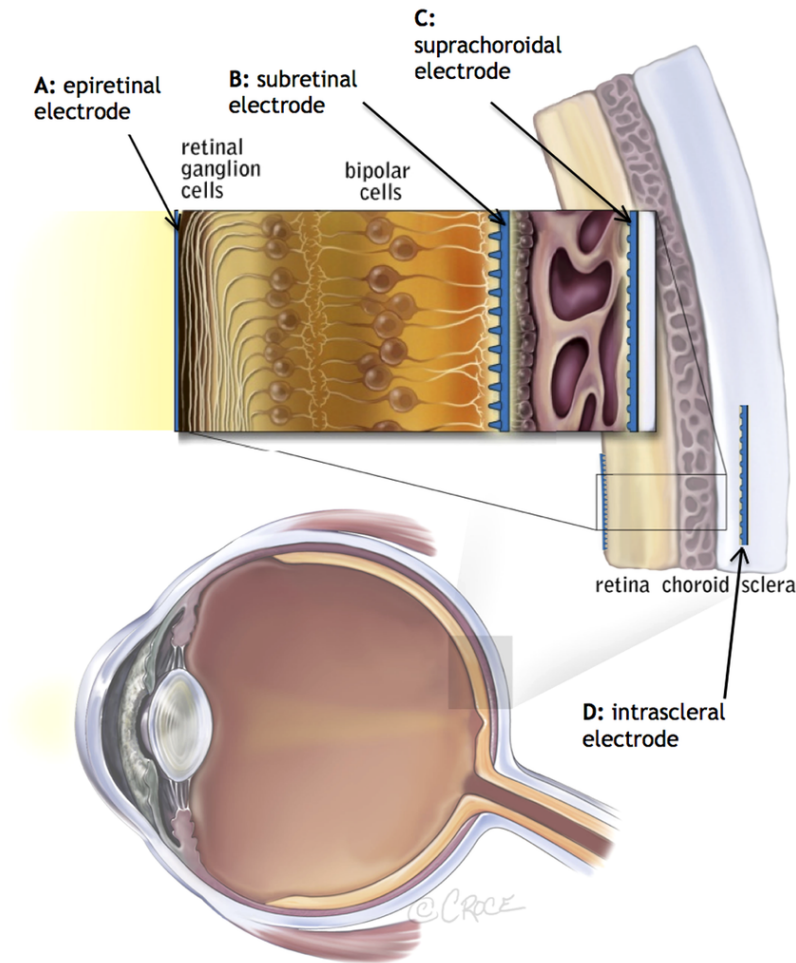


Figure 1.7: Possible electrode implantation sites in the different layers of the retina (Ayton et al., 2014).

Epiretinal

Stimulating electrodes positioned on the inner surface of the retina (the ganglion cell side), they provide epiretinal stimulation. The epiretinal approach, used by Humayun et al. (2003), Sekirnjak (2006) and Margalit et al. (2011), has the advantage of being surgically easier to place. That is, the implanted arrays can be seen with an ophthalmoscope and, according to Tsai et al. (2009), epiretinal stimulation may have a lower safe stimulation threshold compared to the subretinal approach, which allows the use of smaller electrodes, potentially increasing the visual resolution (Sim et al., 2014). The Argus implant (Second Sight Medical Products, Inc. Sylmar, CA USA) is one example of an epiretinal device being developed. It captures the image from an external camera attached to a pair of glasses (see Figure 1.8). The image is then sent wirelessly to the implant (Ong and

da Cruz, 2012). Yanai et al. (2007) presented a 16 electrode epiretinal implant (Argus I) and tested it in three subjects with RP. Users of this implant were able to discriminate which electrode was being activated, and distinguish between rows and columns of activated electrodes. In the experiments of Yanai et al. (2007), the subjects had a fairly good performance (above chance) when counting and detecting white objects on a black background, as well as recognizing object shapes. Argus II is currently the project at the most advanced stage, implementing epiretinal stimulation with a 60 electrode array implanted on the inner retina. It has reported letter recognition in human patients (Luo and Da Cruz, 2014; Ong and da Cruz, 2012). The epiretinal retinal prostheses IRIS VRS (Pixium Vision) uses a similar stimulation approach (Hornig et al., 2017). The device has been approved for commercialization in Europe.

Subretinal

In subretinal prosthetic retinas, the stimulation electrodes are placed between the photoreceptor layer and the retinal pigment epithelium (Gekeler et al., 2004). By having subretinal electrodes, the existing information-reduction functions in the inner nuclear layer can still be used (Dowling, 2009). Subretinal stimulation has a greater chance of activating bipolar cells, which might reduce distortion of the retinotopic map of the visual scene (Sim et al., 2014); it has been used by Zrenner et al. (1997) and Stingl et al. (2013). Electrical stimulation of bipolar cells results in graded potentials, as opposed to the action potentials of RGCs. It has the advantage of a higher spatial correspondence with the perceived image and might result in higher visual resolution compared to stimulation of RGCs (Chow and Chow, 1997). The most common example of a subretinal prosthesis is the optoelectronic approach, which typically uses microphotodiode arrays to make a direct transduction from light to electricity. Three examples of optoelectronic prostheses are the Artificial Silicon Retina (Chow et al., 2010, 2004), the PRIMA system (Palanker et al., 2005) and the Retina Implant AG (Ong and da Cruz, 2012). Stingl et al. (2013), from the team that developed the Retina Implant AG successfully tested an optoelectronic device called Alpha-IMS in human patients. It has been commercially available in Europe since July 2013, and features 1500 microphotodiodes and subretinal electrodes (Luo and Da Cruz, 2014). Pixium Vision's PRIMA system comprises only 378 electrodes within a 2x2 mm implant and is currently under clinical trials (Palanker et al., 2005; Smith et al., 2015).

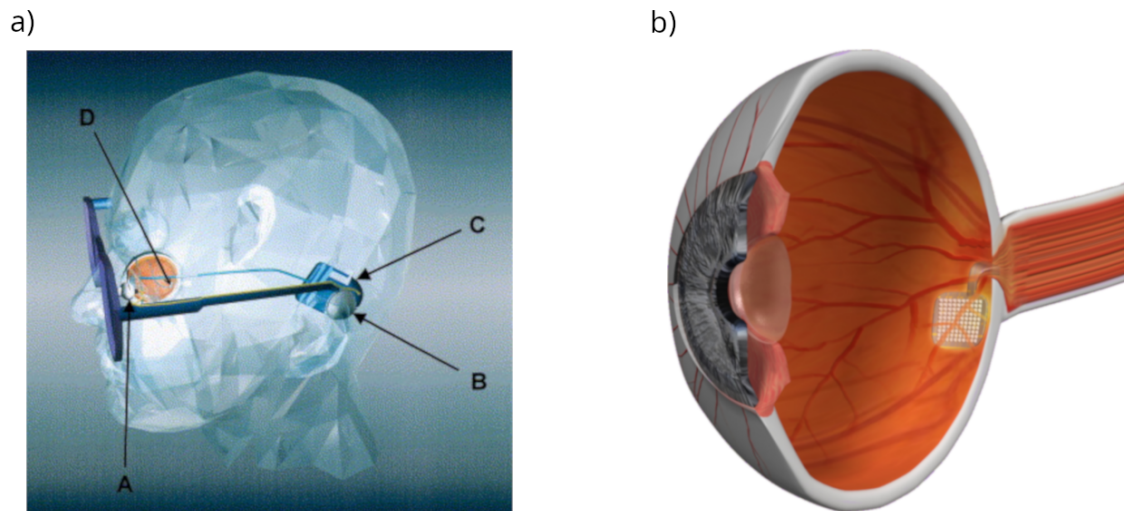


Figure 1.8: Schematic showing the concept of the retinal prosthesis. **(a)** A camera in the glass frame (A) receives an image, which is processed and sent by a wireless transmitter (B) to the receiver (C), finally, an electrode array (D) implanted in the retina stimulates the RGCs. **(b)** An electrode array on the retina. Subfigure (a) taken from Humayun et al. (2003), subfigure (b) taken from equinoxgraphics.net.

Suprachoroidal

The Bionic Eye, developed by Bionic Vision Technologies, (Melbourne, Australia) works by stimulating electrodes in the suprachoroidal space (between the choroid and the sclera). It has a multielectrode array to deliver electrical extracellular stimuli to the retina. A prototype containing only 24 electrodes was tested in three patients with RP (Ayton et al., 2014). Fujikado et al. (2011) have also successfully tested a suprachoroidal device with 2 patients, achieving some simple tasks like object recognition.

Although several prosthetic retina devices have been successfully tested in human patients, and some of them have already received marketing approval in Europe and the US (Sim et al., 2014), there are still many aspects to improve for the prosthetic eyes to restore functional vision to its users. Among these aspects is image fading, produced when an image or stimulus is fixed on the retina for about one second without temporal stimulus changes; the perceived image begins to fade within seconds of receiving such stimulation until there is no more visual percept (Arend, 1973; Freeman and Fried, 2011; Hsieh and Colas, 2012; Martinez-Conde et al., 2006; Pérez Fornos et al., 2012). Other shortcomings of existing retinal prostheses are low-resolution and poor robustness. According to Weiland et al. (2014), "even if a high density, long-lasting implant can be made, ques-

tions remain about how to deploy such a system to achieve high-resolution on a consistent basis". This is to say, that although some groups have tried to increase the number of electrodes, this does not mean that the patients will have higher resolution precepts (Guenther et al., 2012). Moreover, high resolution vision has not been achieved by any of the implants tested in patients (Weiland et al., 2014).

1.4.2 Image fading

Image fading refers to the effect produced after a stimulus is presented to the stabilized retina for about 1 to 3 seconds. When no temporal stimulus changes can occur on the retina, the image begins to fade within a few seconds until visual perception is lost (Arend, 1973; Levy-Schoen, 1968; Martinez-Conde et al., 2006).

Tulunay-Keesey (1982) studied the retina's response to a stabilized image, presenting a real-light image motionless on the retina. They then compared the fading of such an image with an afterimage. The same comparison was made several times, trying to understand whether the afterimage and the fading of the stabilized image are caused by the same mechanism (Burbeck and Kelly, 1984; Ditchburn and Drysdale, 1973; Koenderink, 1972). The work of Hsieh and Colas (2012) suggested that fading is not only related to the retina, but that it also occurs in the cortex.

To avoid image fading, human eyes perform small, quick movements known as fixational eye movements. Martinez-Conde et al. (2006) analysed microsaccades, which are one of three types of fixational movements. They found a clear relationship between microsaccades and image visibility. In their experiment, a decrease in microsaccade rate, probability and magnitude was followed by a fading of the visual stimulus. Rucci et al. (2007) showed that fixational eye movements improve the discrimination of high spatial frequency stimuli, but not of low frequency. A paper by Poletti (2010) tried to determine if the reduction of image fading is actually due to microsaccades or if microsaccades are just a secondary symptom of image fading. They concluded that there is no causal relationship between image fading and microsaccades, which contradicts Martinez-Conde et al. (2006). Mccamy et al. (2014) discussed the difference between fading reversal and fading prevention, and analysed the influence of microsaccades and drift on preventing fading. The inability of retinal prostheses users to move the position of the stimulus (mi-

crosaccades) in order to refresh the faded image, as a person with healthy vision would do, causes perceptual visual fading. In contrast, the prosthesis stimulates the retina in the exact same locations, causing the stimulus to be stabilized and resulting in desensitization of RGCs and fading (Stingl et al., 2013). This is one of the biggest challenges for the development of functional retinal prostheses.

A phenomenon believed to contribute to image fading is known as desensitization and occurs when retinal cells adapt to prolonged or repetitive activation of the synaptic network, thus leading to reduced sensitivity. Freeman and Fried (2011) examined the relation between fading and desensitization and they encountered two phases of desensitization, one at a time of around 176 milliseconds and the other at a time of 14 seconds. These two phases coincide in time with the fading observed in human subjects, which had a rapid decay at around hundreds of milliseconds, followed by a slow decay for the duration of the stimulus (Pérez Fornos et al., 2012). The implementation of a time-varying stimulus could help to reduce desensitization, as demonstrated by Davuluri and Weiland (2014).

Jensen and Rizzo (2007) proposed an anodic first biphasic pulse stimulus with a current of 1.5 times the threshold at frequencies from 1.5 to 65 Hz delivering trains of ten pulses for subretinal stimulation. Their findings show that for frequencies higher than 15-20 Hz, there is a response reduction of more than 50%, i.e. the tenth pulse elicits a response with at least 50% less amplitude than the first pulse. When stimulating at 10 Hz and above, the response reduction from the second pulse is greater than 40%. Similar findings to those of Jensen and Rizzo are reported by Freeman and Fried (2011); they performed epiretinal stimulation with trains of ten cathodic first biphasic pulses at low frequencies (2 to 16 Hz) and obtained a response decay of more than 50% for frequencies above 8 Hz. Likewise, Im and Fried (2016) found that lower frequencies show less response decay in the first pulses, however, they found an important difference between the response of ON RGCs and OFF RGCs. They observed a reset behaviour in the response of ON RGCs that was not present in OFF RGCs response. This is in contrast to the work of Tsai et al. (2009) who stimulated subretinally with trains of 4 biphasic square pulses at frequencies of 50 to 200 Hz. At the highest frequency (200 Hz), they reported a decay of the response rate from 80% in the first pulse, to 70% in the second and above 50% in the 4th pulse. Such differences between studies suggest that further work should be done to clarify how RGCs adapt to stimuli of different frequencies. There is a consensus on the idea that there is

response decay during stimulation, but it is not clear what mechanism controls the decay rate or which stimulation methods could reduce the decay.

Some experiments support the hypothesis that perceptual fading originates in the early visual pathway, particularly the RGCs, and that it could be due to adaptation of the retinal network (Benav et al., 2008; Kotulak and Schor, 1986; Millodot, 1967). Adaptation of RGCs and image fading can be prevented by synchronously moving the stimulus with the natural eye movements (Zrenner et al., 2011). The ability to maintain visibility of an image depends on the interaction of different components of eye movements, rather than one component alone. Also, a reduction of perceptual fading can be achieved not only by moving the image on the retina, but by intermittently illuminating a stabilized image (Ditchburn et al., 1959).

According to Hsieh and Colas (2012), the phenomenon of visual fading can be present without adaptation of RGCs, but it is still not completely understood how much influence retinal adaptation has on the phenomenon. Furthermore, it is unknown if cortical adaptation influences perceptual fading when adaptation of RGCs occurs, thus the phenomenon might be caused by components in both the retina and the visual cortex (Hsieh and Colas, 2012). Experiments on saccadic movements suggest that fading may originate in the lateral geniculate nucleus (Clarke and Belcher, 1962).

Perceptual visual fading has been observed in human patients whose retina was electrically stimulated. Pérez Fornos et al. (2012) studied how 9 patients implanted with a retinal stimulation system perceived the brightness of a visual stimulus. According to their results, the patients perceive on average only 50% of the stimulus's brightness after 2.5 seconds. Stingl et al. (2013) claimed that their subretinal electronic implant does not have this problem of perceptual fading because it does not prevent the eye from making fixational movements, while implants like the one used by Pérez Fornos et al. (2012) presented a stimulus that is fixed on the retina. The epiretinal implant presented by Yanai et al. (2007) does not thoroughly study the fading effect. However, the subjects in their experiments showed better performance when they were allowed to move the head (thus, the camera), which could have refreshed the image in a similar way as microsaccades do in healthy eyes.

In an attempt to reduce response decay, Davuluri and Weiland (2014) proposed a time-varying stimulus. Their protocol consisted of a 20 Hz stimulus of biphasic square pulses

of variable pulse width, selected randomly between 0.1 and 20 ms. The stimulation was applied for 120 s, during which the time-varying pulses were delivered at intervals of 1 second followed by 1 second of no stimulus. They found a response decay in their control group (fixed pulse width) where, after 10 seconds, the strength of the response was 50% of the initial strength and it kept decaying for the rest of the 120 seconds until reaching almost 20% of the initial strength (a decay of 80%). However, the time-varying stimulus showed a significantly smaller decay ($p < 0.05$), where the electrically evoked response strength was 80% after 10 seconds and 50% at the end of the stimulus. This result could be related to the notion of prediction, in the sense that the cells stop responding when they can predict the upcoming stimuli, so their response is reduced less when being stimulated in an unpredictable, random fashion. The response of RGCs to electrical stimulation has been widely evaluated for both extracellular and intracellular stimuli. It has been demonstrated that charge-balanced biphasic pulses are the safest neural stimulation method and it is in fact the most used stimulus type (Margalit et al., 2011).

The ultimate goal of this body of works is to find ways to increase the efficacy of electrical stimulation of RGCs, thus improving retinal prosthesis. The first part of this thesis attempts to answer if it is possible to improve the response of RGCs to electrical pulses by adjusting the stimulation parameters, and specifically explores the hypothesis that adaptation of RGCs to electrical pulses can be reduced by applying temporally irregular stimuli. Then we present the hypothesis that RGCs have different temporal electrical receptive field (ERF) due to their particular dendritic field and because of the intrinsic differences between RGC types. The particular goals on the last sections of this thesis are to (i) recover the temporal ERF of individual RGCs, (ii) to recover the temporal ERF of different morphological types of RGCs, and (iii) to selectively stimulate cells from specific morphological classes based on their temporal ERF.

1.5 Thesis Outline

This thesis is divided into 6 chapters. Chapter 1 is an introduction to the research field studied in this PhD project. It presents a literature review, background and previous work on which this project bases its methods and dissertations. The three main topics of which the thesis is comprised are covered in Chapters 2 to 5, which cover methods and results.

Chapter 2 is a study of the responses of RGCs to electrical stimulation with irregular inter-pulse intervals. It explores the adaptation of the neurons to periodic biphasic pulses, and presents an attempt to reduce the adaptation by changing the temporal features of the stimulus. Chapter 3 presents, in detail, the procedures followed during the morphological classification of the different RGCs analysed in this work. Chapter 4 uses the responses of individual RGCs to reconstruct their temporal receptive fields. It then uses a clustering algorithm to group the cells by the different features of their receptive fields. Chapter 4 also makes a comparison of the receptive fields of each cell with the receptive fields of the same morphological cell type, i.e., types A, B, C, and D. In Chapter 5 the waveforms extracted from the receptive fields of each cell are used to stimulate itself and other cells. This explores the responses of each cell to the waveforms resulting from their own receptive fields, and from the receptive fields of other cells in the same and different morphological types. Finally, Chapter 6 presents a discussion and general conclusion for the thesis.

Chapter 2

Irregularly timed electrical pulses reduce adaptation of retinal ganglion cells

Abstract

Objective. Retinal prostheses aim to provide visual percepts to blind people affected by diseases caused by photoreceptor degeneration. One of the main challenges presented by current devices is neural adaptation in the retina, which is believed to be the cause of fading - an effect where artificially produced percepts disappear over a short period of time, despite continuous stimulation of the retina. We aim to understand the neural adaptation generated in retinal ganglion cells (RGCs) during electrical stimulation. *Approach.* Current visual prostheses use electrical pulses with fixed frequencies and amplitudes modulated over hundreds of milliseconds to stimulate the retina. However, in nature, neuronal spiking occurs with stochastic timing, hence the information received naturally from other neurons by RGCs is irregularly timed. We used a single epiretinal electrode to stimulate and compare rat RGC responses to stimulus trains of biphasic pulses delivered at regular and random inter-pulse intervals (IPI), the latter taken from an exponential distribution. *Main results.* Our observations suggest that stimulation with random IPIs result in lower adaptation rates than stimulation with constant IPIs at frequencies of 50 Hz and 200 Hz. We also

found a high proportion of lower amplitude action potentials, or spikelets. The spikelets were more prominent at high stimulation frequencies (50 Hz and 200 Hz) and were less susceptible to adaptation, but it was not clear if they propagated along the axon. *Significance.* Using random IPI stimulation in retinal prostheses reduces the decay of RGCs and this could potentially reduce fading of electrically induced visual perception.

Keywords: Adaptation, electrical stimulation, inter-pulse interval, irregular stimulation, perceptual fading, rat, retinal ganglion cells.

2.1 Introduction

Mammalian retinas consist of several cell types: photoreceptors, amacrine cells, bipolar cells, horizontal cells, and retinal ganglion cells (RGCs). In total, there are more than 60 subtypes of cells within these groups, which form a complex network that receives, processes, and transmits light to the brain through the axons of RGCs. The information travels through the optic nerve in the form of action potentials or spikes (Masland, 2012). Bipolar cells form the main pathway between photoreceptors and RGCs, mostly acting as excitatory inputs. Amacrine cells provide integration in the network with cell types from different levels, such as feedback to bipolar cells, inhibition or modulation of RGC activity, and connectivity with other amacrine cells (Asari and Meister, 2014; Euler et al., 2014; Masland, 2001, 2012). Electrical stimulation of the retina can generate action potentials and modulate the membrane potentials of RGCs and inner retinal neurons (Cameron et al., 2013; Sekirnjak, 2006).

Degeneration of retinal photoreceptors in diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD) is the major cause of adult blindness in developed countries (Hyman et al., 1983; Wright et al., 2010). RP refers to a set of hereditary retinal diseases that cause blindness due to a progressive loss of rod and cone photoreceptor cells (Hartong et al., 2006). AMD results from degeneration of the macular (central) region of the retina, and also results in progressive loss of photoreceptors (Medeiros and Curcio, 2001; Mehta, 2015; Mullins et al., 2000). There is currently no medical treatment capable of reversing the effects of RP or AMD, but various groups around the world have

been working to replace the function of the photoreceptors with prosthetic retinal implants. The idea behind retinal implants is to stimulate surviving retinal neurons with electrical impulses via a grid of stimulating electrodes (Guenther et al., 2012; Hadjinicolaou et al., 2015a; Luo and Da Cruz, 2014). The electrical impulses generate responses in RGCs, providing the patient with an artificial perception of vision in the form of small spots of light called phosphenes.

Retinal prostheses have been successfully tested in patients (Ayton et al., 2014; Hu-mayun et al., 2012; Stingl et al., 2013; Zrenner et al., 2011, 1997), with results showing that some participants were able to perform simple daily life tasks (e.g. recognise nearby objects, detect if lights were on or off in a room, etc). However, there remain many challenges that need improvement before retinal implants can allow the users to rely on retinal prostheses for independent living (Chuang et al., 2014; Luo and Da Cruz, 2014; Ong and da Cruz, 2012; Stingl et al., 2013; Yanai et al., 2007). Among these challenges is image fading, characterized by the inability to perceive a static image for prolonged periods (Pérez Fornos et al., 2012; Stronks et al., 2013). Due to image fading, the phosphenes induced by electrical stimulation begin to fade within a fraction of a second or up to several seconds, which leads to the loss of visual perception (Arend, 1973; Freeman and Fried, 2011; Hsieh and Colas, 2012; Martinez-Conde et al., 2006; Pérez Fornos et al., 2012; Stronks et al., 2013). Neural adaptation in the retina and visual cortex are thought to drive this fading (Hsieh and Colas, 2012; Millodot, 1967). Although it is still not fully understood how much influence retinal adaptation has on the phenomena of fading, there is evidence suggesting that adaptation of the RGCs could contribute to perceptual fading, and that adaptation of RGCs and image fading could be reduced by using dynamic stimuli that change their patterns frequently (Benav et al., 2008; Kotulak and Schor, 1986; Millodot, 1967; Zrenner et al., 2011).

Adaptation provides a natural way for a neuron to avoid transmitting predictable and therefore redundant information (Deneve, 2008; Liu and Wang, 2001). It has also been suggested that non-periodic stimulation pulses affect the relation between the level of adaptation and the temporal properties of the output spikes (Liu and Wang, 2001). Moreover, natural spikes in cortical and retinal neurons have stochastic intervals, rather than periodic (Gerstein, 1960; Kuffler et al., 1957; Moore et al., 1966; Wong et al., 2014), but current visual prostheses generally use fixed frequency electrical pulses, with amplitudes

modulated over hundreds of milliseconds, to stimulate the retina (Ayton et al., 2014; Hadjinicolaou et al., 2015a; Pérez Fornos et al., 2012; Stingl et al., 2013; Yue et al., 2016; Zrenner et al., 2011). Our hypothesis is that stimulating RGCs with biphasic electrical pulses that are irregularly distributed in time (with random inter-pulse intervals (IPIs)) will reduce the effect of adaptation in the retina. To test the hypothesis, RGCs were recorded in vitro using patch clamp methods while stimulating the retina with a platinum epiretinal electrode. The electrode was used to electrically stimulate RGCs of rat retina with trains of biphasic pulses delivered with periodic or random IPIs, where the intervals were sampled from an exponential distribution. When recording voltage during electrical stimulation, the recording detects the stimulus in the form of artifacts. After artifact removal, action potentials were extracted from the recordings and the spike rate for the duration of the trains was calculated. We examined the RGC spike rates in response to trains of electrical pulses at a mean rate of 10 Hz, 50 Hz, and 200 Hz with random IPIs and compared them with the responses to constant IPIs at the same rates. Our observations suggest that stimulation with random IPIs leads to lower adaptation rates than stimulation with constant IPIs.

As introduced in Chapter 1, the aims of this thesis are to increase the efficacy of electrical stimulation of RGCs and to explore ways to target specific RGC types during stimulation. In this chapter, we explore a non conventional stimulation protocol to reduce adaptation thus increasing efficacy of the stimulus. We also explore the response of ON and OFF cells to the same stimulus looking for a difference in the response between the two types, hence selectively stimulating either ON or OFF cells. Our results suggest that both aims could be achieved by using the stimulation protocols described in this chapter.

2.2 Methods

2.2.1 Animal preparation

Female pigmented Long-Evans rats between 10 and 18 weeks of age and with an average weight of 0.2 kg were used. The rats were anaesthetised with 100 mg/kg of ketamine and injected with 10 mg/kg of xylazine for muscle relaxation. One eye was then enucleated and an incision was made in the cornea prior to placing the eye in a petri dish filled with carbogenated Ames' solution (Sigma-Aldrich, St. Louis, MO USA). This process was

repeated on the second eye. All procedures performed in this study were in accordance with the ethical standards of the Animal Care and Ethics Committee of The University of Melbourne. All rats were sacrificed by intracardiac injection of 1 ml of Lethabarb (325 mg of pentobarbital sodium) immediately after enucleation of both eyes. All institutional guidelines for the care and use of animals were followed. Only female rats were used because, due to male rat temperament, our lab only stocks females.

Next, the cornea was cut out of the eye and the lens separated from the ciliary body using medical forceps; the lens was then removed from the eye cup to give direct access to the posterior chamber. As much vitreous humour as possible was removed from the eye cup, taking care not to harm the retina. Subsequently, the eye cup was inverted and the retina separated, and kept in the carbogenated Ames' solution. Using a razor blade, the retina was cut in four equal pieces. A single piece of tissue was mounted, ganglion cell layer up, on a cover slip and held in place with a stainless steel harp. The cover slip formed the bottom of a perfusion chamber (RC-26GLP, Warner Instruments, Hamden, CT USA), which was then placed under an upright microscope for visualisation and manipulation. The chamber was continuously perfused with carbogenated Ames' solution at a rate of 3 to 8 ml/min at room temperature ($\sim 24^{\circ}\text{C}$), as explained in detail previously (Cloherty et al., 2012; Hadjinicolaou et al., 2015b; Maturana et al., 2016; Wong et al., 2012). Light response was tested under room temperature, therefore confirmed to be enough to activate photoreceptors. The dissections were performed under dim light.

2.2.2 Whole cell recordings

The current-clamp technique was used to record membrane potentials using methods described previously (Cloherty et al., 2012; Hadjinicolaou et al., 2015b; Hamill et al., 1981; Maturana et al., 2016; Wong et al., 2012). A small hole in the inner limiting membrane was made with a sharp glass pipette to expose RGCs for recording. A recording pipette filled with 7 μL of internal solution was mounted on a pipette holder controlled by a motorised micromanipulator (Sutter Instrument, Novato, CA USA). The initial pipette resistance in the bath was 5-10 $\text{M}\Omega$. Prior to recording, the pipette resistance was compensated with a bridge balancing circuit on the amplifier. Neural signals were amplified (NPI instruments SEC-05X), digitised (National Instruments) and stored for offline analysis. The internal

solution was made of 90 % potassium gluconate (KG), 5 % energy cache (in mM: KCl 5, EGTA 5, HEPES 10, ATP-Na 2, GTP-Na 0.25), 2.5 % biocytin and 2.5 % Alexa Fluor (Thermo Fisher Scientific Inc.).

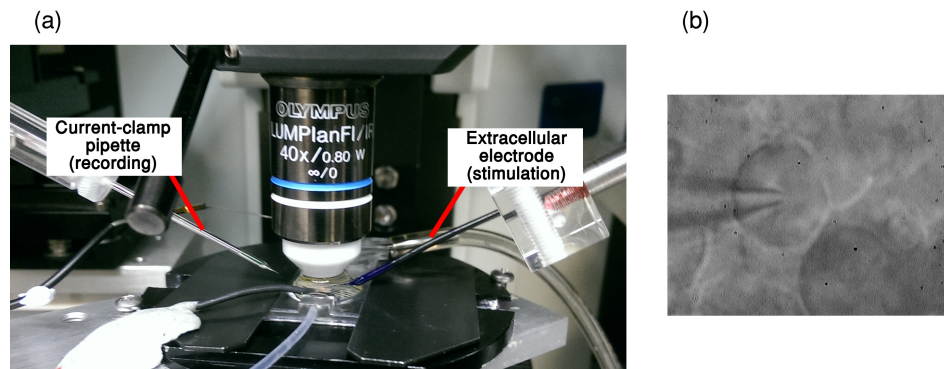


Figure 2.1: Stimulation and recording set-up. **(a)** Retinal tissue is mounted, RGC side up under the lens. A single extracellular, platinum stimulating electrode and an intracellular recording pipette are shown prior to insertion. **(b)** A RGC during whole cell intracellular recording. At the center of the image is the RGC being recorded, the pipette can be seen in contact with the RGC's soma.

2.2.3 Light response

To distinguish ON, OFF and ON-OFF ganglion cells, the patch-clamped cells were stimulated with full-field light for 3 s. Figure 2.2 shows the responses of example ON and OFF cells in response to light stimulation. RGCs that responded with a burst of spikes to the full-field light exposure were classified as ON cells. The burst generally lasted approximately 500 ms after the beginning of the light stimulus. Cells classified as OFF RGCs were those that triggered a spike burst after the 3 s of stimulation, when the light was turned off. ON-OFF cells were RGCs that spiked to both, light on and light off transitions. RGCs were not tested for direction selectivity, hence, it is a possibility that if any of the recorded RGCs was direction-selective, it would have been included in the study.

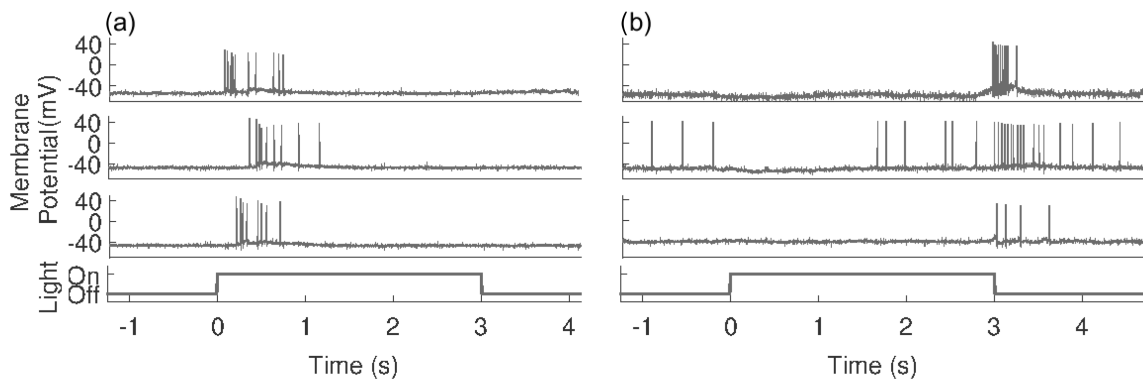


Figure 2.2: Response to full-field light stimulation of **(a)** three example ON cells, and **(b)** three example OFF cells.

2.2.4 Blocking action potentials

In some experiments the sodium channel blocker tetrodotoxin (TTX) was used at a concentration of $1 \mu\text{M}$ to block sodium channels without affecting the membrane potential of the cells (Narahashi, 1964). TTX was dissolved in a bottle of Ames' solution, which replaced the regular Ames' solution.

2.2.5 Electrical stimulation

A single platinum stimulating electrode was placed on the inner limiting membrane approximately $40 \mu\text{m}$ away from the target RGC's soma prior to patching the cell. The RGC was then patched for whole cell recording. To get a consistent stimulation amplitude (A_s) for the whole experiment, we conducted a stimulus calibration. The calibration consisted of stimulating the cell with constant IPI trains of biphasic pulses at 10 Hz, 50 Hz, and 200 Hz. A value for A_s was chosen as the lowest current amplitude that resulted in the maximum spike rate during stimulation with constant IPIs (Figure 2.3a). During this stimulus calibration, the amplitude of each train was initially set to $50 \mu\text{A}$ and was increased or decreased in steps of $10 \mu\text{A}$ until A_s was identified. This amplitude was then used in the main experiment for both regular and irregular pulse trains.

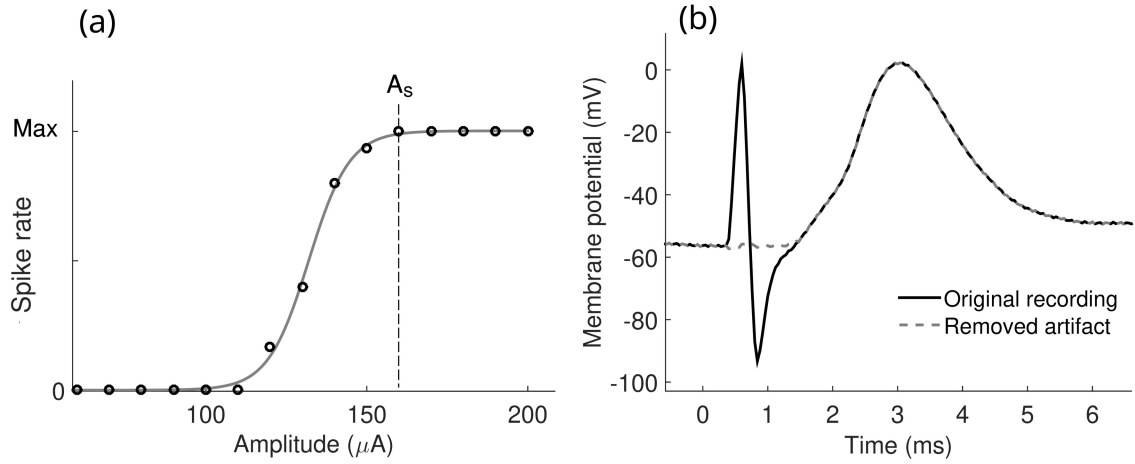


Figure 2.3: Stimulation amplitude and artifact removal. **(a)** Stimulus calibration of an example OFF cell at 10 Hz. Circles represent the spike rate at each stimulation amplitude. A_s was the amplitude that maximized the spike rate when stimulating with constant IPI stimulation. **(b)** A recording of a spike with artifact (solid line) and without artifact (dashed line). The stimulus was a biphasic pulse of 200 μ s per phase and 20 μ s between phases.

The stimulation trains consisted of biphasic, charge balanced, anodic-first pulses. Each pulse had an amplitude of A_s and a duration of 200 μ s per phase, with a 20 μ s gap between phases. Each train was 3 s long and contained either 10, 50, or 200 pulses per second. Anodic-first pulses facilitated data analysis because action potentials did not overlap with the positive peak of the stimulus artifact, hence making it easier to remove artifacts and detect spikes.

The trains were constructed according to one of the following two paradigms:

Constant Inter-Pulse Intervals: The inter-pulse interval was set equally for all pulses according to the stimulation frequency. An example constant IPI stimulus train at 10 Hz is presented in Figure 2.4c.

Random Inter-Pulse Intervals: The inter-pulse interval had a distribution set by a homogeneous Poisson process with the desired mean rate. In a homogeneous Poisson process, the number of pulses in a fixed interval of time has a Poisson distribution, and the time between consecutive pulses ($IPI = x$) is chosen from an exponential distribution. Its probability density function is given by:

$$P(x; \mu) = \frac{1}{\mu} e^{-\frac{x}{\mu}} \quad \forall x \geq 0, \quad (2.1)$$

where μ is the mean of the distribution ($\mu = \frac{1}{f}$). Random IPIs for each train were calculated using the exponential distribution from Equation 2.1, with μ being the mean IPI for each rate (10, 50, or 200 pulses per second), e.g. for 10 pulses per second, $\mu = 0.1$ s. To avoid pulses overlapping, a minimum IPI was set to be the total length of each pulse (420 μ s). Each sequence of random IPI biphasic pulses is used only once, generated in MATLAB from an exponential distribution. To ensure truly random and unique sequences are used, the seed of MATLAB's random number generator is randomized at the startup. An example random IPI stimulus train at 10 Hz is presented in Figure 2.4d.

Each cell was stimulated with 30 trains of random and constant IPI pulses at each frequency (10 Hz, 50 Hz, and 200 Hz). In total, every cell was stimulated with 180 trains lasting 3 s each. Rest periods consisted of 5 s of no stimulation between trains. The type (random or constant IPI) and frequency of train stimulation were randomised over the experiment.

2.2.6 Data analysis

To detect spikes and analyse the membrane potential, stimulus artifacts, which lasted up to 1.2 ms from stimulus onset, were removed from the recordings. All artifacts from a recording were aligned in time and their average waveform was calculated. Then, the average waveform was aligned with each individual artifact and scaled until the minimum difference between averaged artifact and actual artifact was achieved. This was repeated for all artifacts. Figure 2.3b shows an example of recording with and without artifact removal. After artifact removal, a default threshold for spike detection was set as -20 mV (each recording was visually inspected to validate the threshold). Any peak above threshold within 5 ms from a biphasic pulse onset was counted as a spike.

A moving average of the spike rate during the 3 s of each train was calculated using Equation 2.2. That is, a moving window of 300 ms, shifted 50 ms at each iteration was used to analyse the data.

$$r(t_i) = \frac{1}{Y} \int_{t_i}^{t_i+Y} \langle \rho(\tau) \rangle d\tau, \quad (2.2)$$

where $r(t_i)$ is the average spike count for all trials during a period $Y = 300$ ms, $t_i = (i - 1)50$, $i = 1, 2, \dots, 55$, $\rho(\tau)$ represents the spike sequence for a single trial, and $\langle \rho(\tau) \rangle$ is the average over all trials. Due to the nature of the stimulus with random IPIs, the moving average spike rate declines steeply during the last 300 ms of recording. This is an effect of the sliding window and does not relate to the adaptation of the cell. Therefore, our results omit the last 300 ms of stimulation when presenting the moving average data.

To quantify the amount of adaptation we looked at two measures based on the spike rate from Equation 2.2. Firstly, we fitted the spike rate to a first order exponential curve $f(t) = ke^{-\frac{rt}{\tau}}$, where r represents the spike rate and k is a constant. The exponential decay rate can be quantified with the time constant (τ), where a smaller τ means a faster decay rate, indicating more rapid adaptation. τ_C represents the decay rate of a response to constant IPI stimulation, while τ_R represents the decay rate of a response to random IPI stimulation. Secondly, Equation 2.3 provides a quantitative measurement of the adaptation. Adaptation rate (R_A) is the ratio of the difference in spike rate measured at $T = 2s$ from stimulus onset and the initial spike rate, where $R(t)$ is the spike rate at time t . All significance tests are two sample one tailed t-tests, calculated using MATLAB's function `ttest2` (MATLAB R2014b, The MathWorks), where $t(df)$ is the t-value of the test with df degrees of freedom. Outliers were identified using the interquartile range rule.

$$R_A = \frac{R(T) - R(0)}{R(T) + R(0)} \quad (2.3)$$

2.3 Results

Stimulation and recording were conducted on 27 RGCs. Limitations in holding the patch clamp on cells meant that some of the cells were not stimulated at all frequencies. Seventeen cells were stimulated with biphasic electrical pulses at 10 Hz, 23 cells at 50 Hz, and 25 cells at 200 Hz. 12 cells were classified as ON, 8 were classified as OFF, 4 were classified as ON-OFF, and 3 were unclassified due to a lack of response to light. The lack of light response may have resulted from damage to the photoreceptors during dissection.

An example of the response to constant (blue) and random IPI (red) stimulation of an OFF cell is shown in Figure 2.4a-d. A common observation throughout the recordings was the presence of spikelets, or low amplitude action potentials (generally less than half the amplitude of normal spikes, see Figure 2.4e and the section below titled Spikelets). It is not the purpose of this paper to present spikelets as a contribution to reducing adaptation of RGCs or visual fading. However, in the pursuit of thoroughness, we believe that it is essential to report their regular occurrence. Analysis of only the large amplitude spikes will be presented initially as the main focus of our study and the bulk of the relevant results, then we include an analysis with both, spikes and spikelets.

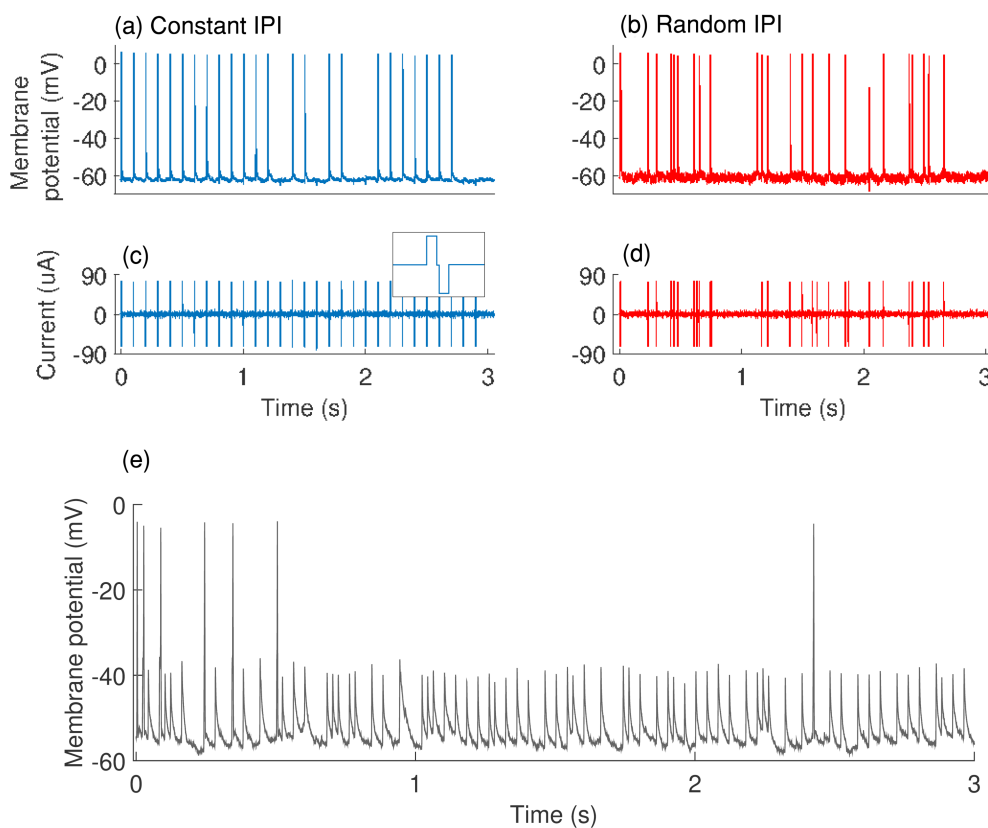


Figure 2.4: Response of an OFF cell to **(a)** 10 Hz constant IPI stimulation and **(b)** 10 Hz random IPI stimulation. Stimulation train with **(c)** constant IPI at 10 Hz and **(d)** random IPI at 10 Hz. The number and shape of the biphasic pulses are the same in both trains (c and d). Insert in (c) shows one pulse from the stimulation train. **(e)** Membrane potential of an example ON-OFF cell during 50 Hz constant IPI stimulation. The recording presents 7 spikes and 66 spikelets in response to the stimulus. Stimulus artifacts have been removed from the recordings.

Figure 2.5 illustrates a raster plot for a representative cell responding to 10 Hz, 50 Hz, and 200 Hz stimulation. When observing the rasters, there is only a small difference

in the number of spikes between the 10 Hz constant IPI stimulation and the random IPI stimulation. This cell's spike rate is reduced in response to constant IPI stimulation at 50 Hz and 200 Hz (blue) after a few hundred milliseconds. A decay is also observed in the responses to random IPI stimulation at 50 Hz and 200 Hz (red) but it is less pronounced.

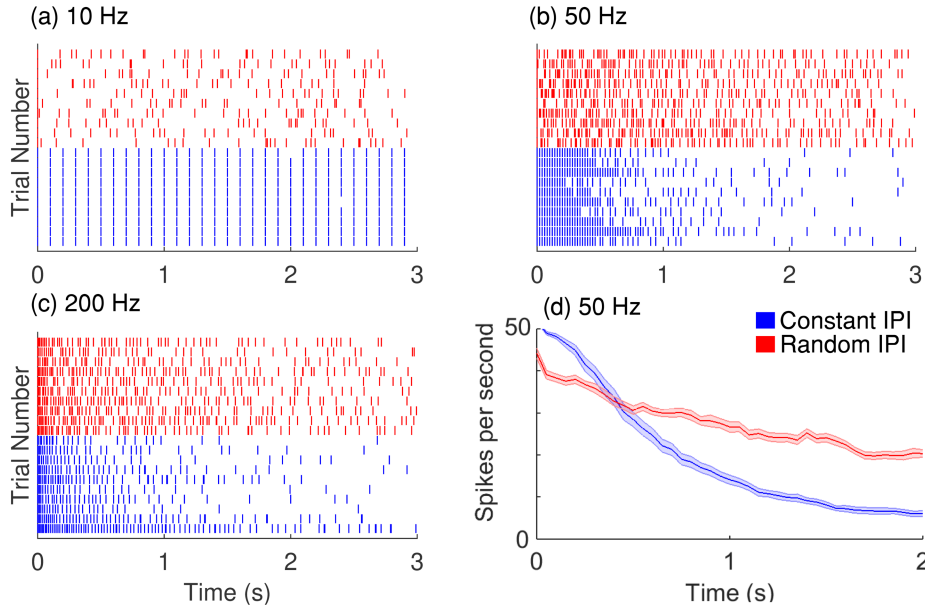


Figure 2.5: Raster plot of the response of a representative ON cell to **(a)** 10 Hz, **(b)** 50 Hz, and **(c)** 200 Hz electrical stimulation. Red raster represents full spikes in response to random IPI stimulation. Blue raster represents full spikes in response to constant IPI stimulation. **(d)** Moving average of the spike rate of this cell in response to constant and random IPI stimulation at 50 Hz.

Figure 2.6 compares the spike rate averaged over all cells in the population for regular and Poisson distributed pulses for each of the three stimulation frequencies (10 Hz, 50 Hz, and 200 Hz). Figure 2.6a shows the mean spike rate over 3 s in response to stimulation with constant IPI and random IPI for each frequency (10 Hz, 50 Hz, and 200 Hz). The number of spikes per train with constant IPI stimulation at high frequencies (50 and 200 Hz) was shown to be significantly lower than with random IPI stimulation at the same frequencies ($t(1378) = 1.7924, p = 0.037$ and $t(1538) = 6.3623, p = 1.3 \times 10^{-10}$, respectively). However, the number of spikes is not significantly different in response to constant IPI and random IPI stimulation at 10 Hz ($t(1018) = 0.712, p = 0.238$).

As illustrated in Figure 2.5, the spike rate is higher during the first 300 ms for all frequencies and protocols. Figure 2.6b shows the spike rate during this initial period, where only the response to 200 Hz stimulation is significantly different between IPI protocols

($t(1538) = 2.6024, p = 0.005$). A comparison between Figures 2.6a and 2.6b translates into a greater reduction in the number of spikes after the first 300 ms of stimulation at 50 Hz and 200 Hz (higher adaptation with constant IPIs).

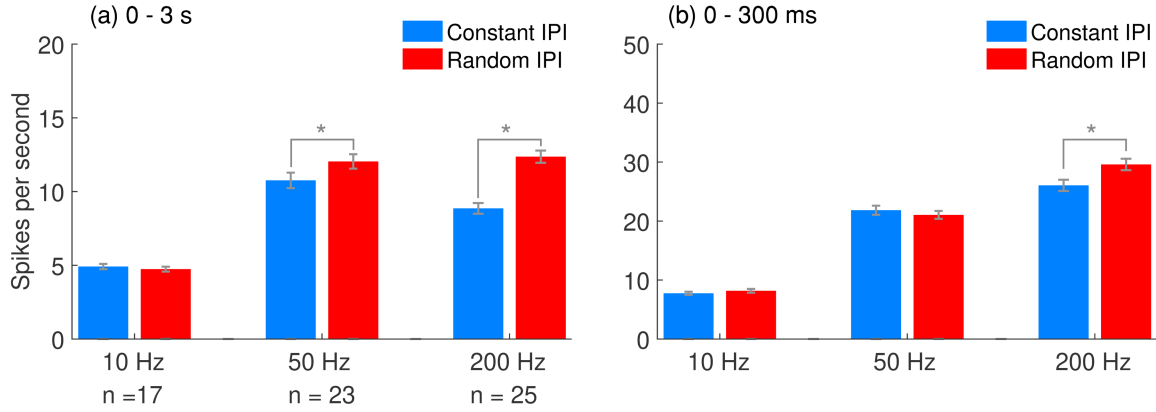


Figure 2.6: Average spike rate for all cells in response to constant IPI and random IPI stimulation during (a) the whole stimulation duration, and (b) the first 300 ms of stimulation. Spike rate is averaged over n cells depending on the frequency; error bars represent the s.e.m. A star (*) represents $p < 0.05$.

Figure 2.7a,c,e shows the moving average of the spike rate for all cells during stimulation. All cells showed a reduction in the spike rate during the stimulation period. For stimulation at 50 Hz and 200 Hz, the reduction in the spike rate was more pronounced. Moreover, the spike rate in response to constant IPIs decayed faster ($\tau_C(50Hz) = 2.34$ s, $\tau_R(50Hz) = 3.42$ s, $\tau_C(200Hz) = 1.24$ s and $\tau_R(200Hz) = 1.75$ s) than in response to random IPI stimulation at 50 Hz and 200 Hz (blue vs red lines, Figure 2.7c,e). This difference in the rate of adaptation did not occur for 10 Hz stimulation where the adaptation to random IPI stimulation and constant IPI stimulation was very similar (Figure 2.7a).

Figure 2.7b,d,f illustrates the response of all ON and OFF cells (averaged across cells) to constant and random IPI stimuli at 10 Hz, 50 Hz, and 200 Hz. The subplots in Figure 2.7b,d,f depict a clear difference between spike rates in the responses of ON and OFF cells to stimulation with different frequencies. There is no significant difference in the spike rate between random IPI and constant IPI stimulation at 10 pulses per second. A slower rate of adaptation to random IPIs than to constant IPIs was observed for the 50 Hz and 200 Hz stimuli, regardless of the cell type (ON or OFF). At 50 Hz, the difference in spike rate between responses to random and constant IPIs is higher for ON cells. At 200 Hz, the adaptation in OFF cells follows an exponential decay for both protocols, however, it occurs

faster with constant IPI stimulation.

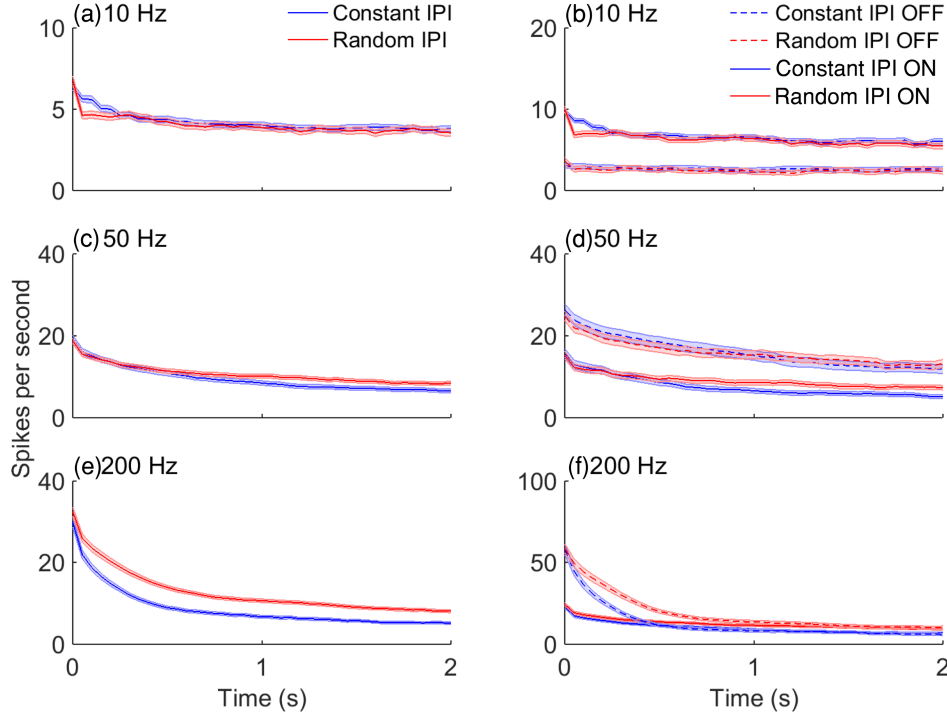


Figure 2.7: Moving average of spike rate over all cells for the duration of the stimulus train at **(a)** 10 Hz, **(c)** 50 Hz, and **(e)** 200 Hz. Red: random IPIs, blue: constant IPIs. Average spike rate of all ON ($N = 12$) and all OFF ($N = 8$) RGCs in response to stimulation at **(b)** 10 Hz, **(d)** 50 Hz, and **(f)** 200 Hz. The shaded areas represent the s.e.m.

Almost every cell showed a slower decay rate with random IPI (τ_R) stimulation than with constant IPI stimulation (τ_C) at 50 Hz and 200 Hz (Figure 2.8). At 10 Hz, the results suggest no difference between random IPIs and constant IPIs. Every marker in Figure 2.8a represents the decay rate of the response of a single cell to random IPI stimulation (τ_R) against constant IPI stimulation (τ_C). Markers that fall on the diagonal line indicate a cell whose decay rate is the same for constant IPI and random IPI stimulation, $\tau_C = \tau_R$. A marker below the diagonal indicates a cell that has a faster decay rate when stimulated with constant IPIs, i.e., less adaptation to random IPI stimulation. The average τ_R and τ_C over all cells are shown in Figure 2.8c, and subplot f shows an example of the exponential fit from which τ_R and τ_C are taken. This is the exponential fit of the recordings in Figure 2.5d. Figure 2.8b shows more rapid adaptation for random IPI stimulation at 10 Hz, but a lower adaptation ratio at 50 Hz and 200 Hz when stimulating with random IPIs. The adaptation ratio (Figure 2.8e) using the unitless difference over sum calculation of Equation

3 is consistent with the calculations of τ_C and τ_R . It shows a faster adaptation to constant IPI than to random IPI stimulation at 50 Hz and 200 Hz. Figures 2.8b and 2.8d suggest that the spike rate after adaptation was higher in response to random IPIs at 50 Hz, and 200 Hz. Outliers have been identified and removed from the data presented in subplots a-d.

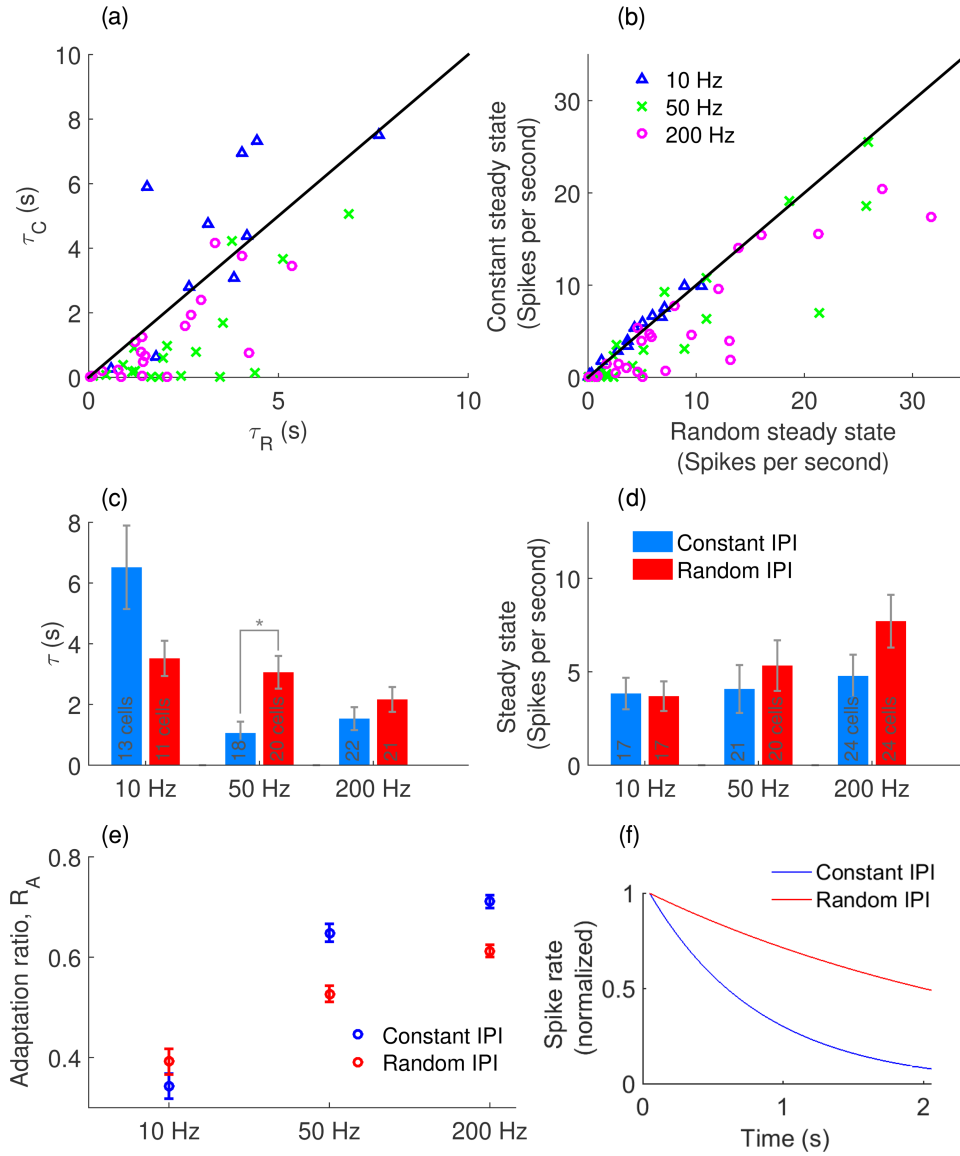


Figure 2.8: Adaptation. **(a)** Time constant of the exponential fit in response to random IPI stimulation (τ_R) and constant IPI stimulation (τ_C). Each marker represents one cell: note that the time constants are most often larger for random IPIs, especially at 50 Hz and 200 Hz stimulation. **(b)** Spike rates during the steady state in response to stimulation with random IPIs vs constant IPIs. **(c)** Average time constant in response to random IPI stimulation (τ_R) and constant IPI stimulation (τ_C). A star (*) represents $p < 0.05$. **(d)** Average spike rate during the steady state in response to random IPI stimulation and constant IPI stimulation. Upon inspection of the average spike rate of each cell, the spike rate at steady state was chosen as the mean value between $t=1.5$ s and $t=2$ s. **(e)** Ratio of the difference in spike rate measured at 2 s from stimulus onset and the initial spike rate (Equation 2.3). **(f)** Example exponential fit of the recordings in Figure 2.5d. Error bars represent s.e.m.

This study was done with anodic-first biphasic pulses, however, we have also explored the performance of irregular IPI stimulation with cathodic-first biphasic pulses. We have

stimulated 5 different cells with cathodic-first pulses. The results show consistency with the response to anodic-first stimulation. Figure 2.9a-c show the spike rate over the recorded cells during stimulation. The spike rate at 50 Hz and 200 Hz is higher when the cells are stimulated with random IPI stimulation. The time constant is also significantly higher for random IPI stimulation at 50 Hz and 200 Hz, indicating that the decay is slower.

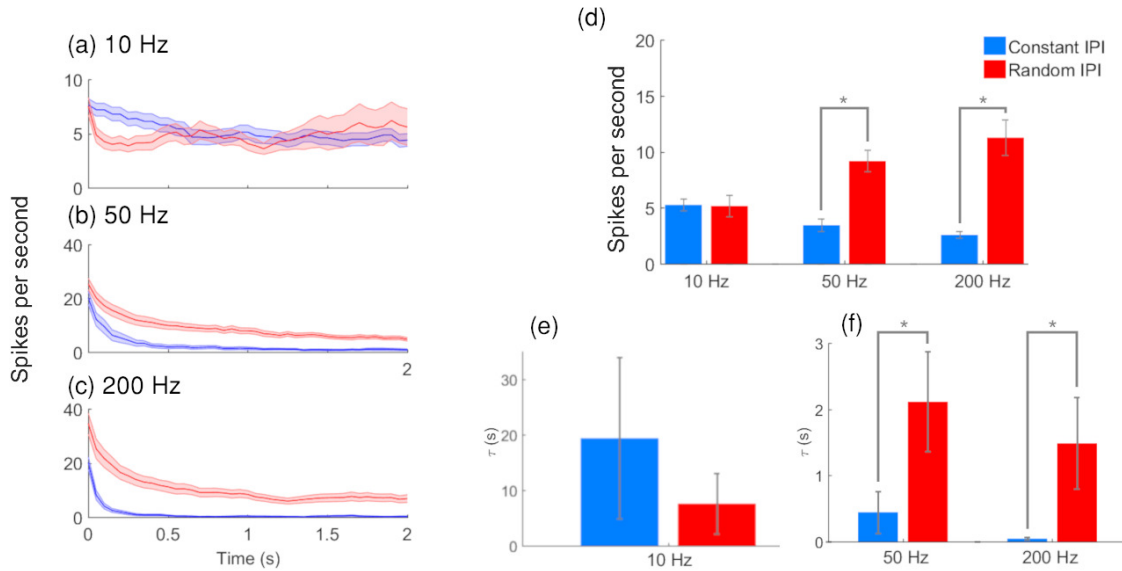


Figure 2.9: Cathodic first stimulation. Spike rate over time in response to stimulation at **(a)** 10 Hz, **(b)** 50 Hz, and **(c)** 200 Hz. **(d)** Mean spike rate during stimulation. Time constant of the exponential fit in response to stimulus at **(e)** 10 Hz, **(f)** 50 Hz, and 200 Hz. Error bars represent s.e.m. A star (*) represents $p < 0.05$.

Spikelets

Two types of spikes with different amplitudes were observed in 18 of 27 cells (Figure 2.10). When the peak membrane potential of a spike was lower than -20 mV (upon visual validation of the distribution, Figure 2.10c-e), it was classified as a spikelet. Spikelets were not considered in the data presented in Figures 2.5 - 2.8. However, they were observed in our recordings (e.g., the recording in Figure 2.4d presents 66 spikelets and only 7 spikes). It is not clear whether these spikelets transmit along the axon but we decided to analyse their characteristics to give a full description of the data collected.

Figure 2.10a-b illustrates the response of an OFF cell with both spikes (peak at ~ 35 mV) and spikelets (peak at ~ -20 mV). Figure 2.10a contains a segment of the recording where a cell was stimulated consecutively 3 times. The first biphasic pulse elicited a spike

(circle), the second biphasic pulse resulted in a spikelet (cross) and the third biphasic pulse did not generate any response from the cell. Artifacts of the 3 biphasic pulses are marked with a black triangle. The response to every biphasic pulse was aligned at stimulus onset and superimposed in Figure 2.10b. The figure shows the presence of spikes (black lines), spikelets (purple lines), and no response (gray lines) within the same stimulus train. It also illustrates that the latency of the spikes and spikelets was similar. The distribution of spike amplitudes for an example OFF cell is shown in Figure 2.10c-e. In this example, full spikes had an amplitude of ~ -10 mV, while spikelets presented a peak amplitude at ~ -40 mV. Note that the recordings in Figure 2.10a-b were taken from a different cell to those in Figure 2.10c-e. The proportion of spikelets vs spikes tended to increase with higher stimulation frequencies.

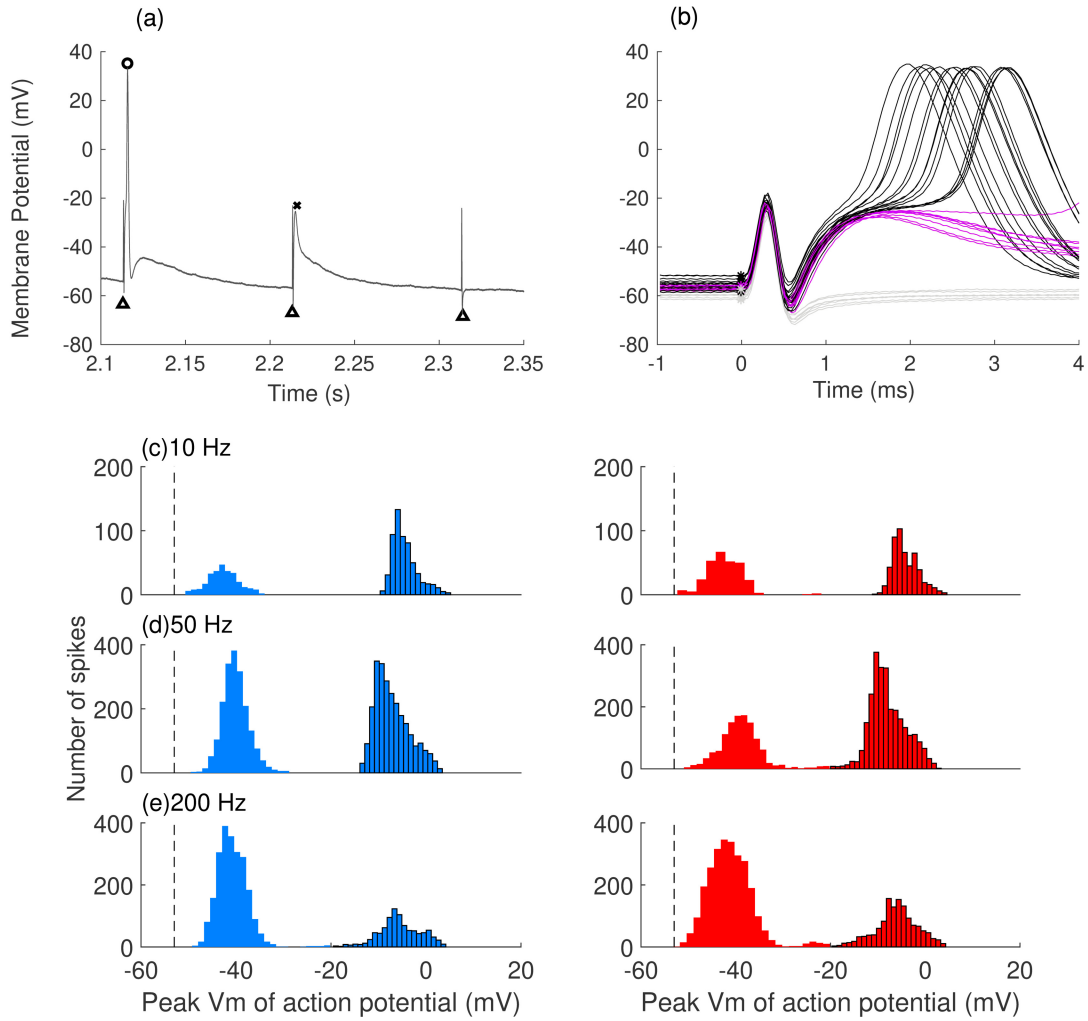


Figure 2.10: Membrane potential of an OFF cell in response to constant IPI electrical stimulation at 10 Hz. **(a)** Segment of 250 ms of recording with a spike (circle), a spikelet (cross), and no response. Black triangles indicate the time of stimulation. Artifacts were not removed in this recording. **(b)** The superimposed response of the same cell as in (a) to every biphasic pulse within 3 s of stimulation: stimulus onset is at 0 ms (asterisk). **(c-e)** Distribution of the amplitudes of spikes and spikelets of an example OFF cell. Action potentials in response to constant IPI stimulation (blue), and random IPI stimulation (red) at **(c)** 10 Hz, **(d)** 50 Hz, and **(e)** 200 Hz. Action potentials with the highest peak were considered to be spikes, while action potentials in the lower distribution were classified as spikelets. The black dotted line is the resting membrane potential.

Figure 2.11a shows the number of spikelets per second for both regular and Poisson distributed pulses at each stimulation frequency. While the number of spikelets per second in response to 10 Hz is comparatively small, higher frequencies have a greater spikelet rate, particularly in response to constant IPI stimulation. Moreover, Figure 2.11b shows that the proportion is less than 1 spikelet per spike in response to stimulation at 10 Hz, but it is significantly higher in response to 50 Hz ($t(166) = 2.4625, p = 0.007$), and 200

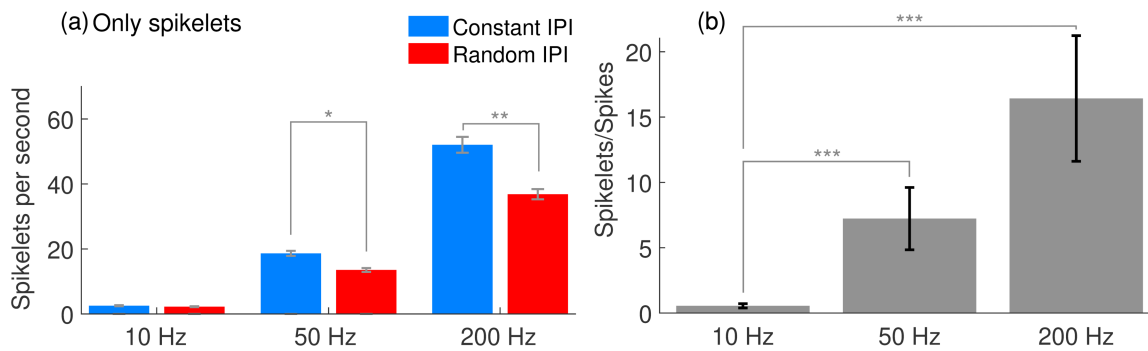


Figure 2.11: Number of spikelets **(a)** Average number of spikelets per second for all cells in response to constant IPI and random IPI stimulation during the duration of the stimulus. A star (*) represents $t(1378) = 5.2275, p = 9.9 \times 10^{-8}$, two stars (**) represent $t(1538) = 5.2338, p = 9.4 \times 10^{-8}$. **(b)** Proportion of spikelets per spike in response to constant IPI and random IPI stimulation at 10 Hz, 50 Hz, and 200 Hz, three stars (***) represent $p < 0.01$. Error bars represent s.e.m.

Hz ($t(166) = 2.5477, p = 0.006$). This is reflected in Figure 2.12, in which we repeat the analysis presented in Figures 2.6b, 2.7, and 2.8b but this time taking into account both the spikes and the spikelets. Results show that the response decay is much smaller for 50 Hz and 200 Hz when spikes and spikelets are counted together than when only spikes are considered, regardless of the stimulation protocol (compare Figure 2.7 and Figure 2.12a-c and e). Figure 2.12d presents the number of spikes and spikelets per second for the 3 different stimulation frequencies. While adaptation is reduced when spikelets are included, there is a significantly higher firing rate with constant IPI stimulation than with random IPI, which is the opposite to what was observed when only spikes were considered.

With 50 Hz and 10 Hz stimulation, a small difference in the spikelet/spike ratio was observed between ON and OFF cells. See table 2.1 for number of spikelets per spike observed in ON and OFF cells.

Table 2.1: Spikelets per spike ratio in ON and OFF cells:

Light response	Frequency (Hz)	Constant IPI	Random IPI
OFF	10	0.42	0.4
	50	0.75	0.58
	200	5.2	2.4
ON	10	0.25	0.2
	50	1.9	1.1
	200	4.5	2.3

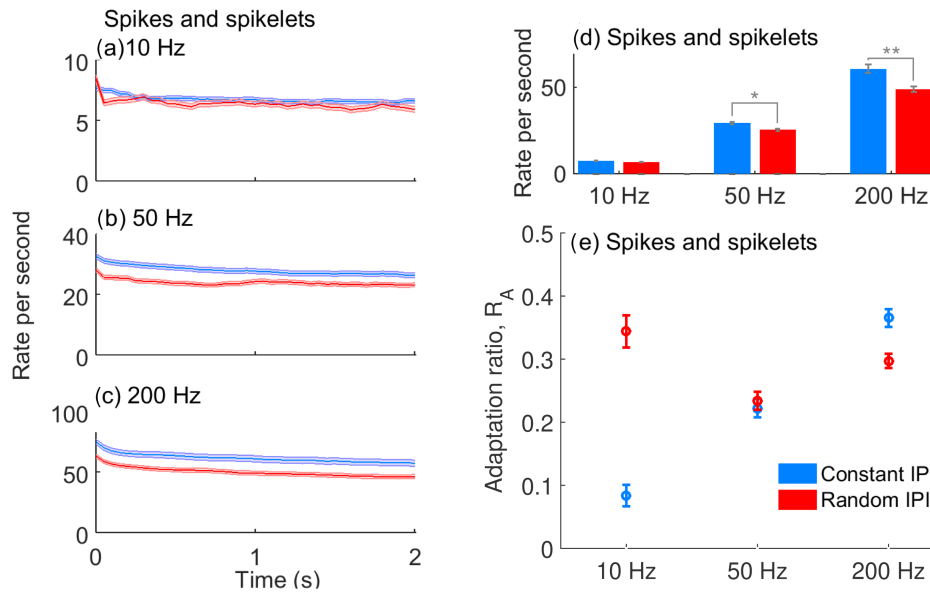


Figure 2.12: Moving average of spike rate (spikes and spikelets) for all cells for the duration of the stimulus train at **(a)** 10 Hz, **(b)** 50 Hz, and **(c)** 200 Hz. Red: random IPI, blue: constant IPI. The shaded areas represent the s.e.m. **(d)** Average number of spikelets and spikes per second for all cells in response to constant IPI and random IPI stimulation during the duration of the stimulus. A star (*) represents $t(1378) = 3.8277, p = 6.8 \times 10^{-5}$, two stars (**) represent $t(1538) = 4.0219, p = 3.0 \times 10^{-5}$. **(e)** Ratio of the difference in spike rate (spikes and spikelets) measured at 2 s from stimulus onset and the initial spike rate (Equation 3). Error bars represent s.e.m.

The use of the sodium channel blocker TTX in the bath solution helped us explore the nature of spikelets and the effect of sodium channels on the presence of spikelets. Application of TTX abolished normal spike responses to light stimulation. Figure 2.13a-b shows the response to light of an ON cell before and after TTX was applied, respectively. Four cells were stimulated with the usual protocol of random and regular electrical stimulation before and after the application of TTX (Figure 2.13c-d). Both spikes and spikelets were abolished in all four cells as a result of blocking the sodium channels. The insert in Figure 2.13c provides a closer look at the response to two biphasic pulses: the first pulse elicited a full action potential, the second pulse evoked a spikelet. There was no presence of spikes or spikelets in response to electrical stimulation when the TTX was present (Figure 2.13d), indicating that the spikelets require sodium channel activation.

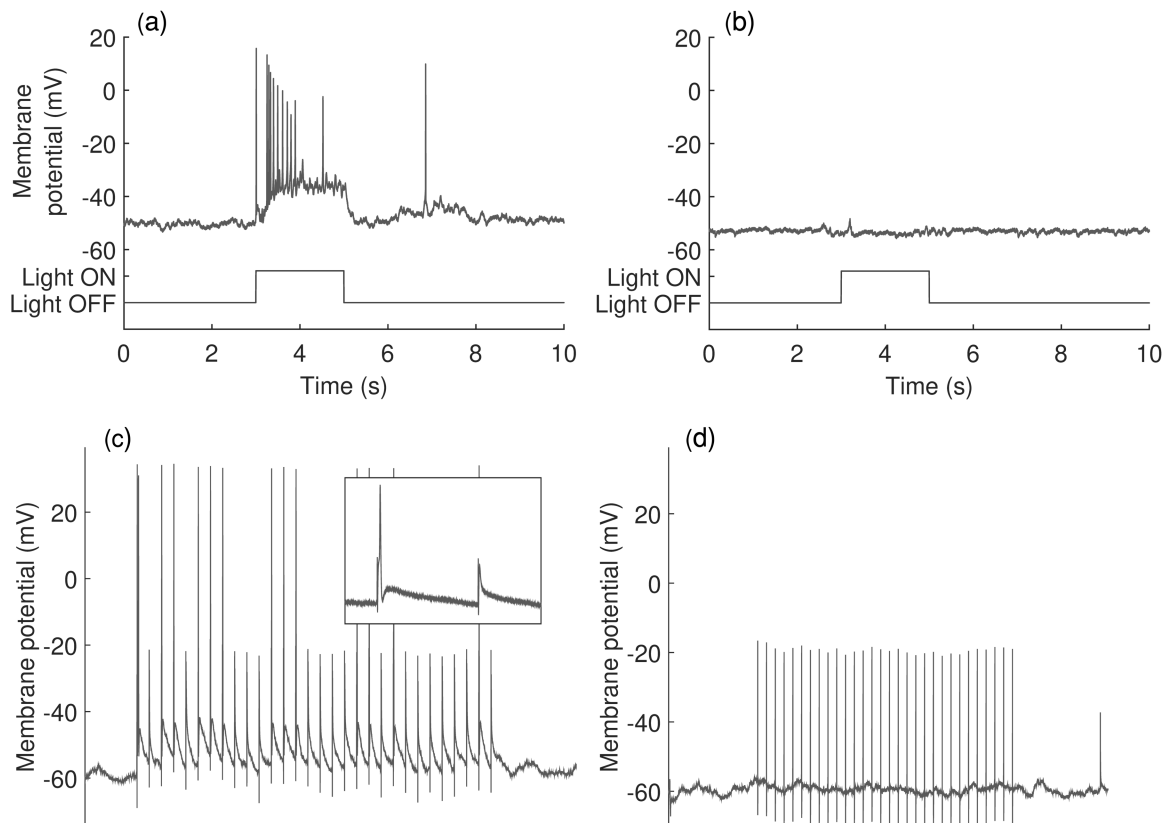


Figure 2.13: Membrane potential of an ON RGC in response to full-field light stimulation for 2 seconds **(a)** before application of TTX and **(b)** after application of TTX. Response of a RGC to constant IPI stimulation at 10 Hz **(c)** before application of TTX and **(d)** after application of TTX. Artifacts were not removed from these recordings.

2.4 Discussion and Conclusion

We observed higher mean spike rates for full action potentials in the responses of RGCs to high stimulation frequencies (50 Hz and 200 Hz) with random IPIs compared to constant IPIs ($t(1378) = 1.7924, p = 0.037$). No significant difference was observed with stimulation at 10 Hz between random IPIs and constant IPIs. The results also show a higher decay rate soon after stimulus onset in response to constant IPI stimulation, compared to the response to random IPI stimulation at 50 Hz and 200 Hz. Conversely, stimulation at 10 Hz shows a higher decay rate with random IPIs than with constant IPIs. We also observed the presence of spikelets, which when taken into account, result in a higher spike rate for 50 Hz and 200 Hz stimulation when using constant IPIs compared to random IPIs. Stimulation with brief bursts of pulses, alternating with periods of inactivity, also shows a reduction in spikelets at 50 and 200 Hz.

When considering full action potentials, it is evident that stimulation at higher rates (50 and 200 pulses per second) generates significantly faster adaptation when equally spaced IPIs are used, than it does when pulses are Poisson distributed (Figures 2.5, 2.7, and 2.8). Such differences in the rate of adaptation translate into a higher spike rate when stimulating with random IPIs (Figure 2.6a). This is not true for stimulation frequencies of 10 Hz. Although no other study has explored stimulation with random IPIs in the retina, some studies have explored random, or pseudo-random IPIs in electro-myostimulation (Franken et al., 1993; Guimaraes et al., 1994) and deep brain stimulation (Feng et al., 2007; Grill and Dorval, 2015; Tass, 2001). In addition, there has been previous work in the retina with random stimulus amplitudes (white noise) (Freeman et al., 2010; Maturana et al., 2016; Sekhar et al., 2016), and time varying stimuli (Davuluri and Weiland, 2014), showing that regular stimulation of RGCs, as is the case with constant IPIs, might drive RGCs to lower spike rates and more rapid adaptation than with irregular stimulation. Previous retinal studies have reported a reduction in the responses to continuous stimulation (constant IPI pulse trains) at frequencies ranging from 1.5 Hz to 200 Hz (Davuluri and Weiland, 2014; Freeman and Fried, 2011; Jensen and Rizzo, 2007; Tsai et al., 2009). Such response decay is magnified at higher frequencies, generally above 50 Hz (Ahuja, 2007; Jensen and Rizzo, 2007; Tsai et al., 2011a,b). Our results support these findings, and provide a way of reducing the decay in spike rate for stimulation frequencies of 50 Hz and 200 Hz. Note that although the difference in τ was not significant at 200 Hz, τ_R was higher than τ_C in

all but one cases (Figure 2.8a). Another interesting finding was that the spike rate was higher in OFF RGCs than in ON cells at 50 Hz and 200 Hz stimulation frequencies. In contrast, the spike rate at 10 Hz was higher in ON cells (Figure 2.7b,d,f). This is interesting because both cell types preserved the difference in adaptation between constant IPIs and random IPIs at 50 Hz and 200 Hz, but presented a different response at 10 Hz, i.e. the spike rate in response to stimulation at 10 Hz is higher in ON cells than in OFF cells, but at 50 Hz and 200 Hz, the spike rate is lower in ON cells. This could suggest that the responses of OFF cells to higher frequency stimulation have a higher spike rate than ON cells, and faster adaptation, while ON cells have a relatively higher spike rate in response to lower frequencies. Im and Fried (2016) have observed a reduced adaptation to constant stimulation at less than 10 Hz, but only in ON cells. This coincides with our observations in two ways: first, stimulation of ON cells at low frequencies is more effective than stimulation of OFF cells. Second, it is possible to reduce adaptation of RGCs in some fashion by altering the stimulus. The coincidence with Im and Fried (2016) becomes more evident when we analyse the difference in spikelet vs spike ratio between ON and OFF cells. For the low frequency of 10 Hz, ON cells showed a ratio of 0.25 spikelets per spike (constant IPI) and 0.2 spikelets per spike (random IPI). On the other hand, OFF cells had a ratio of 0.42 spikelets per spike (constant IPI) and 0.4 spikelets per spike (random IPI). The information loss in OFF cells at 10 Hz is twice as high as in ON cells, regardless of the IPI protocol. Moreover, at 50 Hz, the information loss is much higher for ON cells, having almost 2 spikelets per spike with constant IPI, i.e. one third of the information is lost in the response of ON cells at 50 Hz. OFF cells had only 0.75 spikelets per spike at 50 Hz with constant IPI stimulation. This could suggest a possibility for selective stimulation of a specific RGC type.

We have explored different methods to quantify adaptation. The time constant τ_R and τ_C (Figure 2.8a and c) of the response to random IPIs and constant IPIs, respectively, was taken from an exponential fit of the spike rate (Figure 2.8f). These τ values combined with the spike rate after adaptation (steady state, Figure 2.8b and d) show that adaptation is reduced when the stimulus has random IPIs, instead of constant IPIs at 50 Hz and 200 Hz. The adaptation rate A_R provides an index of the difference in spike rate between two different times in the stimulation sequence. Our calculations of A_R also suggest a higher rate of adaptation in response to constant IPIs at higher frequencies. Indirect activation of RGCs was not considered in this work, only short-latency action potentials (within 5 ms

from the stimulus onset) were taken into account.

The duration of RGCs action potentials and refractory period at room temperature is generally no more than 1 ms, hence our highest stimulation frequency of 200 Hz should not have any issue in activating the neurons, if adaptation did not exist (Fohlmeister et al., 2010). This is evidently not the case, and Tsai et al. (2009) has previously reported the effect of high stimulation frequencies (50 Hz and 200 Hz) on the inactivation of sodium channels and its effect on response decay. Sodium channel inactivation would prevent a neuron from spiking after a small number of consecutive stimulation pulses, one of the possible mechanisms behind the success of random IPI is that some of the biphasic pulses will be separated by a longer period, which would give time for the sodium channels to recover from inactivation (Tsai et al., 2009). On the other hand, some pulses might be too close together and fall within the refractory period of the neuron in response of the previous pulse.

The presence of small amplitude spikes, or spikelets, in RGCs and other neurons has been previously explored (Cai et al., 2011; Hu et al., 2009; Tsai et al., 2011b). These studies suggest that the possible cause for spikelets is a lack of sodium channel activation during high frequency stimulation, which subsequently accounts for the neuron's failure to produce a full size action potential (somatodendritic) when repeatedly stimulated with more than 50 pulses per second. However, other studies have emphasized the existence of electrical currents in the retina, like axo-axonal gap junctions, which could cause an inhibition in the neuron, thus preventing it from firing a full action potential (Schmitz et al., 2001). Hu et al. (Hu et al., 2009) argue that the action potential initiation occurs in the distal axon initial segment (AIS) while the sodium channels in the proximal AIS promote backpropagation to the soma, which translates into a full action potential. High frequency stimulation leads to a decrease in the number of open sodium channels, which might account for the failure to elicit full action potentials while allowing the presence of spikelets (Figure 2.11a), and could also be a cause of adaptation in some RGCs. Stimulating with brief bursts of pulses, interleaved by longer periods of inactivity could prevent the inactivation of sodium channel currents, thus minimizing adaptation of RGCs (Hu et al., 2009; Tsai et al., 2011a,b), as suggested by Figure 2.11a, which shows a reduction in spikelets at 50 Hz and 200 Hz with random IPIs. Random IPIs would have some brief bursts alternating with periods of inactivity.

Our recordings show similar shapes and latencies between spikes and spikelets (Figure 2.10), with the only differences being, firstly, the obvious peak amplitude. The second difference is a hyper polarization, observed only in the full action potentials, but absent in the spikelets. Hyper polarization is due to rectifying potassium channels, and it only happens when the cell is highly depolarized, as occurs when a full spike is elicited (Hodgkin and Huxley, 1952). Spikelets observed in our recordings have a much lower membrane potential during the peak than the full action potentials. We believe the reason for the lack of hyper polarization in spikelets is, simply, the low level of depolarization, i.e. the peak amplitude during depolarization.

Because of the similar shapes and latencies of spikes and spikelets (Figure 2.10), our results agree with the concept of spikelets being the result of action potential initiation and subsequent failure. In any case, we can conclude that both have the same origin. Through blockage of sodium channels we also show that spikelets and spikes result from direct activation of the neuron and depend on the activation of sodium channels. Moreover, spikelets do not appear exclusively in response to high frequency stimulation, as suggested previously (Hu et al., 2009; Tsai et al., 2011a): we report spikelets in response to 10 Hz, 50 Hz and 200 Hz stimulation. Figure 2.11b suggests that the increased number of spikelets is in proportion with the number of spikes produced at higher frequencies. More than half of spikes do not reach the amplitude of full action potentials instead they remain as (or are converted into) spikelets, leading to a massive loss of information at high frequencies if spikelets are not transmitted through the RGC's axon. If this were the case, only 1/16 potential spikes would be transmitted at 200 Hz, and about 1/7 at 50 Hz. Such information loss could be one of the explanations for the perceptual fading patients experience with retinal prostheses. On the other hand, if spikelets do transmit, then perhaps adaptation does not occur in the RGC, but instead at higher levels of the visual pathways (e.g. the lateral geniculate nucleus, or the visual cortex). A future study determining if the spikelets propagate or not, could tell if spikelets should be ignored, or counted as part of the RGC response to electrical stimulation. However, this is a non-trivial experiment as it requires simultaneous recording from the axon and the soma. A recent modelling study from Meng et al. (Meng et al., 2018) suggests that the spikelets do not propagate.

Figure 2.12 illustrates that adaptation to electrical stimulation in RGCs was mainly caused by the inability of the neuron to elicit full spikes, rather than a lack of general

responsiveness (compare Figure 2.6a with Figure 2.12d for 200 Hz). It is important to note that when taking spikelets into account the adaptation of neurons to random IPIs at 200 Hz was less pronounced than the adaptation to constant IPI stimulation at the same frequency (compare Figure 2.7 with Figure 2.12a-c). At 50 Hz, the decay rate was similar for both protocols. At 10 Hz, spikelets did not have much influence on the adaptation of the RGCs. The frequency dependency of spikelets is consistent with Cai et al.'s findings (Cai et al., 2011), which suggest that RGCs could respond to stimulation frequencies up to 600 Hz if spikelets are included in the spike count. The benefit of using random IPIs for avoiding adaptation is abolished if spikelets are considered, and in fact, the overall spike rate is lower for random IPIs than for constant IPIs. While spikelets could be generated in the dendrites (Oesch et al., 2005), or in the axon (Davie et al., 2008; Hu et al., 2009; Schmitz et al., 2001) of RGCs, it could also be a result of activity (or lack of it) in the inner retinal networks that generate inhibition of RGCs.

Fading of visual perception is a phenomenon observed in the human visual system under normal conditions, even when the visual perception is due to light, and not to electrical stimulation. The natural mechanism to overcome visual fading to a fixed visual stimulus consists of small eye movements (microsaccades), which continuously update the stimulus received by the eye (Martinez-Conde et al., 2006; Poletti, 2010; Rucci et al., 2007). This principle is adopted by the developers of alpha-IMS, whose implant is attached to the eye, hence the natural eye movement updates the image and could potentially help prevent the fading of visual percepts. However, some subjects still reported perceptual fading of electrically evoked phosphenes (Stingl et al., 2013). This same principle has been recently implemented in Argus II clinical trials to improve the localization of evoked percepts, however, no mention of reduced perceptual fading has been reported (Caspi et al., 2018).

While response decay to visual stimulation has been found in the retina and visual cortex (Ibbotson, 2005), adaptation to electrical stimulation has only been shown in the retina (Baccus and Meister, 2002; Chander and Chichilnisky, 2001; Horsager et al., 2009; Jensen and Rizzo, 2007; Manookin and Demb, 2006; Rieke, 2001). Hence, exploration of the effect in the visual cortex and lateral geniculate nucleus of irregular stimulation of RGCs could help demonstrate the hypothesis that reducing adaptation in the early visual pathways has an effect on the perceptual fading generated by the higher visual pathways. Inner retinal neurons and RGCs have been successfully stimulated with subretinal and epiretinal stim-

ulation, therefore the stimulus presented here could be easily applied to clinical trials and eventually translate to real life applications such as retinal prostheses (Boinagrov et al., 2014; Stett et al., 2000). Furthermore, stimulation with epiretinal prostheses using irregular temporal patterns, such as random IPI stimulation, could evoke spikes in RGCs that resemble the natural responses to visual stimuli (Wong et al., 2014). Overcoming perceptual fading is one of the necessary improvements in the development of retinal prostheses (Pérez Fornos et al., 2012; Stronks et al., 2013). It has been proposed previously that a pseudorandom temporal pattern between biphasic pulses could reduce the desensitization of the retina, or "restore the retinal sensitivity" in retinal prostheses (Roy et al., 2017). The present work demonstrates the assumption at a cellular level.

Current retinal prostheses generally use cathodic-first biphasic pulses with a duration of 0.46 ms per phase, and a varying gap between phases from 0 to 1 ms. Nevertheless, while the presence of an inter-phase gap improves the efficacy of the electrical pulses, any gap larger than 0.4 ms has been suggested to have only marginal effects on the efficacy of the stimulus (Pérez Fornos et al., 2012; Stronks et al., 2013; Weitz et al., 2014). We have chosen anodic-first stimulation to facilitate artifact removal and data analysis. However, we have also repeated the experiment as a proof of concept with cathodic-first biphasic stimulation on 5 different cells and our results hold under the inverted polarity condition (Figure 2.9), moreover, the difference in adaptation between constant IPI and random IPI stimulation seems to be larger with cathodic first pulses. This could suggest that random IPI stimulation might be more effective with cathodic-first pulses, than with anodic-first pulses. Furthermore, a previous experiment has shown no significant differences in latency or amplitude of electrically elicited evoked potentials in human patients with anodic-first pulses in comparison with cathodic-first (Stronks et al., 2013). The pulse width (0.2 ms per phase) and inter-phase gap (20 μ s) used in our experiments are within the optimal parameter values for direct activation of rat RGCs, as reported previously (Hadjinicolaou et al., 2015a). Adjusting the phase width and inter-phase gap duration will have an effect on the activation threshold and the charge required to stimulate the RGCs, and should be adjusted accordingly for testing with human patients, but it will not affect the performance of the irregular IPI protocol presented here.

It is clear that when measuring only full spikes there is a significant difference in the response to the two different protocols used (constant and random frequency stimulation).

Although the reason for these differences are not fully understood, our results indicate that using stimulation with an exponential distribution of IPIs may limit adaptation and could lead to better efficacy in future generations of retinal prostheses.

Chapter 3

Morphological analysis

3.1 Introduction

In the previous chapter, a non-conventional protocol that might improve the efficacy of retinal stimulation and might provide selective stimulation of ON and OFF cells has been found. In the following chapters, we look further into selective stimulation of RGCs, not only targeting cells according to their light response, but also depending on their morphological classification.

3.1.1 Neurons in the retina

Mammalian retinas consist of several types of neurons: photoreceptors, amacrine cells, bipolar cells, horizontal cells, and retinal ganglion cells (RGCs), arranged in 3 main neuronal layers (Figure 3.1). Bipolar cells form the main pathway between photoreceptors and RGCs, mostly acting as excitatory inputs. They transmit their stimulus properties to RGCs. This pathway builds the initial foundation for retinal response selectivity. RGCs all have their dendrites in the inner plexiform layer (IPL), as shown in Figure 3.1 (Masland, 2012). The dendrites of each RGC arborize in one of two layers of the IPL, or both: (1) the inner IPL (closer to the RGC layer), (2) the outer IPL (closer to the inner nuclear layer) or (3) both layers (bistratified). This difference in stratification allows the RGCs to connect specifically with particular types of bipolar cells, which have their axon terminals in either

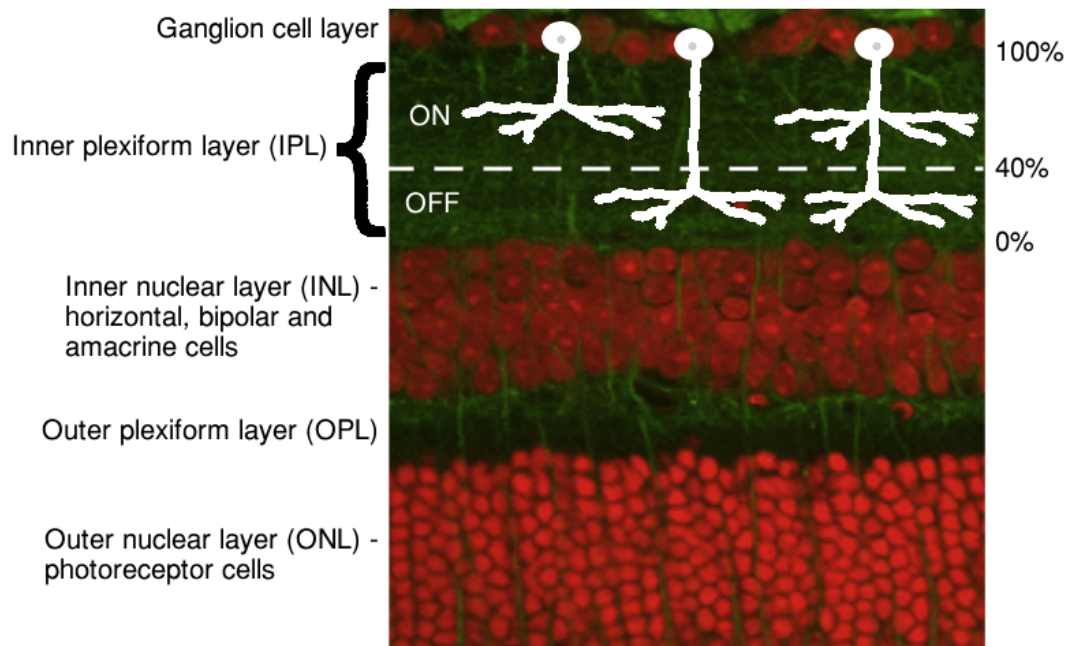


Figure 3.1: Layers of the retina and stratification of RGCs. In the IPL, from left to right, is a schematic of an ON-RGC, an OFF-RGC, and an ON-OFF-RGC. The response of RGCs to light corresponds to the level of stratification of the dendritic arborizations. Dashed line represents the 40% border between the inner IPL and the outer IPL. The border between INL and IPL is the 0% plane and the border between the RGC layer and the IPL is the 100% plane. Figure adapted from Masland (2012).

the inner or outer part of the IPL (Figure 3.2). RGCs that stratify in the inner IPL respond with increased spike rates to brightness increments (light-ON) and to general increases in light intensity (Sanes and Masland, 2015). These are referred to as ON-RGCs. Bipolar cells that have axon terminals in the outer IPL connect with OFF-RGCs, which respond to brightness decrements (light-OFF) and general decreases in light intensity. RGCs that have dendrites in both layers of the IPL (bistratified cells) respond to both brightness increments and decrements and are, therefore, called ON-OFF RGCs (Figure 3.1). In addition to specific connections with bipolar cells, the selective responses of RGCs are influenced by connections with amacrine cells through inhibition of their responses via lateral (between other amacrine cells) and vertical (to RGCs and bipolar cells) synapses (Figure 3.3). All these types of responses to light stimuli result in various functional RGC subtypes that are not only sensitive to light transitions, but also feature direction selectivity, centre-surround interactions, and sustained or transient spiking activity.

Along with the different physiological types, RGCs have been classified by their mor-

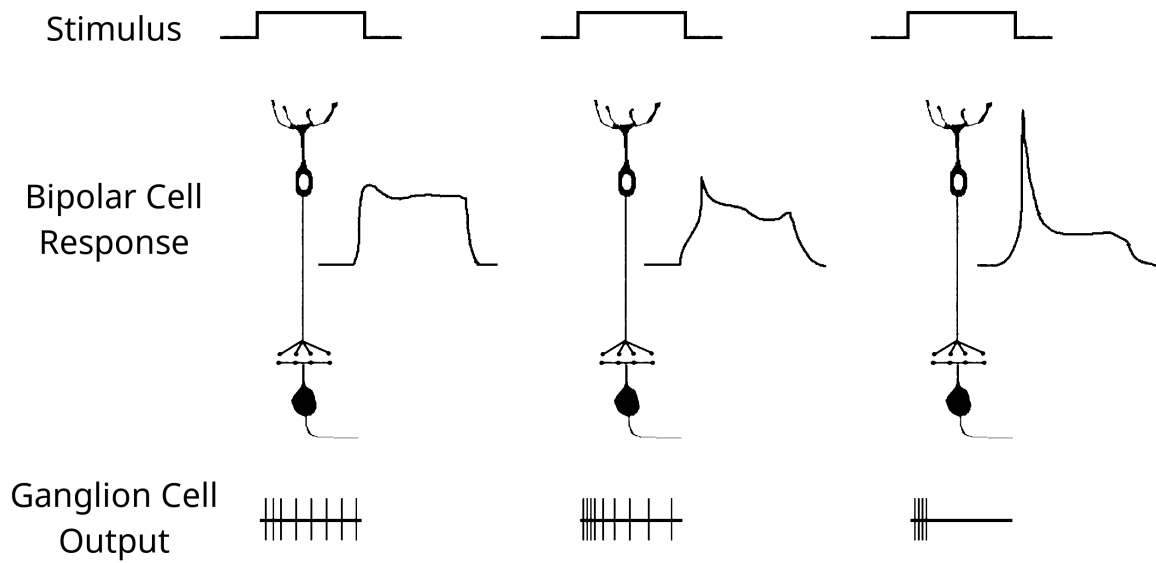


Figure 3.2: Approximation of the activation of RGCs in response to graded potentials of bipolar cells. Contribution of amacrine cells is ignored. Figure adapted from Masland (2012).

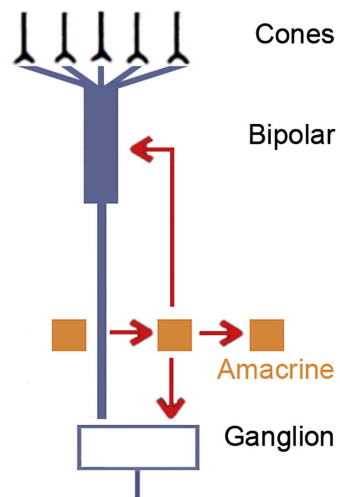


Figure 3.3: Circuitry of amacrine cells. Amacrine cells receive input from bipolar cells and other amacrine cells, send feedback signals to bipolar cells, and output to other amacrine cells and RGCs. Figure taken from Masland (2012).

phology. Specifically, the size of the soma, the area of the dendritic field, and the stratification pattern in the IPL (Sun et al., 2002b). Four morphological classes of rat RGCs have been proposed and are identified by the first letters of the Latin alphabet (Huxlin and Goodchild, 1997). Class A RGCs have large cell body (soma) sizes and large dendritic fields. This class, in turn, is divided into two subclasses, A1 (inner), and A2 (inner or outer). Cells in group B have small somas and small dendritic field sizes and are subdivided into four subclasses: B1, B2, B3, and B4. Subclass B3 is the only one with variable stratification levels (i.e. the dendrites can be either inner or outer). Class C is characterized by having small-medium sized somas, and medium-large sized dendritic fields. There are four subclasses of C RGCs, C1, C2, C3, and C4. Subclasses C2 and C4 can have inner or outer stratification, while C1 and C3 always stratify in the inner IPL. Finally, bistratified RGCs in rat belong to class D. They generally have small somatic sizes, and are subdivided in D1 when the dendritic field is small, and D2 when the dendritic field is large. Huxlin and Goodchild (1997), Sun et al. (2002b), and Wong et al. (2012) present comprehensive compendia of the different morphological classes in rodents. Figure 3.4 shows a diagram with representative cells from each RGC class. A cells have been suggested to be homologous to the alpha cells in other species (e.g. the cat), while B cells could be homologous to beta cells in the cat retina.

There is a direct relationship between the morphological classes of RGCs and the different physiological characteristics of the cells (Cleland et al., 1975). Not only do the responses to light vary between different morphological classes but the responses to electrical stimuli also differ (Wong et al., 2012). Moreover, by using two-photon calcium imaging to record large populations of RGCs, Baden et al. (2016) have found a wide variety of functional outputs in RGCs, which do correspond to the previously identified morphological classification, but result in more cell classes than the morphological studies. Taking into account the variability in morphology, light response, electrical response, and distribution of RGCs in the retina, it is believed that there are more than 30 different RGC classes in the mammalian retina. Each of these cell classes carries a unique selection of visual information to the higher visual pathways (Baden et al., 2016; Masland, 2012; Sanes and Masland, 2015).

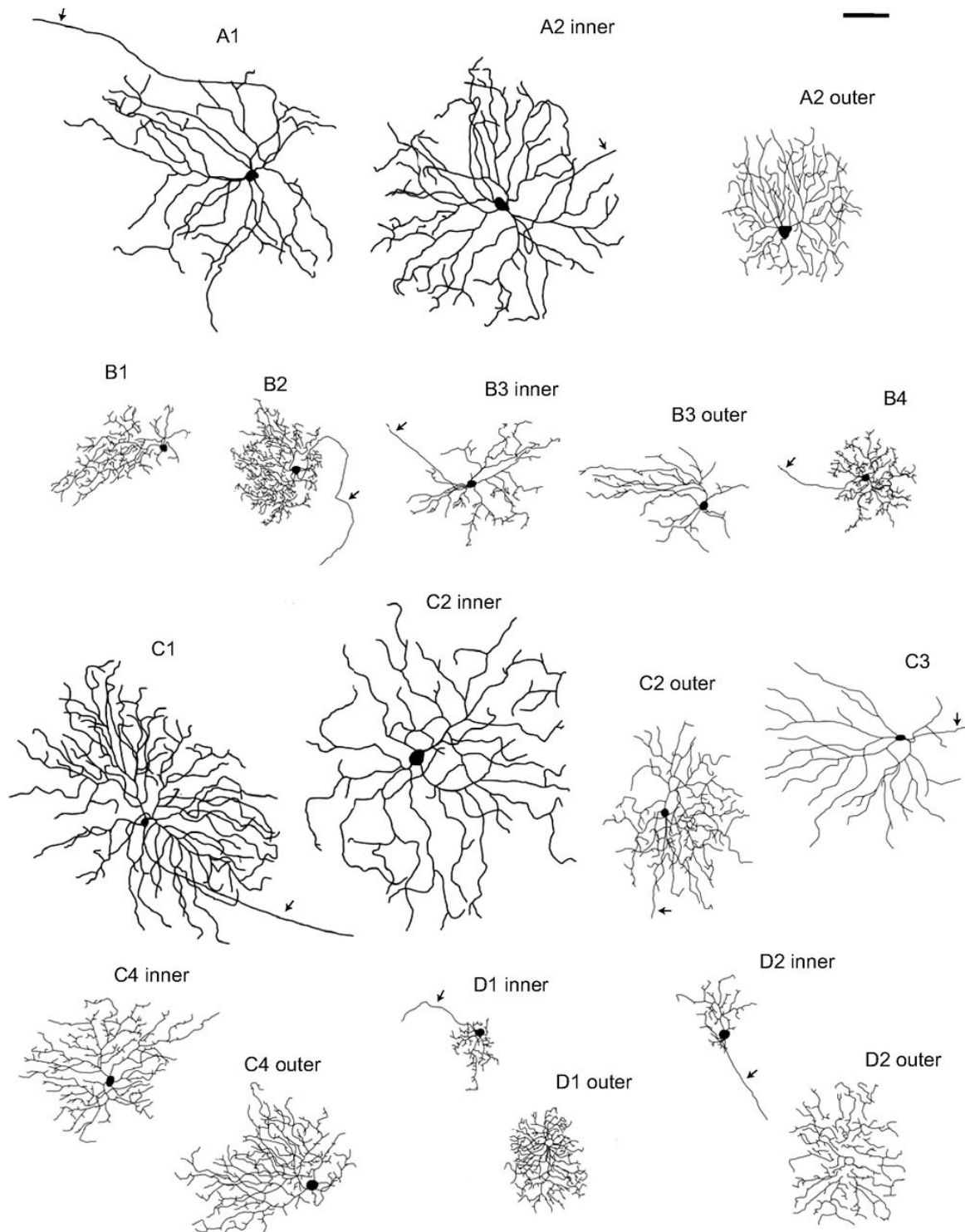


Figure 3.4: Morphological types and subtypes of RGCs. Drawings of individually recorded rat RGCs representative of their morphological class. Figure from Wong et al. (2012).

3.1.2 Cell recording

The activity of neurons reported in this work was obtained from intracellular whole cell recording of the membrane potential. Intracellular recording involves breaking the membrane and filling each cell's soma with a solution that replicates the internal chemistry of a nerve cell. When the solution also includes a fluorescent dye, the soma, dendrites and axon of the neuron become visible under fluorescent light, and can be imaged with the help of a confocal microscope for morphological analysis (Cloherty et al., 2012; Hadjinicolaou et al., 2015a; Maturana et al., 2016; Wong et al., 2012).

In the late 1970's, Neher et al. (1978) suggested an improved form of the Voltage-Clamp technique; a method to record individual channel current flow from the cellular membrane by creating a seal between a glass pipette and a small patch of the membrane. Later, they reviewed the patch-clamp technique and the variants that arose after they published their original method (Sakmann and Neher, 1984). The most influential published work became Hamill et al. (1981), which demonstrates the ability to increase the resistance of the seal by applying small negative pressures (suction) to the pipette. They reported a resistance as large as $60\text{ G}\Omega$, thus calling it a "Giga-seal".

An important practice for the success of this method, according to Hamill et al. (1981) is to ensure that the pipette is free of dirt by applying positive pressure prior to inserting the pipette in the bath and using each pipette only once. Once achieving a Giga-seal, different patch-clamp configurations can be performed; besides the "cell-attached" configuration (the original method described by Neher and Sakmann), Hamill et al. (1981) proposed "outside-out patch", "inside-out patch" and "whole cell recording" as alternate configurations. The latter, illustrated in Figure 3.5, is of special relevance for being the method used in the present project.

The aim of this chapter is to present a detailed description of the methods used to classify the recorded RGCs per light response (ON, OFF and ON-OFF) and in morphological types (A, B, C and D). The methods described here are relevant to all other chapters of the thesis. Dissection and tissue preparation has been described in detail in Chapter 2.

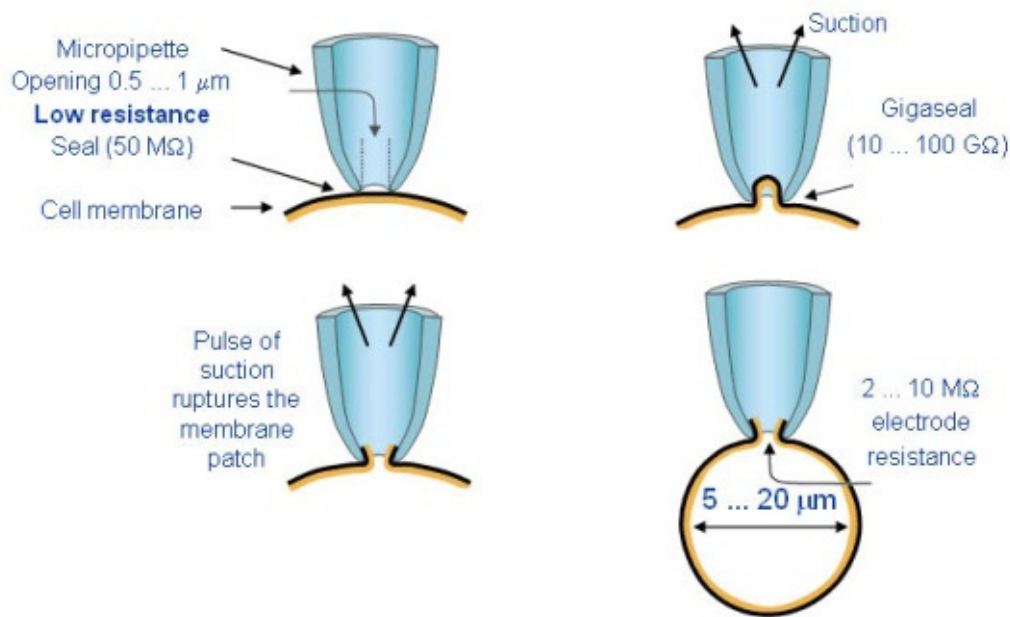


Figure 3.5: Whole cell recording. While intracellular solution flows from it, a glass micropipette is lowered onto the membrane of a RGC, the flow is stopped and suction, or negative pressure, is applied to form a Giga-seal. Once the Giga-seal is formed, the membrane is *broken* by rapidly applying 1 ml of negative pressure. Figure taken from <http://shermanlab.uchicago.edu/patch.html>

3.2 Methods

3.2.1 Light response

To distinguish between ON, OFF and ON-OFF RGCs, the retina was stimulated with full-field light for 3 s. Figure 2.2 shows the responses of example ON and OFF cells to light stimulation. RGCs that responded with a burst of spikes to the full-field light exposure were classified as ON cells. The burst generally lasted around 500 ms after the beginning of the light stimulus. Cells classified as OFF RGCs were those that triggered a spike burst after the 3 s of stimulation, when the light was turned off. ON-OFF cells were RGCs that spiked to both, light on and light off transitions. Dissection and tissue preparation were performed under mesopic conditions, however, the ambience light in the laboratory was not strictly controlled and the light response of some cells might have been affected (Tikidji-hamburyan et al., 2015). This issue is solved by the 3D reconstruction of the cells, which give us a second tool to establish the type of light response of each cell.

3.2.2 Morphological classification

To confirm cell morphological classification, the following technique was used to scan the cells and analyse their morphology. During current-clamp the RGCs were filled with intracellular solution, which included 2.5% Alexa Fluor 488 dye (Thermo Fisher Scientific). Following recording and stimulation of the RGCs, the retinal tissue, still on the chamber, was infused with 2 μ l sulphorodamine (red dye). As each cell had just been patched, the Alexa 488 dye had not faded away, so it was possible to see the cell under fluorescent light without further treatment. Subsequently, the tissue was placed under the confocal microscope (Olympus FluoView FV1200) to perform the 3D reconstruction of the cell using the microscope's dedicated software FV10-ASW v4.2a. First the filled RGC was located and focused, then the depth of the RGC layer and the inner nuclear layer were recorded in the software. Next, the software was set up for automatic scanning of the cell by progressively imaging planes of the tissue in the X-Y axis, with increases in the Z-axis of 1.31 μ m, starting from the RGC layer (plane 0). The scanning was conducted using two different channels, channel 1 (green) detected the RGCs dyed with Alexa 488, and channel 2 (red) detected the extracellular matrix stained by the red dye.

Once the RGCs were scanned, each cell was loaded into the open source image processing software Fiji (ImageJ), then the morphology was reconstructed and analysed as follows. Firstly, the contrast and brightness in channel 1 were adjusted to clearly show the dendrites and soma of the cell in all the layers, then all the scanned layers were superimposed (addition along Z axis; Z-projection) to have a resulting top view 2D image of the soma and dendrites of the cell (Figure 3.6). Using the Z-projection, the soma size was determined by tracing a region on the perimeter of the cell's soma, and fitting it with an ellipse. The diameter of the soma was estimated using the major axis of the ellipse (Figure 3.6b).

To estimate the Dendritic Field (DF) size, the tips of the dendrites were connected by hand using a cursor to form a region, which was fitted with an ellipse (Figure 3.6a). The area of the ellipse was then used to calculate the diameter of the DF, assuming a circular DF. Secondly, channel 1 and channel 2 were merged and 3D projected. This resulted in a 3D image that could be rotated to show a lateral view of the tissue (Figure 3.6c). As shown previously in the schematic of Figure 3.1, the stratification level ($s(x)$) could now be

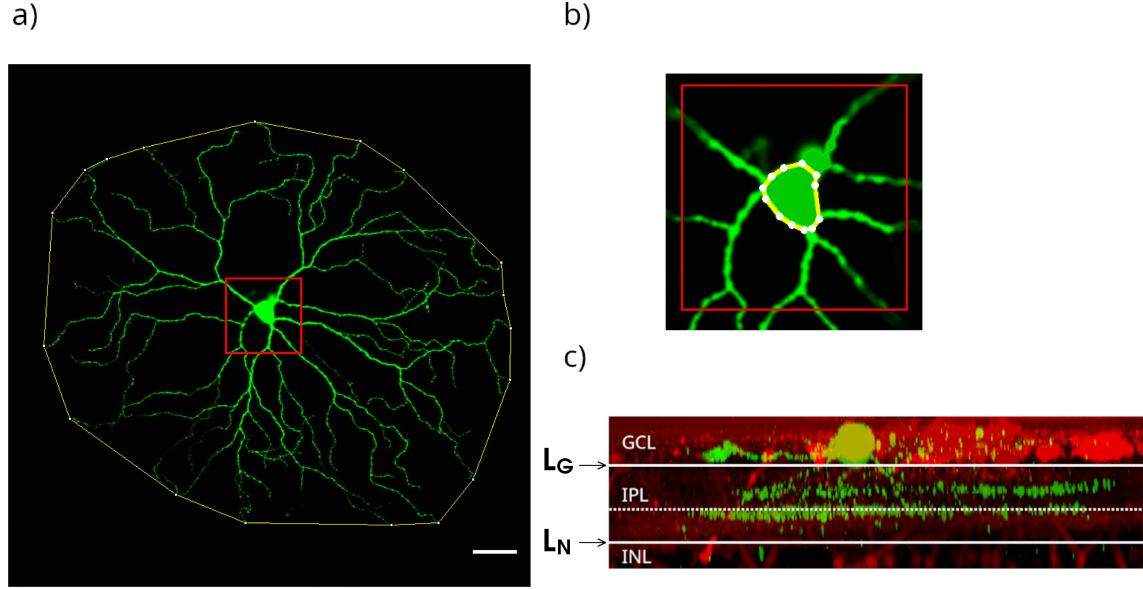


Figure 3.6: Analysing the morphology of a RGC. **(a)** The most distal parts of the dendritic tree are marked and the area is then fit with an ellipse (not shown), whose area is used to estimate a diameter, assuming a circular shape. **(b)** The soma is outlined with the cursor and fitted with an ellipse. The major axis of that ellipse is taken as the diameter of the soma. **(c)** Vertical projection of a RGC (green) and other cells and tissue (red). This projection helps us know if the cell is bistratified or monostatified in the inner plexiform layer (IPL). Solid white lines delimit the IPL (L_G and L_N), dashed line marks the 40% division between the inner and outer regions of the IPL.

determined from the lateral view of the cell as a percentage of the IPL from equation 3.1. Note that in Figure 3.1 the RGC layer represents 100%, but the actual scanning of the cell progresses from the RGC layer (plane 0) to the inner nuclear layer (final plane). Equation 3.1 deals with this by subtracting the calculated percentage from 100. By doing this the results presented here are in agreement with previous literature, and with Figure 3.1.

$$s(x) = 100 - 100 \frac{L_G - x}{L_G - L_N}, \quad (3.1)$$

where x represents the depth of the tip of one dendrite, L_G represents the ganglion cell layer, and L_N represents the border between the IPL, and the inner nuclear layer. Typically, the depths of three dendritic terminals on different regions of the dendritic tree were calculated. The average of the three dendritic tips determined the stratification level. The inner region of the IPL is the region where $s(x) \geq 40\%$ and corresponds to the ON cells. The outer region of the IPL is where $s(x) \leq 50\%$. To deal with the 10% overlap, in the few cases in which the stratification level was between 40%, and 50%, the light

response was used to determine whether it belonged to the inner or outer region of the IPL. This classification method was established previously and repeated in a number of studies (Hadjinicolaou et al., 2015a; Huxlin and Goodchild, 1997; Maturana et al., 2016; Sun et al., 2002b; Wong et al., 2012). L_N , L_G and x are found by inspecting the top view of each plane (slice) of the reconstructed image, as illustrated in Figure 3.7 and Table 3.1.

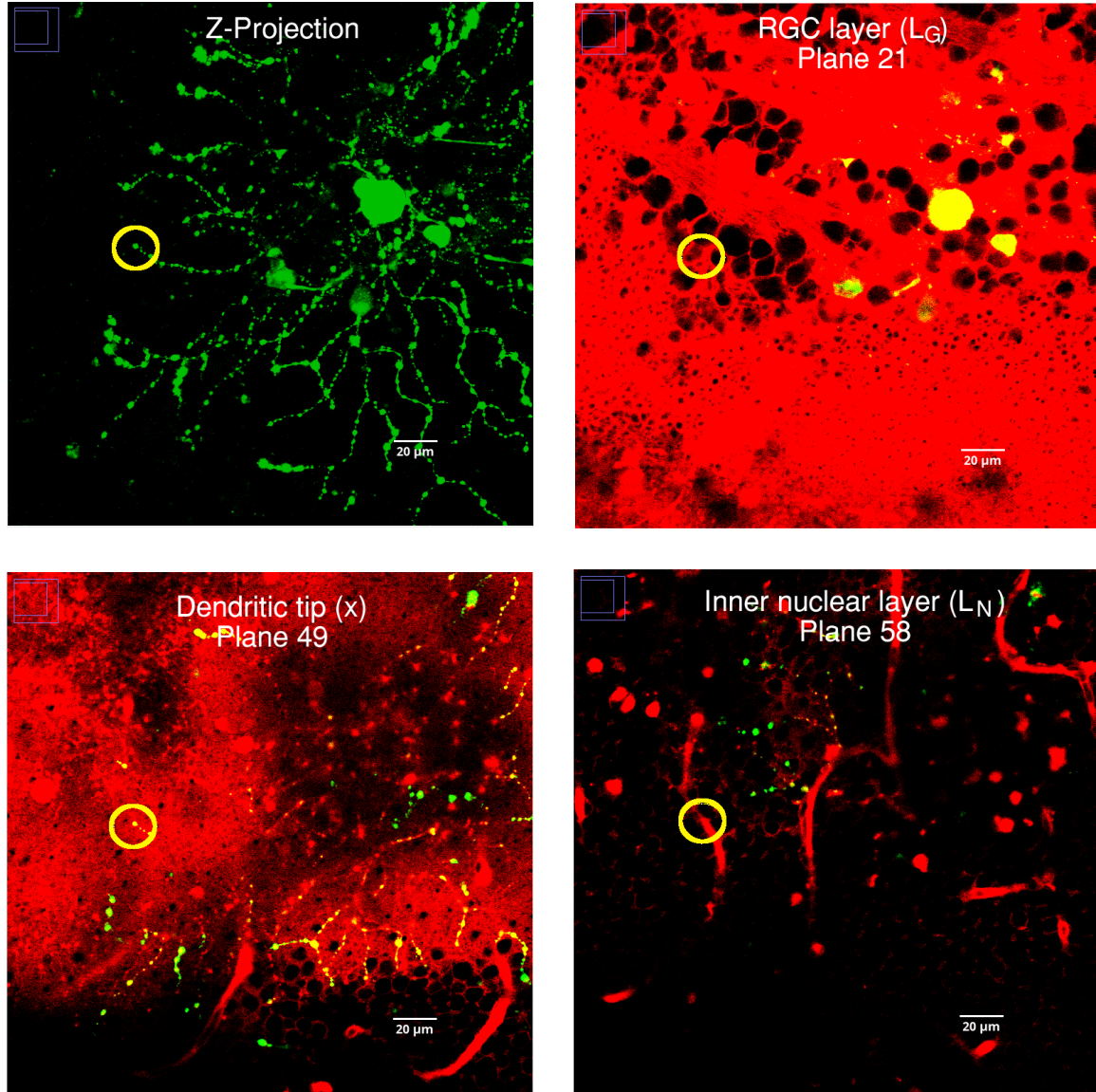


Figure 3.7: Stratification analysis of an example OFF cell. The Z-projection of the planes provides a top view of all the dendrites (top left), the dendritic tip marked by a yellow circle has been chosen to measure its depth in the IPL. Plane 21 of the scanned RGC shows the RGC layer (L_G) at the same X,Y coordinates as the chosen dendritic tip (top right). Location along the z-axis of the chosen dendritic tip (plane 49; bottom left). Plane 58 of the scanned cell shows the border between the IPL and the inner nuclear layer (L_N).

Table 3.1: Analysis of the stratification of the cell in Figure 3.7

L_G	L_N	x	$s(x)$ [%]
21	58	49	24.32

3.3 Results

Based on their light responses, from a total of 44 recorded RGCs, 15 were classified as ON cells, 21 as OFF cells, and 7 as ON-OFF. The remaining cell did not show responses to light stimulation, which may have resulted from damage to the photoreceptors during dissection on that single occasion. The light responses of 36 cells corresponded to the level of stratification found in the morphological analysis (Table 3.2). The stratification level could not be measured for 6 cells due to the lack of a high quality image. The 2 remaining cells were classified as ON-OFF according to their light responses, but were monostatified, and their morphology corresponded to the subclasses A2o and B4, with stratification levels of 31% and 41%, respectively. These two cells were included in their morphological class and subclass in Table 3.3, hence the mismatch in the number of ON-OFF cells and the number of D-type RGCs. From the morphological analysis, 15 cells were found to belong to class A, 10 cells were classified as B cells, 8 cells were class C, and 5 were bistratified, belonging to class D. Finally, 6 cells could not be morphologically classified because either the analysis resulted in ambiguous findings (they could have been cells of 2 different classes), or because the image was unclear or damaged.

Table 3.2: Classification of cells according to their morphological characteristics.

Class	No. of cells (% [*])	Soma diameter [μm]	Range	DF diameter [μm]	Range
A	15 (34.1)	23 ± 4	18 - 33	296 ± 53	196 - 364
B	10 (22.7)	17 ± 4	13 - 28	165 ± 37	108 - 211
C	8 (18.2)	15 ± 3	12 - 18	334 ± 67	233 - 458
D	5 (11.4)	20 ± 2	17 - 22	232 ± 49	188 - 314
NC ⁺	6 (13.6)				

^{*} Percentage of the total number of cells.

⁺ Not classified.

Table 3.3: Cell stratification per morphological class.

Class	Subclass	Cell no.	Stratification [%] Mean $\pm$ SD (n)
A	A1	1	60 ± 0 (1)
	A2i	2	64 ± 8 (2)
	A2o	12	33 ± 14 (10)
B	B2	1	61 ± 0 (1)
	B3i	1	83 ± 0 (1)
	B4	6	19 ± 14 (6)
	NS ⁺	2	-
C	C1	4	57 ± 11 (3)
	C2o	2	26 ± 0 (1)
	C3	2	66 ± 18 (2)
D	D2	5	27 ± 3 / 52 ± 4 (5)

⁺ Unable to assign a subclass.

3.3.1 Class A

The 3D reconstruction of a representative A cell is shown in Figure 3.8. The figure shows the sum of all the slices along the Z-axis so it represents, in 2D, the 3D image of the cell seen from above. The bottom figure is the vertical projection of the same cell, the ganglion cell layer, the IPL and the inner nuclear layer can be appreciated, as well as the soma, axon, and dendritic tree of the RGC. It is clear that this RGC is monostратified. This cell stratifies in the inner section of the IPL (60.2%), has a large dendritic field of $358\ \mu\text{m}$ in diameter, and a medium soma diameter of $18\ \mu\text{m}$.

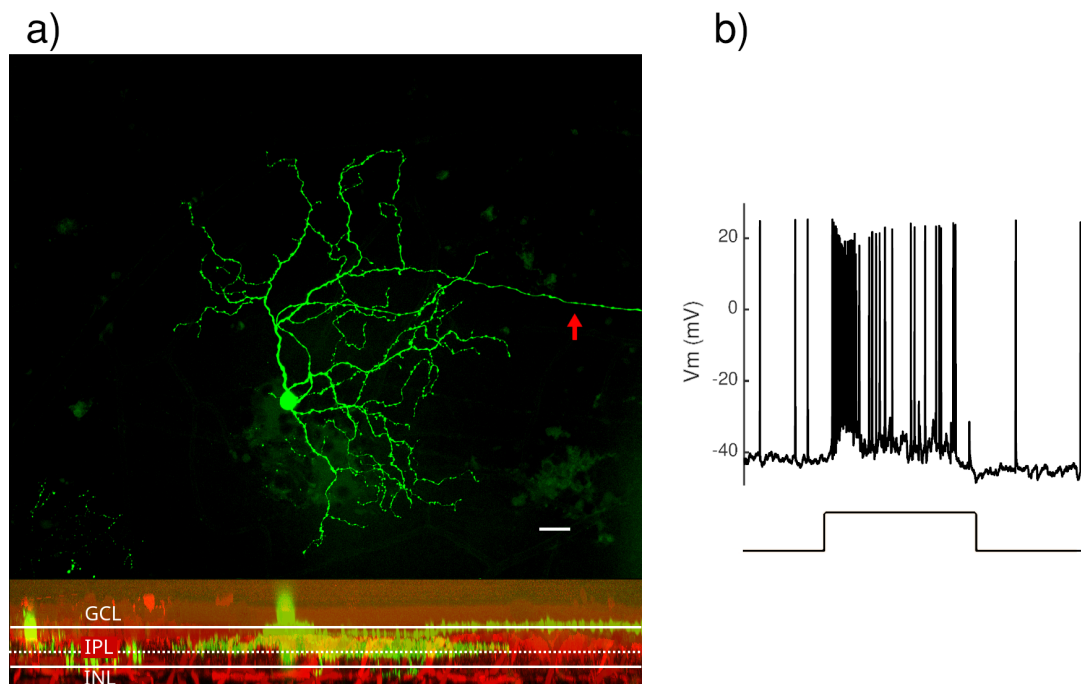


Figure 3.8: 3D reconstruction of a representative RGC from the morphological class A, subclass A1 (ON cell). **(a)** Top view (top), lateral view (bottom). Scale bar represents $50\ \mu\text{m}$. A red arrow indicates the axon. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer. Solid white lines delimit the IPL, dashed line marks the 40% division between the inner and outer regions of the IPL. **(b)** Membrane potential of the RGC in response to a 3 s full-field light pulse.

3.3.2 Class B

Figure 3.9 shows a representative B cell. It has a small soma and medium to small dendritic tree area. The ellipse fitted to the dendritic field of the cell in Figure 3.9 had a diameter of $183\ \mu\text{m}$ while its soma had a diameter of $19\ \mu\text{m}$. This cell belongs to the subclass B4, which is characterized as being an OFF cell and stratifying in the outer section of the IPL. This example cell has a stratification depth of 47%.

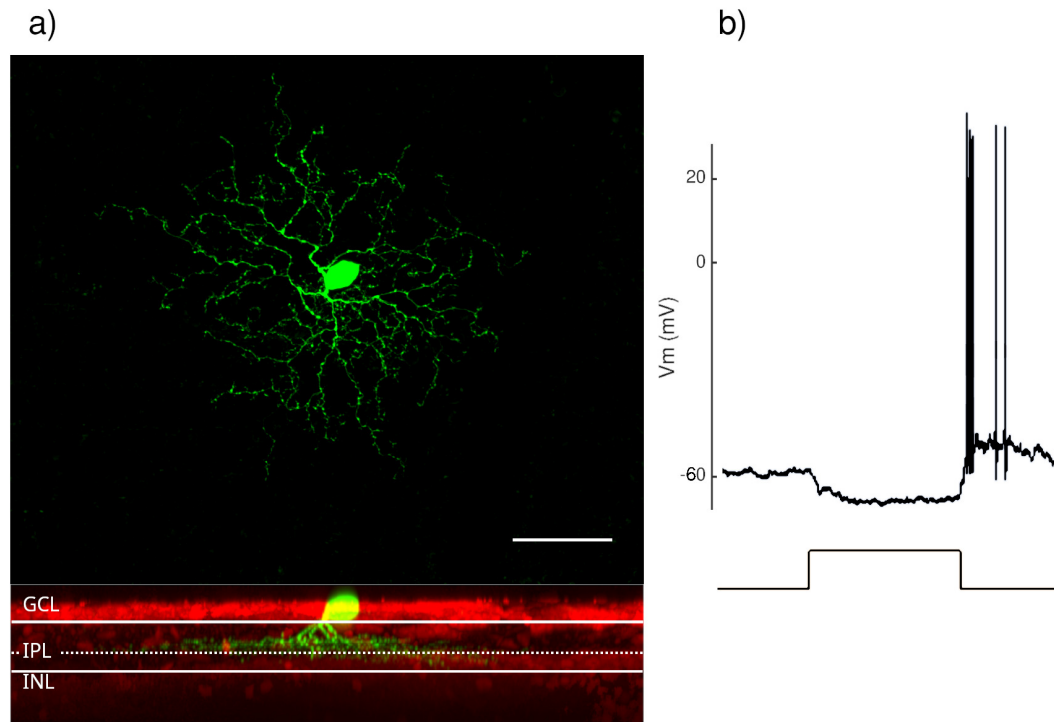


Figure 3.9: Representative RGC from the morphological class B, subclass B4 (OFF cell). **(a)** 3D reconstruction, top view (top), lateral view (bottom). Scale bar represents $50\ \mu\text{m}$. The axon of this cell could not be recovered during the reconstruction. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer. Solid white lines delimit the IPL, dashed line marks the 40% division between the inner and outer regions of the IPL. **(b)** Membrane potential of the RGC in response to a full-field light pulse.

3.3.3 Class C

Figure 3.10 shows the 3D reconstruction of a representative C RGC. This is an ON cell classified as C1, with 18 μm soma diameter and a dendritic field diameter of 348 μm . It stratifies in the inner IPL (69%).

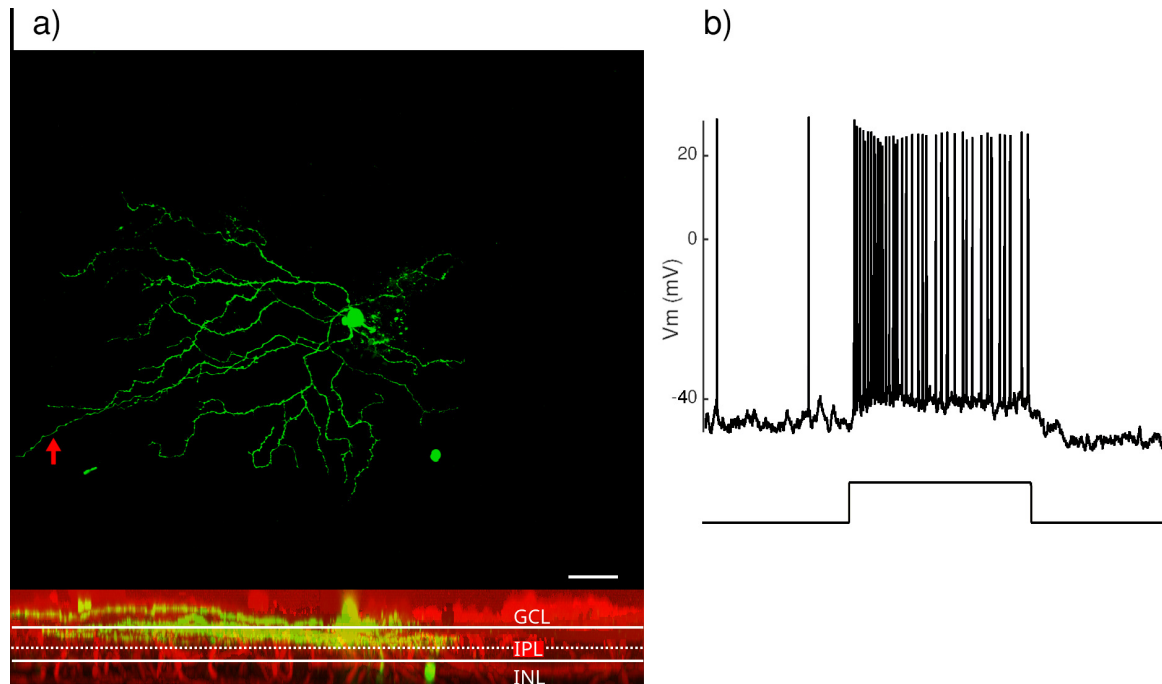


Figure 3.10: Representative RGC from the morphological class C, subclass C1 (ON cell). **(a)** 3D reconstruction, top view (top), lateral view (bottom). Scale bar represents 50 μm . A red arrow indicates the axon. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer. Solid white lines delimit the IPL, dashed line marks the 40% division between the inner and outer regions of the IPL. **(b)** Membrane potential of the RGC in response to a full-field light pulse.

3.3.4 Class D

Figure 3.11 shows the 3D reconstruction of an example D cell. All the cells in class D are bistratified, therefore they spike in response to light transitioning from off to on and from on to off. This cell was classified as a D2 cell, with 19 μm soma diameter and a dendritic field diameter of 419 μm and 295 μm in the inner and outer layers of the IPL, respectively. It stratifies in the inner IPL at 57% of the total depth and in the outer IPL at 27%.

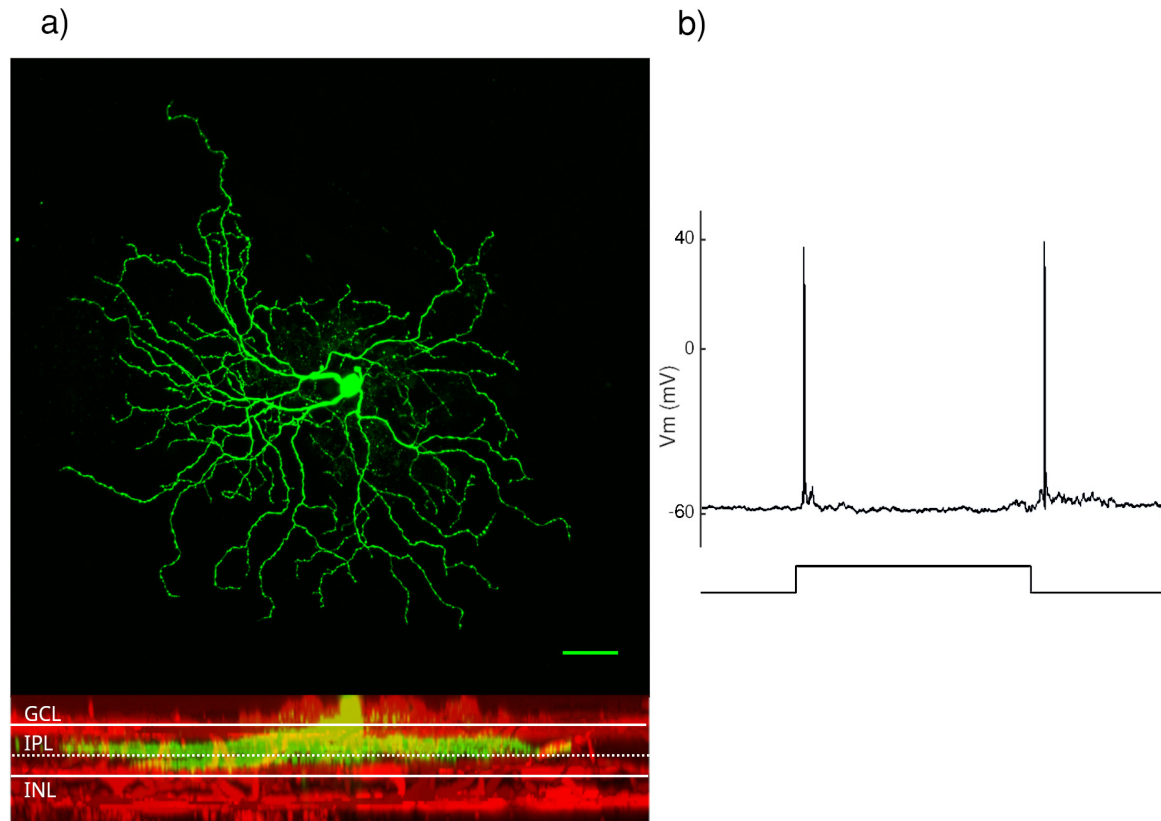


Figure 3.11: 3D reconstruction of a representative RGC from the morphological class D, subclass D2 (ON-OFF cell). Top view (top), lateral view (bottom). Scale bar represents 50 μm . The axon of this cell could not be recovered during the reconstruction. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer. Solid white lines delimit the IPL, dashed line marks the 40% division between the inner and outer regions of the IPL. **(b)** Membrane potential during full-field light stimulation. It showed activity in the form of short burst of spikes to ON and OFF transitions.

3.3.5 Polemic ON-OFF cells

There were two cells whose response to light had an ON-OFF behaviour, but only one stratification level upon morphological analysis. Having their dendrites stratifying at only one depth makes the cells unlikely to be candidates for class D. The first cell (cell I) had

the morphological characteristics of an A2o cell: very large soma ($33\ \mu\text{m}$ of diameter) and large dendritic tree ($354\ \mu\text{m}$) stratifying in the outer segment of the IPL (31%). Figure 3.12 shows the 3D reconstruction of cell I and its membrane potential in response to light stimulation. The membrane potential of the cell dropped immediately after the transition from lights off to lights on, then it elicited a brief burst of spikes. It then went back to a steady condition until the transition from lights on to lights off created a second burst of spikes. Because of its morphology and the delay before the spikes at the on transition, this cell was classified as an A cell. It appears that the transition from lights off to lights on caused hyperpolarization and suppression of spikes, followed by a rebound depolarization (Bleeck et al., 2008).

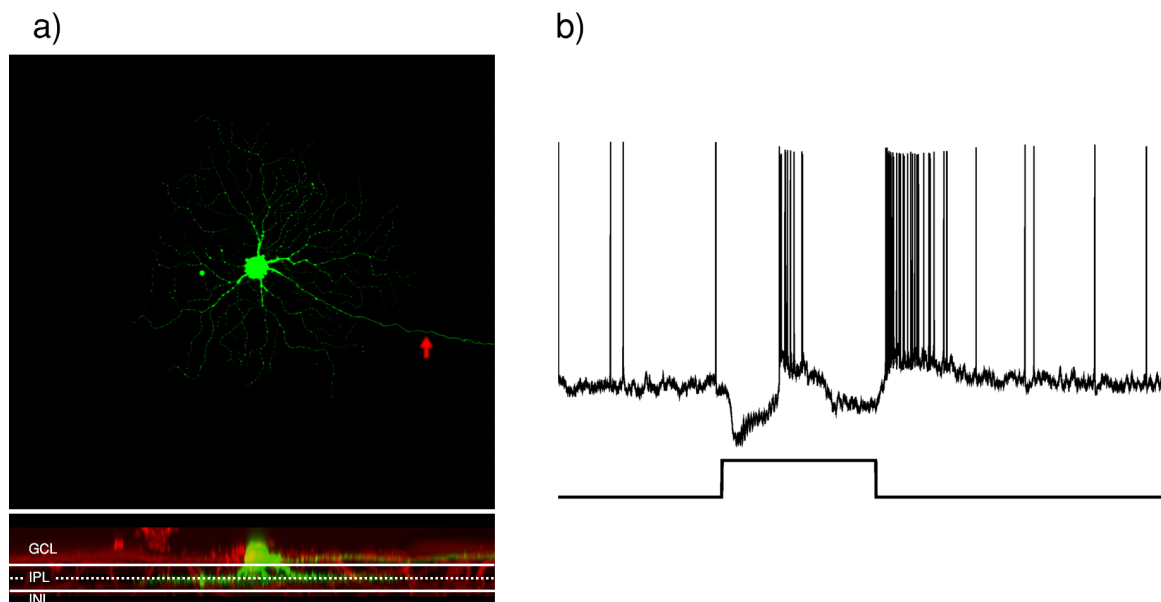


Figure 3.12: Cell I, classified as an A2o cell: **(a)** 3D reconstruction, top view (top), lateral view (bottom). Note that dendrites extended to a single level of the IPL. A red arrow indicates the axon. GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer. Solid white lines delimit the IPL, dashed line marks the 40% division between the inner and outer regions of the IPL. **(b)** Membrane potential during full-field light stimulation. It showed activity in the form of depolarizations and spikes to ON and OFF transitions, although the membrane potential hyperpolarized immediately after the lights were turned on. Given the suppression of spikes and hyperpolarization at lights on, it is clear that the cell is not an ON RGC.

The second cell (cell II) was harder to classify because its 3D reconstruction was not clear, most likely due to the tissue being damaged after the electrical stimulation and recording of the cell. Similarly to cell I, the light response of cell II belonged more to a bistratified dendritic tree than to a monostратified cell (Figure 3.13b), but the scanning only showed stratification in one level of the IPL (Figure 3.13a). Figure 3.14 and Table 3.4 show

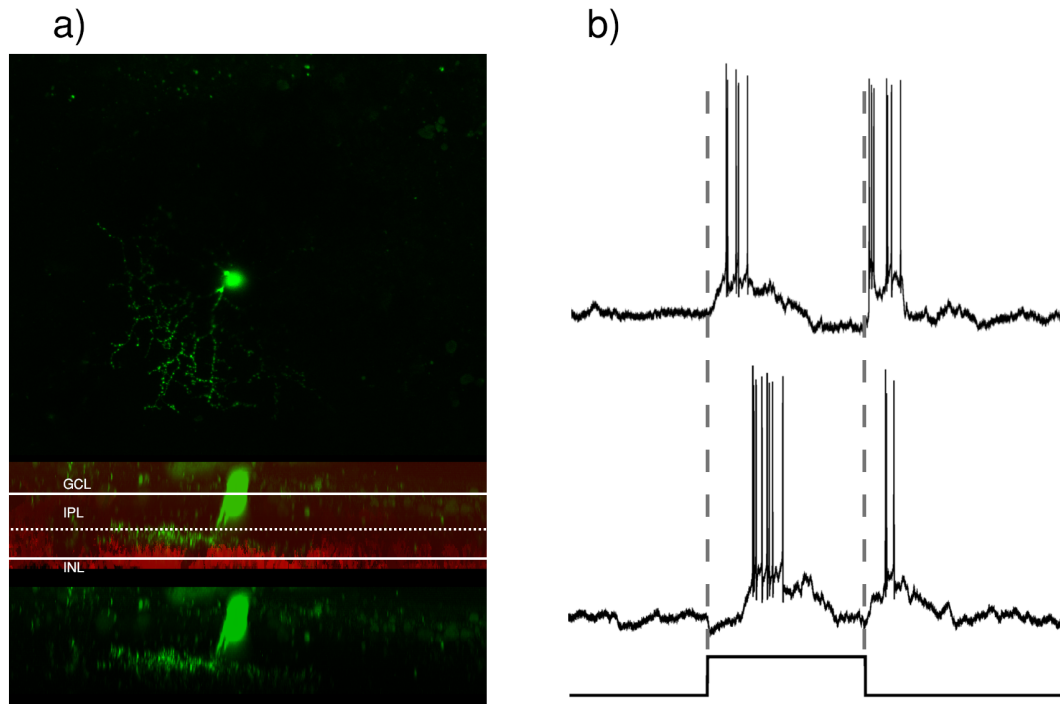


Figure 3.13: Cell II, classified as a B4 cell: **(a)** 3D reconstruction, top view (top) and lateral view (middle and bottom). The red color was removed from the lateral view in the bottom subfigure to have a clearer view of the monostratified dendritic tree. Note that the dendrites, although the image is blurry and tilted, arborized in a single layer of the IPL. **(b)** Two different recordings of the membrane potential in response to full-field light stimulation. It showed activity in response to both ON and OFF transitions.

in detail the measurement of cell II's stratification. Despite the unexpected response to light ON and OFF, cell II was classified as a B cell, because of the size of its soma and dendritic tree.

Table 3.4: Analysis of the stratification of cell II

	LG	LN	x	s(x) [%]
1	11	53	35	42.86
2	11	51	33	45
3	12	56	40	36.36
Mean	11.33	53.33	36	41.27

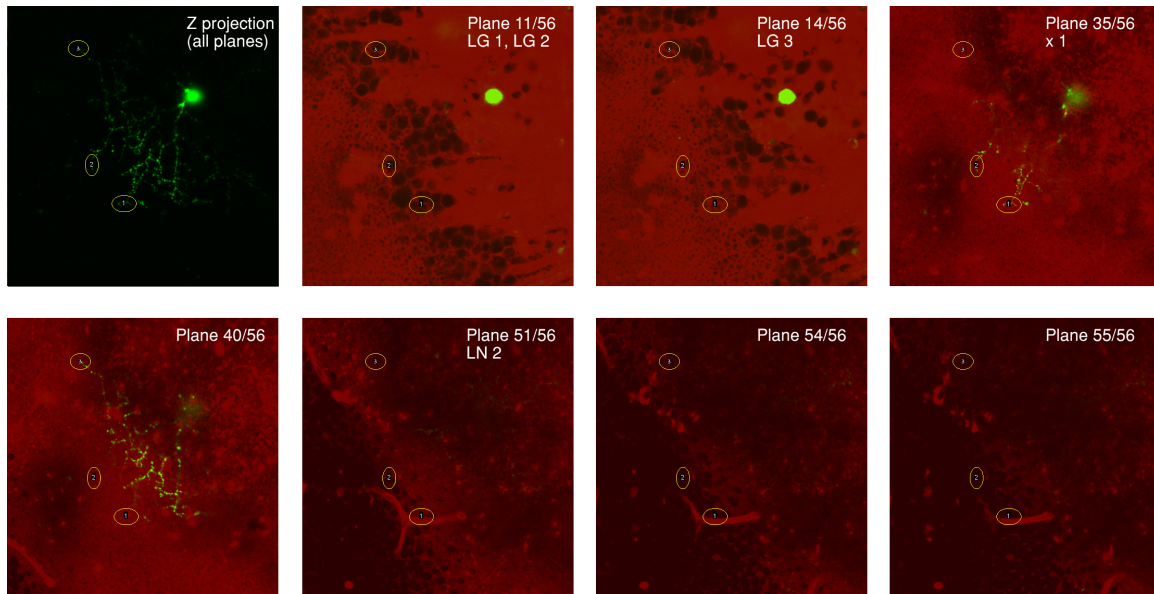


Figure 3.14: Cell II, top view of the Z-projection and planes 11, 14, 35, 40, 51, 54 and 55. The lowest plane is the closest to the RGC layer. Circles marked 1-3 are the chosen dendritic tips. Percentage of stratification is the average stratification of those 3 dendritic tips. In the figure, the RGC layers at points 1 and 2 were found on plane 11. The RGC layer at point 3 was on plane 14. The actual location of the dendritic tip (1) was on plane 35. The border between the IPL and the inner nuclear layer at point 2 was on plane 51.

3.4 Conclusions

Having a clear, complete and universal classification of the different neurons is a sound scientific necessity - the BRAIN initiative in the USA sees the development of an atlas of the neural categories as one of its fundamental goals (Insel et al., 2013). Such a classification would provide researchers in different parts of the world, and different times, with the possibility to know whether they are studying the same neuron types. It would also allow evolutionary comparisons between different species, including humans and animal models (Sanes and Masland, 2015). There is, however, not just one approach to classifying cells, and the possible classification types are broader than would be ideal. Macosko et al. (2015) propose a classification of RGCs based on gene expression, profiling individual cells through an analysis of their mRNA transcripts. Regular spacing is another possible classification, which consists of grouping RGCs by their sparseness under the assumption that neurons are not positioned randomly with respect of members of the same type, and that cells of the same type are evenly spaced (Rockhill et al., 2000). A different classification approach is to categorize cells by their physiological properties, such as direction selectivity, ON or OFF response to light, transient or sustained spiking, etc (Wei and Feller,

2011; Wong et al., 2012). Finally, the morphological classification consists of grouping the neurons according to quantitative or qualitative morphological characteristics, such as the size and shape of the somas and dendritic trees, the widths of the axon's initial segment and the density of the dendrites, among many others (Ramón y Cajal, 1972; Tauchi et al., 1992). Because there are so many different criteria and overlapping characteristics, a conclusive consensus on the classification of RGCs has not been reached, however, there is agreement that there are at least 20 different types in the mammalian retina, including primates (Masland, 2012; Wässle, 2004). Moreover, the different types of classification are not mutually exclusive, since it is common to be able to map already known physiological types onto types discovered through a different classification (Rockhill et al., 2002). An obvious example of this is that the dendrites of ON responding RGCs stratify in the inner part of the IPL.

Rat RGCs have been classified by Huxlin and Goodchild (1997) into 3 morphological groups, A, B and C. There are 14 subtypes within these 3 groups, but this classification was later extended to a 4th group to include the bistratified cells as class D RGCs, making a total of 16 RGC types (Sun et al., 2002b). According to Jelinek et al. (2011) there could be an infinite number of morphological features. We have followed the same approach as Huxlin and Goodchild (1997) and Sun et al. (2002b), which focus on the size of the soma, stratification of the dendritic tree, and the size of the dendritic tree, which are quantitative characteristics. The classification of RGCs into different morphological classes will be used in later chapters to compare the electrical receptive fields between cell classes, and to study the excitation and suppression of neural activity in different cell groups.

Chapter 4

Receptive fields

The electrical receptive field (ERF) of a neuron can refer to the spatial characteristics of the electrical stimuli that activate a neuron, and as the temporal characteristics of the electrical signal that elicits a response (Maturana et al., 2018, 2016). The visual receptive fields of a cell can be recovered by first visually stimulate the subject with white noise images (Gaussian noise), and then reconstructing the 2D features of the visual space that activate the cell (Chichilnisky, 2001; Hubel et al., 1962), the same process can be followed to recover the ERFs. Maturana et al. (2016) described the temporal ERFs of single retinal ganglion cells (RGCs) by applying epiretinal biphasic electrical stimuli with varying amplitudes. The amplitude was sampled from a Gaussian distribution, thus producing a random stimulus that was effectively a form of electrical white noise. Then they analysed the spiking response of the cell with a technique called spike-triggered covariance (STC), in order to recover the temporal ERF (Schwartz et al., 2006). The spatial ERF, in contrast, looks into the spatial locations from where a neuron is better activated by electrical stimulation (Maturana et al., 2018). Receptive fields describe the spatial and temporal properties of sensory neurons. Schwartz et al. (2006) introduced the use of STC to estimate the linear filters of a model to describe receptive fields (Figure 4.1). An example of the distribution of raw stimulus set and stimuli that elicited a response (spike-triggered stimulus) in an STC model is shown in Figure 4.2. The figure shows the nonlinearity associated to the response.

Based on the hypothesis that RGCs have different temporal ERFs due to their particular dendritic field and because of the intrinsic differences between RGC types, the goals of this chapter are to recover the temporal ERF of individual RGCs and the ERF of each

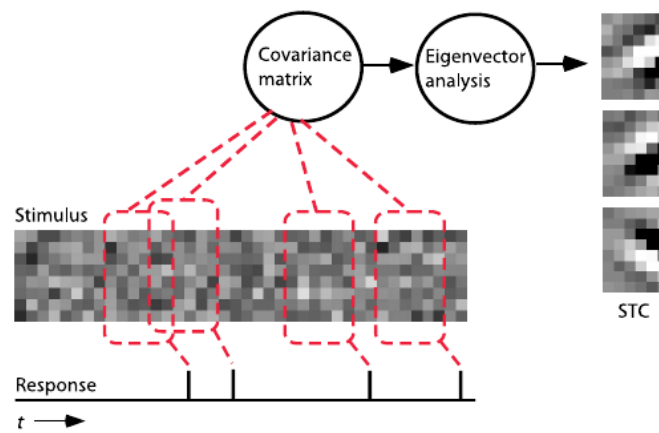


Figure 4.1: Spike-triggered covariance. An example stimulus matrix through time. The response shows the time of spike, and a block of the stimulus previous to every spike forms the spike-triggered stimuli. The covariance matrix of the spike-triggered stimuli is calculated, then an eigenvector analysis of the covariance matrix is performed to determine the STC, the result is the features of the stimulus that most contributed to the spiking response (Schwartz et al., 2006).

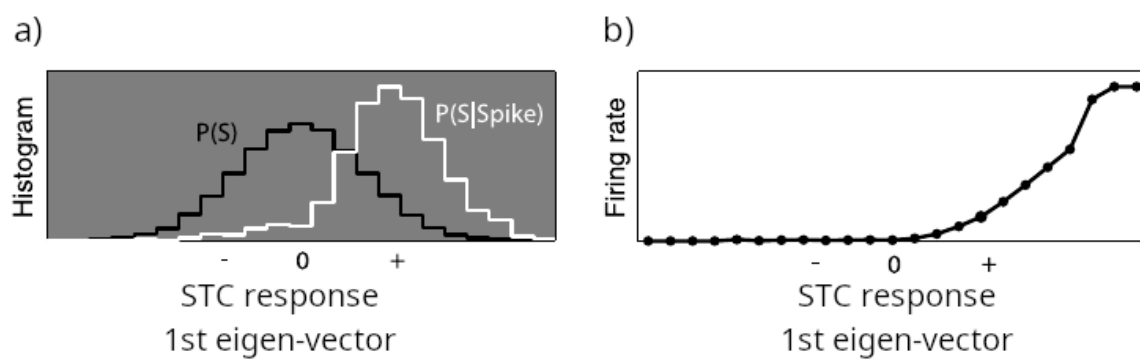


Figure 4.2: Example of a model's nonlinearity. **(a)** Probability of raw stimulus (black) and the probability of spike-triggered stimulus (white). **(b)** Ratio of stimulation vs spikes, the line represents the nonlinearity of the filter. Figure adapted from Schwartz et al. (2006).

morphological class based on the features of the stimulus that preferentially activate single neurons and cells as a part of a morphological type. Also to explore the use of the different ERFs as a method of morphological classification of RGCs. For simplicity and to avoid confusion, the "temporal receptive field" will be called "waveform" from now on.

4.1 Methods

The methods for animal preparation and patch-clamp recording are similar to those described in Chapters 2 and 3. In short, rat retina was dissected and a piece of tissue mounted under the microscope. Then, a RGC was located and exposed, an extracellular platinum electrode placed on the inner limiting membrane approximately $50\ \mu\text{m}$ away from the cell, and the RGC was patched for recording of its membrane potential (recording sampling rate of 25 kHz).

Subsequently, the following extracellular stimulation protocol was performed: a total of 44 RGCs were stimulated with Gaussian white noise for periods of 30 seconds as illustrated in Figure 4.3 (RZ5D BioAmp Processor, Tucker Davis-Technologies, $10\ \Omega$ output impedance, stimulation sampling rate was 48 KHz). The stimulation was repeated until at least 1000 stimulus-driven spikes had been detected. This meant that each cell was stimulated for a total of around 60 periods of 30 seconds. In some cases, less than 60 periods were required. The resting time between white noise periods was at least 4 seconds (see Figure 4.3). Intracellular data is included and analysed in this chapter.

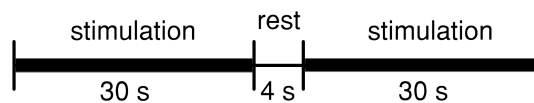


Figure 4.3: Schematic of the stimulation. Each period of 30 seconds of white noise stimulation was followed by at least 4 seconds of resting time, in which no current was applied to the tissue.

4.1.1 Gaussian white noise stimulation

The Gaussian white noise consisted of constant current stimulation with random amplitude (Figure 4.4a-b), with the amplitude updated every $t_A = 150\ \mu\text{s}$ as sampled from a Gaussian distribution with a chosen standard deviation (A_{std}). To get a consistent A_{std} during the

whole experiment, we conducted a stimulus calibration, which consisted of stimulating each cell for 10 seconds with Gaussian white noise prior to running the protocol. The standard deviation was initially set to $5 \mu\text{A}$. The recording was analysed to detect and count spikes, then the process was repeated with increments of $5 \mu\text{A}$ until the set value of A_{std} resulted in 1 spike per second higher than the spontaneous activity of the cell. Figure 4.4c shows a segment of an example recording during white noise stimulation after A_{std} had been set (in this example $A_{std} = 40 \mu\text{A}$). The recording shows a noisy signal (noise in this example is $\sim 40 \text{ mV}_{pk-pk}$), which is caused by the stimulation artifacts. Due to the nature of the stimulus, artifact removal was not possible, hence we limited stimulation to amplitudes that produced an artifact lower than the action potentials, otherwise, spike detection was not possible. When A_{std} could not be found before the stimulus artifact covered the spikes, the cell was discarded and a new cell was patched. This meant that, although the same piece of tissue could still be used, we had to make a new hole in the inner limiting membrane at a different location and locate a new RGC for recording and stimulation. In cases where we attempted to patch a cell in the proximity of the first failed RGC, 2D reconstruction and morphological analysis was no longer possible.

Spikes were detected using threshold crossings of -20 mV , upon visual validation of the stimulus artifact. When the stimulus artifact was higher than -20 mV , the threshold was increased. Any peak found during the white noise stimulation that was higher than the threshold was considered a spike (Figure 4.4c). We ensured not to count the same spike twice by assigning what we called a "no-spike period" of $15 \mu\text{s}$, which meant that when two peaks were found within a period of $15 \mu\text{s}$, only one was counted. Randomly chosen recordings from each cell were visually inspected before the automatic spike detection to check the algorithm did not include a real spike that was too close to another one, or to ensure that the same spike was not counted twice, even if the second peak was found more than $15 \mu\text{s}$ apart from the first one. Due to the nature of the analysis, spikes from spontaneous activity were taken into account. This should not affect the results. Each spike during the spontaneous activity was elicited after a period of random and independent stimuli, which when analysed among other periods of random, independent stimuli, results in those segments being averaged out. In other words, the principal eigenvectors of the covariance matrix of random, independent vectors, is just Gaussian noise.

A similar process was followed to recover the waveform of the 43 RGCs subjected to in-

tracellular stimulation. Intracellular data was collected using the current-clamp electrode for simultaneous recording and stimulation. This resulted in direct stimulation of the neuron's soma. Two significant differences in the stimulation protocol with intracellular stimulation existed:

- The duration of a single stimulation amplitude (t_A) was 300 μ s.
- The stimulation and recording sampling rates were both 25kHz.
- The STC analysis was performed using only the changes in amplitudes, instead of every single sample of the stimulus, i.e., 330 changes of amplitude, corresponding to 99 ms of stimulation, and the stimulation matrix was built with those 330 different values, instead of the 2475 samples that would otherwise create such a stimulus.

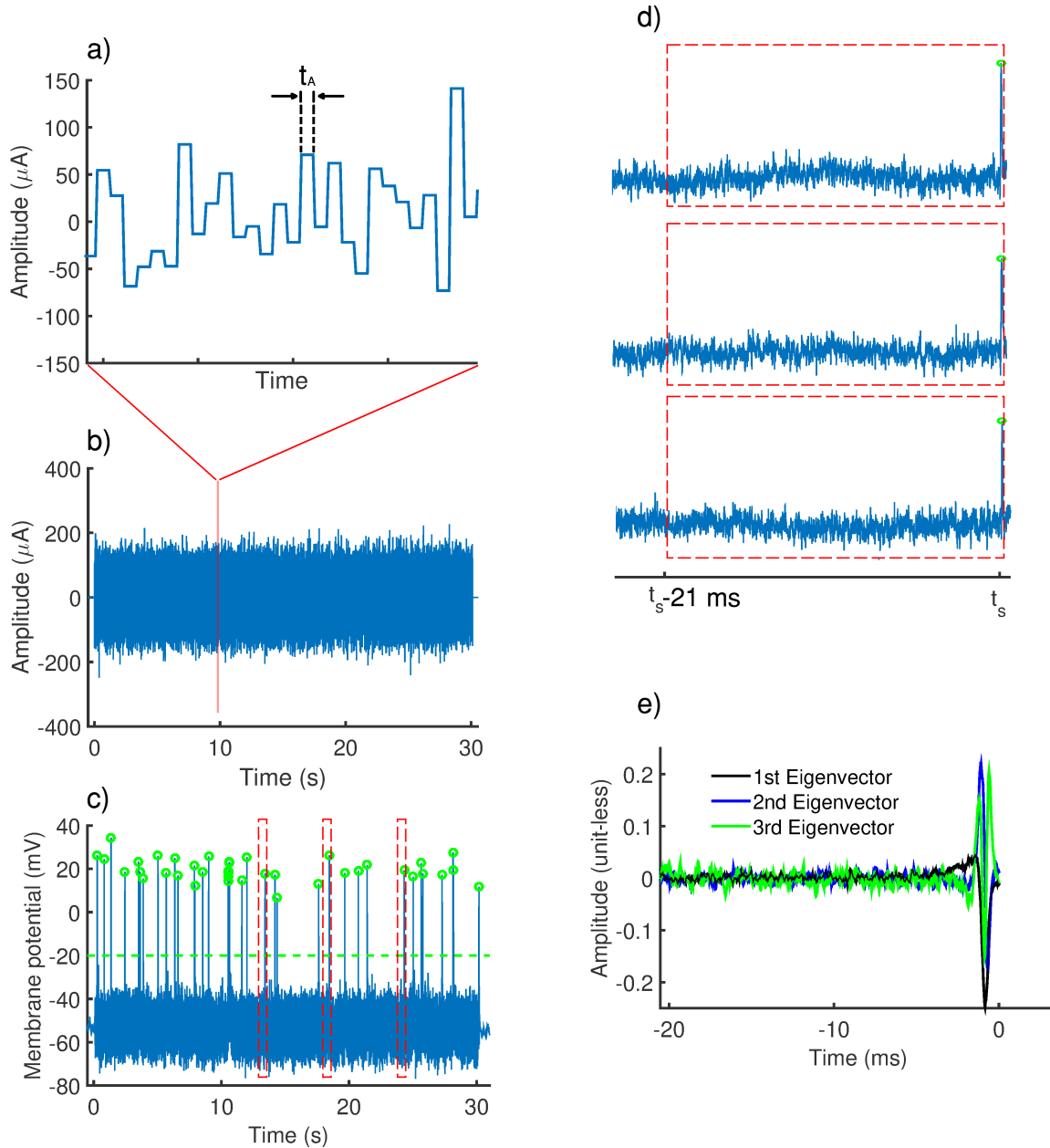


Figure 4.4: Stimulation protocol and STC analysis. **(a)** Short segment of the white noise stimulus waveform from subfigure **b**, the amplitude is sampled randomly from a Gaussian distribution, each amplitude value is held for $t_A = 150\mu s$. **(b)** Stimulus waveform during 30 seconds of Gaussian white noise extracellular stimulation. **(c)** Example recording of the membrane potential during extracellular electrical stimulation. Subplot **c** shows the ongoing electrical changes caused by the stimulus, spikes are marked by green dots, the green dotted line marks the threshold for spike detection, noise between -80 and -40 mV is the artifact of the stimulus waveform, red squares mark the section of stimulus waveform analysed before three example spikes in (d). **(d)** Close up of the section before the three spikes marked in subplot **c**, the segment of the stimulus waveform between $t_s - 21\text{ms}$ and t_s (where t_s is the time of occurrence of each spike) is a vector, which is stored in a matrix among the vectors from the rest of the spikes for STC analysis (i.e., one spike forms a vector, multiple spikes form a matrix). For intracellular stimulation, the stimulus segment used to calculate the STC was 99 ms, instead of 21. **(e)** The three main eigenvectors resulting from the STC analysis of the segments from subplot **d**, all spikes from all recordings of the cell were considered for the STC analysis.

4.1.2 Spike-Triggered Covariance

Upon detection of spikes in all the recordings from one neuron, a stimulus matrix was created by assigning one row per spike, and each row consisting of all the samples in the stimulus applied during the 21 ms prior to the peak of that action potential (1027 samples and 140 changes of amplitude; Figure 4.4c-d). Then, the stimulus matrix was evaluated with a method called Spike-Triggered Covariance (STC) (Schwartz et al., 2006). First the covariance matrix of the stimulus matrix was evaluated and its eigenvectors were calculated. The eigenvectors were ordered according to their eigenvalues, from higher to lower. The eigenvector that corresponded to the highest eigenvalue was then stored and called the "waveform" of that neuron (Figure 4.4e). The waveform is the components of the stimulus that had the most influence on the generation of the spike.

To ensure the significance of the principal eigenvector, we only used cells that elicited at least 1000 spikes during all the repetitions of white noise stimulation. Nevertheless, a significance test was performed. It consisted of shifting the time of the spikes a random number of samples, and repeating the STC analysis, but with the new spike times. This was repeated 999 times with a new random shift for every repetition. The STC for each of the 999 repetitions was the result of chance, hence the waveform should not have much influence on the generation of the spikes. This results in low principal eigenvalues. The waveform of the original recording (prior to the random temporal shift) was statistically significant if it was not generated by chance, i.e. if it had an influence on the generation of the spikes, therefore its principal eigenvalue was higher than the eigenvalues from the other 999 repetitions. Figure 4.5 illustrates the result of the significance test for an example cell. The eigenvalues of STC on the original recording are in blue, and the eigenvalues of all the 999 repetitions are in black, ordered from high to low on each repetition. The error bar represents the s.e.m of the highest eigenvalue from each repetition.

4.1.3 Waveform analysis

We refer to the preferred waveform of each cell as the waveform (Figure 4.6). The waveforms of all cells were analysed to extract several parameters, which were then used as the input to a "batch K-means clustering algorithm" (Lloyd (1982); MacQueen (1965); MAT-

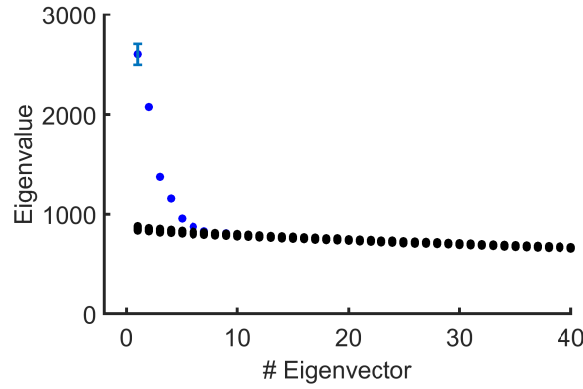


Figure 4.5: Significance test for an example cell's waveform. Every dot is the eigenvalue of its corresponding eigenvector, ordered from high to low (eigenvector 1 has the highest eigenvalue). Blue dots represent the eigenvalues of the original recording. Black dots represent the eigenvalues of 999 different analysis when the stimulus spike-time was randomly shifted in time. Error bar represents s.e.m.

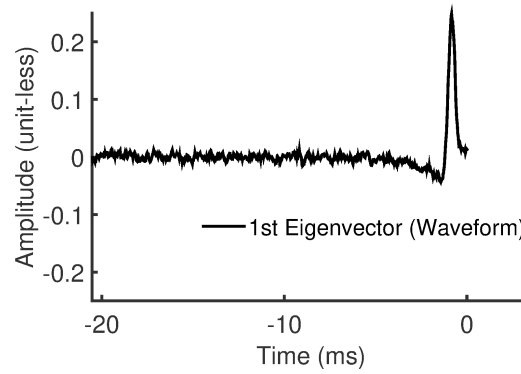


Figure 4.6: The waveform from the example in Figure 4.4e. The 1st eigenvector has been extracted and multiplied by -1. All the waveforms were oriented with a positive peak for easier comparison. Time 0 is when the spikes occurred.

LAB R2017a, The Mathworks, Inc.). All the waveforms were arranged in a matrix with one waveform per row, then the covariance matrix was calculated, and its eigenvectors were found. The three eigenvectors with the highest eigenvalue were extracted and labeled $e1$, $e2$, and $e3$. Figure 4.7a-b show the eigenvalues in ascending order, and Figure 4.7c-d show $e1$, $e2$, and $e3$ of both intracellular and extracellular waveforms. Finally, each waveform was projected into the eigenvectors $e1$, $e2$, and $e3$, resulting in the vectors $v1$, $v2$, and $v3$, which were later used to do the K-means cluster analysis (Equation 4.1).

$$v_i = w'_j \times e_i \quad (4.1)$$

The rising time of the waveforms was taken from a two-term exponential curve fit. A

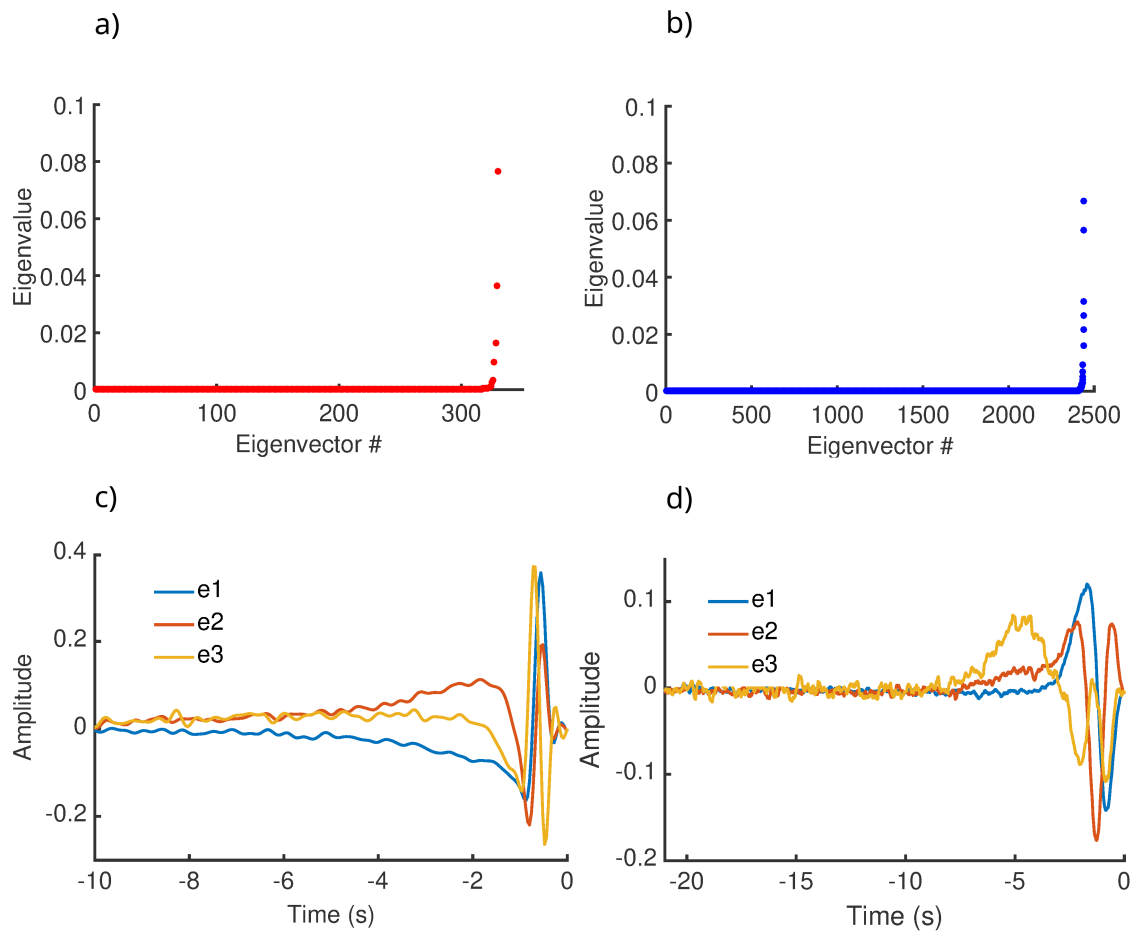


Figure 4.7: Analysis of the covariance matrix of all the waveforms. **(a)** Eigenvalues of the covariance matrix in ascending order, intracellular stimulation. **(b)** Eigenvalues of the covariance matrix in ascending order, extracellular stimulation. **(c)** The 3 eigenvectors corresponding to the highest eigenvalues, intracellular stimulation. The x-axis represents time where 0 is the last stimulus sample before the spikes. **(d)** The 3 eigenvectors corresponding to the highest eigenvalues, extracellular stimulation. The x-axis represents time of the stimulus, where 0 is the last stimulus sample before the spikes.

curve described by Equation 4.2 was fit to each waveform, and the parameters τ_1^{-1} and τ_2^{-1} were used as input variables to the K-means clustering algorithm (Figure 4.8a). The amplitude at the peak, or height, of the waveforms was also used as an input parameter for the clustering algorithm.

$$f(t) = ae^{t/\tau_1} + be^{t/\tau_2} \quad (4.2)$$

To determine the number of clusters K in the K-means algorithm, an Error Sum of Squares (ESS; Equation 4.3) was optimized for the lowest number of clusters with the lowest ESS (Celebi et al., 2013). In Figure 4.8b, the ESS is plotted as a function of K . We considered the 'elbow' for both intracellular and extracellular protocols to be between 4 and 6 clusters, hence we performed the analysis with $K = 4, 5$, and 6. After that analysis, we considered that 4 was the best choice of K for extracellular stimulation for several reasons, e.g. with higher K there were clusters with only 1 element. Similarly, the choice of K for intracellular stimulation was $K = 3$. The morphological characteristics, class, and type of light response of the cells were not used as input variables in the cluster classification algorithm, therefore, the clusters do not depend on those parameters.

$$ESS = \sum_{k=1}^K \sum_{i=1}^N (x_i - c_k)^2, \quad \text{where } N = \text{number of cells} \quad (4.3)$$

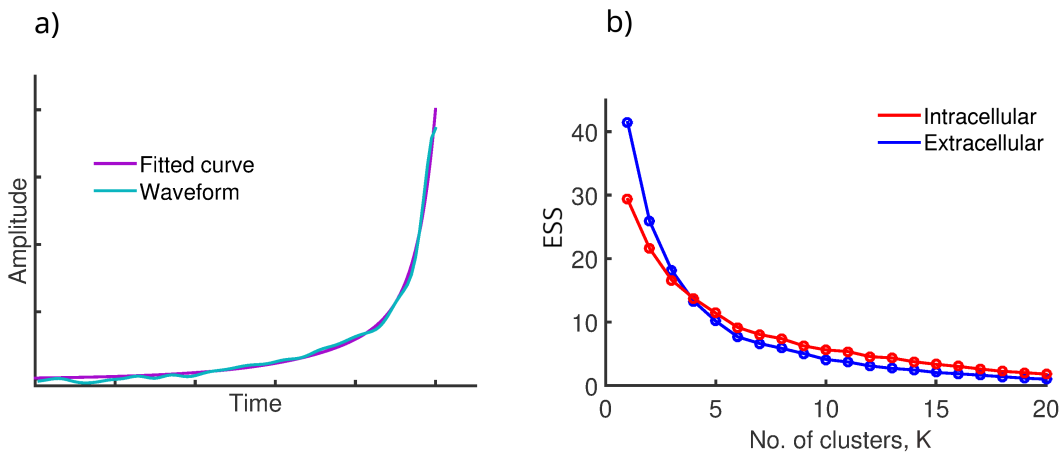


Figure 4.8: K-means clustering parameters. **(a)** Rising part of the waveform of an example cell (cyan), exponential curve fit from equation 4.2 (purple). **(b)** Error Sum of Squares per number of clusters for intracellular and extracellular data.

4.2 Results

When analysing the waveforms resulting from both intracellular and extracellular stimuli, we always found a biphasic artifact occurring after the spike initiation (circled in Figure 4.9). The cause of this artifact is not clear, although it is clear that the spike triggering mechanism does not depend on this part of the waveform, because it always occurs after the action potential has started. Figure 4.9 shows an example waveform with the artifact and the spike, note how the artifact occurs after the action potential has initiated. We chose to perform the STC analysis from the time of spike initiation, rather than from the peak of the action potential, since only stimuli prior to action potential initialization affected the spike.

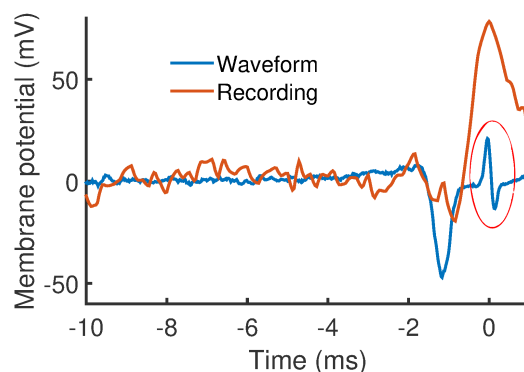


Figure 4.9: Waveform from the example recording in Figure 4.4e (blue), superimposed with 10 ms of recording prior to a spike (red). The y-axis corresponds to the recording (red), the y-axis corresponding to the waveform (blue) was omitted. The x-axis is aligned and proportional in both lines, hence it is evident how the artifact occurred after the spike had been initiated. The biphasic artifact (circled) was removed from all the waveforms before the K-means analysis.

4.2.1 Intracellular stimulation

43 cells were stimulated with intracellular stimulation. From their morphology, 8 of those RGCs were classified as class A, 1 cell was class B, 22 class C, and 12 of them belonged to class D. The K-means analysis of their waveforms resulted in 3 different clusters. Figure 4.10a illustrates the waveforms of all the cells classified by cluster. As shown in Figure 4.10b, the clusters correlate well with the morphological class: cluster 1 made up 75% A cells, cluster 2 made up 58% D cells, and 59% of C cells were assigned to cluster 3. Each cluster was made predominantly from the same morphological class (Figure 4.10c).

Class B is a special case, due to having only 1 RGC of this class. Hence, 100 % of B cells belonged to cluster 1, which means that 60% of the cells in cluster 1 were A cells, and 76% of cluster 3 were C cells. There were almost twice as many C cells as D type RGCs, therefore cluster 2 seems to be formed mostly of C cells in Figure 4.10c, however, as in Figure 4.10b, D cells proportionally fall mainly into cluster 2.

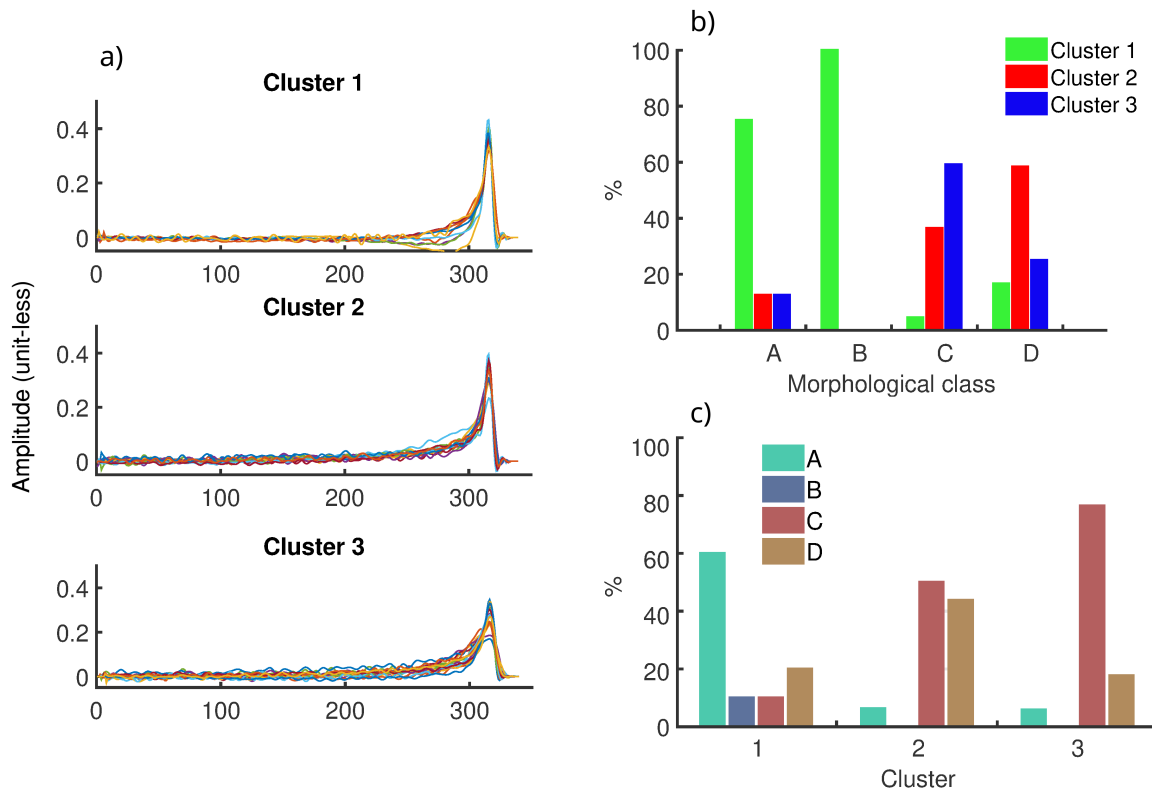


Figure 4.10: Distribution of intracellularly stimulated RGCs in the clusters. **(a)** Waveforms of the cells classified in each cluster. Waveforms are aligned at the peak. **(b)** Percentage of cells of each morphological class in clusters 1, 2 and 3. **(c)** Proportion of cells from each morphological class per cluster.

It is clear, then, from Figure 4.10 that the cluster division coincides with the morphological classification of RGCs, for instance, the majority of cells that form cluster 1 are A class RGCs, and class C RGCs contribute mainly to cluster 3. While cluster 2 is formed of both C and D cells, the majority of D cells belong to cluster 2. Figures 4.11 to 4.13 present each RGC with its morphological class organized in clusters. Figure 4.11 shows the segregation in v1, v2, and the latency of the RGCs. The figure shows very little overlap between clusters, and a clear segregation of cells. This means that the shape of the waveforms depend to some extent on the morphological characteristics of each RGCs, which could suggest a possible preference of each morphological type to a particular stimulus. Figure

4.12 illustrates v_2 , the height of the waveforms, τ_1 , and τ_2 as functions of v_1 .

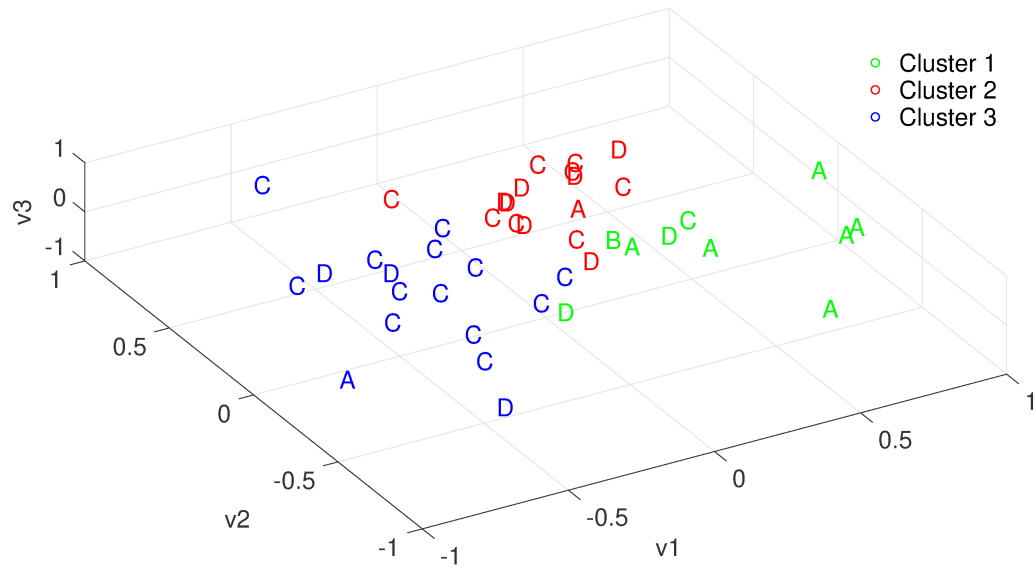


Figure 4.11: Separation of intracellular stimulation cells in cluster 1 (green), cluster 2 (red), and cluster 3 (blue). Each letter represents a RGC and indicates its morphological class. X, Y, and Z axis correspond to variables v_1 , v_2 , and v_3 , respectively.

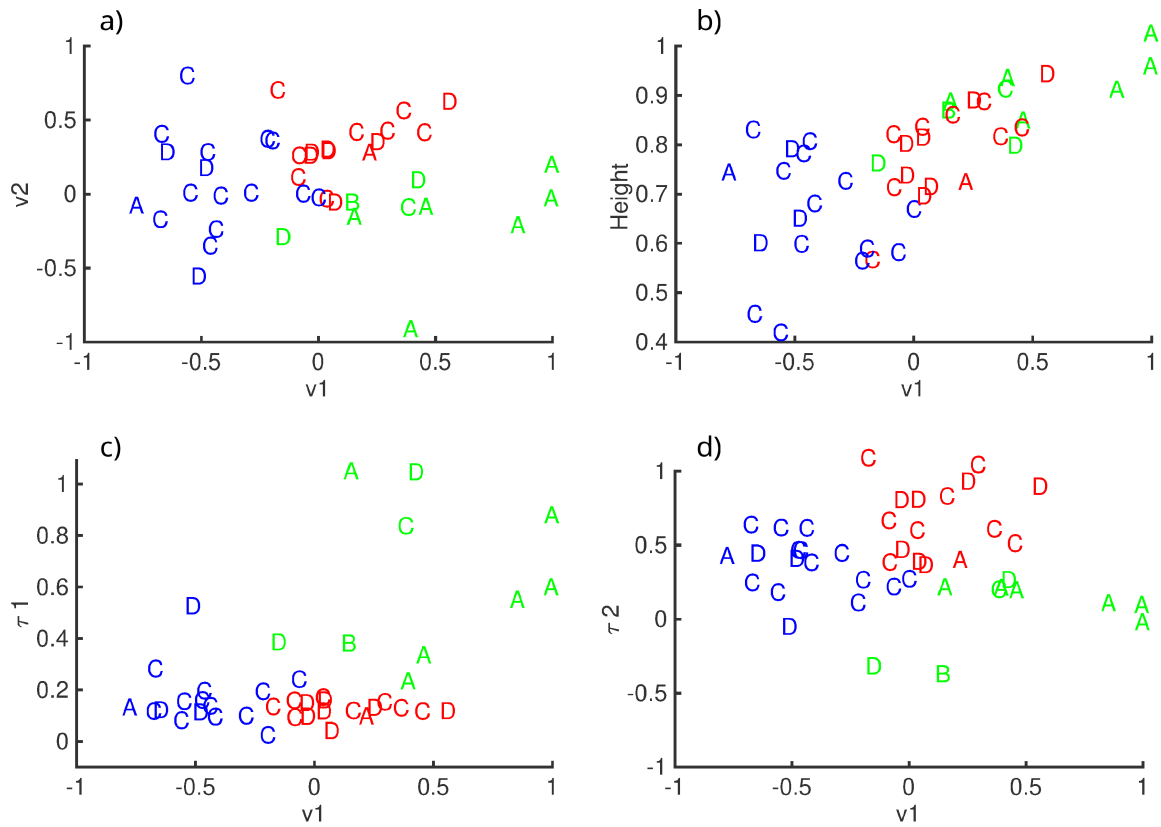


Figure 4.12: Separation of intracellular stimulation cells in cluster 1 (green), cluster 2 (red), and cluster 3 (blue). Each letter represents a RGC and indicates its morphological class. Variables **(a)** v_2 , **(b)** height, **(c)** τ_1 , and **(d)** τ_2 , plotted as functions of v_1 .

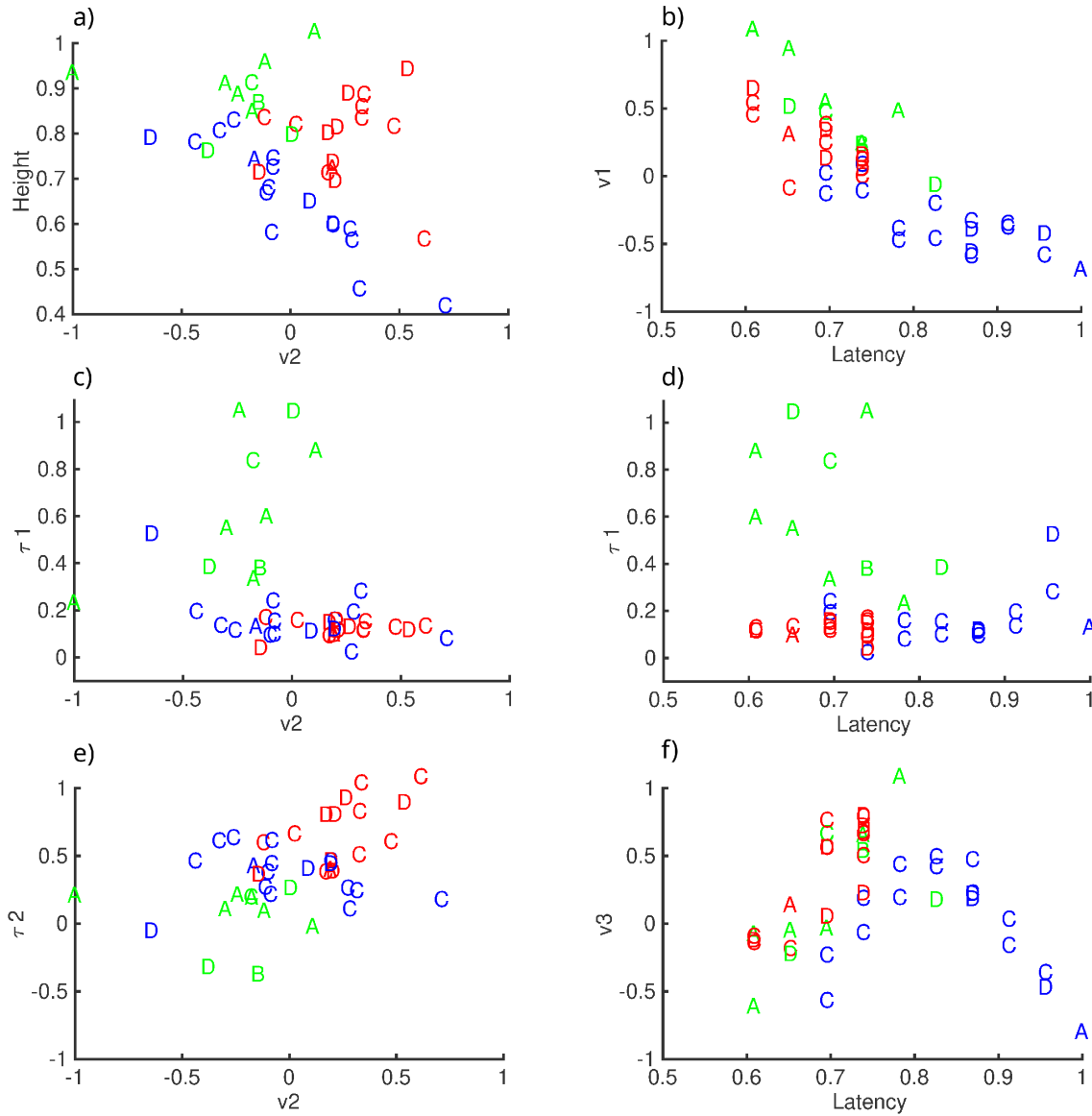


Figure 4.13: Separation of intracellular stimulation cells in cluster 1 (green), cluster 2 (red), and cluster 3 (blue). Each letter represents a RGC and indicates its morphological class. Variables **(a)** height, **(c)** τ_1 , and **(e)** τ_2 , plotted as functions of v_2 , and **(b)** v_1 , **(d)** τ_1 , and **(f)** v_3 , plotted as functions of the latency.

Further analysis was done by attempting to use other variables, like the latency and height as inputs for the clustering algorithm, but the inclusion of such variables showed no difference in the results. For that reason, we removed those variables from the K-means algorithm. Nevertheless, in Figure 4.13 we plotted the latency as a parameter to show its little effect on the clustering. Subplot (b) shows correlation between Latency and v_1 , it could be that the little influence of Latency in the clusters was already implicit in v_1 . As in Figure 4.21, Figure 4.14 illustrates the Gaussian distribution of the raw intracellular stimulus and the distribution of the spike-triggered stimulus set in the response of two

example cells to white noise stimulation. The ratio of stimulus vs response (yellow line) shows the nonlinearity of the filter that extracts the preferred waveform to stimulate a cell. In contrast with the data from extracellular stimulation (Figure 4.21), the two examples in Figure 4.14 show the distribution of the spike-triggered stimulus fully overlapping with the raw stimulus. This means that there was a range of amplitudes that elicited a response, whereas Figure 4.21 might suggest that there was a lack of higher amplitude stimulation to convey all the amplitudes that would elicit a response.

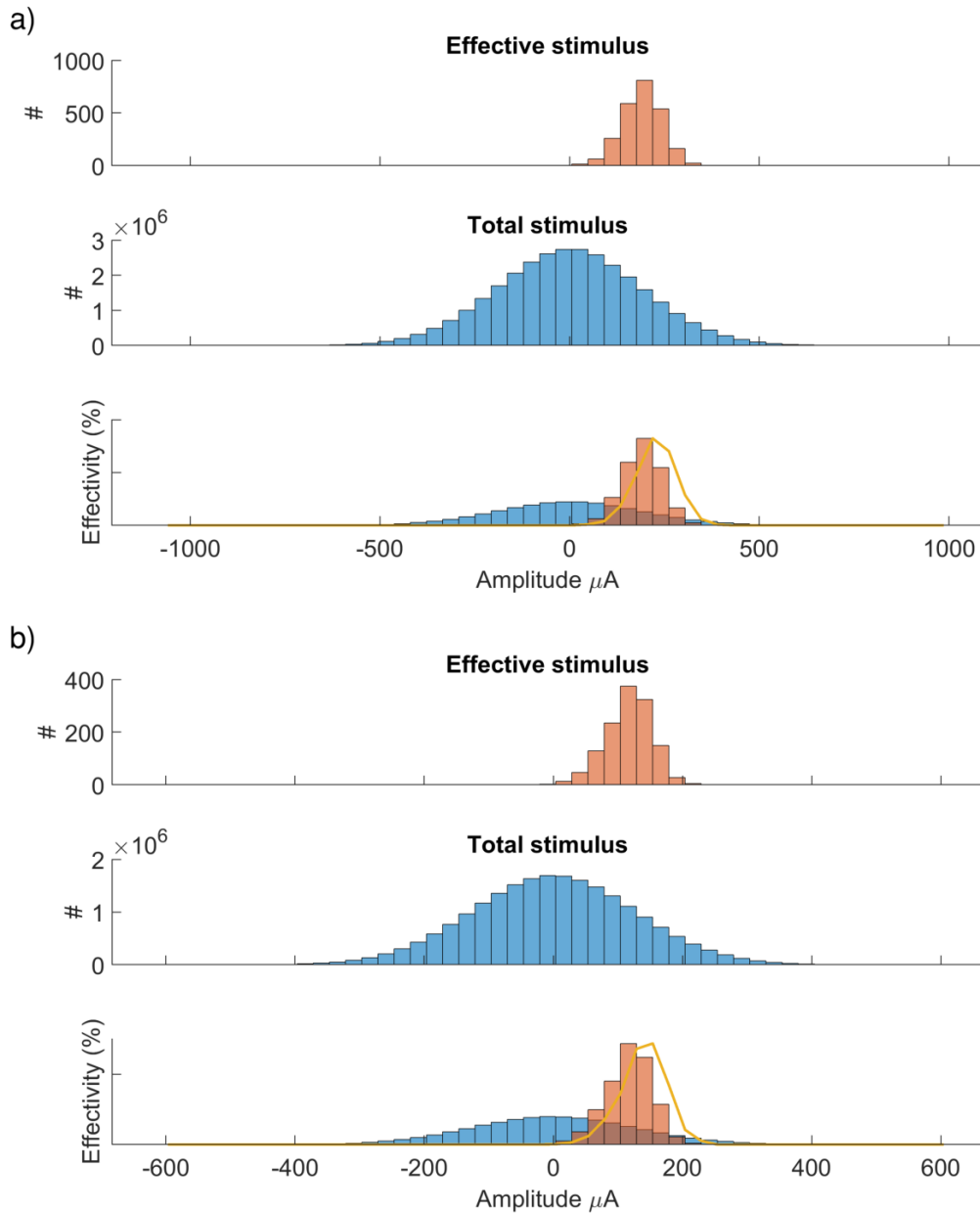


Figure 4.14: Intracellular stimulation. **(a)** Number of spikes elicited per stimulus amplitude on an example cell (top), distribution of different amplitudes in the applied stimulation train for the example cells (middle), efficacy of the stimulus applied at each different amplitude (bottom). The yellow line represents the ratio of stimulation vs spikes. The peak of the yellow curve indicates the amplitudes with the highest efficacy. **(b)** Same format as (a), but for another example cell.

4.2.2 Extracellular stimulation

The 44 waveforms from the extracellular stimulation protocol are presented in Figure 4.15a, they have been separated into the 4 clusters resulting from the K-means clustering algo-

rithm. Figure 4.15b-d show the distribution of the different RGC morphological classes into each cluster and the distribution per type of light response. There were 15 RGCs in morphological class A, 10 in class B, 8 in class C, and 5 in class D. Although theoretically, all ON-OFF cells belong to morphological class D, we encountered 7 ON-OFF cells, but could only classify 5 of them as D cells (see Chapter 3), hence, there is a difference in the distribution of D cells and ON-OFF cells in subplots (b) and (d). A flat line at the end of some of the waveforms in Figure 4.15a is the result of horizontal alignment of the waveforms.

The light response of the different cells stimulated with extracellular stimulation was compared with the cluster analysis (Figure 4.15d) and, just as with the morphological class, there was no relation between the light response and the cluster grouping of the cells. Most clusters had a similar number of cells from each functional type: cluster 1 had $\sim 10\%$ of ON cells and $\sim 5\%$ of OFF cells, cluster 2 made up $\sim 30\%$ of ON cell and $\sim 25\%$ of OFF and ON-OFF cells, cluster 3 had $\sim 20\%$ of ON and $\sim 40\%$ of OFF and ON-OFF, and finally, cluster 4 made up almost the same $\sim 30\%$ of each type. This means that the functional type does not define the shape of the preferred waveform of a particular cell, and most likely the response to electrical stimulation does not depend on the stratification level, but on the size and shape of the dendritic tree.

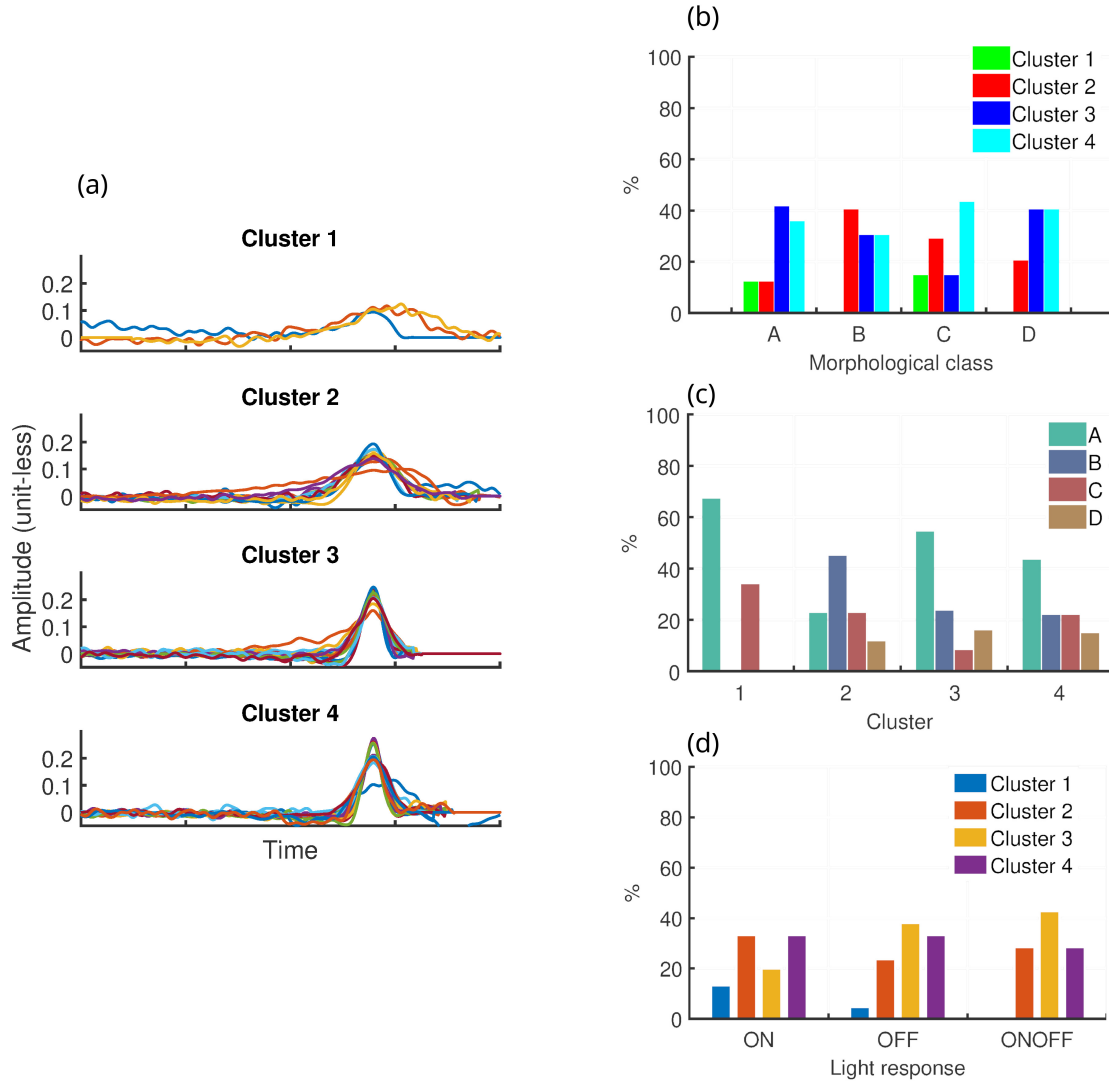


Figure 4.15: Distribution of RGCs subjected to extracellular stimulation in the clusters. **(a)** Waveforms of the cells classified in each cluster. Waveforms are aligned at the peak. **(b)** Percentage of cells of each morphological class in clusters 1, 2, 3 and 4. **(c)** Proportion of cells from each morphological class per cluster. **(d)** Distribution of functional cell types between clusters.

The grouping of RGCs in clusters is shown in Figure 4.16, where each cell is plotted in function of variable v_1 against v_2 , v_3 , and height. The variable that seems to contribute the most to the clusters is v_1 (Clusters 2 and 3), while v_2 and v_3 play a decisive role into choosing between Clusters 1 and 4 for the rest of the RGCs, this was expected, because v_1 is the principal eigenvector of the covariance matrix, i.e. the direction of the largest variance in the data. τ_1 does not affect the clustering, which means that the slope of the waveform plays a small role in the segregation of cell responses.

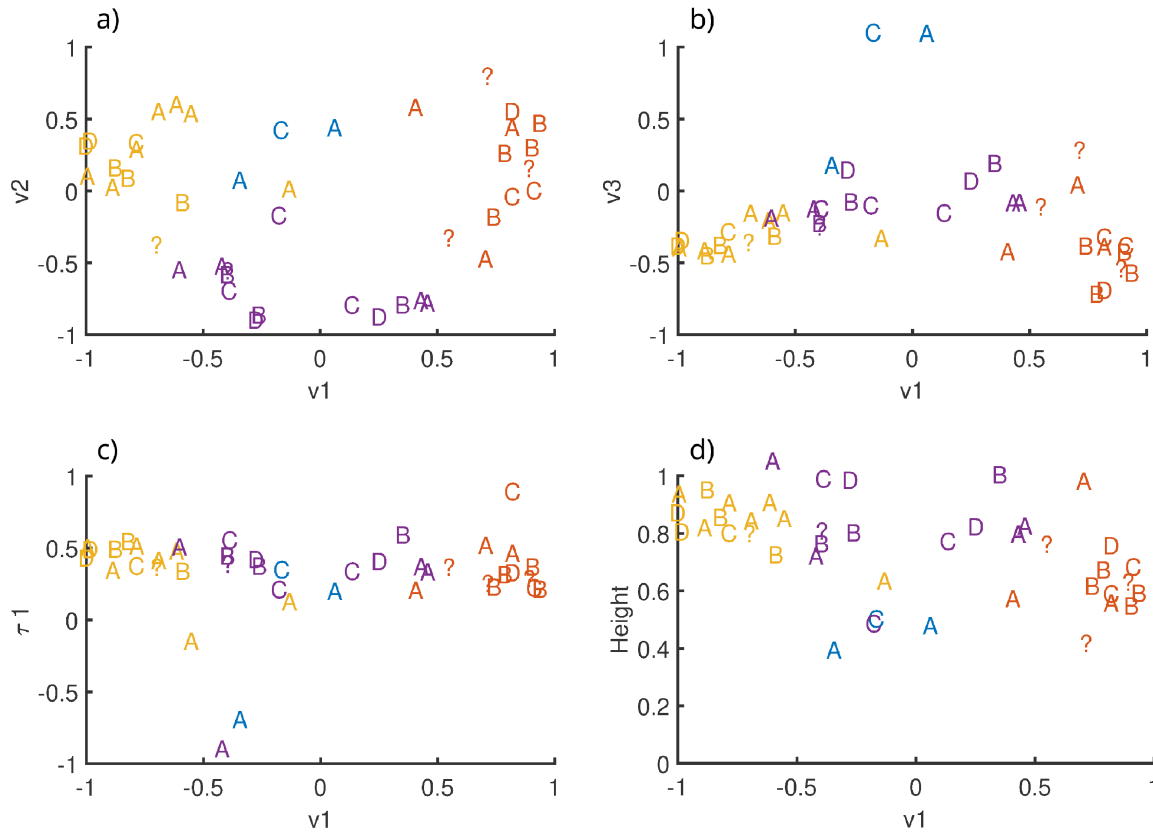


Figure 4.16: Separation of extracellular stimulation cells in cluster 1 (blue), cluster 2 (orange), cluster 3 (yellow), and cluster 4 (purple). Each letter represents a RGC and indicates its morphological class. Variables **(a)** v_2 , **(b)** v_3 , **(c)** τ_1 , and **(d)** height, plotted as functions v_1 .

4.2.3 Influence of latency on the temporal receptive field

Latency was the time between the peak of each waveform and the time of the spikes. There was no major difference in the cluster distribution when including the latency as an input to the K-means algorithm, hence it was removed from the input parameters. However, there seems to be an important correlation between the latency and the clustering, as seen in Figures 4.18 and 4.19a. The latter shows a significant difference in the latencies of clusters 2, 3 and 4, while cluster 1 has a wider latency range. Figure 4.17 illustrates that there are only 3 cells in cluster 1 (7% of the total number of RGCs), and two of those cells have a much higher latency than the majority of RGCs, therefore, we can say that also for cluster 1, latency might be an important factor. When v_1 was plotted as function of latency, it suggested that, at least for lower latencies, v_1 was linearly proportional to the latency, which can be interpreted as the latency having high influence in the shape of

the waveform and the clustering segregation. In other words, latency was not an input to the clustering algorithm, but the clusters were compared to the latency of each waveform and the results show coincidence between the latency of the waveforms and the cluster distribution. The correlation between clusters and latency is evident in Figure 4.18, and it becomes more obvious in Figure 4.18c, which could imply that latency plays an important role in the shape of the preferred waveform. It would be helpful, however, to know if latency depends on morphological characteristics of the RGCs, or on different factors.

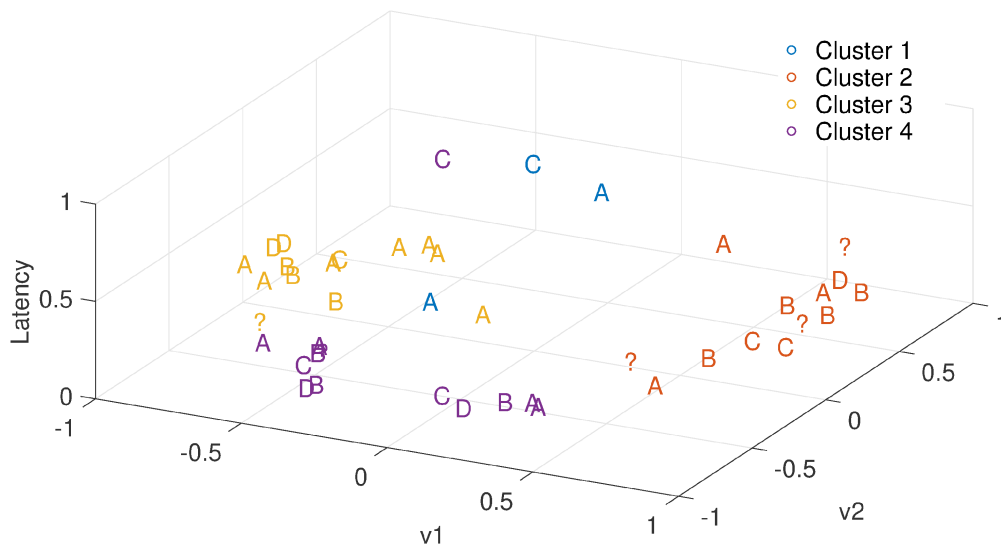


Figure 4.17: Separation of extracellular stimulation cells in cluster 1 (blue), cluster 2 (orange), cluster 3 (yellow), and cluster 4 (purple). Each letter represents a RGC and indicates its morphological class. X, Y, and Z axis correspond to variables v1, v2, and latency, respectively.

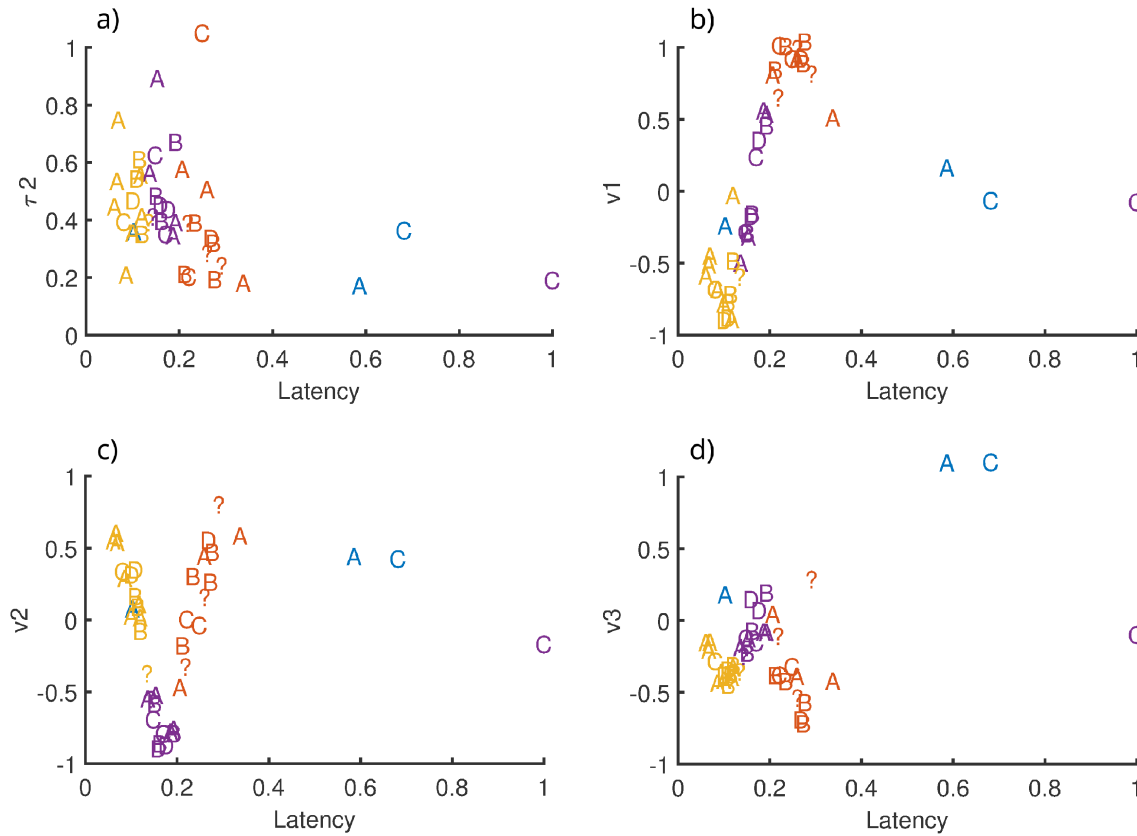


Figure 4.18: Separation of extracellular stimulation cells in cluster 1 (blue), cluster 2 (orange), cluster 3 (yellow), and cluster 4 (purple). Each letter represents a RGC and indicates its morphological class. Variables **(a)** τ_2 , **(b)** v_1 , **(c)** v_2 , and **(d)** v_3 , plotted as functions of the latency.

Although the stimulating electrode was carefully located at a close distance to the soma ($\sim 50 \mu\text{m}$), the distance was not always exactly $50 \mu\text{m}$. Most importantly, the nature of the experiment did not allow the placement of the extracellular electrode at a precise location relative to the exit point of the axon. Thus, the latency could be dependent more on the location of the electrode than on the morphological or intrinsic electrical properties of the RGCs (Figure 4.19b). For instance, in the hypothetical case of Figure 4.19b, electrodes 1 and 2 are equally separated from the soma. However, electrode 1 is not directly above any dendrite, while electrode 2 is above various branches. This means that the stimulus from electrode 1 would need a higher amplitude to stimulate the cell, and would take longer to produce a spike than a stimulus from electrode 2, because the stimulus itself would take longer to reach a dendrite, the soma, or the axon. Electrode 2 would be directly stimulating the dendritic tree, and potentially generating a dendritic spike. Moreover, electrodes 3, 4, and 5 are all at the same distance from the soma, nevertheless, they would stimulate the RGC in different ways. A stimulus from electrode 3 would have to travel a longer distance

to be able to stimulate the neuron, but electrodes 4 and 5 would be directly stimulating the axon, and dendrites, respectively. The latency of the waveform resulting from a stimulation with electrode 4 would obviously have shorter latency than one resulting from the stimulation with electrode 3. Furthermore, electrode 4, would probably even produce a shorter latency spike than electrode 1, or 2, due to direct axonal stimulation. Meng et al. (2018) found a relationship in the latency and the spike probability of RGCs in response to anodic-first biphasic stimulation, as shown in Figure 4.20. Lower latency spikes were in the range of amplitudes to which the cell responded with higher probability.

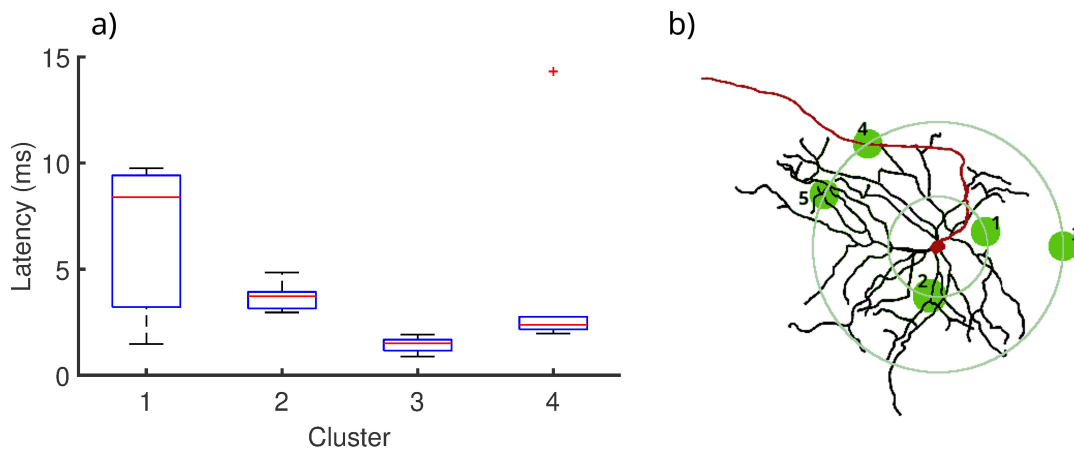


Figure 4.19: Latency of the response to extracellular stimulation. **(a)** Time between the peak of the waveforms and the occurrence of the spikes (latency) per cluster. On each box, the central mark indicates the median, and the bottom and top edges of the box indicate the 25th and 75th percentiles, respectively. The whiskers extend to the most extreme data points not considered outliers, and the outliers are plotted individually using the '+' symbol. (MATLAB 2017a, The Mathworks, Inc.). **(b)** Hypothetical placement of extracellular electrodes on an example RGC. The soma and axon of the example A class RGC are in red, the dendritic tree is black. Green dots represent possible placement of epiretinal or subretinal electrodes, light green concentric circle indicate distance to the soma is equal between electrodes 1 and 2, and between electrodes 3, 4, and 5. Figure of RGC adapted from Wong et al. (2012).

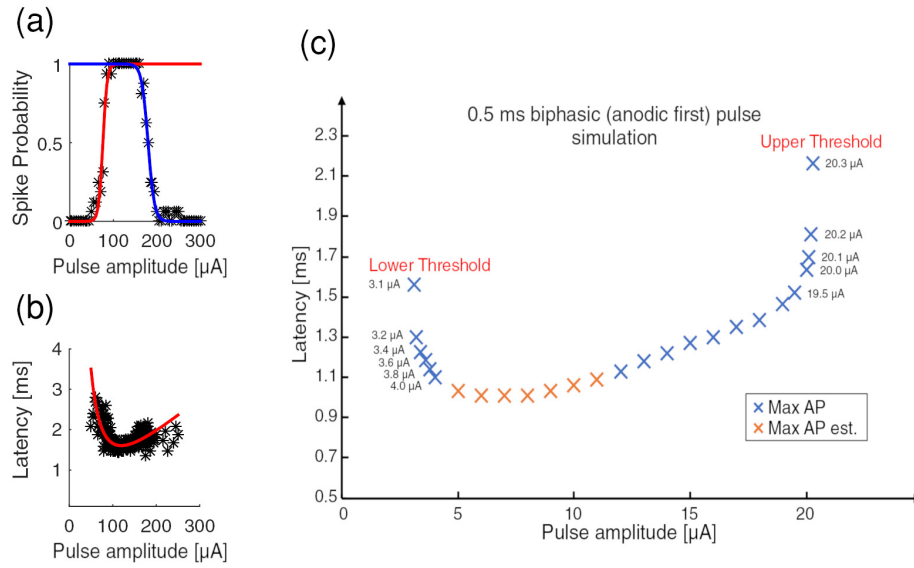


Figure 4.20: Lower and upper amplitude stimulation thresholds **(a)** Activation probability of a cell in response to biphasic pulses. Experimental data points (black stars). A positive sigmoid curve was fitted to the lower amplitude data (red), and a negative sigmoid to the higher amplitudes (blue). **(b)** Same data as in (a) plotted in function of amplitude versus latency of spikes. **(c)** Simulation of somatic action potentials, amplitude versus latency as in the experimental data in (b). Figure taken from Meng et al. (2018).

Similar to Figure 4.2, the distribution of stimulus in two example recordings is shown in Figure 4.21. The figure shows the Gaussian distribution of the raw stimulus and the distribution of the spike-triggered stimulus set. It also shows the ratio of stimulus vs response, which illustrates the nonlinearity of the filter that extracts the preferred waveform to stimulate a cell. Figure 4.21b does not show the ideal scenario, having the distribution of successful stimuli shifted to the right, instead of overlapping with the distribution of all the applied stimuli means that the upper threshold was not reached (compare Figure 4.21 against Figure 4.14). The issue could be solved by applying a wider range of stimulus amplitudes.

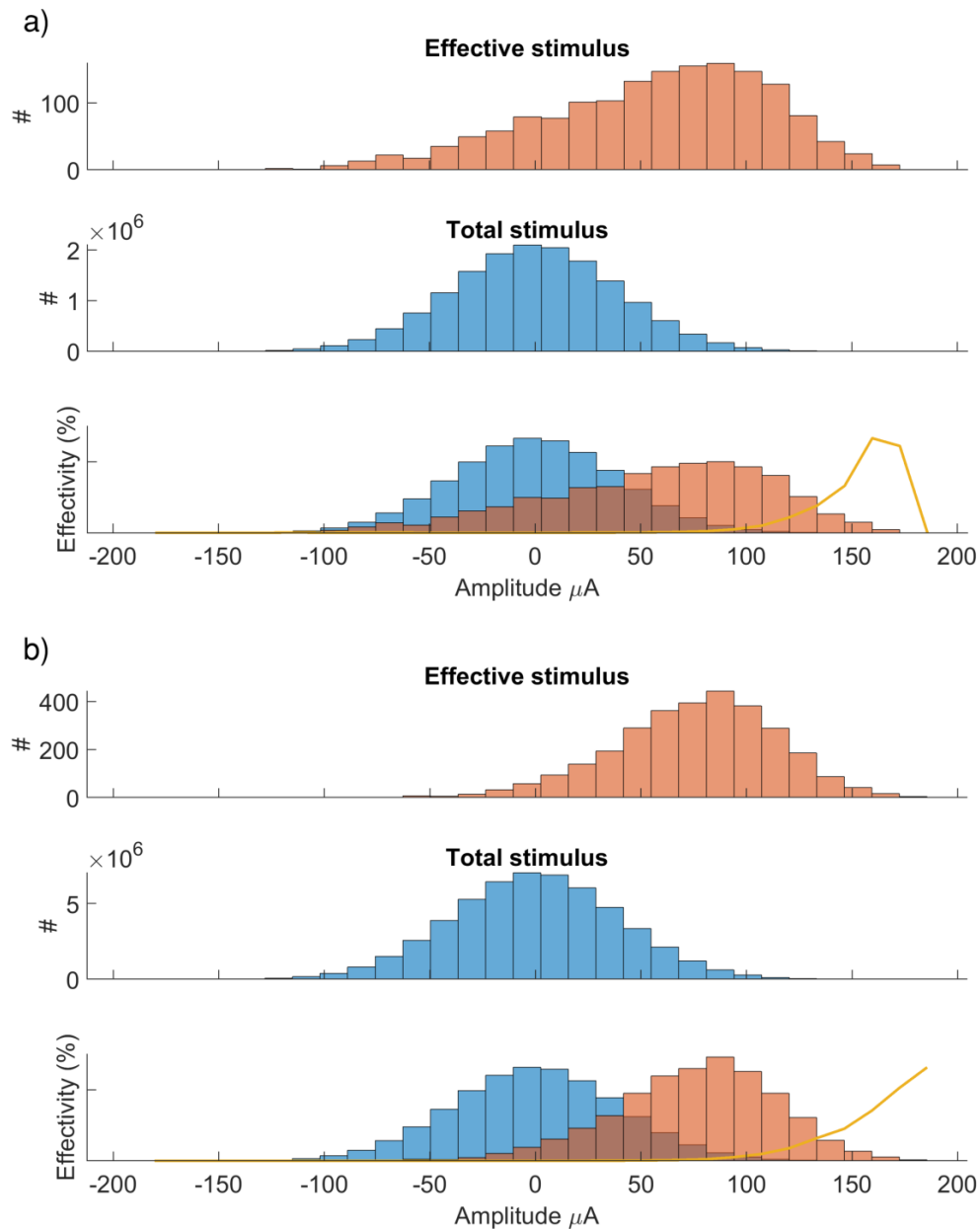


Figure 4.21: Extracellular stimulation. **(a)** Number of spikes elicited per stimulus amplitude on an example cell (top), distribution of different amplitudes in the applied stimulation train for the example cells (middle), efficacy of the stimulus applied at each different amplitude (bottom). The yellow line represents the ratio of stimulation vs spikes. The peak of the yellow curve indicates the amplitudes with the highest efficacy. **(b)** Same conventions as (a), but for another example cell.

4.3 Discussion and conclusion

Discrete Gaussian white noise was used for extracellular stimulation of 44 cells, and intracellular stimulation of 43 cells. The response of the RGCs to the white noise stimulation was analysed and the waveform of each cell were recovered. Once done, the features of the ERFs were used to group the cells into different clusters: 4 clusters for extracellular stimulation and 3 clusters for intracellular stimulation.

Analysis of the responses of RGCs to white noise has been done before with light stimulation (Chichilnisky, 2001; Pillow and Simoncelli, 2003; Schwartz et al., 2002) and electrical stimulation (Freeman et al., 2010; Marmarelis and Naka, 1972; Maturana et al., 2016). While electrical stimulation has been randomized in a white noise fashion, it was generally done by applying charge balanced biphasic pulses with amplitudes sampled from a Gaussian distribution (Maturana et al., 2016), or binary square-pulses with a fixed amplitude (Freeman et al., 2010). They could effectively extract features of the stimulus that describe an ideal activation of RGCs. For example, Maturana et al. (2016) were able to optimize the spatial and temporal characteristics of multi-electrode stimulation to obtain higher efficacy with multiple electrodes for a given power. The stimulation was done using pure white noise by Marmarelis and Naka (1972), who used the Wiener theory for non-linear system identification to extract features of RGCs such as latency, frequency response, and non-linearities. Their results suggested that the studied RGCs had an underdamped response and a low-pass filter-like nonlinearity with a cut-off frequency of 11 Hz. They also found that the model and white noise electrical stimulation can predict the response of RGCs. We apply a stimulus similar to that used by Marmarelis and Naka (1972), but the complexity of the STC analysis we use increases exponentially with the number of samples in the stimulus, hence we limited the Gaussian white noise and chose to hold each amplitude for a short period of time. We have identified the features of the stimulus that were preferred by individual neurons and have extracted metrics to compare among different RGCs. We believe our system can more accurately describe the receptive fields of neurons than binary or biphasic stimulation. Moreover, it has been suggested that the traditional stimulation protocols using rectangular waveforms are not ideal in activating visual and cortical neurons (Foutz and McIntyre, 2010; Lee and Im, 2018; Wongsarnpigoon and Grill, 2010). In fact, the most efficient waveform shape is a Gaussian waveform, similar to the shape observed in our recovered waveforms (Sahin and Tie, 2007). The validation of our model

is done in Chapter 5.

This technique has shown the capacity to segregate cells by morphology. That is, with intracellular stimulation the classification based on the electrical receptive fields of the RGCs correlates quite accurately with their morphological classification. We can say that RGCs from the morphological class A, are more likely to be in Cluster 1, cells with a morphology class D are more likely to be in Cluster 2, and those in class C are more likely to be in Cluster 3. Wong et al. (2012) found distinct physiological features between A-cells (particularly A2), and D-cells (bistratified). A-RGCs were found to have the smallest membrane time constant and the lowest input resistance. Here we have found that the features of the stimulus that most likely activate A-cells could be grouped in Cluster 1, which had a slower charging curve and a higher peak amplitude. Conversely, Cluster 2 grouped most of the D-RGCs, which were found to have the longest membrane time constant and the highest input resistance (Wong et al., 2012). The differences in the intrinsic properties of the neurons may account for the ability to extract and classify RGCs in different morphological types by their ERF.

In the case of extracellular stimulation, latency played an important role in defining the waveform that would ideally stimulate different cells as observed by Maturana et al. (2016). In fact, latency was the variable that seemed to have the largest impact on the differentiation of waveforms - much more so than the morphological characteristics of the cells or their responses to light. Latency seemed to depend greatly on the location of the electrode relative to the RGC's axon and dendrites, this is a hypothetical conclusion which would need to be quantified in future experiments. Although it would not be a trivial exercise, it would be an interesting future experiment to control the placement of the stimulating electrode with respect to the dendritic tree of the recorded axons, e.g. placing the stimulating electrode always above the dendrites, at a certain distance from the soma. This would allow us to know whether the results from intracellular stimulation extend to extracellular stimulation. Such an experiment would also confirm that the morphological type did in fact set the parameters for the waveform with extracellular stimulation, as it does for intracellular stimulation. In any case, our experiments confirm that it would not be possible to use the waveforms, or a special shape of waveform to target specific cells in a clinical environment, because neither the cell class (morphology) nor the cell type (light response) have much input into the shape of the waveform when the location of the electrodes is not controlled,

as is the case with neuroprosthetic visual arrays in a clinical setting.

Chapter 5

Selectivity

Retinal ganglion cells (RGCs) are feature detectors that send highly processed visual information to the brain (Sanes and Masland, 2015). More than 20 distinct types of RGCs have been identified in most mammalian retinas with diverse responses to various features (Dowling and Dowling, 2016c; Wässle, 2004). At the extreme RGCs respond oppositely to the exact same stimulus: for example, Kuffler (1953) identified some RGCs that responded with an increased spike activity when the retina was exposed to a brightness increment (ON cells), and other RGCs that were excited or had an increase in spike rate when exposed to a brightness decrement (OFF cells). Ideally, in order to convey all the necessary information from a visual scene through electrical stimulation of the RGCs, some neurons should be stimulated with some features of the image, and inhibited with other features. Kotsakidis et al. (2018) reported differences in the responses of RGCs to intracellular and extracellular stimulation, as well as differences in the responses to light, particularly a narrower window of 'preferred' electrical stimuli in ON cells than in OFF cells. Previously, Twyford et al. (2014) designed a stimulus with a high frequency waveform ($\sim 2kHz$) that selectively stimulates ON or OFF cells by changing the envelope of the stimulus waveform. Similarly, Maesoon Im might have found two different approaches for selective stimulation of ON (Im and Fried, 2016) and OFF (Im et al., 2018) cells by inhibiting the decay of network mediated responses. It is possible to explore selectivity to different mechanisms, e.g. to either activate network mediated responses or direct responses (Boinagrov et al., 2014), or different morphological types (Fried et al., 2009; Jensen and Rizzo, 2008).

In principle, the waveforms recovered in Chapter 4 represent the electrical current pro-

file to which each cell is sensitive (given a constraint on power), i.e. statistically, the spikes of each cell were elicited according to how much of that particular waveform was embedded in the random stimulus, with power in any other component of the stimulus being irrelevant to its probability of spiking. In this case a cell's own waveform should be optimal for stimulating it, among all waveforms of equal power. In this section of the project, we stimulated some of the recorded RGCs with their own waveform, and all the previously recovered waveforms from other cells in an attempt to test our hypothesis that (given waveforms of equal mean power, i.e. $\int g(t)^2 dt = E$): (a) a cell would respond more frequently when stimulated with its own waveform than with the waveforms recovered from other neurons, (b) cells from a particular morphological class should respond more frequently to a stimulus using waveforms from the same morphological class than to stimuli with waveforms from a different class, and (c) selectivity for different classes could be improved by understanding how each cell responds to the different waveforms.

5.1 Methods

In Chapter 4, the waveforms were recovered for 44 RGCs under extracellular stimulation and 43 RGCs from intracellular stimulation. 16 out of the 44 cells stimulated with extracellular stimulation were presented with all the previously recovered waveforms, including their own, while 27 out of the 43 stimulated with intracellular stimulation were stimulated with the previous waveforms, as described below. The same methods for dissection of rat eyes, retina preparation, perfusion, intracellular recording and stimulation, and extracellular stimulation described in the previous chapters were followed.

Following the successful recovery of a RGC's waveform, a stimulus train consisting of all the previously recovered waveforms (including the currently patched cell) was injected into the tissue through the same extracellular electrode. Figure 5.1 shows an ensemble of the 44 different waveforms. The waveforms in the train were arranged in random order at a rate of 10 waveforms per second, and each of them was scaled by an *amplitude* factor. The amplitude factor initially ranged from 0 to 1000 in increasing steps of 100 (i.e., $[0, 100, 200, \dots, 1000]$). An example of the stimulus train is presented in Figure 5.2. The cell was stimulated with the train of waveforms at least 5 times, then the range of amplitude factor was narrowed according to the response of the cell. For example, if the threshold

amplitude factor (see Figure 5.3) was found between amplitude factors of 200 and 600, the range of amplitudes was changed to 200-600 with steps of 50, new trains of waveforms were constructed and the cell stimulated. The stimulus range covered the dynamic range of the cell as densely as possible with a minimum amplitude factor step size of 10. New trains were generated every time the cell was stimulated to ensure a random order of waveforms and amplitudes. The same train was never used more than once in any case. Each RGC was stimulated with new trains until the quality of the patch decreased, or the cell's responses had deteriorated.

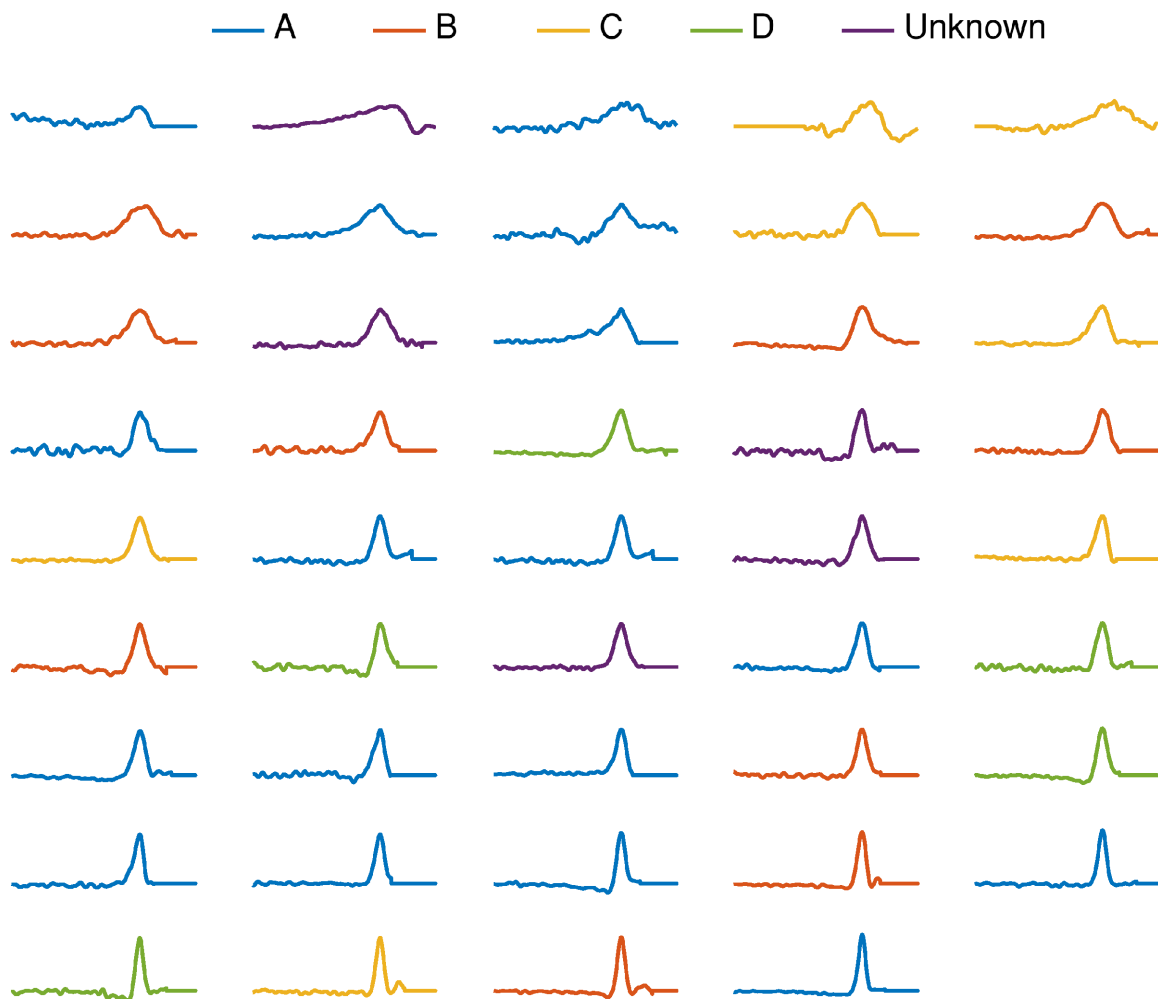


Figure 5.1: Ensemble of all the 44 extracellular stimulation waveforms. The waveforms were arranged in increasing order by the amplitude of their peak. The morphological class of the RGC to which each waveform belonged is indicated by the colour of the line.

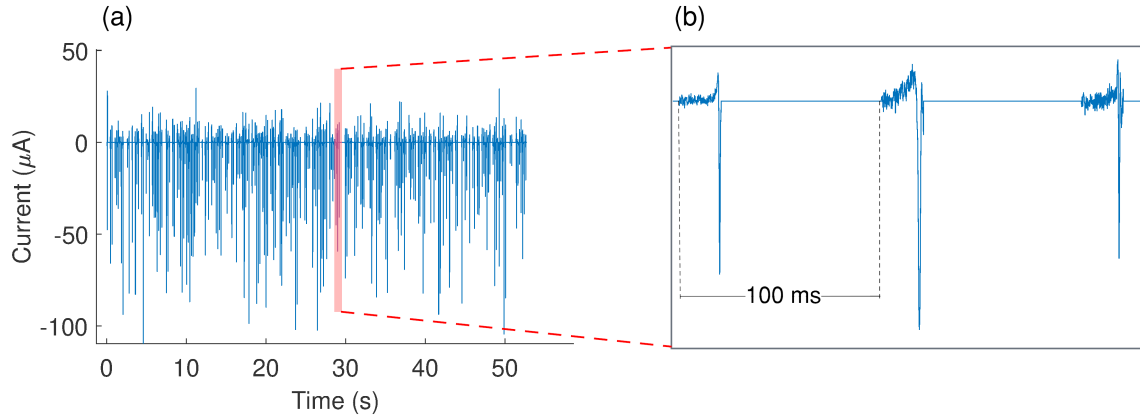


Figure 5.2: Example stimulus train for extracellular stimulation. **(a)** The complete train generated for one stimulation repetition. It consists of all the waveforms scaled and randomly arranged. **(b)** Close up on a small period of the train. Three waveforms at different *amplitudes* are shown. The waveforms are separated by a 100 ms period.

5.1.1 Data analysis

For each stimulated cell, the efficacy of the response to each waveform was evaluated. A sigmoid function described by Equation 5.1, where x is the amplitude factor, was fit to the response probability to each waveform, which varied between 0 and 1 (see Figure 5.3). Each point of data in the figure represents the efficacy of the stimulus at that amplitude factor, i.e. when the waveform was multiplied by an amplitude factor of x , the efficacy was the probability of eliciting a spike in response to that waveform at that amplitude. A data point at the bottom of the curve (Efficacy = 0) means that none (0%) of the stimuli with that amplitude factor elicited a spike, similarly, a circle with Efficacy = 1 means that every time the cell was stimulated with a waveform multiplied by that amplitude factor, it elicited a spike, hence it had 100% of efficacy, or saturation. The threshold of each cell in response to each waveform was defined as the amplitude that resulted in 50% of efficacy.

$$f(x) = (1 + e^{-a(x+b)})^{-1} \quad (5.1)$$

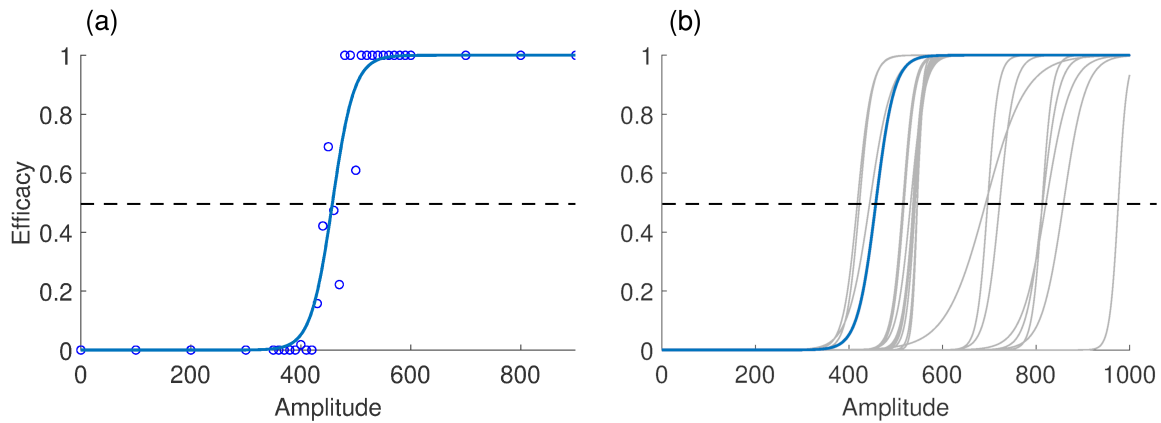


Figure 5.3: Efficacy of the response of an example RGC to waveforms. A sigmoid curve was fit to the data. **(a)** Efficacy of response to its own waveform multiplied by each amplitude factor (blue circles), curve fit (blue line). **(b)** One curve fit to the response of the example RGC to each waveform, its own waveform (same as (a), blue), and all the waveforms from other cells (grey). Threshold amplitude factor is the amplitude to which efficacy is 0.5 (dotted line).

5.2 Results

5.2.1 Extracellular stimulation

Sixteen RGCs were stimulated with the train of waveforms from the extracellular stimulation protocol. To reach this stage, a neuron had to be stimulated with white noise and its waveform recovered, then it was ready to be stimulated with the waveform sequences from other cells. It was difficult for neurons to last long enough to allow the whole protocol and it was attempted many more times than were successful. Six cells belonged to morphological class A, 8 to class B, and 2 to class D. RGCs in this section were labelled with a number from 1 to 16 (refer to Table 5.1). An example recording is shown in Figure 5.4. The artifact of the waveforms can be seen on the recording in Figure 5.4a and the detected action potentials are marked by a green circle.

Table 5.1: Summary of cells simulated with the train of waveforms:

Cell No.	Morphological Class	Cluster
1	A	3
2	A	4
3	A	3
4	A	3
5	A	4
6	A	3
7	B	2
8	B	4
9	B	4
10	B	3
11	B	2
12	B	2
13	B	3
14	B	3
15	D	3
16	D	4

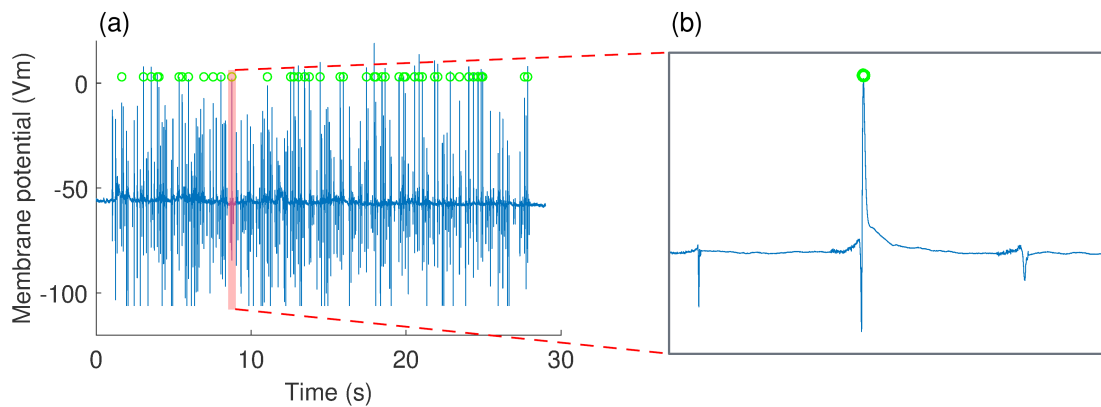


Figure 5.4: Example recording of the membrane potential of a RGC in response to a stimulus with a 30 s train. **(a)** Recording during the whole stimulation time. Green circles mark the detection of a spike in response to a waveform. **(b)** Close-up on a small period of the recording. A spike was elicited in response to the second waveform in the segment, while the two other waveforms did not elicit spikes.

Responses per morphological class

We have compared the response of each cell to the waveforms from the different morphological classes. A patched cell was stimulated with all the waveforms from previously recorded cells (train of waveforms) following the recovering of its waveform. The ensemble of waveforms that conformed the train are shown in Figure 5.1. In Figure 5.5 they are grouped by morphological class.

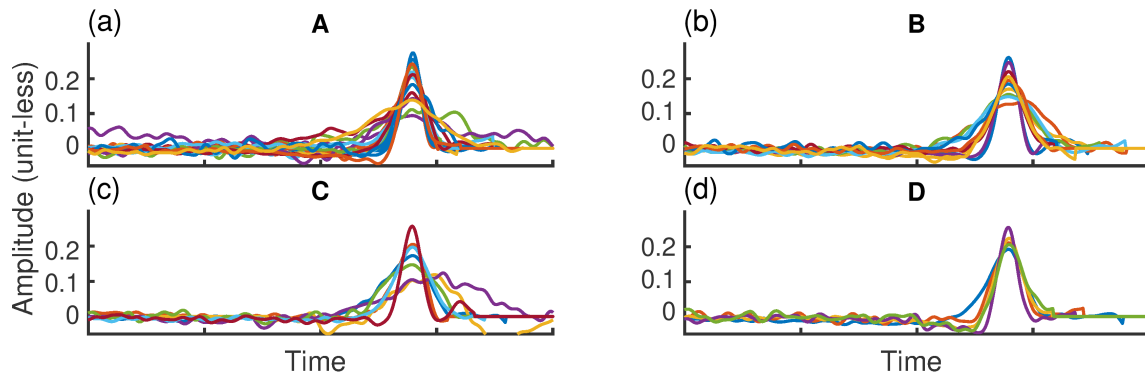


Figure 5.5: All the waveforms from the recovered waveform of each recorded cell. The waveforms are aligned at the peak, and grouped by morphological class (a) A, (b) B, (c) C and (d) D.

The response of a RGC to a waveform was judged by the amplitude factor that multiplied the waveform to achieve 50% efficacy. Hence, if a RGC had lower threshold in response to one waveform, the RGC was considered to respond better to that waveform. We compared the response of each RGC to its own waveform and to all waveforms. Figure 5.6 shows an example cell from each morphological class, the response of each example RGC to its own waveform, and the mean of the response to all the waveforms of each of the morphological classes.

Figure 5.6 illustrates that some cells had very different thresholds for different stimulation waveforms relative to the dynamic range of the waveforms (e.g. Figure 5.6c). Others had thresholds that were mostly very similar compared to the dynamic range of individual waveforms (e.g. Figure 5.6b). For cells with different thresholds, it should be easy to find an amplitude that reliably stimulates the cell using its preferred waveform but avoid stimulation with its non-preferred waveforms. For cells with similar thresholds, this is likely to be more difficult or unreliable. To quantify the variability in thresholds to different test waveforms, x , relative to the best waveforms in terms of the dynamic range, we used the measure of

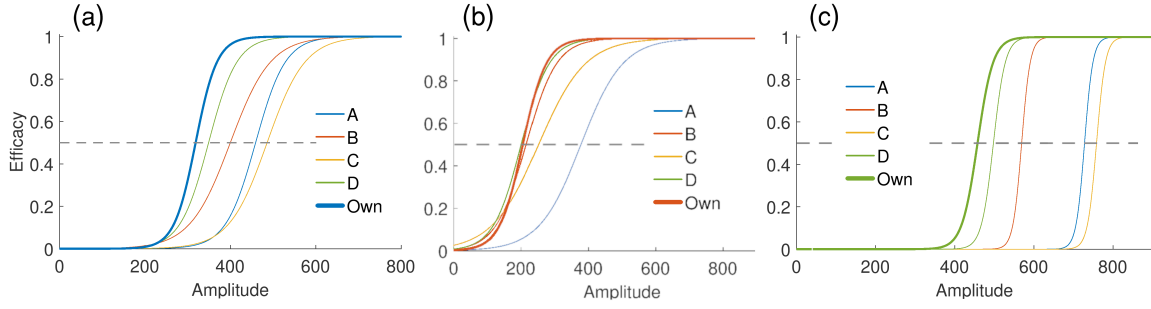


Figure 5.6: Efficacy of response of 3 example cells to their own waveform (thick coloured line) and the mean response to waveforms from each morphological class. **(a)** Example A cell, **(b)** example B cell, **(c)** Example D cell. Thin lines in each sub-figure are the average of the responses to all the waveforms from a morphological class. Dotted lines correspond to 50% efficacy.

threshold difference ($D(Th_x)$) given by Equation 5.2, where DR is the dynamic range of the best threshold of each cell, defined as the range of amplitude factors that took the best sigmoid to vary from 10% Efficacy to 90% Efficacy; Th_x is the threshold (50% Efficacy) of the response of a cell to a test waveform (its own waveform, all other waveforms, all the waveforms of the same class, or all the waveforms from all the other classes); Th_{best} is the lowest threshold in the response of that cell to all waveforms.

$$D(Th_x) = \frac{Th_x - Th_{best}}{DR}, \quad (5.2)$$

The threshold difference ($D(Th_x)$) was used in Figure 5.7 to better visualize the response of each cell to their own waveform, and to get an idea of variation in thresholds between cells. Figure 5.7a compares, for each cell, the threshold difference in response to its own waveform (x-axis, $D(Th_x = \text{threshold in response to its own waveform})$), against the threshold difference of the average response to all other waveforms (y-axis, $D(Th_x = \text{mean of the thresholds in response to all other waveforms})$). Similarly, Figure 5.7b compares the threshold difference in response to all waveforms of its own type (x-axis) against all waveforms from all other types (y-axis). In Figure 5.7a, a dot above the diagonal means that a cell was more sensitive to its own waveform than to the other waveforms, i.e. had lower threshold under that condition. A value of 0 in either axis would mean that $D(Th_x) = 0$, which in turn means that Th_x was the best sigmoid (i.e. the lowest threshold). It becomes evident, from Figure 5.7a, that all cells, except for 1 type A RGC had lower activation thresholds in response to their own waveform than to other waveforms, and the blue dots below the diagonal in Figure 5.7b suggest that A cells respond better to the waveforms of

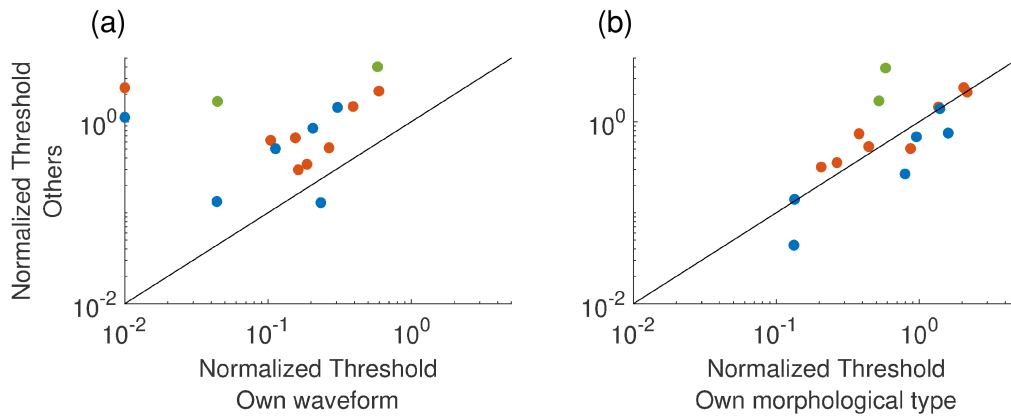


Figure 5.7: Difference in threshold of each RGC in response to **(a)** their own waveform and all other waveforms, and **(b)** all waveforms of their own morphological class and all other morphological classes. Colours indicate to which morphological class each RGC belongs: blue, A; red, B; green; D.

other morphological types.

We wanted to know if the RGCs from each type had waveforms to which other cells in the same morphological class responded better, so we compared the threshold of the response of each cell to waveforms of their own morphological class, and waveforms of all other morphological classes. To obtain the sigmoid (therefore the threshold) of the response to two or more waveforms (the sigmoid fit to the response to all waveforms of morphological class A, for example), first we fit a sigmoid to the response of each waveform (as in Figure 5.3) and we calculated the average of parameters a and b (see Equation 5.1). The response of all RGCs from each morphological class to their own waveform, and to the waveforms of other classes are shown in Figure 5.8. It is evident from Figure 5.8, and in confirmation of Figure 5.7, that type A RGCs are not preferentially activated by waveforms of other A cells, in contrast, they seem to respond better to waveforms of type D RGCs. Type D waveforms, in fact, seem to activate all cells, regardless of their morphological type, including D cells. B cells have a low threshold when stimulated with waveforms from their own morphological class, but also have a low threshold in response to D type waveforms. In general, we can observe that waveforms from morphological type A need to be multiplied by a higher amplitude factor to activate a cell, contrary to type D waveforms, which can activate cells when multiplied by lower factors.

Figure 5.9a-c shows, for 3 example cells, the variation in fits across their own class and across all other classes. Then, Figure 5.9d presents the mean efficacy ratio per morphological class. We defined the mean efficacy ratio as the ratio of the difference between the

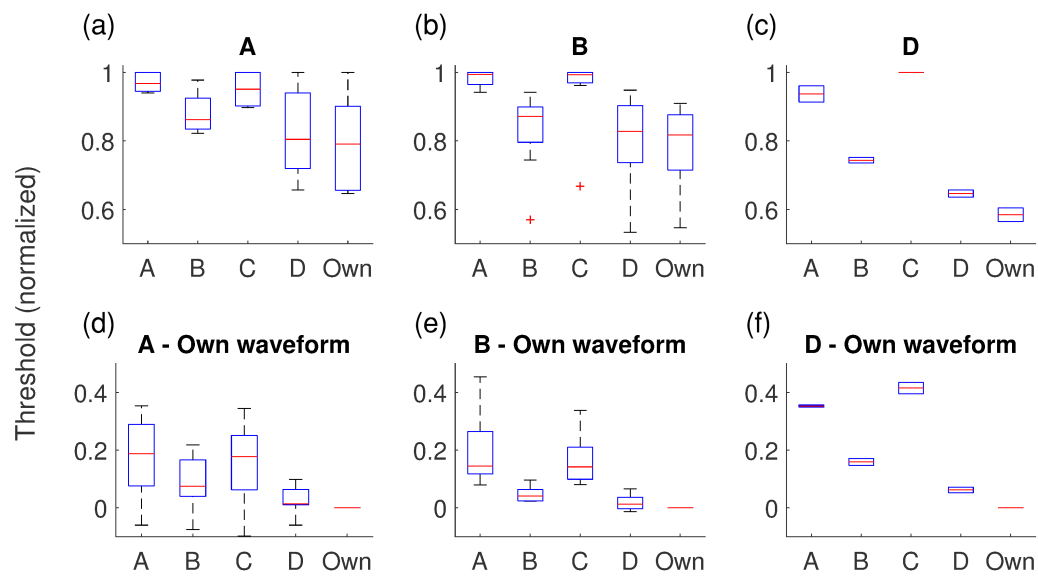


Figure 5.8: Threshold of the response of cells to waveforms from each morphological class and to their own waveform. RGCs of morphological class **(a)** A, **(b)** B (a '+' indicates an outlier), and **(c)** D. And the difference between thresholds of the responses to waveforms of each morphological class and the threshold of the response to their own waveform (**(d)**, **(e)** and **(f)**). In sub-figures **(d)**, **(e)** and **(f)**, the value *Own* is at zero, as expected, because it is the threshold in response to a RGC's own waveform minus itself. The normalized threshold for each value is obtained dividing the threshold of each cell to a particular waveform by the highest threshold of any cell to that particular waveform.

threshold of a cell in response to waveforms from the same morphological class, and in response to other morphological classes, see Equation 5.3, where MR is the mean efficacy ratio, $\mu(X_{own})$ is the mean threshold of RGCs of a morphological class in response to the waveforms of the same morphological class, and $\mu(X_{other})$ is the mean threshold in response to the waveforms of the other morphological classes.

$$MR = \frac{\mu(X_{own}) - \mu(X_{other})}{\mu(X_{own}) + \mu(X_{other})}, \quad (5.3)$$

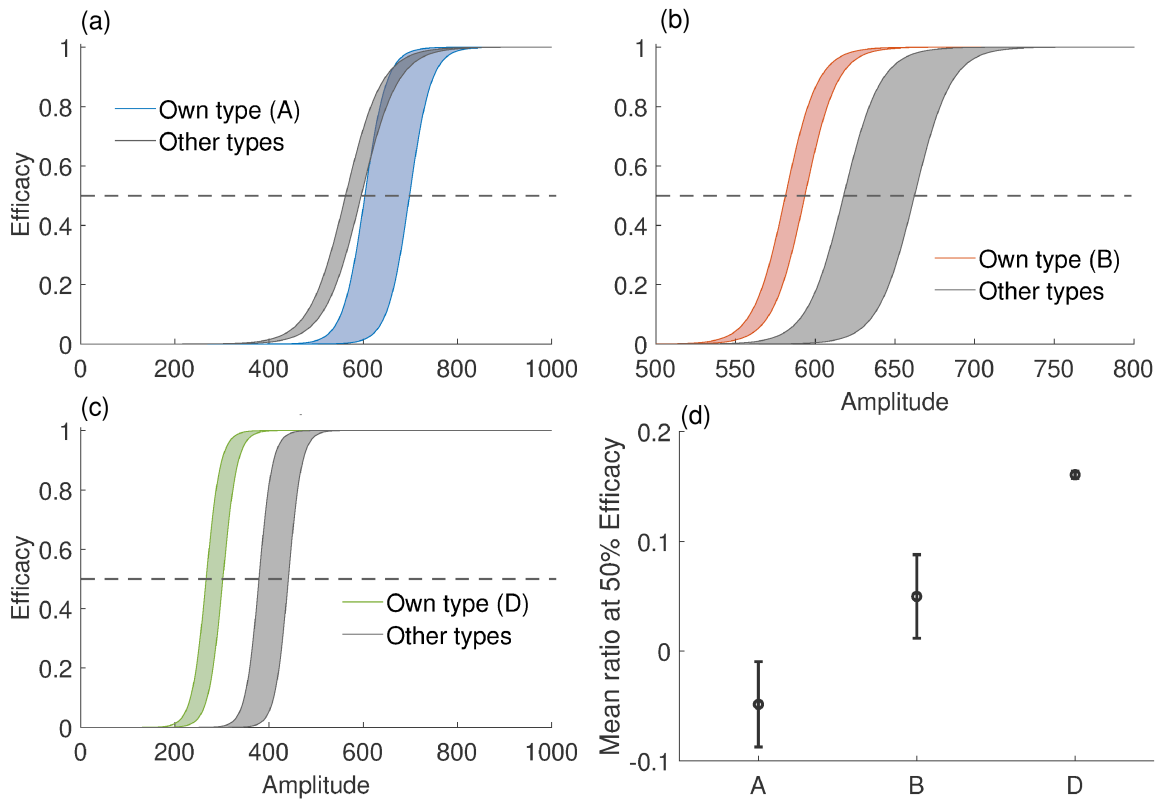


Figure 5.9: Mean efficacy of the response of 3 example cells to the waveforms from their own morphological class and the other classes. **(a)** Example A cell, **(b)** B cell and **(c)** D cell. **(d)** Average mean efficacy ratio of each morphological class ($\mu(MR)$, see Equation 5.3). Error bars are the standard deviations across morphological types' MR .

Summary of response per morphological type

A summary of the response of each cell per morphological type is presented in Table 5.2. The threshold of each cell to the waveforms from the different morphological types is shown, as well as the threshold in response to each cell's own waveform. Green values

correspond to a cell having a lowest threshold (better) response to its own waveform, as compared to the average responses of the waveforms per morphological type. As expected, most of the cells have a lower threshold in response to their own waveform (green value), which might seem like a trivial result, but it confirms the functionality of our model and proves that the waveform extracted through the STC analysis is, in fact, the preferred stimulus to activate each cell.

Table 5.2: Threshold of recorded cells in response to the waveforms of each morphological class:

Class	Cell	A	B	C	D	its own waveform
A	1	164 ± 6	164 ± 0	161 ± 7	160 ± 0	148
	2	125 ± 4	123 ± 0	120 ± 4	125 ± 0	133
	3	486 ± 74	420 ± 91	436 ± 30	319 ± 18	314
	4	659 ± 55	550 ± 19	618 ± 27	534 ± 12	524
	5	157 ± 16	141 ± 5	164 ± 11	131 ± 3	129
	6	461 ± 47	401 ± 25	488 ± 39	351 ± 9	320
B	7	154 ± 25	134 ± 9	158 ± 11	126 ± 0	126
	8	235 ± 15	218 ± 7	246 ± 17	211 ± 0	206
	9	562 ± 74	418 ± 58	554 ± 58	386 ± 0	364
	10	379 ± 92	216 ± 8	253 ± 13	202 ± 6	207
	11	660 ± 42	589 ± 6	645 ± 41	596 ± 7	574
	12	212 ± 16	192 ± 4	204 ± 11	201 ± 8	187
	13	212 ± 17	193 ± 9	225 ± 16	177 ± 0	176
	14	273 ± 13	260 ± 6	276 ± 11	249 ± 2	251
D	15	730 ± 76	571 ± 66	760 ± 70	499 ± 40	459
	16	411 ± 55	331 ± 33	450 ± 38	286 ± 18	254

Response to waveforms per waveform cluster

In Chapter 4 we clustered cells according to similarity in their waveform. This showed only weak correspondence to morphological class. Thus an alternative hypothesis to the one considered in the previous section, is that cells belonging to the same waveform cluster (Figure 5.10) would show better stimulation efficacy when stimulated by waveforms from

their own cluster compared to those from a different cluster. To test this, we reanalysed the previous data according to waveform cluster.

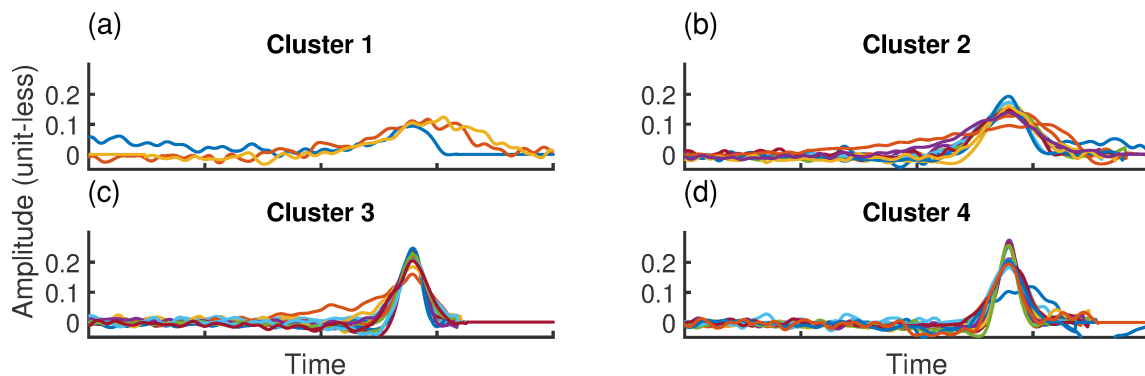


Figure 5.10: All the waveforms from the recovered waveform of each recorded cell. The waveforms are aligned at the peak, and grouped by waveform cluster: **(a)** Cluster 1, **(b)** Cluster 2, **(c)** Cluster 3 and **(d)** Cluster 4.

Similar to the previous section, here we also compared the threshold in response to their own waveforms and the waveforms from each cluster. In Figure 5.11 we show the sigmoid fits of the response of a cell from Cluster 2 (Figure 5.11a), a cell from Cluster 3 (Figure 5.11b), and from Cluster 4 (Figure 5.11c) to all the waveforms from each waveform cluster (thin lines). Cells from Cluster 1 were excluded because the recording occurred early in the data collection process, hence there were not enough cells stimulated with Cluster 1 waveforms. The data is averaged across all waveforms from each waveform cluster. The thick lines in Figure 5.11 are the sigmoid fit of the response of each cell to its own waveform. The mean response by waveform cluster was also calculated (Figure 5.12). The figure shows 3 example cells and the response to waveforms from its own cluster, against waveforms from all other clusters. Figure 5.12d shows the mean efficacy ratio (Equation 5.3). A value above zero means that RGCs in that cluster responded better (lower threshold) to the waveforms from the same waveform cluster, than to the waveforms from other waveform clusters.

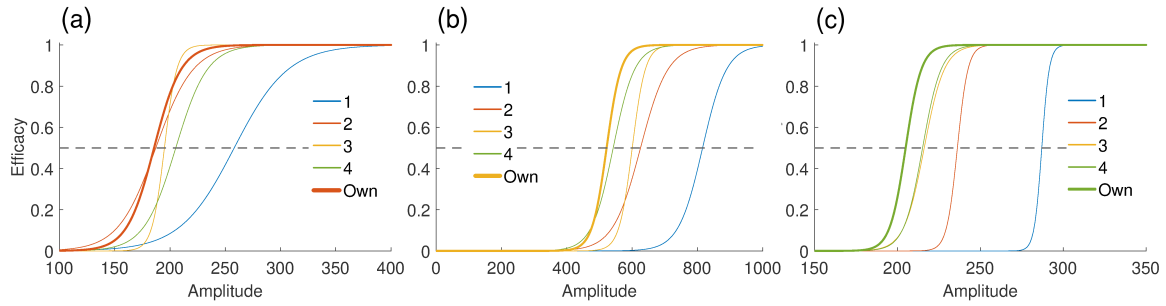


Figure 5.11: Efficacy of response of 3 example cells to their own waveform (thick coloured line) and the mean response to waveforms from each waveform cluster. **(a)** Example cell from Cluster 2, **(b)** example cell from Cluster 3, **(c)** Example cell from Cluster 4. Thin lines in each sub-figure are the average of the response to all the waveforms from a waveform cluster. Dotted lines correspond to 50% efficacy.

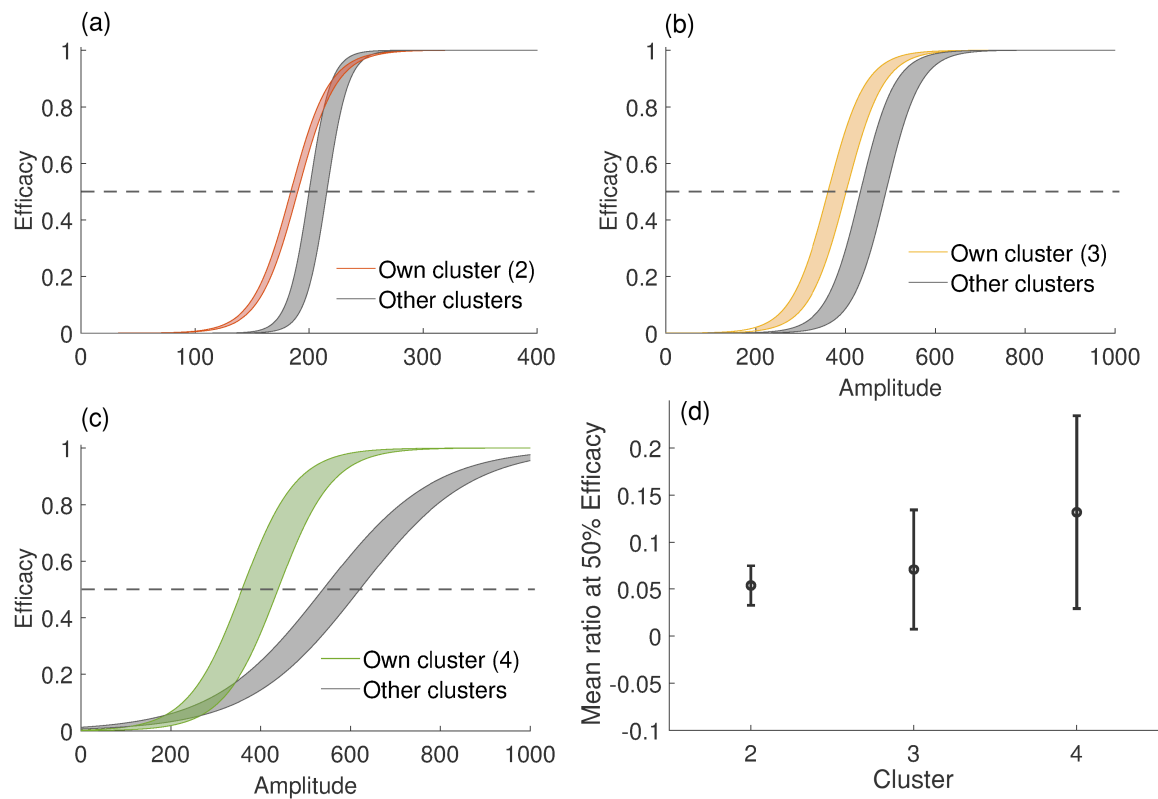


Figure 5.12: Mean efficacy of the response of 3 example cells to the waveforms from their own waveform cluster and the other waveform clusters. **(a)** Example cell from Cluster 2, **(b)** Cluster 3 cell and **(c)** Cluster 4 cell. **(d)** Mean efficacy ratio of cells from each waveform cluster. Error bars are the standard deviations across all cells. See Equation 5.3.

We can see from previous figures that cells tend to have lower thresholds in response to waveforms from the same cluster. This is confirmed in Figure 5.13b, which is the comparison of every cell's response to its own waveform cluster and other classes, using the metric from Equation 5.2. Theoretically each cell's waveform is the RGC's preferred stim-

ulus, so cells should have a lower threshold in response to their own waveform, and that is confirmed by the values above the diagonal in Figure 5.13a. The response of all RGCs from each cluster to their own waveform, and to the waveforms in other waveform clusters are shown in Figure 5.14.

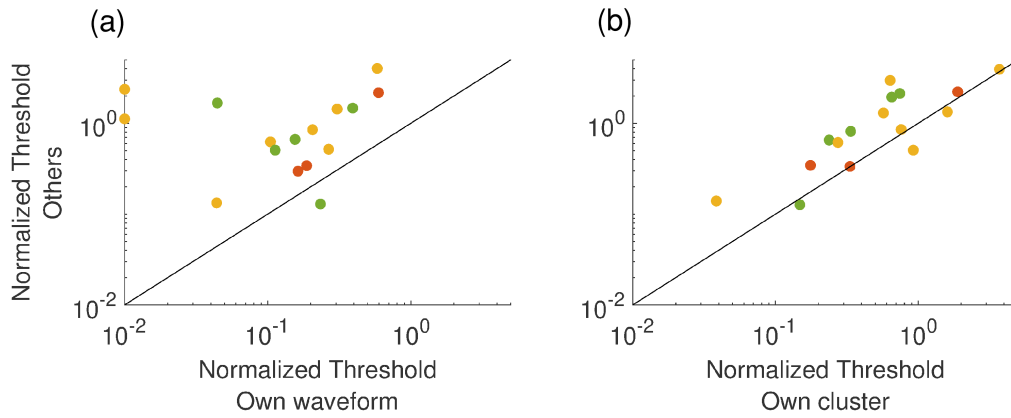


Figure 5.13: Difference in threshold of each RGC in response to **(a)** their own waveform and all other waveforms, and **(b)** all waveforms of their own waveform cluster (cluster) and all other waveform clusters (clusters). Colours indicate to which waveform cluster each RGC belongs: red, Cluster 2; yellow, Cluster 3; green, Cluster 4.

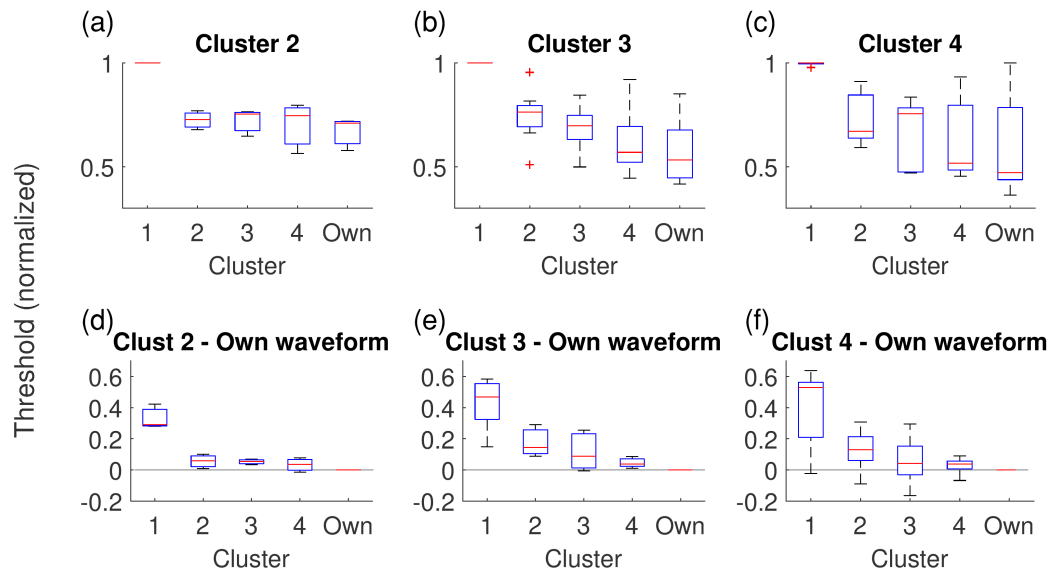


Figure 5.14: Threshold of the response of cells to waveforms from each waveform cluster and to their own waveform. RGCs from cluster **(a)** 2, **(b)** 3, and **(c)** 4. The difference between threshold of the response to waveforms of each waveform cluster and threshold of the response to their own waveform (**(d)**, **(e)** and **(f)**). In sub-figures **(d)**, **(e)** and **(f)**, the value *Own* is at zero, as expected, because it is the threshold in response of a RGC's own waveform, minus itself.

Summary of response per waveform cluster

A summary of the response of each cell per waveform cluster is presented in Table 5.3. The threshold of each cell to the waveforms from the different clusters is shown, as well as the threshold in response to each cell's own waveform. Green values correspond to a cell having a lower threshold (better response) in response to its own waveform, as compared to the average responses of the waveforms per cluster. See the explanation of the green values on the previous section.

Table 5.3: Threshold of recorded cells in response to the waveforms of each waveform cluster:

Cluster No.	Cell No.	Cluster 1	Cluster 2	Cluster 3	Cluster 4	its own waveform
2	7	218 ± 37	148 ± 11	141 ± 8	123 ± 12	126
	11	809 ± 96	622 ± 17	618 ± 37	603 ± 24	574
	12	260 ± 60	189 ± 3	196 ± 2	207 ± 5	187
3	1	174 ± 9	166 ± 6	147 ± 1	160 ± 2	148
	3	623 ± 72	482 ± 60	471 ± 110	345 ± 22	314
	4	817 ± 88	627 ± 24	602 ± 59	544 ± 8	524
	6	768 ± 117	509 ± 36	383 ± 20	375 ± 13	320
	10	491 ± 178	250 ± 12	332 ± 112	218 ± 10	207
	13	314 ± 31	227 ± 13	184 ± 7	183 ± 5	176
	14	353 ± 30	288 ± 12	251 ± 4	255 ± 5	251
	15	977 ± 0	742 ± 48	668 ± 98	543 ± 51	459
4	2	130 ± 3	121 ± 2	111 ± 2	124 ± 2	133
	5	279 ± 21	165 ± 13	133 ± 5	138 ± 5	129
	8	289 ± 6	238 ± 10	218 ± 12	217 ± 12	206
	9	773 ± 7	504 ± 43	592 ± 86	400 ± 40	364
	16	699 ± 84	469 ± 57	328 ± 27	317 ± 25	254

5.2.2 Intracellular stimulation

Responses per morphological class

With intracellular stimulation, 27 cells of the 43 recovered waveforms reached this stage in the experimental protocol. Four were A cells, 14 were C cells and 9 were D cells. As in subsection 5.2.1, Figure 5.15 shows the sigmoid fit to the response of 3 example cells to the waveforms of their own morphological type, and the other morphological types (there is no figure corresponding to a B cell because the only B type RGC could not be stimulated with a train of waveforms). Figure 5.15d shows the mean efficacy ratio of all the RGCs in each morphological type, where a value below zero means that RGCs from a morphological cell type responds badly (high threshold) to waveforms from that morphological type, and it responds better to waveforms from other morphologies. A value above zero means that RGCs from a particular morphological type respond better to waveforms from their morphological type than to other waveforms. With extracellular stimulation, we saw that A type RGCs had a higher threshold in response to A type waveforms, than to other types. Similarly, with intracellular stimulation A type cells have higher thresholds to A type waveforms. Figure 5.15a confirms this by showing the threshold in response to A type cells (blue) after the threshold in response to all other types (gray). Figure 5.15b and c show significantly lower threshold in response to waveforms from the cell's own type. This means that this example cell, could potentially be selectively stimulated. The analysis was repeated by waveform cluster (Figure 5.16), and considering that intracellular stimulation showed a correlation between morphology and clusters, we can say that Figure 5.16d confirms the results from Figure 5.15d (note that the value for A and the value for Cluster 1 are both below zero in the two figures).

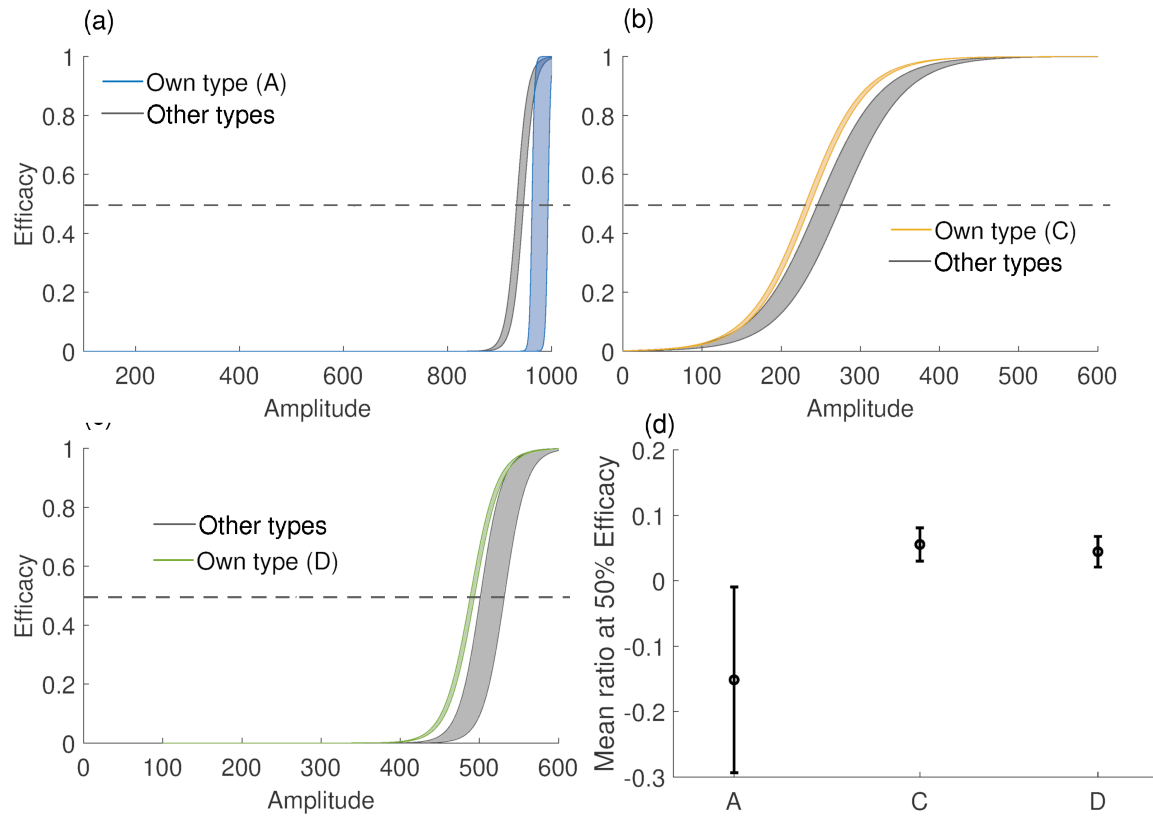


Figure 5.15: Mean efficacy of the response of 3 example cells to the waveforms from their own morphological class and the other classes. **(a)** Example A cell, **(b)** C cell and **(c)** D cell. **(d)** Shaded regions are the standard error of the mean boundaries. Mean efficacy ratio of each morphological class (see Equation 5.3). Error bars are the standard deviation across all cells.

Responses per waveform cluster

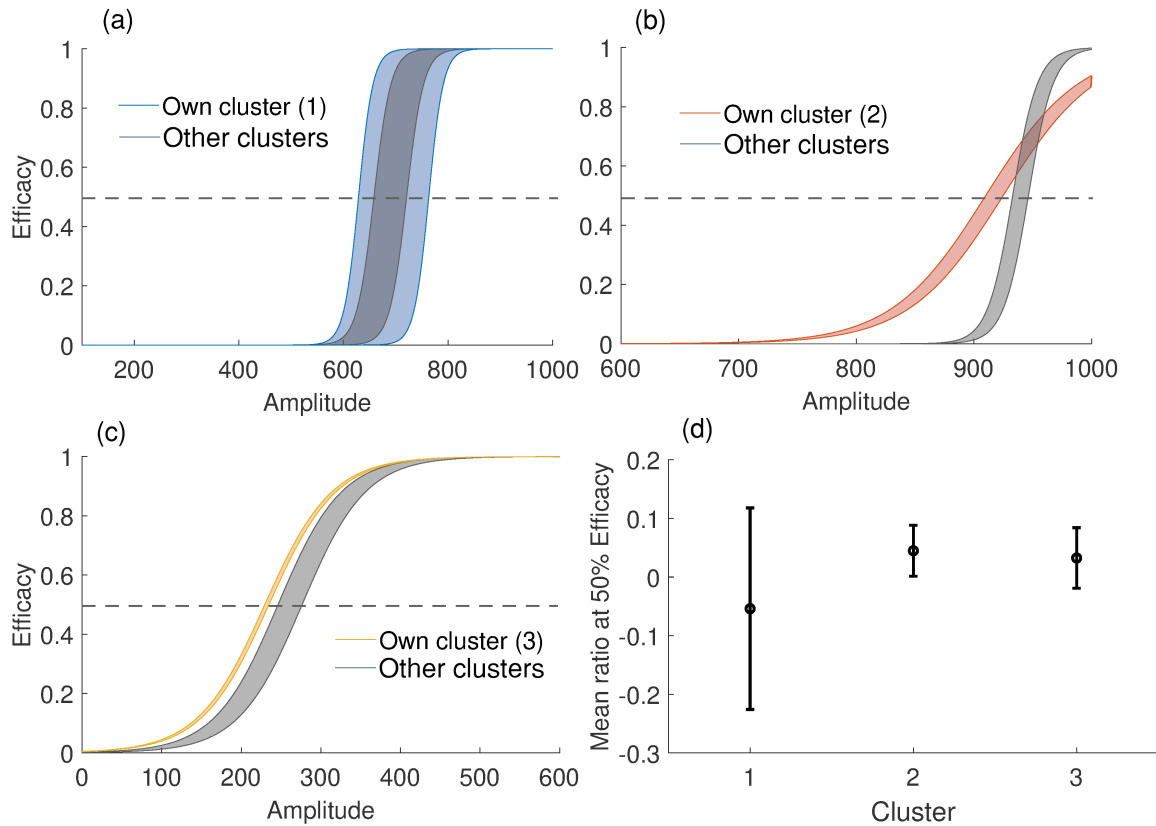


Figure 5.16: Mean efficacy of the response of 3 example cells to the waveforms from their own waveform cluster and the other clusters. **(a)** Example cell from Cluster 1, **(b)** Cluster 2 and **(c)** Cluster 3. **(d)** Shaded regions are the standard error of the mean boundaries. Mean efficacy ratio of each waveform cluster (see Equation 5.3). Error bars are the standard deviation across all cells.

5.3 Discussion and conclusions

Having shown that each RGC can have a particular waveform, we developed the hypothesis that different groups of RGCs, be it different morphological classes, or different clusters of waveform, might show higher responses to the waveform recovered from cells within the same class, compared to the waveform from other cell types. To test our hypothesis, we arranged the ensemble of waveforms recovered in Chapter 4 (Figure 5.1) into trains of varying waveform amplifications. This process allowed us to calculate the efficacy of each waveform to activate individual RGCs, i.e. how sensitive each cell was to each waveform. Then we compared the sensitivity of each cell in response to its own waveform and to other waveforms. We also compared the efficacy of waveforms corresponding to different

morphological classes and different waveform clusters.

Thanks to the retinal network, RGCs have highly selective responses to different visual stimuli. Some RGCs, for example, respond particularly to the direction of movement, or to lights transitioning from on to off, or off to on (Kuffler, 1953; Masland, 2011). For electrical stimuli to deliver a message to the brain in a form appropriate to drive visual perception, it would have to maintain the variety of features to which different RGCs respond. Differential activation of neurons in the retina has been tried before: Fried et al. (2009) showed different activation thresholds between RGC types, and Jensen and Rizzo (2008) found a different type of response in type II RGCs in a retinal degenerative mouse model. However, these studies have had minimal applicability to visual prosthetic devices. To explain this, in natural vision ON and OFF cells are activated in different patches of the scene and are not activated simultaneously (Tian, 2008). Yet, during electrical stimulation ON and OFF cells are activated simultaneously, thus potentially providing a very mixed set of signals. Any mechanism that allows the ON and OFF cells to be stimulated separately offers the potential to greatly improve the artificial images perceived by people wearing prosthetic vision devices (Tsai et al., 2012). The first study to find a functionally different response between two different RGC types to the same stimulus was Cai et al. (2013). They used high frequency (2 kHz) sinusoidal stimulation to activate and inhibit ON RGCs and OFF RGCs, respectively, by using the exact same stimulus. A more recent work obtained different responses from ON and OFF RGCs by injecting a reactivating cathodic pulse, which prevented the response decay on ON cells, but had no effect on OFF cells (Im and Fried, 2016). Moreover, Im et al. (2018) found that increasing the stimulus duration had an important decreasing effect on the response of ON cells, which was not observed in OFF cells. This could potentially have significant implications, due to the fact that light stimulation, and visual scenes could be replicated by electrical stimulation if the method improves (Margolis and Detwiler, 2007).

We have compared the waveforms from each type of light response, but we did not see any pattern that could segregate ON or OFF cells. Similarly, selectivity between ON and OFF cells was tested with unsatisfactory results. Thus, we focused our analysis to selectivity of morphological type. We have found that RGCs from morphological type A tend to have higher stimulation thresholds, both with intracellular and extracellular stimulation. Furthermore, although we found a preferential stimulation of almost every cell to its own

ERF, A-RGCs responded worse to the waveforms of other A-RGCs than to the waveforms of different morphological types. This unexpected result could be due to the larger cell bodies of A-type cells as compared to the other morphological types (Huxlin and Goodchild, 1997). Again, the difference was apparent in both extracellular and intracellular stimulation. It might be that the very large cell bodies and the large dendritic trees, covering many millimetres of the retina create substantial variation in electrical activation properties such that more variation occurs within A-cells than in all other retinal cells combined.

Interestingly, almost every cell in the extracellular stimulation protocol responded better to the waveforms of D-RGCs. The only exceptional morphological characteristic of D-cells is that they have bistratified dendrites in the IPL. Perhaps this bistratification gives D-cells more similarities with various other retinal cells. Wong et al. (2012) made a survey of RGCs and compared their morphological classification with their intrinsic electrophysiological characteristics. Some of the properties they measured were the resting membrane potential, the input resistance of a cell (calculated according to Ohm's law from the change in steady state membrane potential when applying some current stimulation), and the membrane time constant (the time constant of the charging curve of the membrane potential in response to a current step pulse, (Johnston and Wu, 1994)). In their study, Wong et al. (2012) found that bistratified cells (the D-morphological type) had lower resting membrane potentials than all other types, they also exhibited higher input resistance and membrane time constant, hence a higher membrane capacitance than monostратified cells. Those findings suggest that it would take more energy to activate bistratified RGCs than other types of RGCs, as the shape of the waveform would have to take longer for the membrane potential to reach threshold and elicit an action potential, therefore, the waveform would most likely stimulate other morphological types, as is the observation in our recordings (Figures 5.7, 5.8 and 5.9d). Therefore, having dendrites that stratify to two different layers of the IPL in the retina, D-RGCs might respond to a wider range of electrical stimulation conditions than neurons that stratify to a single depth in the IPL.

Even though A2 cells in rat and the equivalent alpha cells in cat retinas are very large, their properties have been tuned to have a rapid response to their synaptic inputs (low membrane time constant), which would decrease the RGC's sensitivity to equivalent inputs compared to other RGC types (O'Brien et al., 2002; Wong et al., 2012). According to O'Brien et al. (2002) and Wong et al. (2012) A2 cells and alpha cells also have the lowest

input resistance, thus giving them the highest conductance. Therefore, the white noise stimulus that elicited A-RGCs responses might be expected to have more energy, and the energy in the recovered waveforms is normalized during the STC analysis, i.e. energy in waveforms from A-RGCs is the same as the energy from B-RGCs, C-RGCs and D-RGCs, even though A-RGCs need more energy to elicit a response. As seen in Chapter 3, most of the cells from morphological type A in our recordings belong to the morphological subgroup A2, which would suggest that the poor performance of the waveforms recovered from A-RGCs in activating other A-RGCs is expected (Figures 5.7b, 5.9 and 5.15).

The size of the sample in this segment of the work is unfortunately too small to give any definite results, but it gives an insight about the potential selectivity of RGCs by their morphological type. Data collection for this project should continue until a significant sample size is achieved, which would allow us to build a stimulus library describing the different morphological types. At this moment, we can conclude that the waveforms of the different RGCs, although they do not seem distinct when viewed by morphology (Figure 5.5), have hidden features that affect the sensitivity of the cells. By defining the differences in preferred stimuli for the different morphological types, a stimulus could target specific cells and convey to the brain only a particular aspect of the visual scene. Such achievement would have an important impact on the efficacy of retinal prostheses, given that each morphological type has particular physiological properties and responds distinctly to specific attributes of a visual scene (Dowling and Dowling, 2016c; Masland, 2011; Wong et al., 2012).

Chapter 6

Conclusions

We explored adjustments to the parameters of the stimuli to reduce adaptation of retinal ganglion cells (RGCs) to electrical stimulation (Freeman and Fried, 2011; Horsager et al., 2009). We have found significant differences in spike rate and speed of adaptation between stimulation with random interpulse intervals (IPIs) and stimulation with constant IPIs at frequencies of 50 Hz and 200 Hz. Our results suggest that stimulating the neurons with charge balanced biphasic pulses from a Poisson distribution, i.e. with IPIs sampled from an exponential distribution, may limit adaptation of RGCs. Random IPI stimulation would potentially reduce perceptual fading, hence lead to better efficacy in future retinal prostheses (Tsai et al., 2011a).

A recurrent observation in the recordings from Chapter 2 was the presence of action potentials with smaller (but constant) amplitudes than normal spikes. These types of mini-spikes have been reported previously and are called “spikelets”. They are thought to not propagate along the axon to postsynaptic neurons, hence they are frequently ignored in the analysis of neural responses (Meng et al., 2018; Oesch et al., 2005; Tsai et al., 2011a). We have found, however, that if spikelets are considered among spikes as the responses of RGCs, then there is virtually no adaptation whatsoever, regardless of the type of IPI used to stimulate (random or constant). Moreover, the overall spike rate during stimulation is higher in response to constant IPI stimuli than in response to random IPI stimuli, as opposed to when the spikelets are not considered in the response. This leads to the conclusion that the mechanisms preventing a response from becoming a full action potential are also intimately involved in the adaptation to electrical stimulation (Tsai et al., 2011a). The fact

that people with bionic eyes perceive fading suggests that the spikelets do not influence perception but more research is required at the neurophysiological level to confirm this notion (Pérez Fornos et al., 2012). We have attempted to study the cause of spikelets and whether they propagate along the axons of RGCs, but the only finding in that regard is that spikelets, as spikes, disappear when the sodium channels are blocked, which is consistent with the hypothesis that spike elicitation failure may be due to sodium channels inactivation during electrically evoked responses (Tsai et al., 2011b). It would be of interest to retinal prostheses to further study the mechanisms responsible for spikelets, and to understand what causes an action potential to become a spikelet, instead of a normal-amplitude spike. One possible avenue for such studies is to try and identify a chemical that, when applied to the tissue would reduce spiking thresholds. In this way, the spikelets may become full spikes, and therefore by inference, the perceived images in implanted patients should not fade.

The temporal electrical receptive fields (tERF), or waveform, recovered from the responses to intracellular stimulation have shown that RGCs can be classified in their respective morphological classes by using a K-means clustering algorithm (Celebi et al., 2013; Lloyd, 1982; MacQueen, 1965). Quite outside the aims of prosthetic vision, this finding may be quite useful for retinal physiologists as an addition method for classifying cell types. Unfortunately, extracellular stimulation did not result in waveforms with a clear correlation between clusters and morphological classes. Due to the retinal prostheses using only extracellular stimulation and recording, the classification technique presented here is unlikely to be useful in a clinical environment (Hadjinicolaou et al., 2015a). However, there are hints of possible selectivity in RGCs to the recovered waveform, both with extracellular and intracellular stimulation. Cells from B and D morphological types had lower thresholds when stimulated with the waveform recovered from cells in the same morphological class. A-RGCs on the contrary, did not seem to share the same temporal features in their waveform as other A-type neurons. Further studies involving a larger data set might determine whether the waveforms could preferentially stimulate cells from a specific morphological class.

Future work includes increasing the data collection of intracellular and extracellular white noise stimulation, as well as conducting experiments where the location of the stimulating electrode is controlled, hence understanding if latency has any relation with the

position of the electrode. It would also be a good exercise to test the response of cells to repetitive presentations of the same waveform, integrating the waveforms found in chapters 4 with our findings from chapter 2. Finally, there is a deep interest in understanding the cause of spikelets and testing how far along the axon they can propagate, if this is the case. This would require a single cell to be simultaneously patch-clamped at the soma and the axon, which is a non trivial experiment in wild type RGCs, but is more likely to be achieved in cultured cells.

The first aim of this project was to improve efficacy of electrical stimulation of RGCs, which was achieved by reducing action potential decay through irregular stimulation at 50 and 200 pulses per second in Chapter 2. The second aim of this work was to explore selective activation of different RGCs. This was tested in Chapter 2 and our findings suggest a likely selectivity of ON cells to 10 Hz stimulation, and OFF cells to 50 Hz stimulation, similar to the recent findings of Im and Fried (2016) and Im et al. (2018). Selectivity was further explored in Chapters 4 and 5, where a cell specific preferred waveform was found and tested on other cells from different morphological types.

Bibliography

- Ahuja, A. K. (2007). An in vitro model of a retinal prosthesis. *IEEE Transactions on Biomedical Engineering*, 55(6):1744–1753.
- Alonso, J.-M. and Chen, Y. (2009). Receptive field. *Scholarpedia*, 4(1):5393.
- Arend, L. E. (1973). Spatial differential and integral operations in human vision: Implications of stabilized retinal image fading. *Psychological Review*, 80(5):374–395.
- Asari, H. and Meister, M. (2014). The Projective Field of Retinal Bipolar Cells and Its Modulation by Visual Context. *Neuron*, 81(3):641–652.
- Ayton, L. N., Blamey, P. J., Guymer, R. H., Luu, C. D., Nayagam, D. A., Sinclair, N. C., Shivdasani, M. N., Yeoh, J., McCombe, M. F., Briggs, R. J., Opie, N. L., Villalobos, J., Dimitrov, P. N., Varsamidis, M., Petoe, M. A., McCarthy, C. D., Walker, J. G., Barnes, N., Burkitt, A. N., Williams, C. E., Shepherd, R. K., and Allen, P. J. (2014). First-in-human trial of a novel suprachoroidal retinal prosthesis. *PLoS ONE*, 9(12):1–27.
- Baccus, S. A. and Meister, M. (2002). Fast and slow contrast adaptation in retinal circuitry. *Neuron*, 36(5):909–919.
- Baden, T., Berens, P., Franke, K., Román Rosón, M., Bethge, M., and Euler, T. (2016). The functional diversity of retinal ganglion cells in the mouse. *Nature*, 529(7586):345–350.
- Bae, J. A., Mu, S., Kim, J. S., Briggman, K. L., Seung, H. S., Bae, J. A., Mu, S., Kim, J. S., Turner, N. L., Tartavull, I., Kemnitz, N., Bland, D., Sterling, A. L. R., Park, J., Briggman, K. L., and Seung, H. S. (2018). Digital Museum of Retinal Ganglion Cells with Dense Anatomy and Physiology Resource Digital Museum of Retinal Ganglion Cells with Dense Anatomy and Physiology. *Cell*, 173:1293–1306.
- Benav, H., Wilke, R., Stett, A., and Zrenner, E. (2008). Simulation of spatial and temporal characteristics of perceptions elicited by a subretinal implant. *Investigative Ophthalmology and Visual Science*, 49(13):3013.
- Bleeck, S., Ingham, N. J., Verhey, J. L., and Winter, I. M. (2008). Rebound depolarization in single units of the ventral cochlear nucleus: A contribution to grouping by common onset? *Neuroscience*, 154(1):139–146.

- Boinagrov, D., Pangratz-Fuehrer, S., Goetz, G., and Palanker, D. (2014). Selectivity of direct and network-mediated stimulation of the retinal ganglion cells with epi-, sub- and intraretinal electrodes. *Journal of Neural Engineering*, 11(2).
- Brindley, G. S. and Lewin, W. S. (1968). The sensations produced by electrical stimulation of the visual cortex. *Journal of Physiology*, 196(2):479–493.
- Burbeck, C. a. and Kelly, D. H. (1984). Role of local adaptation in the fading of stabilized images. *Journal of the Optical Society of America. A, Optics and image science*, 1(2):216–20.
- Cai, C., Ren, Q., Desai, N. J., Rizzo, J. F., and Fried, S. I. (2011). Response variability to high rates of electric stimulation in retinal ganglion cells. *Journal of Neurophysiology*, 106:153–162.
- Cai, C., Twyford, P., and Fried, S. (2013). The response of retinal neurons to high-frequency stimulation. *Journal of Neural Engineering*, 10(3):036009.
- Cameron, M. A., Suaning, G. J., Lovell, N. H., and Morley, J. W. (2013). Electrical Stimulation of Inner Retinal Neurons in Wild-Type and Retinally Degenerate (rd/rd) Mice. *PLoS ONE*, 8(7):e68882.
- Caspi, A., Roy, A., Wuyyuru, V., Rosendall, P. E., Harper, J. W., Katyal, K. D., Barry, M. P., Dagnelie, G., and Greenberg, R. J. (2018). Eye movement control in the Argus II retinal-prosthesis enables reduced head movement and better localization precision. *Investigative Ophthalmology and Visual Science*, 59(2):792–802.
- Celebi, M. E., Kingravi, H. A., and Vela, P. A. (2013). A comparative study of efficient initialization methods for the k-means clustering algorithm. *Expert Systems with Applications*, 40(1):200–210.
- Chander, D. and Chichilnisky, E. J. (2001). Adaptation to temporal contrast in primate and salamander retina. *Journal of Neuroscience*, 21(24):9904–9916.
- Chichilnisky, E. J. (2001). A simple white noise analysis of neuronal light responses. *Network: Computation in Neural Systems*, 12(2):199–213.
- Cholkar, K., Patel, A., Dutt Vadlapudi, A., and K. Mitra, A. (2012). Novel Nanomicellar Formulation Approaches for Anterior and Posterior Segment Ocular Drug Delivery. *Recent Patents on Nanomedicine*, 2(2):82–95.
- Chow, A. Y., Bittner, A. K., and Pardue, M. T. (2010). The artificial silicon retina in retinitis pigmentosa patients (an American Ophthalmological Association thesis). *Transactions of the American Ophthalmological Society*, 108:120–154.
- Chow, A. Y. and Chow, V. Y. (1997). Subretinal electrical stimulation of the rabbit retina. *Neuroscience Letters*, 225:13–16.
- Chow, A. Y., Chow, V. Y., Packo, K. H., Pollack, J. S., Peyman, G. a., and Schuchard, R. (2004). The artificial silicon retina microchip for the treatment of vision loss from retinitis pigmentosa. *Archives of ophthalmology*, 122(4):460–9.

- Chuang, A. T., Margo, C. E., and Greenberg, P. B. (2014). Retinal implants: A systematic review. *British Journal of Ophthalmology*, 98(7):852–856.
- Clarke, F. and Belcher, S. (1962). On the localization of troxler's effect in the visual pathway. *Vision Research*, 2(1-4):53–68.
- Cleland, B. Y. B. G., Levick, W. R., and Wassle, H. (1975). Physiological identification of a morphological class of cat retinal ganglion cells. *Journal of Physiology*, 248:151–171.
- Cloherly, S. L., Wong, R. C. S., Hadjinicolaou, A. E., Meffin, H., Ibbotson, M. R., and O'Brien, B. J. (2012). Epiretinal electrical stimulation and the inner limiting membrane in rat retina. In *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference*, volume 2012, pages 2989–2992, San Diego, California, USA.
- Coombs, J., van der List, D., Wang, G. Y., and Chalupa, L. M. (2006). Morphological properties of mouse retinal ganglion cells. *Neuroscience*, 140(1):123–136.
- Davie, J. T., Clark, B. A., and Hausser, M. (2008). The Origin of the Complex Spike in Cerebellar Purkinje Cells. *Journal of Neuroscience*, 28(30):7599–7609.
- Davuluri, N. S. and Weiland, J. D. (2014). Time-varying pulse trains limit retinal desensitization caused by continuous electrical stimulation. In *2014 36th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, volume 2014, pages 414–417, Chicago, USA. IEEE.
- Demb, J. B. and Singer, J. H. (2015). Functional Circuitry of the Retina. *Annual Review of Vision Science*, 1(1):263–289.
- Deneve, S. (2008). Bayesian Spiking Neurons I: Inference. *Neural Computation*, 20(1):91–117.
- Devries, S. H. and Baylor, D. a. (1997). Mosaic arrangement of ganglion cell receptive fields in rabbit retina. *Journal of Neurophysiology*, 78(4):2048–2060.
- Ditchburn, R. W. and Drysdale, A. E. (1973). Perception of structure in flashes and in afterimages. *Vision Research*, 13(12):2423–2433.
- Ditchburn, R. W., Fender, D. H., and Mayne, S. (1959). Vision with controlled movements of the retinal image. *Journal of Physiology*, 145(1):98–107.
- Dowling, J. E. (2009). Current and future prospects for optoelectronic retinal prostheses. *Eye*, 23(10):1999–2005.
- Dowling, J. L. J. and Dowling, J. E. (2016a). Beyond the Retina—Lateral Geniculate Nucleus and Visual Cortex: Amblyopia. In *Vision : how it works and what can go wrong*, chapter 5, pages 105–133. MIT Press, Cambridge, MA, USA.
- Dowling, J. L. J. and Dowling, J. E. (2016b). Capturing light - The photoreceptors: Retinitis pigmentosa and age-related macular degeneration. In *Vision: How it works and what can go wrong*, chapter 3, pages 45–71. The MIT Press, Cambridge, MA, USA.

- Dowling, J. L. J. and Dowling, J. E. (2016c). Visual pathways, eye development, and retinal organization: visual deficit and blindness. In *Vision: How it works and what can go wrong*, chapter 1, pages 1–20. MIT Press, Cambridge, MA, USA.
- Euler, T., Haverkamp, S., Schubert, T., and Baden, T. (2014). Retinal bipolar cells: Elementary building blocks of vision. *Nature Reviews Neuroscience*, 15(8):507–519.
- Feng, X.-J., Shea-Brown, E., Greenwald, B., Kosut, R., and Rabitz, H. (2007). Optimal deep brain stimulation of the subthalamic nucleus—a computational study. *Journal of Computational Neuroscience*, 23(3):265–282.
- Fohlmeister, J. F., Cohen, E. D., and Newman, E. A. (2010). Mechanisms and Distribution of Ion Channels in Retinal Ganglion Cells: Using Temperature as an Independent Variable. *Journal of Neurophysiology*, 103(3):1357–1374.
- Foutz, T. J. and McIntyre, C. C. (2010). Evaluation of novel stimulus waveforms for deep brain stimulation. *Journal of Neural Engineering*, 7:10.
- Franken, H. M., Veltink, P. H., Tijssmans, R., Nijmeijer, H., and Boom, H. B. K. (1993). Identification of Passive Knee Joint and Shank Dynamics in Paraplegics Using Quadriceps Stimulation. *IEEE Transactions on Rehabilitation Engineering*, 1(3):154–164.
- Freeman, D. K. and Fried, S. I. (2011). Multiple components of ganglion cell desensitization in response to prosthetic stimulation. *Journal of Neural Engineering*, 8(1):16008.
- Freeman, D. K., Rizzo, J. F., and Fried, S. I. (2010). Electric stimulation with sinusoids and white noise for neural prostheses. *Frontiers in Neuroscience*, 4(FEB):2–4.
- Fried, S. I., Lasker, A. C. W., Desai, N. J., Eddington, D. K., and Rizzo, J. F. (2009). Axonal Sodium-Channel Bands Shape the Response to Electric Stimulation in Retinal Ganglion Cells. *Journal of Neurophysiology*, 101(4):1972–1987.
- Fujikado, T., Kamei, M., Sakaguchi, H., Kanda, H., Morimoto, T., Ikuno, Y., Nishida, K., Kishima, H., Maruo, T., Konoma, K., Ozawa, M., and Nishida, K. (2011). Testing of semichronically implanted retinal prosthesis by suprachoroidal-transretinal stimulation in patients with retinitis pigmentosa. *Investigative Ophthalmology and Visual Science*, 52(7):4726–4733.
- Gekeler, F., Kobuch, K., Schwahn, H. N., Stett, A., Shinoda, K., and Zrenner, E. (2004). Subretinal electrical stimulation of the rabbit retina with acutely implanted electrode arrays. *Graefe's Archive for Clinical and Experimental Ophthalmology*, 242(7):587–596.
- Gerstein, G. L. (1960). Analysis of firing patterns in single neurons. *Science*, 131(3416):1811–1812.
- Gregory-Evans, K., Pennesi, M. E., and Weleber, R. G. (2013). *Chapter 40 - Retinitis pigmentosa and allied disorders*. W.B. Saunders, fifth edit edition.
- Grill, W. M. and Dorval, A. D. (2015). Non-regular electrical stimulation patterns for treating neurological disorders. *US Patent 8,923,981*, pages 1–16.

- Guenther, T., Lovell, N. H., and Suaning, G. J. (2012). Bionic vision: System architectures - A review. *Expert Review of Medical Devices*, 9(1):33–48.
- Guimaraes, a. C., Herzog, W., Hulliger, M., Zhang, Y. T., Day, and S (1994). Effects of Muscle Length on the Emg–Force Relationship of the Cat Soleus Muscle Studied Using Non-Periodic Stimulation of Ventral Root Filaments. *Journal of Experimental Biology*, 193:49–64.
- Hadjinicolaou, A. E., Meffin, H., Maturana, M. I., Cloherty, S. L., and Ibbotson, M. R. (2015a). Prosthetic vision: Devices, patient outcomes and retinal research. *Clinical and Experimental Optometry*, 98(5):395–410.
- Hadjinicolaou, A. E., Savage, C. O., Apollo, N. V., Garrett, D. J., Cloherty, S. L., Ibbotson, M. R., and O'Brien, B. J. (2015b). Optimizing the Electrical Stimulation of Retinal Ganglion Cells. *IEEE Transactions on Neural Systems and Rehabilitation Engineering*, 23(2):169–178.
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv European Journal of Physiology*, 391(2):85–100.
- Hartong, D. T., Berson, E. L., and Dryja, T. P. (2006). Retinitis pigmentosa. *Lancet*, 368(9549):1795–1809.
- Hattar, A. S., Liao, H., Takao, M., Berson, D. M., and Yau, K. (2002). Published by : American Association for the Advancement of Science Linked references are available on JSTOR for this article : Melanopsin-Containing Retinal Ganglion Cells : Architecture , Projections , and Intrinsic. *Science, New Series*, 295(5557):1065–1070.
- Hodgkin, A. and Huxley, A. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology*, 117(4):500–544.
- Hornig, R., Dapper, M., Le Joliff, E., Hill, R., Ishaque, K., Posch, C., Benosman, R., LeMer, Y., Sahel, J.-A., and Picaud, S. (2017). Pixium Vision: First Clinical Results and Innovative Developments. In *Artificial Vision*, pages 99–113. Springer International Publishing, Cham.
- Horsager, A., Greenwald, S. H., Weiland, J. D., Humayun, M. S., Greenberg, R. J., McMahon, M. J., Boynton, G. M., and Fine, I. (2009). Predicting visual sensitivity in retinal prosthesis patients. *Investigative Ophthalmology and Visual Science*, 50(4):1483–1491.
- Hsieh, P. J. and Colas, J. T. (2012). Perceptual fading without retinal adaptation. *Journal of Experimental Psychology: Human Perception and Performance*, 38(2):267–271.
- Hu, C., Hill, D. D., and Wong, K. Y. (2013). Intrinsic physiological properties of the five types of mouse ganglion-cell photoreceptors. *Journal of Neurophysiology*, 109(7):1876–1889.
- Hu, W., Tian, C., Li, T., Yang, M., Hou, H., and Shu, Y. (2009). Distinct contributions of Nav1.6 and Nav1.2 in action potential initiation and backpropagation. *Nature Neuroscience*, 12(8):996–1002.
- Hubel, B. Y. D. H., Wiesel, a. D. T. N., Hubel, D. N., Wiesel, T. N., Hubel, B. Y. D. H., and Wiesel, a. D. T. N. (1962). Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. *Journal of Physiology*, 160(1):106–154.

- Humayun, M. S., Dorn, J. D., Da Cruz, L., Dagnelie, G., Sahel, J. A., Stanga, P. E., Cideciyan, A. V., Duncan, J. L., Elliott, D., Filley, E., Ho, A. C., Santos, A., Safran, A. B., Ardit, A., Del Priore, L. V., and Greenberg, R. J. (2012). Interim results from the international trial of second sight's visual prosthesis. *Ophthalmology*, 119(4):779–788.
- Humayun, M. S., Weiland, J. D., Fujii, G. Y., Greenberg, R., Williamson, R., Little, J., Mech, B., Cimmarrusti, V., Van Boemel, G., Dagnelie, G., and De Juan, E. (2003). Visual perception in a blind subject with a chronic microelectronic retinal prosthesis. *Vision Research*, 43(24):2573–2581.
- Huxlin, K. R. and Goodchild, a. K. (1997). Retinal ganglion cells in the albino rat: Revised morphological classification. *Journal of Comparative Neurology*, 385(2):309–323.
- Hyman, L. G., Lilienfeld, A. M., Ferris, F. L. I., and Fine, S. L. (1983). Senile macular degeneration: a case-control study. *American Journal of Epidemiology*, 118(2):213–227.
- Ibbotson, M. R. (2005). Physiological Mechanisms of Adaptation in the Visual System. In Clifford, C. W. G. and Rhodes, G., editors, *Fitting the Mind to the World: Adaptation and After-Effects in High-Level Vision*, pages 17–45. Oxford Univ Press, Oxford.
- Im, M. and Fried, S. I. (2016). Temporal properties of network-mediated responses to repetitive stimuli are dependent upon retinal ganglion cell type. *Journal of Neural Engineering*, 13(2).
- Im, M., Werginz, P., and Fried, S. I. (2018). Electric stimulus duration alters network-mediated responses depending on retinal ganglion cell type. *Journal of Neural Engineering*, 15(3).
- Insel, T. R., Landis, S. C., and Collins, F. S. (2013). Research priorities. The NIH BRAIN Initiative. *Science*, 340(6133):687–8.
- Jelinek, H. F., Ristanović, D., and Milošević, N. T. (2011). The morphology and classification of alpha ganglion cells in the rat retinae: A fractal analysis study. *Journal of Neuroscience Methods*, 201(1):281–287.
- Jensen, R. J. and Rizzo, J. F. (2007). Responses of ganglion cells to repetitive electrical stimulation of the retina. *Journal of Neural Engineering*, 4(1):S1–6.
- Jensen, R. J. and Rizzo, J. F. (2008). Activation of retinal ganglion cells in wild-type and rd1 mice through electrical stimulation of the retinal neural network. *Vision Research*, 48(14):1562–1568.
- Johnston, D. and Wu, S. M.-S. (1994). *Foundations of Cellular Neurophysiology*. The MIT Press.
- Kels, B. D., Grzybowski, A., and Grant-Kels, J. M. (2015). Human ocular anatomy. *Clinics in Dermatology*, 33(2):140–146.
- Koenderink, J. J. (1972). Contrast Enhancement and the Negative Afterimage. *Journal of the Optical Society of America*, 62(5):685–689.
- Kotsakidis, R., Meffin, H., Ibbotson, M., and Kameneva, T. (2018). In vitro assessment of the differences in retinal ganglion cell responses to intra- and extracellular electrical stimulation. *Journal of Neural Engineering*.

- Kotulak, J. C. and Schor, C. M. (1986). The accommodative response to subthreshold blur and to perceptual fading during the Troxler phenomenon. *Perception*, 15(1):7–15.
- Kuffler, S. W. (1953). Discharge Patterns and Functional of Mammalian Retina. *Journal of Neurophysiology*.
- Kuffler, S. W., Fitzhugh, R., and Barlow, H. B. (1957). Maintained activity in the cat's retina in light and darkness. *The Journal of General Physiology*, 40(5):683–702.
- Lasker, I. (2014). Restoring Vision to the Blind: The Lasker/IRRF Initiative for Innovation in Vision Science. *Translational Vision Science & Technology*, 3(7):1.
- Lee, J.-i. and Im, M. (2018). Non-rectangular waveforms are more charge-efficient than rectangular one in eliciting network-mediated responses of ON type retinal ganglion cells. *Journal of Neural Engineering*, 15:17.
- Levy-Schoen, A. (1968). *Eye movements and vision*, volume 6. Plenum Press, New York.
- Lin, B. and Peng, E. B. (2013). Retinal Ganglion Cells are Resistant to Photoreceptor Loss in Retinal Degeneration. *PLoS ONE*, 8(6).
- Liu, Y. H. and Wang, X. J. (2001). Spike-frequency adaptation of a generalized leaky integrate-and-fire model neuron. *Journal of Computational Neuroscience*, 10(1):25–45.
- Lloyd, S. P. (1982). Least Squares Quantization in PCM. *IEEE Transactions on Information Theory*, 28(2):129–137.
- Lucas, R. J., Douglas, R. H., and Foster, R. G. (2001). Characterization of an ocular photopigment capable of driving pupillary constriction in mice. *Nature Neuroscience*, 4(6):621–626.
- Luo, Y. H. and Da Cruz, L. (2014). A review and update on the current status of retinal prostheses (bionic eye). *British Medical Bulletin*, 109(1):31–44.
- Macosko, E. Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A. R., Kamitaki, N., Martersteck, E. M., Trombetta, J. J., Weitz, D. A., Sanes, J. R., Shalek, A. K., Regev, A., and McCarroll, S. A. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell*, 161(5):1202–1214.
- MacQueen, J. (1965). Some methods for classification and analysis of multivariate observations. *Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability*, 1(14):281–297.
- Manookin, M. B. and Demb, J. B. (2006). Presynaptic Mechanism for Slow Contrast Adaptation in Mammalian Retinal Ganglion Cells. *Neuron*, 50(3):453–464.
- Margalit, E., Babai, N., Luo, J., and Thoreson, W. B. (2011). Inner and outer retinal mechanisms engaged by epiretinal stimulation in normal and rd mice. *Visual Neuroscience*, 28(2):145–154.
- Margolis, D. J. and Detwiler, P. B. (2007). Different Mechanisms Generate Maintained Activity in ON and OFF Retinal Ganglion Cells. *Journal of Neuroscience*, 27(22):5994–6005.

- Marmarelis, P. Z. and Naka, K.-I. (1972). White-Noise Analysis of a Neuron Chain : An Application of the Wiener Theory. *Science*, 175.
- Martinez-Conde, S., Macknik, S. L., Troncoso, X. G., and Dyar, T. A. (2006). Microsaccades counteract visual fading during fixation. *Neuron*, 49(2):297–305.
- Masland, R. H. (2001). The fundamental plan of the retina. *Nature Neuroscience*, 4(9):877–886.
- Masland, R. H. (2011). Cell populations of the retina: The proctor lecture. *Investigative Ophthalmology and Visual Science*, 52(7):4581–4591.
- Masland, R. H. (2012). The Neuronal Organization of the Retina. *Neuron*, 76(2):266–280.
- Maturana, M. I., Apollo, N. V., Garrett, D. J., Kameneva, T., Cloherty, S. L., Grayden, D. B., Burkitt, A. N., Ibbotson, M. R., and Meffin, H. (2018). Electrical receptive fields of retinal ganglion cells: Influence of presynaptic neurons. *PLoS Computational Biology*, 14(2):1–32.
- Maturana, M. I., Apollo, N. V., Hadjinicolaou, A. E., Garrett, D. J., Cloherty, S. L., Kameneva, T., Grayden, D. B., Ibbotson, M. R., and Meffin, H. (2016). A Simple and Accurate Model to Predict Responses to Multi-electrode Stimulation in the Retina. *PLoS Computational Biology*, 12(4):e1004849.
- Mazzoni, F., Novelli, E., and Strettoi, E. (2008). Retinal Ganglion Cells Survive and Maintain Normal Dendritic Morphology in a Mouse Model of Inherited Photoreceptor Degeneration. *Journal of Neuroscience*, 28(52):14282–14292.
- Mccamy, M. B., Macknik, S. L., and Martinez-Conde, S. (2014). Different fixational eye movements mediate the prevention and the reversal of visual fading. *Journal of Physiology*, 592(19):4381–4394.
- Medeiros, N. E. and Curcio, C. a. (2001). Preservation of ganglion cell layer neurons in age-related macular degeneration. *Investigative Ophthalmology and Visual Science*, 42(3):795–803.
- Mehta, S. (2015). Age-Related Macular Degeneration. *Primary Care - Clinics in Office Practice*, 42(3):377–391.
- Meng, K., Fellner, A., Rattay, F., Ghezzi, D., Meffin, H., Ibbotson, M. R., and Kameneva, T. (2018). Upper stimulation threshold for retinal ganglion cell activation. *Journal of Neural Engineering*, 15(046012).
- Millodot, M. (1967). Extra-foveals variations of the phenomenon of troxler. *Psychologie Francaise*, 12(3):190 – 196.
- Moore, G. P., Perkel, D. H., and Segundo, J. P. (1966). Statistical analysis and functional interpretation of neuronal spike data. *Annual Review of Physiology*, 28(October):493–522.
- Mullins, R. F., Russell, S. R., Anderson, D. H., and Hageman, G. S. (2000). Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 14(7):835–846.

- Narahashi, T. (1964). Tetrodotoxin Blockage of Sodium Conductance Increase in Lobster Giant Axons. *Journal of General Physiology*, 47(5):965–974.
- Neher, E., Sakmann, B., and Steinbach, J. H. (1978). The extracellular patch clamp: A method for resolving currents through individual open channels in biological membranes. *Pflügers Archiv European Journal of Physiology*, 375(2):219–228.
- Noell, W. K. (1963). Cellular Physiology of the Retina*†. *Journal of the Optical Society of America*, 53(1):36.
- O'Brien, B. B. J., Isayama, T., Brien, B. J. O., Richardson, R., Berson, D. M., Isayama, T., Richardson, R., Berson, D. M., and O'Brien, B. B. J. (2002). Intrinsic physiological properties of cat retinal ganglion cells. *Journal of Physiology*, 538(2002):787–802.
- Oesch, N., Euler, T., and Taylor, W. R. (2005). Direction-selective dendritic action potentials in rabbit retina. *Neuron*, 47(5):739–750.
- Ong, J. M. and da Cruz, L. (2012). The bionic eye: A review. *Clinical and Experimental Ophthalmology*, 40(1):6–17.
- Palanker, D., Vankov, A., Huie, P., and Baccus, S. (2005). Design of a high-resolution optoelectronic retinal prosthesis. *Journal of Neural Engineering*, 2(1).
- Panetsos, F., Sanchez-Jimenez, A., Cerio, E. D.-d., Diaz-Guemes, I., and Sanchez, F. M. (2011). Consistent Phosphenes Generated by Electrical Microstimulation of the Visual Thalamus. An Experimental Approach for Thalamic Visual Neuroprostheses. *Frontiers in Neuroscience*, 5:84.
- Pérez Fornos, A., Sommerhalder, J., da Cruz, L., Sahel, J. A., Mohand-Said, S., Hafezi, F., and Pelizzone, M. (2012). Temporal Properties of Visual Perception on Electrical Stimulation of the Retina. *Investigative Ophthalmology & Visual Science*, 53(6):2720.
- Pillow, J. W. and Simoncelli, E. P. (2003). Biases in white noise analysis due to non-Poisson spike generation. *Neurocomputing*, 52-54:109–115.
- Poletti, M. (2010). Eye movements under various conditions of image fading. *Journal of Vision*, 10(3):1–18.
- Ramón y Cajal, S. (1972). *The structure of the retina*. Charles C. Thomas Publisher.
- Rieke, F. (2001). Temporal contrast adaptation in salamander bipolar cells. *Journal of Neuroscience*, 21(23):9445–9454.
- Rockhill, R. L., Daly, F. J., MacNeil, M. a., Brown, S. P., and Masland, R. H. (2002). The diversity of ganglion cells in a mammalian retina. *Journal of Neuroscience*, 22(9):3831–3843.
- Rockhill, R. L., Euler, T., and Masland, R. H. (2000). Spatial order within but not between types of retinal neurons. *Proceedings of the National Academy of Sciences*, 97(5):2303–2307.
- Rodieck, R. W. and Stone, J. (1965). Analysis of receptive fields of cat retinal ganglion cells. *Journal of Neurophysiology*, 28:832–849.

- Rosenfield, M., Logan, N., and Edwards, K. K. H. (2009). *Optometry : science techniques and clinical management*. Butterworth Heinemann Elsevier.
- Roy, A., Greenberg, R. J., Humayun, M. S., and MacClure, K. H. (2017). Neural stimulation for increased persistence. *US Patent 9,757,563*.
- Rucci, M., Iovin, R., Poletti, M., and Santini, F. (2007). Miniature eye movements enhance fine spatial detail. *Nature*, 447(7146):851–854.
- Sahin, M. and Tie, Y. (2007). Non-rectangular waveforms for neural stimulation with practical electrodes. *Journal of Neural Engineering*, 4:227–233.
- Sakmann, B. and Neher, E. (1984). Patch Clamp Techniques for Studying Ionic Channels in Excitable Membranes. *Annual Review of Physiology*, 46(1):455–472.
- Sanes, J. R. and Masland, R. H. (2015). The Types of Retinal Ganglion Cells: Current Status and Implications for Neuronal Classification. *Annual Review of Neuroscience*, 38(1):221–246.
- Schiller, P., Sandell, J., and Maunsell, J. (1986). Functions of the ON and OFF channels of the visual system. *Nature*, 322:824–825.
- Schmidt, T. M., Chen, S.-k., and Hattar, S. (2011). Intrinsically photosensitive retinal ganglion cells : many subtypes , diverse functions. *Trends in Neurosciences*, 34(11):572–580.
- Schmitz, D., Schuchmann, S., Fisahn, A., Draguhn, A., Buhl, E. H., Petrasch-Parwez, E., Dermietzel, R., Heinemann, U., and Traub, R. D. (2001). Axo-axonal coupling: A novel mechanism for ultrafast neuronal communication. *Neuron*, 31(5):831–840.
- Schwartz, O., Chichilnisky, E. J., and Simoncelli, E. P. (2002). Characterizing neural gain control using spike-triggered covariance. *Advances in Neural Information Processing Systems*, 14:269–276.
- Schwartz, O., Pillow, J. W., Rust, N. C., and Simoncelli, E. P. (2006). Spike-triggered neural characterization. *Journal of Vision*, 6(4):13.
- Sekhar, S., Jalligampala, A., Zrenner, E., and Rathbun, D. L. (2016). Tickling the retina: Integration of subthreshold electrical pulses can activate retinal neurons. *Journal of Neural Engineering*, 13(4):046004.
- Sekirnjak, C. (2006). Electrical Stimulation of Mammalian Retinal Ganglion Cells With Multielectrode Arrays. *Journal of Neurophysiology*, 95(6):3311–3327.
- Sim, S. L., Szalewski, R. J., Johnson, L. J., Akah, L. E., Shoemaker, L. E., Thoreson, W. B., and Margalit, E. (2014). Simultaneous recording of mouse retinal ganglion cells during epiretinal or subretinal stimulation. *Vision Research*, 101:41–50.
- Smith, J., Ward, D., Michaelides, M., Moore, A. T., and Simpson, S. (2015). New and emerging technologies for the treatment of inherited retinal diseases: A horizon scanning review. *Eye (Basingstoke)*, 29(9):1131–1140.

- Soto-Breceda, A., Kameneva, T., Meffin, H., Maturana, M. I., and Ibbotson, M. R. (2018). Irregularly timed electrical pulses reduce adaptation of retinal ganglion cells. *Journal of Neural Engineering*, 15(056017).
- Stett, A., Barth, W., Weiss, S., Haemmerle, H., and Zrenner, E. (2000). Electrical multisite stimulation of the isolated chicken retina. *Vision Research*, 40(13):1785–1795.
- Stingl, K., Bartz-Schmidt, K. U., Besch, D., Braun, A., Bruckmann, A., Gekeler, F., Greppmaier, U., Hipp, S., Hortdorfer, G., Kernstock, C., Koitschev, A., Kusnyerik, A., Sachs, H., Schatz, A., Stingl, K. T., Peters, T., Wilhelm, B., and Zrenner, E. (2013). Artificial vision with wirelessly powered subretinal electronic implant alpha-IMS. *Proceedings of the Royal Society B: Biological Sciences*, 280(1757):20130077–20130077.
- Stronks, C. H., Barry, M. P., and Dagnelie, G. (2013). Electrically elicited visual evoked potentials in Argus II retinal implant wearers. *Investigative Ophthalmology and Visual Science*, 54(6):3891–3901.
- Suematsu, N., Naito, T., Miyoshi, T., Sawai, H., and Sato, H. (2013). Spatiotemporal receptive field structures in retinogeniculate connections of cat. *Frontiers in Systems Neuroscience*, 7(December):1–13.
- Sun, W., Li, N., and He, S. (2002a). Large-scale morphological survey of mouse retinal ganglion cells. *Journal of Comparative Neurology*, 451(2):115–126.
- Sun, W., Li, N., and He, S. (2002b). Large-scale morphological survey of rat retinal ganglion cells. *Visual Neuroscience*, 19(4):483–493.
- Tass, P. A. (2001). Desynchronizing double-pulse phase resetting and application to deep brain stimulation. *Biological Cybernetics*, 85(5):343–354.
- Tauchi, M., Morigiwa, K., and Fukuda, Y. (1992). Morphological Comparisons between Outer and Inner Ramifying Alpha-Cells of the Albino-Rat Retina. *Experimental Brain Research*, 88(1):67–77.
- Tian, N. (2008). ON and OFF Pathways in the Mouse Retina and the Role of Stimulation. In Chalupa, L. M. and Williams, R. W., editors, *Eye, Retina and Visual System of the Mouse*, chapter 29, pages 353–361. MIT Press, Cambridge, MA, USA.
- Tikidji-hamburyan, A., Reinhard, K., Seitter, H., Procyk, C. A., Allen, A. E., Schenk, M., Robert, J., and Münch, T. A. (2015). Europe PMC Funders Group Retinal output changes qualitatively with every change in ambient illuminance. *Nature Neuroscience*, 18(1):66–74.
- Tribble, J. R., Cross, S. D., Samsel, P. A., Sengpiel, F., and Morgan, J. E. (2014). A novel system for the classification of diseased retinal ganglion cells. *Visual Neuroscience*, 31(6):373–380.
- Tsai, D., Chen, S., Protti, D. A., Morley, J. W., Suaning, G. J., and Lovell, N. H. (2012). Responses of Retinal Ganglion Cells to Extracellular Electrical Stimulation, from Single Cell to Population: Model-Based Analysis. *PLoS ONE*, 7(12):e53357.

- Tsai, D., Morley, J. W., Suaning, G. J., and Lovell, N. H. (2009). Direct Activation and Temporal Response Properties of Rabbit Retinal Ganglion Cells Following Subretinal Stimulation. *Journal of Neurophysiology*, 102(5):2982–2993.
- Tsai, D., Morley, J. W., Suaning, G. J., and Lovell, N. H. (2011a). Frequency-dependent reduction of voltage-gated sodium current modulates retinal ganglion cell response rate to electrical stimulation. *Journal of Neural Engineering*, 8(6):12.
- Tsai, D., Morley, J. W., Suaning, G. J., and Lovell, N. H. (2011b). Sodium channel inactivation reduces retinal ganglion cell responsiveness to repetitive prosthetic stimulation. *2011 5th International IEEE/EMBS Conference on Neural Engineering, NER 2011*, pages 550–553.
- Tulunay-Keesey, U. (1982). Fading of stabilized retinal images. *Journal of the Optical Society of America*, 72(4):440–447.
- Twyford, P., Cai, C., and Fried, S. (2014). Differential responses to high-frequency electrical stimulation in on and off retinal ganglion cells. *Journal of Neural Engineering*, 11(2):25001.
- Wässle, H. (2004). Parallel processing in the mammalian retina. *Nature Reviews Neuroscience*, 5(10):747–757.
- Wei, W. and Feller, M. B. (2011). Organization and development of direction-selective circuits in the retina. *Trends in Neurosciences*, 34(12):638–645.
- Weiland, J. D., Member, S., and Humayun, M. S. (2014). Retinal Prosthesis. *IEEE Transactions on Biomedical Engineering*, 61(5):1412–1424.
- Weitz, A. C., Behrend, M. R., Ahuja, A. K., Punita, C., Jianing, W., Wuyyuru, V., Patel, U., Greensberg, R. J., Humayun, M. S., Chow, R. H., and Weiland, J. D. (2014). Interphase gap as a means to reduce electrical stimulation thresholds for epiretinal prostheses. *Journal of Neural Engineering*, 11(1).
- Wong, R. C. S., Cloherty, S. L., Ibbotson, M. R., and O'Brien, B. J. (2012). Intrinsic physiological properties of rat retinal ganglion cells with a comparative analysis. *Journal of Neurophysiology*, 108(7):2008–2023.
- Wong, R. C. S., Garrett, D. J., Grayden, D. B., Ibbotson, M. R., and Cloherty, S. L. (2014). Efficacy of electrical stimulation of retinal ganglion cells with temporal patterns resembling light-evoked spike trains. In IEEE, editor, *Conference proceedings : ... Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual Conference*, volume 2014, pages 1707–1710, Chicago, USA.
- Wongsarnpigoon, A. and Grill, W. M. (2010). Energy-efficient waveform shapes for neural stimulation revealed with a genetic. *Journal of Neural Engineering*, 7.
- Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M., and Bhattacharya, S. S. (2010). Photoreceptor degeneration: Genetic and mechanistic dissection of a complex trait. *Nature Reviews Genetics*, 11(4):273–284.

- Yanai, D., Weiland, J. D., Mahadevappa, M., Greenberg, R. J., Fine, I., and Humayun, M. S. (2007). Visual Performance Using a Retinal Prosthesis in Three Subjects With Retinitis Pigmentosa. *American Journal of Ophthalmology*, 143(5):820–827.
- Yue, L., Weiland, J. D., Roska, B., and Humayun, M. S. (2016). Retinal stimulation strategies to restore vision: Fundamentals and systems. *Progress in Retinal and Eye Research*, 53:21–47.
- Zaidi, F. H. (2009). Non-Rod, Non-Cone Photoreception in Humans: Roles in Vision and Disease. In *Eye and Vision Research Developments : Retinal Degeneration : Causes, Diagnosis, and Treatment*, chapter 11, pages 213–235. Nova Science Publishers, Inc., New York, NY, USA.
- Zaidi, F. H., Hull, J. T., Peirson, S. N., Wulff, K., Aeschbach, D., Gooley, J. J., Brainard, G. C., Gregory-Evans, K., Rizzo, J. F., Czeisler, C. A., Foster, R. G., Moseley, M. J., and Lockley, S. W. (2007). Short-Wavelength Light Sensitivity of Circadian, Pupillary, and Visual Awareness in Humans Lacking an Outer Retina. *Current Biology*, 17(24):2122–2128.
- Zrenner, E., Bartz-Schmidt, K. U., Benav, H., Besch, D., Bruckmann, A., Gabel, V.-P., Gekeler, F., Greppmaier, U., Harscher, A., Kibbel, S., Koch, J., Kusnyerik, A., Peters, T., Stingl, K., Sachs, H., Stett, A., Szurman, P., Wilhelm, B., and Wilke, R. (2011). Subretinal electronic chips allow blind patients to read letters and combine them to words. *Proceedings of the Royal Society B: Biological Sciences*, 278(1711):1489–1497.
- Zrenner, E., Miliczek, K. D., Gabel, V. P., Graf, H. G., Guenther, E., Haemmerle, H., Hoefflinger, B., Kohler, K., Nisch, W., Schubert, M., Stett, A., and Weiss, S. (1997). The development of sub-retinal microphotodiodes for replacement of degenerated photoreceptors. *Ophthalmic Research*, 29(5):269–280.

Appendices

Appendix A

Example stimulus trains from Chapter 2

Here we present an example of constant and random IPI stimuli for a better understanding. Note that for consistency, every sequence at each frequency has exactly the same number of pulses (regardless of it being constant or random IPI) i.e. a sequence of pulses at 10 Hz contains 30 pulses and lasts exactly 3 seconds for constant IPI, but lasts slightly more or less for random IPI. A sequence of pulses at 50 Hz has always 150 pulses, and lasts 3 seconds with constant IPI, but slightly more or less with random IPI. A sequence of pulses at 200 Hz has 600 pulses, and lasts 3 seconds with constant IPI, but slightly more or less with random IPI. Figure A.1 shows 3 example stimulation trains at each frequency with random IPI, and 3 example stimulation trains at each frequency with constant IPI. Each cell was stimulated with 30 trains of random and constant IPI pulses at each frequency (10 Hz, 50 Hz, and 200 Hz). In total, every cell was stimulated with 180 trains lasting 3 s each. Rest periods consisted of 5 s of no stimulation between trains. The type (random or constant IPI) and frequency of train stimulation were randomised over the experiment.

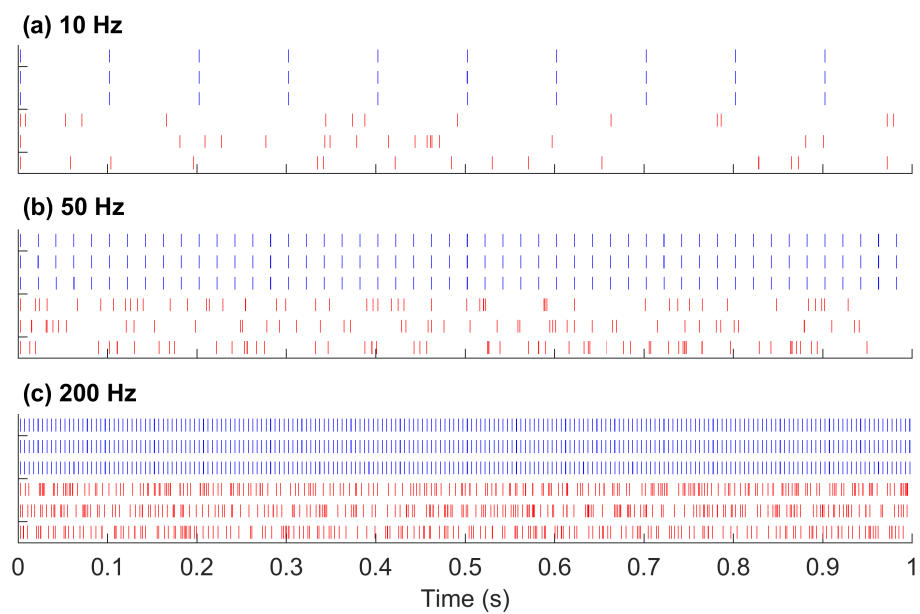


Figure A.1: Example sequences of biphasic pulses with constant IPI (blue) and random IPI (red) at **(a)** 10 Hz **(b)** 50 Hz and **(c)** 200 Hz. The figure presents the first second of stimulation of 18 example sequences, six trains per frequency, where the first three correspond to constant IPI, and the last three to random IPI. Each sequence of random IPI is sampled individually from an exponential distribution and is used only once.

Appendix B

Efficacy of response per class

The efficacy of response of cells 1 to 6 (Class A) to all the waveforms of each cell are presented in Figures B.1 and B.2. The efficacy of response of cells 7 to 14 (Class B) to all the waveforms of each cell are presented in Figures B.3 and B.4. Finally, the efficacy of RGCs 15 and 16 (Class D) are shown in figure B.5. None of the Class C cells reached this state of the protocol.

Morphological class A

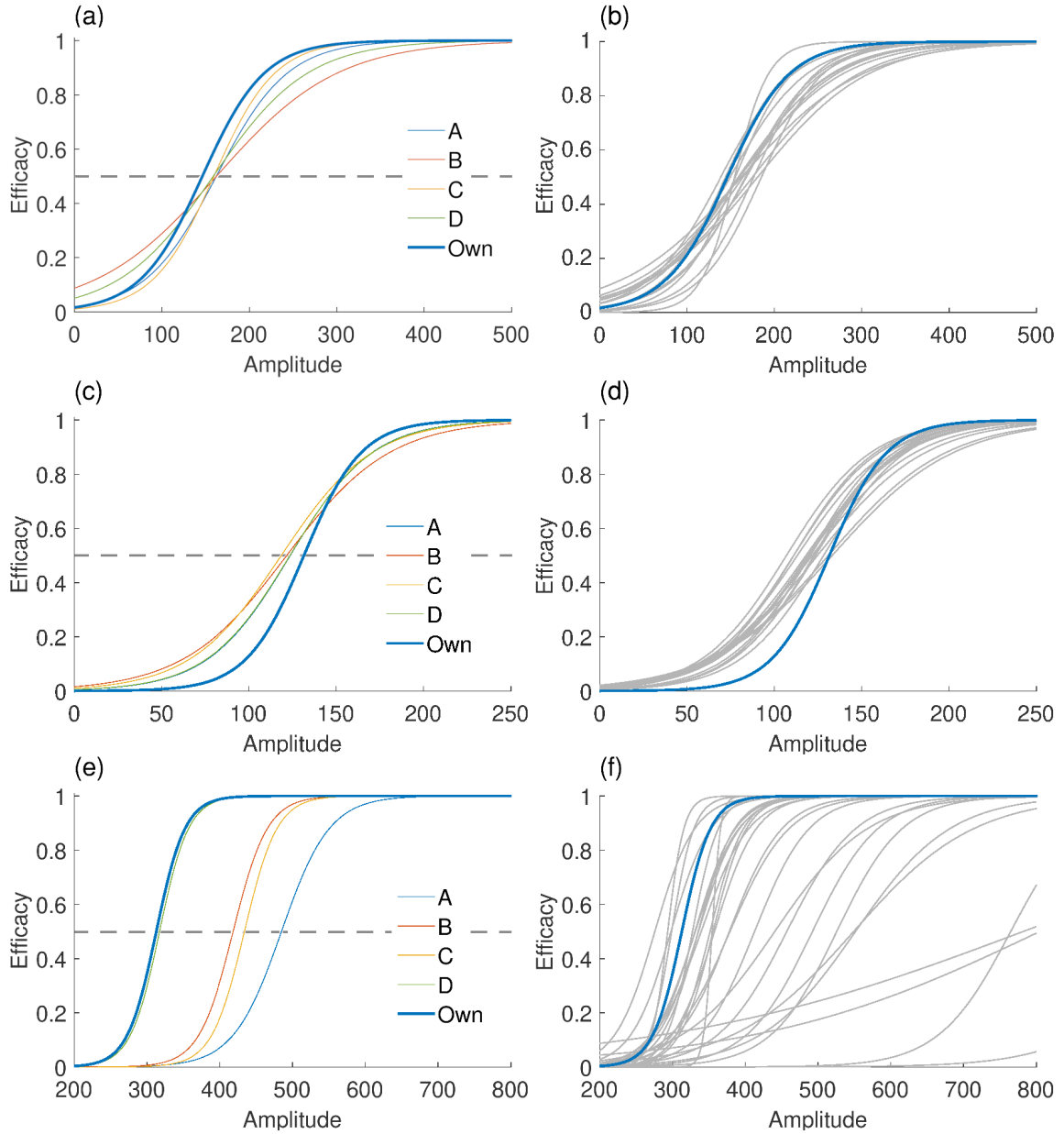


Figure B.1: Stimulus response curves of the first three Class A cell. Colour lines are the average fitted sigmoids to the spike probability of a cell in response to the waveforms from Class A (blue), Class B (red), Class C (yellow), and Class D (green). Thick blue line is the fitted sigmoid of each cell in response to its own waveform. **(a)** Threshold of cell no. 1, **(b)** fitted sigmoid to the spike probability of cell no. 1 to every waveform (grey) and to its own waveform (blue), **(c)** Threshold of cell no. 2, **(d)** fitted sigmoid to the spike probability of cell no. 2 to every waveform (grey) and to its own waveform (blue), **(e)** Threshold of cell no. 3, **(f)** fitted sigmoid to the spike probability of cell no. 3 to every waveform (grey) and to its own waveform (blue). Gray dotted line indicates 50% spike probability.

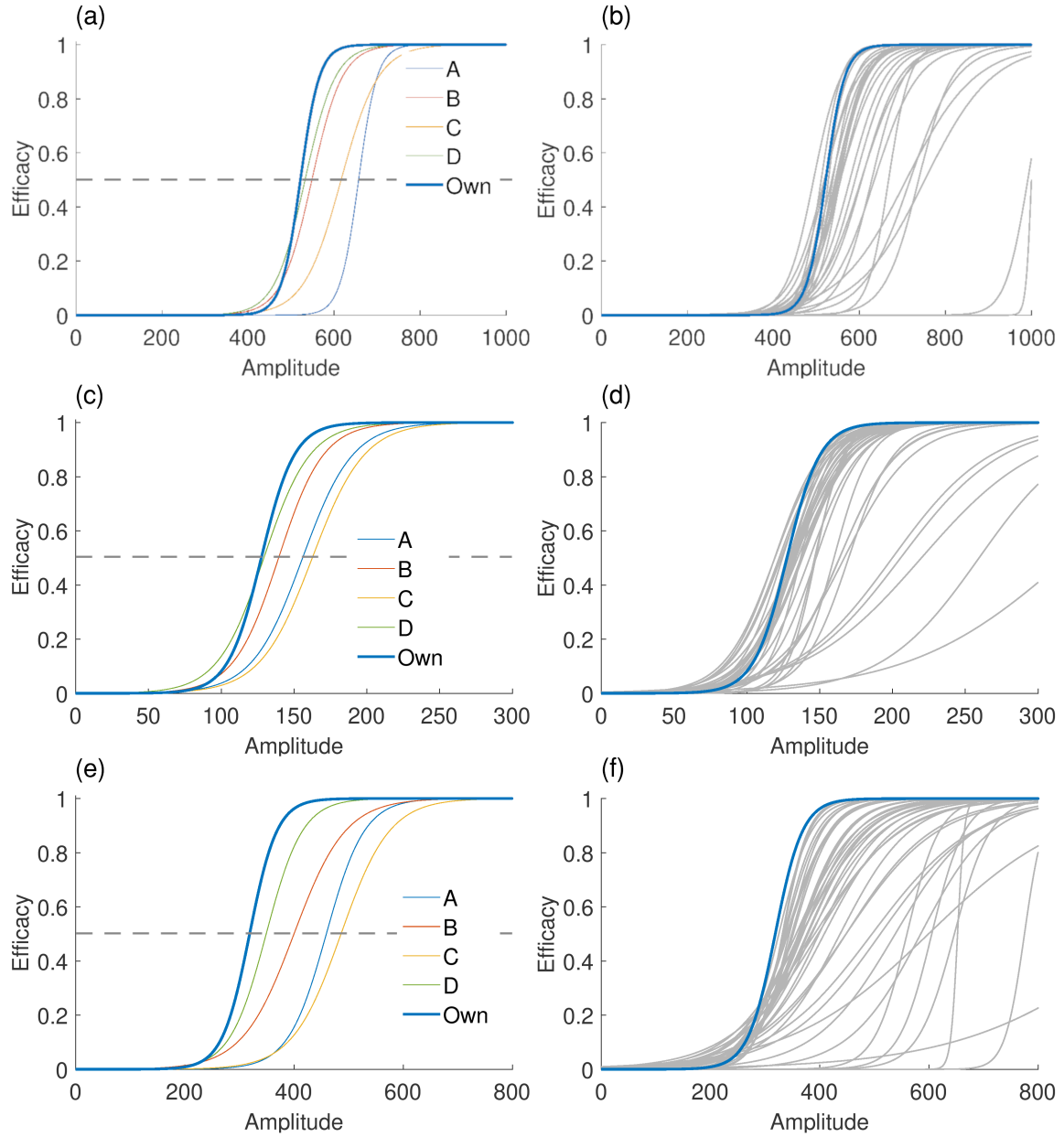


Figure B.2: Stimulus response curves of the last three Class A cell. Colour lines are the average fitted sigmoids to the spike probability of a cell in response to the waveforms from Class A (blue), Class B (red), Class C (yellow), and Class D (green). Thick blue line is the fitted sigmoid of each cell in response to its own waveform. **(a)** Threshold of cell no. 4, **(b)** fitted sigmoid to the spike probability of cell no. 4 to every waveform (grey) and to its own waveform (blue), **(c)** Threshold of cell no. 5, **(d)** fitted sigmoid to the spike probability of cell no. 5 to every waveform (grey) and to its own waveform (blue), **(e)** Threshold of cell no. 6, **(f)** fitted sigmoid to the spike probability of cell no. 6 to every waveform (grey) and to its own waveform (blue). Gray dotted line indicates 50% spike probability.

Morphological class B

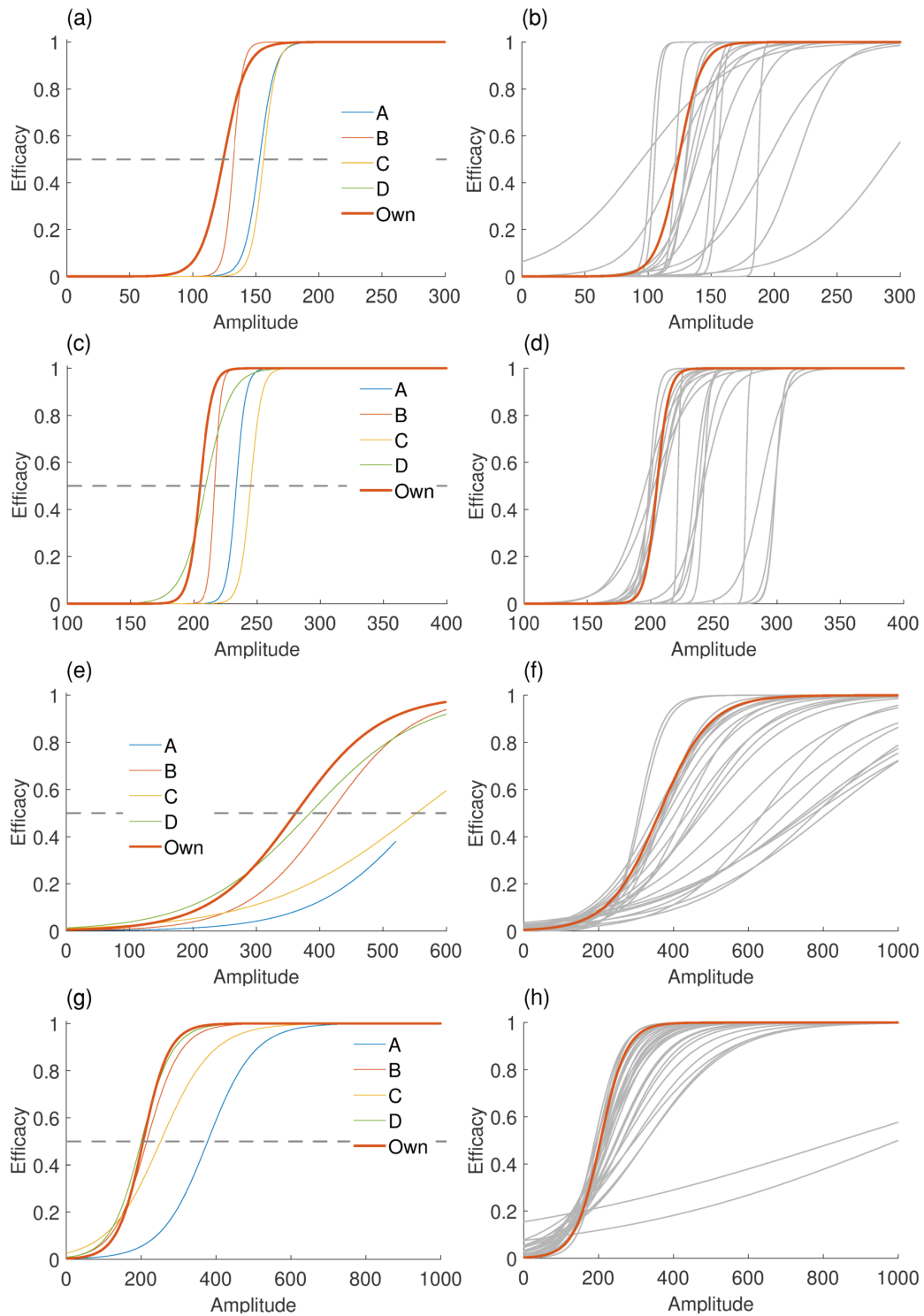


Figure B.3: Stimulus response curves of the first four Class B cell (See caption of Figure B.1). **(a)** and **(b)** fitted sigmoids to the response of cell no. 7, **(c)** and **(d)** fitted sigmoids to the response of cell no. 8, **(e)** and **(f)** fitted sigmoids to the response of cell no. 9, **(g)** and **(h)** fitted sigmoids to the response of cell no. 10.

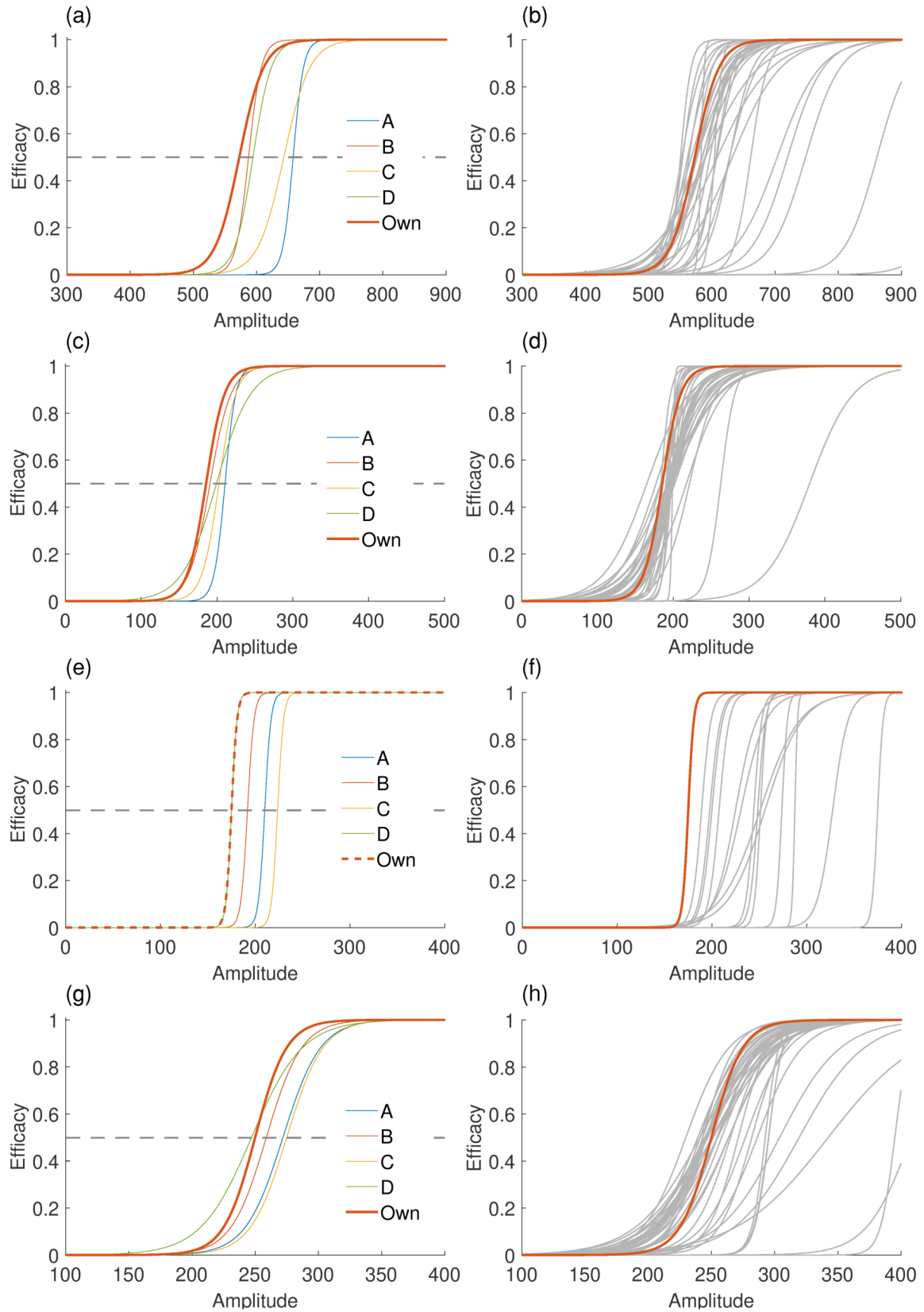


Figure B.4: Threshold of the last four Class B cell (See caption of Figure B.1). **(a)** and **(b)** fitted sigmoids to the response of cell no. 11, **(c)** and **(d)** fitted sigmoids to the response of cell no. 12, **(e)** and **(f)** fitted sigmoids to the response of cell no. 13, **(g)** and **(h)** fitted sigmoids to the response of cell no. 14.

Morphological class D

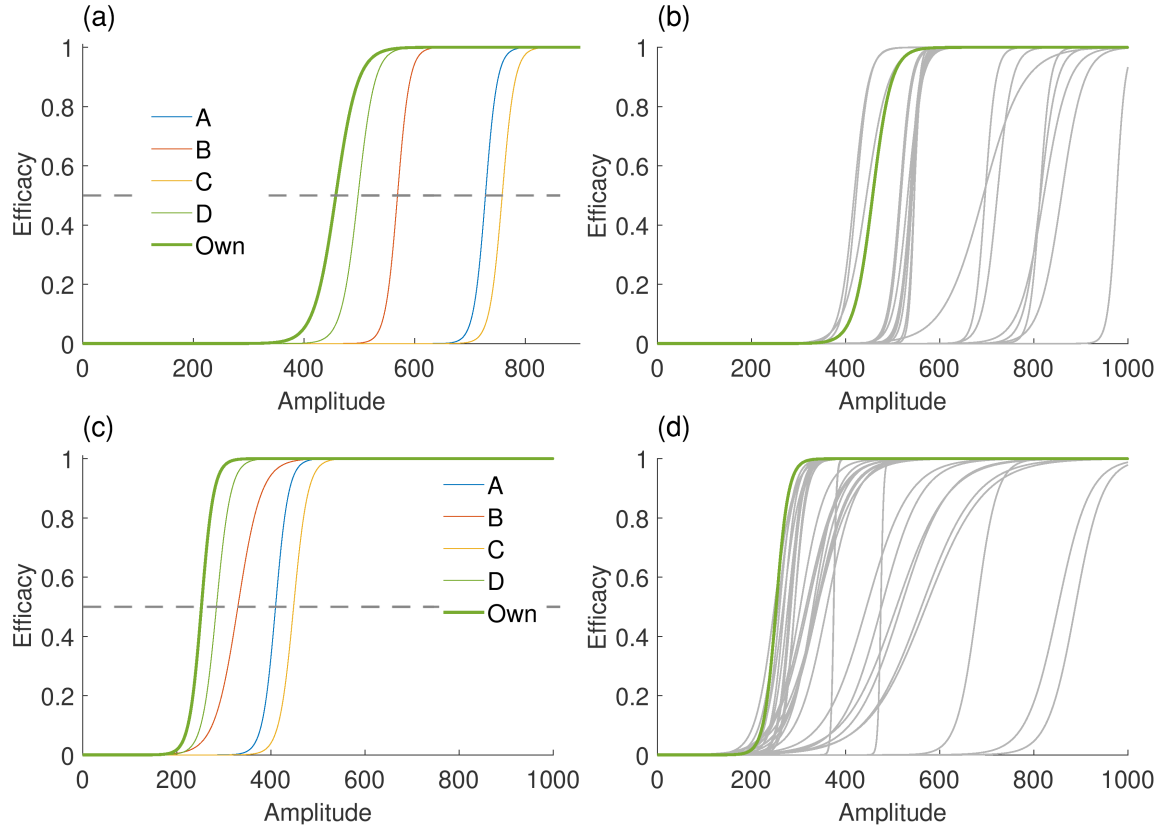


Figure B.5: Stimulus response curves of each Class D cell. Colour lines are the average fitted sigmoids to the spike probability of a cell in response to the waveforms from Class A (blue), Class B (red), Class C (yellow), and Class D (green). Thick green line is the fitted sigmoid of each cell in response to its own waveform. **(a)** Threshold of cell no. 15, **(b)** fitted sigmoid to the spike probability of cell no. 15 to every waveform (grey) and to its own waveform (green), **(c)** Threshold of cell no. 16, **(d)** fitted sigmoid to the spike probability of cell no. 16 to every waveform (grey) and to its own waveform (green). Gray dotted line indicates 50% spike probability.

Appendix C

Efficacy of response per cluster

The efficacy of response of cells to all waveforms in the ensemble is shown in Figure C.1 for Cluster 2, Figure C.2 for Cluster 3 and Figure C.3 for Cluster 4. No RGCs whose waveform belonged to Cluster 1 were stimulated in this section of the protocol.

Cluster 2

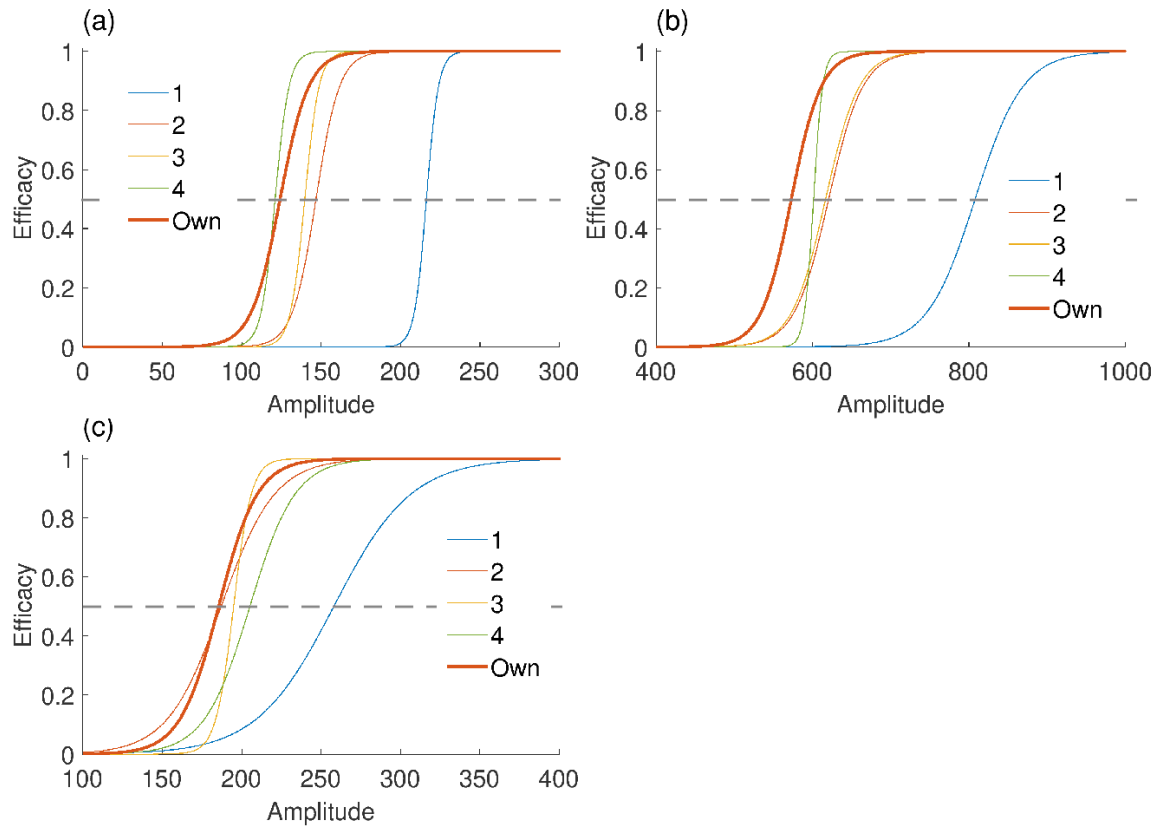


Figure C.1: Stimulus response curves of each cell in Cluster 4. Colour lines are fitted sigmoids to the spike probability of a cell in response to the waveforms from Cluster 1 (blue), Cluster 2 (red), Cluster 3 (yellow), and Cluster 4 (green). Thick red line is the fitted sigmoid of each cell in response to its own waveform. Threshold of **(a)** cell no. 2, **(b)** cell no. 5, **(c)** cell no. 8, **(d)** cell no. 9, **(e)** cell no. 16. Gray dotted line indicates 50% spike probability.

Cluster 3

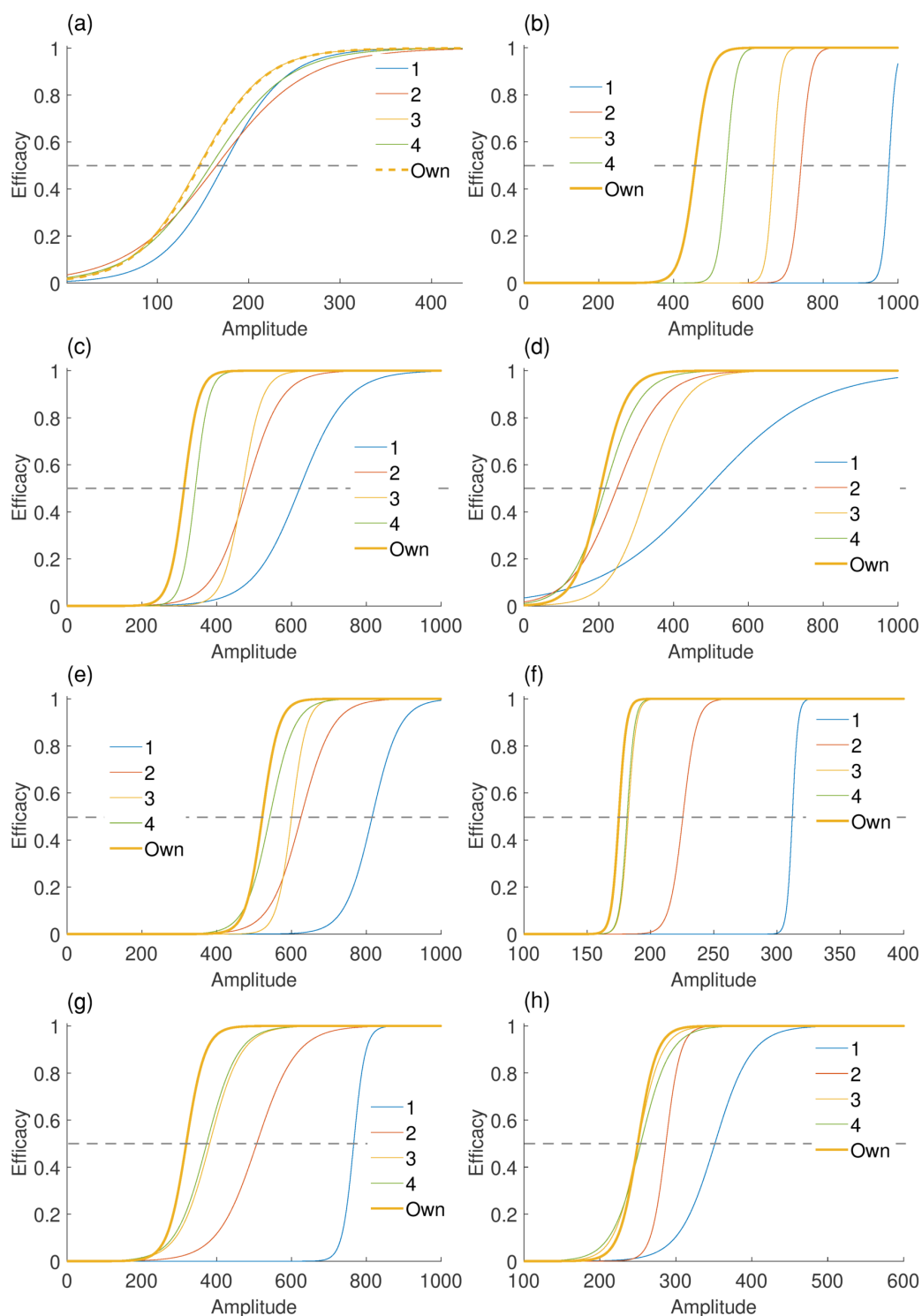


Figure C.2: Stimulus response curves of each cell in Cluster 4. Colour lines are fitted sigmoids to the spike probability of a cell in response to the waveforms from Cluster 1 (blue), Cluster 2 (red), Cluster 3 (yellow), and Cluster 4 (green). Thick yellow line is the fitted sigmoid of each cell in response to its own waveform. Threshold of (a) cell no. 2, (b) cell no. 5, (c) cell no. 8, (d) cell no. 9, (e) cell no. 16. Gray dotted line indicates 50% spike probability.

Cluster 4

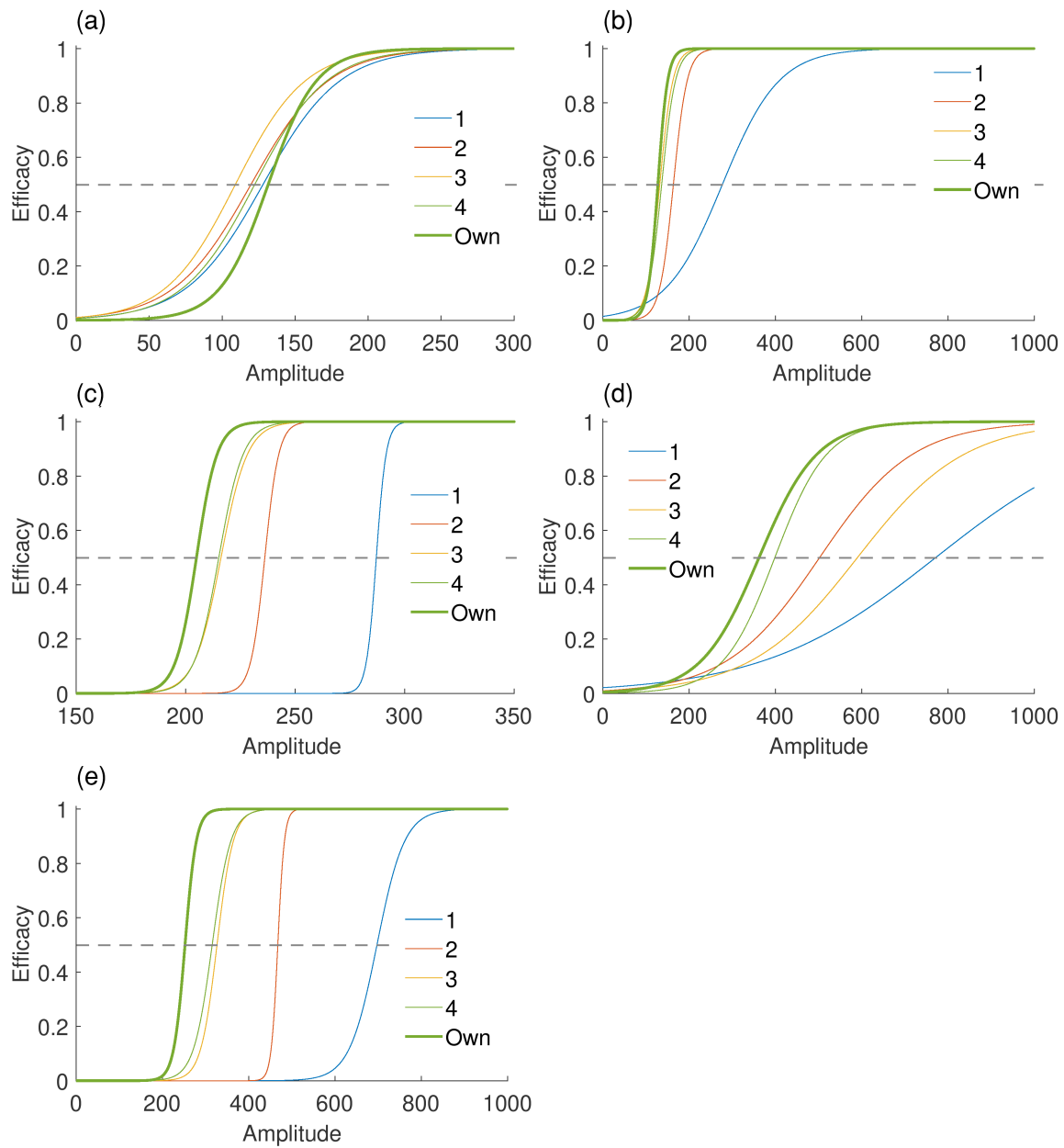


Figure C.3: Stimulus response curves of each cell in Cluster 4. Colour lines are fitted sigmoids to the spike probability of a cell in response to the waveforms from Cluster 1 (blue), Cluster 2 (red), Cluster 3 (yellow), and Cluster 4 (green). Thick green line is the fitted sigmoid of each cell in response to its own waveform. Threshold of (a) cell no. 2, (b) cell no. 5, (c) cell no. 8, (d) cell no. 9, (e) cell no. 16. Gray dotted line indicates 50% spike probability.