Title:
The development of direction selectivity in the mouse retina

Author:
Elstrott, Justin Blake

Acceptance Date:
2009

Series:
UC San Diego Electronic Theses and Dissertations

Degree:
Ph. D., Neurosciences UC San Diego

Permalink:
http://escholarship.org/uc/item/0m208981

Local Identifier:
b6626634

Abstract:
Whether capturing prey or evading predators, an animal's ability to detect movement in the world is critical for its survival. The first cells in the visual pathway that are tuned to detect motion are a subset of retinal ganglion cells called direction-selective ganglion cells (DSGCs). DSGCs respond strongly to motion in the "preferred" direction and weakly to motion in the opposite, or "null" direction. The circuitry underlying this computation consists of precise wiring between inhibitory interneurons, called amacrine cells, and the DSGCs. The role of neural activity in guiding this wiring is not known. We performed a series of experiments to examine whether DSGCs require activity for normal development. First, we tested whether visual experience was required for the establishment of direction selectivity in the retina. We recorded from the retinas of dark-reared mice using large-scale multi-electrode arrays, which allow for the recording from hundreds of neurons simultaneously. We found that DSGCs develop independent of visual experience, and are present as early as eye-opening. Second, to target recordings from DSGCs before eye-opening, we characterized a mouse line where a class of DSGCs expresses a fluorescent reporter. These experiments also allowed for the first complete description of the projection patterns for this cell. Finally, using this transgenic mouse with labeled DSGCs, we showed that DSGCs participate in spontaneous retinal activity before the eyes open, suggesting a possible role for early, spontaneous activity in their development.

Copyright Information:
All rights reserved unless otherwise indicated. Contact the author or original publisher for any necessary permissions. eScholarship is not the copyright owner for deposited works. Learn more at http://www.escholarship.org/help_copyright.html#reuse
The development of direction selectivity in the mouse retina

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neurosciences/Specialization in Computational Neuroscience by Justin Blake Elstrott

Committee in charge:
Professor Massimo Scanziani, Chair
Professor EJ Chichilnisky
Professor Marla B. Feller
Professor David Rapaport
Professor Pamela Reinagel

2009
The Dissertation of Justin Blake Elstrott is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2009
This thesis is dedicated to Joy Bonaguro.

Marrying you was the best decision I’ve ever made.
Every honest researcher I know admits he's just a professional amateur. He's doing whatever he's doing for the first time. That makes him an amateur. He has sense enough to know that he's going to have a lot of trouble, so that makes him a professional.

Charles Franklin Kettering
Table of Contents

Signature Page........................................................................................................ iii
Dedication .............................................................................................................. iv
Epigraph ............................................................................................................... v
Table of Contents ............................................................................................... vi
List of Figures ...................................................................................................... viii
Acknowledgments .............................................................................................. x
Vita and Publications ......................................................................................... xiii
Abstract of the dissertation ............................................................................... xv

I. Introduction ...................................................................................................... 1

  General circuit organization of direction-selective responses
  in retina and primary visual cortex ....................................................... 2
  Development of directional responses in retina
  occurs independent of vision ............................................................ 5
  Development of directional responses in cortex depends on vision 6
  Proposed models for the development of direction selectivity...... 7
  References ................................................................................................. 11

II. Direction selectivity in the retina is established independent
  of visual experience and early patterned activity ............................. 18

  Abstract ...................................................................................................... 18
  Introduction .............................................................................................. 18
  Results ...................................................................................................... 21
  Discussion ............................................................................................... 27
  Methods .................................................................................................... 28
  Acknowledgment ..................................................................................... 33
  References .............................................................................................. 34

III. Genetic identification of an On-Off direction selective
  retinal ganglion cell subtype reveals a layer specific
  subcortical map of posterior motion ................................................. 47
# List of Figures

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 1.1</td>
<td>The distribution of preferred directions for DSGCs in the rabbit retina</td>
<td>16</td>
</tr>
<tr>
<td>Fig. 1.2</td>
<td>Two ways of implementing the correlation model of direction selectivity postulated by Barlow and Levick</td>
<td>17</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2.1</td>
<td>Frequency doubling analysis</td>
<td>40</td>
</tr>
<tr>
<td>Fig. 2.2</td>
<td>Responses of On-Off DSGCs to moving gratings reveal strong direction selectivity in the adult and P14 dark reared mouse</td>
<td>41</td>
</tr>
<tr>
<td>Fig. 2.3</td>
<td>Development of On and Off responses</td>
<td>42</td>
</tr>
<tr>
<td>Fig. 2.4</td>
<td>Comparison of null direction and preferred direction firing rates for Adult and P14 WT DSGCs</td>
<td>43</td>
</tr>
<tr>
<td>Fig. 2.5</td>
<td>Population tuning widths change during development</td>
<td>44</td>
</tr>
<tr>
<td>Fig. 2.6</td>
<td>Distributions of preferred directions for On-Off DSGCs in adult and P14 mice</td>
<td>45</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 3.1</td>
<td>A mosaic of $GFP^+$ On-Off RGCs in $DRD4$-$GFP$ mice</td>
<td>66</td>
</tr>
<tr>
<td>Fig. 3.2</td>
<td>Two photon targeted recording from GFP+ retinal ganglion cells</td>
<td>68</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>2-photon targeted recordings of $GFP^+$ cells reveal a preference for posterior motion</td>
<td>69</td>
</tr>
<tr>
<td>Fig. 3.4</td>
<td>Both bright (right) and less bright (left) GFP+ RGCs show the same directional tuning profile</td>
<td>70</td>
</tr>
</tbody>
</table>
Fig. 3.5  GFP+ On-Off pDSGCs do not target any retinorecipient nuclei other than the dLGN and SC........................................ 71

Fig. 3.6  On-Off pDSGCs form laminar-specific connections in the dLGN................................................................. 73

Fig. 3.7  On-Off pDSGCs form laminar-specific connections in the SC................................................................. 75

Chapter 4

Fig. 4.1  Measuring pDSGCs and non-pDSGCs activity using calcium imaging .................................................. 91

Fig. 4.2  Peak ΔF/F as a function of wave direction for a P11 pDSGC indicate participation in waves in all directions with no apparent bias........................................... 92

Fig. 4.3  Large variability associated with the amplitude of ΔF/F as a function of spike count during a wave.............. 93

Fig. 4.4  Simultaneous calcium imaging and cell-attached recording show that RGCs bursts during retinal waves ........... 94

Fig. 4.5  Spiking properties of a RGC during retinal waves.......... 95

Fig. 4.6  Non-pDSGCs show asymmetric spiking across wave directions ............................................................... 96

Fig. 4.7  pDSGCs show no significant difference in spiking across wave directions................................................. 97
Acknowledgments

A graduate student is lucky to have a supportive mentor. I’ve had the good fortune of working with two talented advisors during my graduate career. Marla Feller and EJ Chichilnisky have different approaches to Science, but the results are equally impressive. Both were willing to invest the time and energy to turn a philosophy student into a neuroscientist. That alone deserves a medal.

Marla is living proof that rigorous science and a generous spirit are absolutely compatible. As a mentor, Marla strikes the perfect balance between hands-on guidance and intellectual respect that makes for happy, independent students. I can only hope that some of Marla’s knack for framing a problem and following through to a published result has rubbed off in the past six years. Marla has advocated for me since day one, and for that I am truly grateful.

EJ generously adopted me into his lab for three years and showed me computational neuroscience at its finest. EJ’s clarity of thought is matched only by his ability to succinctly articulate the essence of a problem. The skills I learned in his lab will benefit me the rest of my life, not the least of which is an appreciation for elegant code (even if I don’t always write it myself).

Of course, half the fun of doing Science are the people you get to work with. Christine Torborg trained me while defending her own Ph.D., a feat I’m just now fully appreciating. Jeff Gauthier made my leap into visual neuroscience an entertaining one, his enthusiasm and talent for displaying data beautifully are inspiring. Martin
Greschner was always generous with his time and intellect, a fact that will surprise none who know him. Greg Field’s deep knowledge of the retina and his willingness to share it benefited me greatly, and Clare Goo’s expert technical assistance made experiments that much easier. I thank Lauren Hruby for indulging me as I rambled on about the merits of LISP. I must give special thanks to Anastasia Anishchenko, whose contributions to my Ph.D. are too numerous to list. Anastasia helped pioneer the mouse preparation in EJ’s lab and is a valued friend and peer.

For my last year of graduate school, I’ve had the privilege to work again with the members of the Feller lab. I thank Aaron Blankenship, Will Barkis, and Kevin Ford for helping an old dog learn new tricks. I also thank Wei Wei and Andy Huberman for being great collaborators and even better friends. I thank Martha Bagnall for sharing her thesis, it always helps to imitate the best.

Finally, I wish to thank my family for their unconditional support. My father’s advice to “do whatever interests you” has proven increasingly prescient with time. Most of all, I thank my wife and best friend, Joy Bonaguro. I suspect Joy has learned more about the retina than some neuroscientists have in the past six years, a testament to her keen intellect and unwavering devotion. I plan on returning the favor.

Chapter 1, in part, is a reprint of the material as it appears in Elstrott, J; Feller, MB. Vision and the establishment of direction-selectivity: a tale of two circuits. Current Opinion Neurobiology, 2009 with permission from all authors. The dissertation author was the primary author of this paper.
Chapter 2, in full, is a reprint of the material as it appears in Elstrott, J; Anishchenko, A; Greschner, M; Sher, A; Litke, AM; Chichilnisky, EJ; Feller, MB. Direction selectivity in the retina is established independent of visual experience and Cholinergic Retinal Waves. Neuron, vol. 58, 2008 with permission from all authors. The dissertation author was the primary author of this paper.

Chapter 3, in full, is a reprint of the material as it appears in Huberman, AD; Wei, W; Elstrott, J; Stafford, BK; Feller, MB; Barres, BA. Genetic identification of On-Off direction selective retinal ganglion cells reveals a layer specific subcortical map of posterior motion. Neuron, vol. 62, 2009 with permission from all authors. The dissertation author was co-first author of this paper in addition to Drs. Andrew Huberman and Wei Wei.

Chapter 4 is original work in preparation as Elstrott, J; Feller, MB. The interaction of direction selective cells and retinal waves and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
Vita and Publications

2003 B.S., Psychology, Tulane University  
   B.A., Philosophy, Tulane University  
2004 M.S., Computational Neuroscience, University of California, San Diego  
2009 Ph.D., Computational Neuroscience, University of California, San Diego

Publications

   * = equal contribution.


## Awards

<table>
<thead>
<tr>
<th>Year</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008</td>
<td>Participant in the Cold Spring Harbor Computational Vision course</td>
</tr>
<tr>
<td>2007</td>
<td>Gordon Research Conference poster prize</td>
</tr>
<tr>
<td>2005-2008</td>
<td>National Science Foundation Graduate Research Fellowship</td>
</tr>
<tr>
<td>2003</td>
<td>Academic Valedictorian for Tulane College</td>
</tr>
<tr>
<td>2003</td>
<td>Phi Beta Kappa</td>
</tr>
<tr>
<td>2003</td>
<td>Senior scholar award psychology</td>
</tr>
<tr>
<td>1998-2003</td>
<td>Tulane University Dean’s List 10 semesters</td>
</tr>
</tbody>
</table>
ABSTRACT OF THE DISSERTATION

The development of direction selectivity in the mouse retina

by

Justin Blake Elstrott

Doctor of Philosophy in Neurosciences/Specialization in Computational Neuroscience

University of California, San Diego, 2009

Professor Massimo Scanziani, Chair

Whether capturing prey or evading predators, an animal’s ability to detect movement in the world is critical for its survival. The first cells in the visual pathway that are tuned to detect motion are a subset of retinal ganglion cells called direction-selective ganglion cells (DSGCs). DSGCs respond strongly to motion in the “preferred” direction and weakly to motion in the opposite, or “null” direction. The circuitry underlying this computation consists of precise wiring between inhibitory interneurons, called amacrine cells, and the DSGCs. The role of neural activity in guiding this wiring is not known. We performed a series of experiments to examine whether DSGCs require activity for normal development. First, we tested whether visual experience was required for the establishment of direction selectivity in the retina. We recorded from the retinas of dark-reared mice using large-scale multi-electrode arrays, which allow for the recording from hundreds of neurons simultaneously. We found that DSGCs develop independent of visual experience, and
are present as early as eye-opening. Second, to target recordings from DSGCs before eye-opening, we characterized a mouse line where a class of DSGCs expresses a fluorescent reporter. These experiments also allowed for the first complete description of the projection patterns for this cell. Finally, using this transgenic mouse with labeled DSGCs, we showed that DSGCs participate in spontaneous retinal activity before the eyes open, suggesting a possible role for early, spontaneous activity in their development.
I. Introduction

Direction selectivity is a basic feature of visual systems that has fascinated neuroscientists for over 100 years (Exner, 1894). Direction selective neurons give larger responses to motion in the preferred direction versus motion in the opposite, null direction. David Hubel first recorded the direction selective responses in primary visual cortex in the awake cat (Hubel, 1959). Soon after, Barlow and Levick characterized direction selective retinal ganglion cells (DSGCs) in the rabbit (Barlow and Levick, 1965). Directional preference implies an asymmetry in wiring, however this asymmetry manifests itself differently in retina versus cortex. While retinal direction selectivity relies heavily upon inhibition induced by motion in the null direction, cortical direction selectivity relies more upon the timing of excitatory and inhibitory inputs stimulated by motion in the preferred direction.

Here we compare the results of recent experiments that reveal a fundamental difference in the establishment of directional circuits in retina and visual cortex. Namely, retinal direction selectivity is established at eye-opening independent of visual experience, while direction selectivity in primary visual cortex (V1) requires visual experience during a critical period around the time of eye-opening. Though direction-selective responses have been found in many other locations throughout the nervous system, including subcortical visual pathways as well as high order cortical areas, we restrict our discussion to direction-selective responses in retina and primary visual cortex.
General circuit organization of direction-selective responses in retina and primary visual cortex

The retina contains three categories of direction-selective ganglion cells (DSGCs): Off-DSGCs, On-DSGCs, and On-Off DSGCs. Off-DSGCs are a recently described cell type with asymmetric dendrites pointing towards the ventral pole of the retina (Kim et al., 2008). These cells respond to decrements of light and prefer motion toward the ventral pole (corresponding to superior motion \textit{in vivo}). Though they project primarily to superior colliculus, little is known about their function. On-DSGCs and On-Off DSGCs were originally described over 40 years ago (Barlow and Levick, 1965). On-DSGCs are large cells consisting of three sub-types which prefer low velocity motion along three cardinal directions: superior, inferior, and posterior (Oyster, 1968) (Figure 1.1A). On-DSGCs project to the accessory optic system and mediate the optokinetic reflex. On-Off DSGCs have four subtypes, each preferring motion in one of the four cardinal directions (posterior, anterior, superior, inferior) (Figure 1.1B) that may map onto the four rectus muscles of the eye (Oyster and Barlow, 1967). Each subtype forms a mosaic that tiles the retina with little overlap (Amthor and Oyster, 1995). Though On-Off DSGCs are ten times more numerous than On-DSGCs and have received the most attention from retinal physiologists, their central projections have only recently been characterized (Huberman et al., 2009). The function of On-Off DSGCs is not known, though it has been hypothesized that they play a role in detecting moving objects in visual space (Vaney et al., 2000). We will limit our discussion to the On-Off DSGCs because their physiology has been most studied.
Models have long been used to guide and interpret the physiological studies exploring the underpinnings of direction selectivity (Clifford and Ibbotson, 2002). The popular “Reichardt detector” exemplifies a correlation model of motion detection, in which a temporal correlation is computed to obtain the motion signal from the outputs of two spatially offset luminance receptors activated sequentially by a moving stimulus (Reichardt, 1961). To compare the circuits that mediate direction selectivity in visual cortex and retina, here we present two other manifestations of the correlation model originally described by Barlow and Levick (Figure 1.2). A directional response is obtained by either facilitating the response to preferred motion and/or inhibiting the response to the null motion. In the Excitatory (Facilitation) model (Figure 1.2A), the outputs of two spatially offset receptors facilitate each other during preferred motion to produce a response that is greater than the linear sum of the responses generated by each receptor separately. In the Inhibitory model (Figure 1.2B), null motion elicits delayed inhibition that “vetoes” the excitatory inputs from subsequent receptors (Torre and Poggio, 1978).

Based on their initial observations, Barlow and Levick concluded that the Inhibitory model was the dominant component of retinal direction selectivity. They predicted that the “symmetry-breaking” component of the circuit that provides null direction inhibition was based on asymmetric inputs from inhibitory interneurons onto excitatory cells. Indeed, several studies have identified a particular interneuron, the starburst amacrine cell (SBAC), as the inhibitory cell providing null-side inhibition to direction-selective ganglion cells (Fried et al., 2002; Fried et al., 2005); for reviews see (Demb, 2007; Fried and Masland, 2007; Zhou and Lee, 2008). A surprising recent result
revealed that SBACs themselves have an asymmetric response to motion, with motion away from their somas causing a larger calcium transient at their distal release sites than motion moving toward the soma (Euler et al., 2002). This has led to the hypothesis that individual processes of SBACs, functioning as independent computational structures, selectively contact DSGC subtypes tuned to different directions of motion. Under this hypothesis, any model for the development of direction-selective circuits in the retina should include selective wiring of SBAC processes onto DSGCs of different preferred directions.

Direction-selective cells in V1 have been explained both with the Excitatory and Inhibitory correlation models described in Figure 1 (Livingstone, 1998). V1 direction-selective neurons are organized into subcolumns within an iso-orientation column, with each subcolumn preferring motion in a different direction. Within a cortical column, V1 neurons show varying directional tuning ranging from a slight directional bias to a strong bias similar to that seen in the retina. This range of tuning may reflect a diverse set of mechanisms for generating directional responses in simple and complex cells (Rust et al., 2005).

In addition to the models above, direction-selective cells in V1 are often described using “Motion energy models” (Adelson and Bergen, 1985). These models, which are formally equivalent to the correlational models (Adelson and Bergen, 1985; van Santen and Sperling, 1985), square and sum the outputs of non-directional receptive fields to create a directional receptive field (Adelson and Bergen, 1985; DeAngelis et al., 1995; Livingstone, 2005). These models formalize Hubel and Weisel’s intuition that cortical direction selectivity can arise from appropriate pooling of non-directional receptive fields
(Hubel and Wiesel, 1962). Unlike retinal DSGCs, which receive asymmetric inhibition, some directional cells in V1 selectively gather excitatory inputs from non-directional neurons in cortex and/or lateral geniculate nucleus (LGN) to create directional preferences (Peterson et al., 2004). In fact, whole cell recordings from direction-selective cortical simple cells reveal that maximal inhibitory currents are elicited by the preferred direction of motion, though they lagged relative to the excitatory inputs (Priebe and Ferster, 2005). These findings indicate that a model based on null-side inhibition is not likely to apply to this subset of V1 directional cells, and a facilitory model based on nonlinear summation of either geniculocortical inputs and/or intracortical circuitry is more appropriate.

**Development of directional responses in retina occurs independent of vision**

How does the selective wiring that underlies direction selectivity in the retina wire up during development? Three recent studies indicate that this process happens prior to eye-opening and independent of vision. (Similar results were found for On DSGCs (Yonehara et al., 2009)). Using either cell-attached recordings from single cells (Chen et al., 2008) or large-scale multi-electrode recordings from populations of DSGCs (Elstrott et al., 2008), robust directional responses were detected before and around the time eye opening in mice reared under control conditions or in the dark. At this young age, neither the glutamatergic inputs (Tian and Copenhagen, 2001) nor the intrinsic excitability of retinal ganglion cells (Chen et al., 2008; Qu and Myhr, 2008; Sun et al., 2008) are fully mature, and hence the firing rate in the preferred direction is lower than in adult animals. In contrast, the firing rate in the null direction is as low as in adult, indicating that the
circuits mediating null-side inhibition are established early in development prior to the maturation of excitatory pathways. Similar results were found in rabbit retina, where DSGCs were detected at eye-opening and the strength of directional tuning was not altered by dark-rearing, although light deprivation did influence the development of other forms of inhibition (Chan and Chiao, 2008).

The results above agree with an earlier study which attempted to bias the distribution of preferred directions for On-Off DSGCs in the rabbit retina (Daw and Wyatt, 1974). Dark-reared rabbits were head-fixed daily from eye-opening to adulthood in a drum with vertical stripes rotating in one direction. The experimenters saw no change in the distribution of DSGC preferred directions in the adult rabbits, suggesting the training had little effect on the development of the DSGCs. Together, these results show that retinal direction selectivity develops independent of visual experience.

Development of directional responses in cortex depends on vision

In sharp contrast to the development of direction selectivity in the retina, direction selectivity in V1 does not emerge until several days after eye-opening (Li et al., 2006). Dark rearing ferrets from one week before until one to seven weeks after eye-opening prevented the formation of direction-selective responses. Visual experience provided later in development did not rescue direction selectivity, demonstrating that the establishment of direction selectivity in V1 requires visual experience during a critical period around eye-opening. This is in contrast to other neuronal response properties in primary visual cortex, such as orientation tuning and ocular dominance, which are detectable at eye-opening in ferret.
Recently, strong evidence has been provided that visual experience does not merely play a permissive role, but is a driving force behind the formation of direction selectivity in V1 (Li et al., 2008). Naïve ferrets, whose eyes had been open for less than 48 hours, were shown orientated gratings that moved back and forth along one axis. Remarkably, direction-selective responses could be detected after as few as 6 hours of training using calcium imaging to monitor responses in individual cortical neurons. In as little as 10 hours, direction-selective maps were induced as assayed by intrinsic signal imaging, a technique sensitive to changes in activity in contiguous neuronal populations with similar response properties. In the course of normal development, direction-selective maps would have become detectable 2-3 days later (Li et al., 2006). The rapid acquisition of direction-selective responses induced by the directional visual stimulus strongly suggests that vision plays an instructive role during normal development (Crair, 1999), possibly through mechanisms such as spike-timing dependent plasticity (Caporale and Dan, 2008) (see below).

**Proposed models for the development of direction selectivity**

Why is the establishment of direction selectivity in V1 more sensitive to visual stimulation than the establishment of direction selectivity in retina? Here we explore the possibility that the answer lies in the details of the circuitry underlying direction selectivity in these two different parts of the visual system.

First consider the retina, where the computation of direction selectivity relies upon strong inhibition of inputs triggered by motion in the null direction. The timing of this inhibition is not critical so long as its duration is sufficient to suppress the subsequent
excitatory inputs (Barlow and Levick, 1965; Taylor and Vaney, 2002). Wiring up null-side inhibition requires matching SBAC processes, which act as independent motion detectors, with DSGCs cells of appropriate directional preference. There are several scenarios under which this precise wiring can occur. In one scenario, molecular markers specific to each DSGC subtype ensure that DSGCs form synapses only with appropriately oriented SBAC processes. A second scenario is that there is an initial bias in the wiring of directional circuits that is reinforced by strengthening of appropriate synapses. In a third scenario, there is initially a uniform distribution of inhibitory synapses that are later pruned into directional circuits by eliminating synapses formed with inappropriate SBAC processes.

Given the current data, any of the above three scenarios are possible. All three rely upon the idea that there is a molecular signature that matches the four quadrants of SBAC processes with the four subtypes of DSGCs. Though there is recent evidence that other types of DSGCs have different molecular signatures (Kim et al., 2008; Yonehara et al., 2009; Yonehara et al., 2008), it remains unknown whether a single SBAC expresses four different markers on its processes as predicted by the model.

In the second and third scenarios, where synaptic strengthening or pruning is involved, a critical role is likely to be played by neural activity. Since retinal direction selectivity is established early in development, independent of visually driven activity, an intriguing possibility is that it relies on spontaneous activity present in the immature retina. This early patterned activity, termed retinal waves, can serve as a directional source of activity prior to the maturation of vision. Retinal waves provide important cues for the establishment of normal retinotopic and eye-specific maps of retinal projections.
Do retinal waves play a role in the establishment of DSGCs? During the first ten days after birth, retinal waves in mouse retina are mediated by a temporary cholinergic circuit comprised of retinal ganglion cells and SBACs, later replaced by a glutamatergic wave generation mechanism (Firth et al., 2005; Zheng et al., 2006). Using a mouse model with disrupted cholinergic waves (β2-nAChR-KO), we found DSGCs with robust tuning present at eye-opening (Elstrott et al., 2008). However, recent studies indicate that under different physiological conditions, β2-nAChR-KO mice can exhibit non-cholinergic propagating waves (Sun et al., 2008), which, if present in vivo, may compensate for the absence of normal wave activity. In addition, glutamatergic waves appear 2-3 days earlier in β2-nAChR-KO mice compared to wild type mice. Therefore additional experiments are required to determine whether correlated spontaneous activity of the developing retina plays a role in the establishment of directional circuits.

In contrast to retinal DSGCs, directional responses in some cortical cells arise primarily from facilitating the excitatory inputs triggered by preferred motion, a nonlinear summation process that relies on the precise timing of those inputs (DeAngelis et al., 1995). White and colleagues (White and Fitzpatrick, 2007) have postulated that development of directional responses in V1 requires two components: (1) maturation of receptive fields in the lateral geniculate nucleus (LGN), to ensure the precise timing of visual inputs; and (2) modification of synaptic strengths in cortex via an activity-dependent plasticity mechanism. The reliance upon retinotopic tuning of mature LGN receptive fields is consistent with the observations that dark rearing prevents the formation of robust center-surround receptive fields in LGN (Hooks and Chen, 2007;
Tavazoie and Reid, 2000). The plasticity mechanism required for the modification of synaptic strengths in V1 may come in the form of spike-timing dependent plasticity (STDP) (Caporale and Dan, 2008). STDP relies upon correlated firing of inputs, but more importantly, it relies upon the order of the activation of those inputs, and therefore provides a potential mechanism for encoding the timing of inputs induced by motion (Mehta et al., 2002). Indeed, STDP has been implicated in the establishment of direction responses induced in subcortical structures (Engert et al., 2002) by visual training. The lack of a detailed understanding of the cortical circuitry mediating direction-selectivity prevents more specific model predictions.

While our understanding of the circuitry behind direction selectivity has greatly improved since the earliest reports over 50 years ago, our journey in solving the developmental questions surrounding this remarkable feature of visual circuits is just beginning. Given the pace of recent discoveries, we may not have to wait another 50 years for the answers.

Acknowledgement

Chapter 1, in part, is a reprint of the material as it appears in Elstrott, Justin; Feller, Marla B. Vision and the establishment of direction-selectivity: a tale of two circuits. Current Opinion Neurobiology, 2009 with permission from all authors. The dissertation author was the primary author of this paper.
References


Fig. 1.1  The distribution of preferred directions for DSGCs in the rabbit retina. A. On-DSGCs prefer motion in three cardinal directions. The number of cells is given in parentheses. B. On-Off DSGCs prefer motion in four cardinal directions. Reprinted with permission from (Oyster, 1968).
Fig. 1.2 Two ways of implementing the correlation model of direction selectivity postulated by Barlow and Levick. A. The Excitatory (or Facilitation) model hypothesizes that the preferred motion elicits excitatory responses from receptor A whose delayed output combines with the excitatory output from B (via an ‘And’ conjunction gate) to produce a response that is greater than expected from the linear sum of the responses generated by the individual receptor inputs. B. The Inhibitory model relies upon delayed inhibition arising from null direction motion to “veto” the excitatory responses of subsequent receptors. Using apparent motion stimuli consisting of two bars flashed with a time delay in adjacent spatial locations, Barlow & Levick found that the apparent motion in the null direction strongly suppressed the response to the second bar. While they also detected facilitation in the preferred direction, the strong null inhibition led Barlow and Levick to favor the Inhibitory model as the primary source of directional responses in the retina. Reprinted with permission from (Barlow and Levick, 1965).
II. Direction selectivity in the retina is established independent of visual experience and early patterned activity

Abstract

Direction-selectivity in the retina requires the asymmetric wiring of inhibitory inputs onto four subtypes of On-Off direction selective ganglion cells (DSGCs), each preferring motion in one of four cardinal directions. The primary model for the development of direction selectivity is that patterned activity plays an instructive role. Here we use a unique, large scale multielectrode array to demonstrate that DSGCs are present at eye-opening, in mice that have been reared in darkness, and in mice that lack early retinal waves. These data suggest that direction selectivity in the retina is established largely independent of patterned activity, and is therefore likely to emerge as a result of complex molecular interactions.

Introduction

Direction selective retinal circuity in the retina extracts motion information from the visual field and initiates reflexive eye movements, stabilizing the visual image on the retina when the animal is in motion (Faulstich et al., 2004; Stahl, 2004). Direction selective ganglion cells (DSGCs), first characterized in the rabbit retina over 40 years ago (Barlow and Hill, 1963), fall into two populations. On DSGCs project to the accessory optic system (Simpson, 1984) and mediate the slow component of the optokinetic reflex (OKR) (Oyster et al., 1972). On-Off DSGCs project to the thalamus (Stewart et al., 1971) and superior colliculus (Vaney et al., 1981) and are thought to mediate the fast component of the OKR (Oyster et al., 1972). DSGCs show robust firing to stimuli
moving in the preferred, but not the null, direction of their receptive fields. On-Off DSGCs, which have been studied most extensively, form four subtypes, each preferring motion in one of the cardinal directions: nasal, temporal, dorsal, or ventral (Oyster and Barlow, 1967). Each subtype of On-Off DSGC forms an independent mosaic that tiles the retina with little dendritic overlap (Amthor and Oyster, 1995). Thus, in the rabbit that each point on the retina falls within the receptive fields of DSGCs sensitive to motion in each of four cardinal directions.

How does direction selectivity emerge during development? The generation of direction selective responses in the retina requires asymmetric wiring of inhibitory starburst amacrine cells (SBACs) onto the On-Off DSGC dendritic arbor (Barlow and Levick, 1965; Fried et al., 2002; Yoshida et al., 2001); for reviews see (Demb, 2007; Taylor and Vaney, 2002, 2003). There are two primary models for how this asymmetric wiring might emerge. In the first model, patterned neural activity, defined as correlated activity in the retina originating from either vision or spontaneous bursts known as retinal waves, provides an instructive cue for the establishment of direction selectivity. In the second model, direction selectivity is “hardwired”, arising from molecular matching between subsets of processes of starburst amacrine cells and DSGCs.

Four recent lines of evidence suggest that patterned activity, rather than molecular cues, is required for the establishment of direction selectivity. First, the establishment of direction selectivity in primary visual cortex requires visual experience during a critical period of development immediately following eye-opening (Li et al., 2006). This distinguishes direction selectivity from other cortical maps such as ocular dominance and orientation tuning, which are both present at eye-opening and are relatively insensitive to
dark rearing (White et al., 2001). Second, work on spike-timing dependent learning rules has indicated that direction selective responses can emerge spontaneously from training with directional moving stimuli (Mehta et al., 2002). Indeed, intensive training with directional stimuli artificially induces direction tuning in tectal neurons, which are not normally direction selective (Engert et al., 2002). Third, the development of sensory-motor reflexes generally requires sensory experience. In the optokinetic reflex, the gain of the visually driven eye movement must be tuned to minimize the extent of “retinal slip” in order to stabilize the visual scene. The optokinetic reflex is first observed in a mouse at postnatal day 21 (P21), approximately 8 days after eyes open (Faulstich et al., 2004), and is diminished after a period of dark rearing (McMullen et al., 2004). Fourth, no molecular-based mechanism has yet been identified that could mediate the asymmetric wiring pattern of inhibitory inputs onto an individual DSGC along one of four distinct cardinal axes. Though molecular gradients that uniquely determine location along the dorsal-ventral (D-V) and nasal-temporal (N-T) axes exist in the retina (Flanagan, 2006; McLaughlin and O'Leary, 2005), DSGCs at the same D-V, N-T location would need to respond differently to the gradients in order to wire the null side inhibition along all four cardinal axes.

The development of direction selectivity in the retina has not been fully explored. Older studies reported that retinal ganglion cells (RGCs) with asymmetric responses to moving stimuli were detected around the time of eye opening in rabbit (Bowe-Anders et al., 1975; Masland, 1977), and that the distribution of preferred directions was not biased by repeated presentations of moving gratings during development (Daw and Wyatt,
1974). Here we directly test the hypothesis that patterned activity induced by early visual responses is necessary for the establishment of On-Off DSGCs.

**Results**

First, we characterize direction selective circuits in adult mice using novel multielectrode arrays that record simultaneously from hundreds of RGCs (Frechette et al., 2005; Litke A.M., 2004; Shlens et al., 2006). Isolated retinas were stimulated with drifting gratings and On-Off DSGCs were identified based on strict criteria (see Methods and Figure 2.1). In agreement with previous studies (Weng et al., 2005), we found robust On-Off DSGCs in the adult mouse. An example of all the On-Off DSGCs recorded simultaneously from a single adult mouse retina (postnatal day 45: P45) is shown in Figure 2.2B. The polar plot in Figure 2.2A summarizes the raw spike trains recorded from a single cell in response to a single period of a drifting grating stimulus that moved across the retina in 16 directions. For each cell, we computed the vector sum of the response, with the length of the vector representing the strength of tuning and the angle of the vector defining the preferred direction of motion (see Methods). Similar to what has been observed in the rabbit retina using in vivo single unit recordings (Oyster and Barlow, 1967), On-Off DSGCs in the mouse retina respond most strongly to motion along each of four roughly perpendicular cardinal directions (Figure 2.2B).

Next, we determined the earliest age at which On-Off DSGCs could be identified. The first light-evoked responses are detected in mouse retina at P10 (Demas et al., 2003; Tian and Copenhagen, 2003), which is three to four days before the eyes open. These early responses require strong light flashes and are much weaker compared to responses
at older ages. The earliest age at which we could elicit robust RGC firing by both increasing and decreasing the illumination was P14 (Figure 2.3). These On and Off responses were still not at the adult level, consistent with previous studies concluding that the anatomy and physiology of RGCs are not fully mature in mice until P28 (Tian and Copenhagen, 2001, 2003), similar to the retinal development in rabbit (Bowe-Anders et al., 1975; Masland, 1977), ferret (Wang et al., 2001), and cat (Rusoff and Dubin, 1977; Tootle, 1993). However, we found that at P14, which is one or two days after the eye-opening, On-Off DSGCs were already present in the retina.

To test the role of visual experience in establishing On-Off DSGCs, we repeated the recordings in P14 dark reared mice. Despite the weakness of the light responses prior to eye-opening, previous studies indicate that light coming through the closed eyelids may provide a relevant pattern of activity for the development of visual circuits. Indeed, visual deprivation by dark rearing during this period alters the refinement of circuits within the retina and in the dorsal LGN (Sernagor et al., 2001; Sernagor and Grzywacz, 1996; Tian and Copenhagen, 2003); (Grubb and Thompson, 2004). To test whether the limited vision that occurs through closed eyelids, or the brief visual experience between eye opening and the P14 experiments were necessary to establish direction selectivity, we assayed direction selective responses in mice that were dark reared from P7 to the time of the experiments. We detected a large number of DSGCs in P14 dark-reared mice (Figure 2.2C-D), indicating that direction selectivity is established early in development and independent of visual experience.

Robust direction selective responses were detected despite the observation that P14 DSGCs had significantly lower firing rates in their preferred directions compared to
adult DSGCs (p < 0.01; Figure 2.4). In contrast, there was no difference between the adult and P14 DSGCs in the null direction firing rates. We would therefore expect P14 DSGCs to have more uniform response curves and, consequently, broader tuning widths. To quantify the tuning width of each cell, we used a fit to its direction-selective response curve (see Methods; Figure 2.5A, insets). In the adult retina, tuning widths varied between 70-150 degrees, with the more broadly tuned cells exhibiting a more uniform distribution of firing rates across stimulus directions (Figure 2.5A). As expected, the tuning width distributions for both the P14 and P14 dark reared were shifted to broader values compared to the adult distribution (Figure 2.5B). Most importantly, though, both normal and dark reared P14 mice exhibited DSGCs with well-defined null directions.

In summary, the presence of directional tuning at P14 indicates that there is sufficient null side inhibition at the time of eye-opening for robust direction selectivity. The null side firing rate is dependent on the strength of the inhibitory inputs onto DSGCs, whereas firing rate for stimuli moving in the preferred direction is determined primarily by the strength of the excitatory inputs. Therefore the lower preferred direction firing rates at P14 compared to adult suggest that effective null side inhibition onto DSGCs is established before the maturation of the excitatory pathways and/or the spike generating mechanisms in DSGCs.

We have excluded the role of visual experience in the establishment of direction selectivity, and concluded that the null-side inhibition provided by SBACs onto DSGCs is wired very early in development. Could some form of patterned activity other than vision instruct this early wiring? One strong candidate is retinal waves: spontaneous bursts of correlated activity that spread across the ganglion cell layer of the retina prior to
eye-opening (for reviews see: (Firth et al., 2005; Torborg and Feller, 2005; Wong, 1999). In particular, cholinergic retinal waves, which appear during the first postnatal week, are generated by the same starburst amacrine cells that underlie the null side inhibition in DSGCs (Zheng et al., 2006; Zheng et al., 2004). SBACs and DSGCs costratify early in development (Stacy and Wong, 2003), and show tightly correlated activity during this stage of retinal waves (Zheng et al., 2006; Zheng et al., 2004; Zhou, 1998). This evidence suggests that cholinergic retinal waves, which are directional in their propagation properties, could provide instructions for the asymmetric wiring between SBACs and DSGCs during the first week after birth (Demb, 2007; Weng et al., 2005).

To determine whether cholinergic retinal waves are necessary for establishing direction selectivity, we examined mice lacking the β2 subunit of the nicotinic acetylcholine receptor (β2 -/-). These mice show no propagating correlated activity during the first postnatal week, in contrast to the patterned activity seen in WT mice during the same period (Bansal et al., 2000; McLaughlin et al., 2003). Even though significant local correlations persist in β2 -/- mice, these mice lack the propagating waves that correlate the firing of SBACs and DSGCs in a sequential manner (Torborg et al., 2004). Characterizing DSGCs in β2 -/- mice therefore allows us to directly test whether early retinal waves play an instructive role in the establishment of direction selectivity.

If patterned activity in the form of cholinergic waves were necessary for establishing DSGCs, we would expect direction selectivity in β2 -/- mice to be grossly disrupted. However, we found well-tuned DSGCs in β2 -/- mice at P14 (Figure 2.5C). This was despite the fact that the DSGCs in P14 β2 -/- mice were fewer in number compared to the P14 WT, consistent with the observation that signaling via nicotinic
acetylcholine receptors (nAChRs) strongly modulates the amplitude of DSGC responses in adult mice (Weng et al., 2005) and rabbits (Kittila and Massey, 1997). Indeed, we found that acute blockade of β2-containing nAChRs with di-hydro-β-erethroidine (8μM) significantly reduced the firing rate of DSGCs in P14 WT mice (n=3 cells, data not shown). Despite the relative sparseness of detected DSGCs, β2 -/- mice showed narrow directional tuning that was not significantly different from adult WT mice (Figure 2.5B). The presence of strong direction selective responses with complete null-side inhibition in β2 -/- mice indicates that the asymmetric wiring underlying the generation of direction selectivity can occur independent of cholinergic retinal waves.

The finding that visual experience and cholinergic retinal waves are not necessary for the establishment of direction selectivity does not completely eliminate a role for neural activity in the process. First, there may be a role for glutamatergic retinal waves, which appear between P11 and P14 (Torborg and Feller, 2005). Glutamatergic retinal waves coexist with visual responses and are thought to be mediated by bipolar cells, whose inputs are symmetrically distributed onto DSGCs (Jeon et al., 2002). Whether SBACs and DSGCs are correlated by glutamatergic waves, is not known. Second, spontaneous activity that locally correlates the firing between SBACs and DSGCs but does not propagate across the retina may still be critical for the establishment of the inhibitory synapses that shape the final light responses of DSGCs (Huang et al., 2007; Maffei et al., 2006; Tao and Poo, 2005).

Thus far we have shown that the establishment of direction selectivity does not require patterned activity in the form of early light responses or cholinergic retinal waves that correlate the firing of starburst amacrine cells and DSGCs. These findings indicate
that molecular markers rather than correlated activity are likely to be critical for establishing DSGCs. We would thus predict that the alignment of the DSGC preferred directions along the four cardinal axes would also be established independent of visual experience.

To assess the development of the cardinal axes, we first characterized the axes in adult mice. By pooling across preparations, we were able to count the fraction of total DSGCs belonging to a 90 degree bin centered at each cardinal direction. Though we were careful to align the retinas in a consistent manner on the array (see Methods), we deliberately chose a large bin size to account for the variability in placement of about ±10 degrees. In the adult, the preferred directions of the DSGCs formed four clear clusters corresponding to the four cardinal directions (Figure 2.2B and 2.6A). Similarly to previous studies of rabbit DSGCs (Oyster, 1968), the fraction of cells belonging to each cardinal direction was not uniformly distributed (Figure 2.6A; \( p < 0.05, \chi^2 \) test of uniformity). However, the distributions along the temporal-nasal and ventral-dorsal axes were roughly symmetric, in that the cells that prefer two opposite directions of motion were roughly the same in number (ratio of temporal to nasal quadrant count: 1.107, \( p = 0.3281 \); ventral to dorsal: 1.583, \( p = 0.0887 \); see Methods).

The pooled P14 WT data also showed a non-uniform distribution of cells among the four cardinal directions (Figure 2.6B; \( p < 0.001, \chi^2 \) test of uniformity). In addition, the representation of opposite directions along the same axis was strongly biased, with temporal and ventral quadrants being significantly overrepresented compared to their opposites (ratio of temporal to nasal quadrant count: 4.154, \( p = 0.0001 \); ventral to dorsal: 3.461, \( p = 0.0001 \)). Figure 2.6C illustrates this asymmetry along both temporal-nasal
and ventral-dorsal axes at P14 by showing the fractions of cells belonging to the two quadrants along each axis.

To further quantify the differences in the distribution of preferred directions of On-Off DSGCs at P14 and in the adult, we compared the relative abundance of cells on a quadrant-by-quadrant basis. We constructed 95% confidence intervals for each adult cardinal direction by resampling the adult data (see Methods), and found that the relative representation of the nasal and ventral directions was significantly changed between P14 and adult (Figure 2.6D). To exclude the influence of a sampling bias, we verified that this effect was robust to variations of the cell selection criteria (see Methods).

What could explain the biases in the distributions of DSGC preferred directions seen at P14? Since RGC differentiation is largely completed by the time of eye opening (Sernagor et al., 2001), the On-Off DSGC mosaics corresponding to the four preferred directions are likely to be formed at P14. It is possible, however, that the four cell types are not equally responsive to light at this age. Indeed, the synaptic contacts between RGCs and bipolar cells only start to form around the time of eye opening (Sherry et al., 2003). It is therefore possible that the bipolar cell inputs onto the four On-Off DSGC mosaics mature in a stereotyped order during development, starting with the temporal and ventral directions of motion.

Discussion

In summary, at the time of eye-opening we observed well-established DSGCs in the mouse retina, with nearly normal tuning and an organized but asymmetrical distribution of the preferred directions. These findings indicate that patterned activity
containing directional information, either from visual experience or from early retinal waves which correlate the firing of starburst amacrine cells and DSGCs, is not necessary to establish the retinal circuits that mediate direction selectivity, though it may be involved in the refinement of these circuits. This is in sharp contrast to the primary visual cortex, where dark rearing during an early critical period prevented the establishment of direction selective cells (Li et al., 2006), and to On-Off segregation in the retina, where dark rearing disrupted the normal development of the dendritic stratification and the receptive fields of On and Off RGCs (Tian and Copenhagen, 2003). In addition, our data suggest that the excitatory drive, presumably from bipolar cell contacts, to the four subtypes of DSGCs emerges in a stereotyped order beginning with DSGCs preferring temporal and ventral motion. Together, these results have implications for understanding how neural circuits are wired during development to perform behaviorally relevant computations, and they show that some components of sensory-motor reflexes are established independent of visual activity.

Methods

Animals

C57BL/6 mice obtained from Harlan labs were used for all recordings. Dark reared litters (mothers and pups) were raised in darkness from P7 to P14 and monitored using infrared goggles. Mice not used for dark rearing experiments were maintained on a 12h light/dark cycle. Adult mice used in the recordings ranged in age from P28 to P45. All procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee and conformed to Salk Institute guidelines.
Acute Retina Preparation

All dissections were performed using dim red ambient light and infrared illumination on the dissection scope to prevent bleaching of the retinas. All mice were dark adapted for at least 60 minutes before the experiment. Animals were anesthetized with an IP injection of 100 mg/kg ketamine and 10 mg/kg xylazine prior to decapitation. After enucleation of the right eye, the eye was transferred to buffered Ames medium. Next, the eye was hemisected posterior to the ora serrata; and the cornea, lens, and vitreous were removed with forceps. We used blood vessel landmarks visible through the retina on the choroid to mark the ventral axis of the eyecup prior to removing the pigment epithelium with forceps. Next, we hemisected the isolated retina along the nasal-temporal axis as indicated by the landmarks. For all recordings, we used retina from the dorsal region of the right eye to minimize the variability across preparations. Stimulus motion was defined in the coordinates of an isolated retina, e.g. dorsal motion moved towards the dorsal part of the retina. These directions are flipped for the intact animal due to the optics of the lens.

Once a piece of dorsal retina had been isolated, it was placed ganglion-cell side down onto a custom-built multielectrode array. Three types of arrays were used: 61-electrode arrays with 5µm diameter electrodes and 60µm spacing, 61 electrode arrays with 5µm diameter electrodes and 30µm spacing, and 512 electrode arrays with 5µm diameter electrodes and 60µm spacing (Frechette et al., 2005; Litke A.M., 2004; Shlens et al., 2006). The piece of retina was aligned so that the nasal-temporal axis was perpendicular to the bottom of the array, with a variability of roughly ± 10 degrees. Once
the piece had been aligned, a dialysis membrane was used to hold the retina in place on the array (Spectra/Por 6 RC dialysis tubing, MWCO 25,000). The array was superfused with Ames’ solution (~ 220 ml/hr) bubbled with 95% O₂ and 5% CO₂ and maintained at 34-35° C, pH 7.4.

**Light Stimulation**

The photoreceptor layer of the isolated piece of retina was stimulated from above with an optically reduced image of a CRT monitor focused with a microscope objective, centered on the array, and refreshing at 120 Hz (gray screen intensity = 1623 491-nm equivalent photons/μm²/sec). For most experiments, the retina was stimulated with 5 repetitions of full-field square wave gratings moving in one of 16 randomly interleaved directions with each presentation lasting 10 seconds followed by 3 seconds of gray screen (800 μm/period, 1.066 sec/period, velocity = 25 degrees/sec for the adult mouse - (Remtulla and Hallett, 1985)). In some 61-electrode recordings, full-field sinusoidal gratings were used (400 μm/period, 0.533 sec/period, velocity = 25 degrees/sec for the adult mouse).

**Data Analysis**

The voltage trace recorded on each electrode was sampled at 20 kHz and stored for offline analysis. Spikes that crossed threshold were sorted according to the principal components of their voltage waveforms on individual electrodes (for arrays with 60μm electrode spacing) or on a set of neighboring electrodes (for arrays with 30μm spacing). Spikes were first projected into the first 5 principal component dimensions where an
expectation maximization algorithm was used to group spikes based on a mixture of Gaussians model (Litke A.M., 2004). All resulting spike clusters were inspected manually in the principal component space.

To verify that each cluster came from a single cell, the rate of refractory period violations was estimated using the number of interspike intervals between 0.5 and 1.2 msec. Only the clusters where refractory period violations represented less than 10% of all spikes were used for the subsequent analysis. In addition, cells with an average firing rate of less that 1Hz, and cells with unstable background firing (a larger than 75% decrease, as measured in a 260 sec window in the beginning and at the end of the recording) were excluded from consideration.

For a given cell, the response to motion in each of the 16 stimulus directions was measured as the mean firing rate during a 10 sec stimulus presentation, averaged across stimulus repetitions. The shape of the resulting tuning curve did not change when median inter-spike-intervals were used instead of the mean firing rates. The tuning curves were fit with the von Mises distribution (Oesch et al., 2005):

\[
R = R_{\text{max}} e^{k \cos(x - \mu)} / e^k,
\]

where \( R \) is the response to motion in a given stimulus direction \( x \) in radians, \( R_{\text{max}} \) is the maximum response, \( \mu \) is the preferred direction in radians (defined below), and \( k \) is the concentration parameter accounting for tuning width. The tuning width of each cell was estimated as the full width at half height (fwhh) of the von Mises fit using the following equation derived from (1):

\[
fwhh = 2\theta,
\]

where
The preferred direction of every cell was determined by a vector sum of the normalized responses to motion in all 16 stimulus directions. Before the summation, the response to motion in each direction on a given repetition was normalized by dividing by the total number of spikes elicited during that repetition across directions. Averaging the resulting vector sums across repetitions yielded the preferred direction vector. For a given cell, the magnitude of the response in the preferred direction could vary from 0 (no direction preference) to 1.

On-Off direction selective cells were isolated based on two criteria. First, the direction selective index was calculated for each cell:

$$D.I. = \frac{(pref - null)}{(pref + null)}$$

where $pref$ is the average response in the preferred direction, defined as the stimulus direction closest to the vector sum, and $null$ is the stimulus direction 180 degrees opposite $pref$. Cells with a $D.I. > 0.6$ were classified as direction selective. Next, the On-Off sensitivity of these cells was quantified based on the power spectra of responses to drifting square wave or sinusoidal gratings (Figure 2.1). Cells with a prominent f1 component ($f2/f1 < 1$) were classified as On DSGCs and excluded from the analysis.

To verify that the distribution of preferred directions among the On-Off DSGCs was not biased by cell selection, we varied independently each of the 5 selection parameters: the minimum average firing rate, maximum firing rate instability, maximum
rate of refractory period violations, minimum value of the direction selectivity index, and the minimum f2/f1 ratio. Depending on the parameter, it could be varied between 25% and up to 300% of its original value without changing the relative fraction of On-Off DSGCs that fall into each of the four quadrants in Figure 2.6A-B.

To compute the 95% confidence intervals (shaded gray in Figure 2.6C) for the ratio of cells in opposing quadrants, sample distributions were created by drawing from either an adult or P14 distribution with replacement, and calculating the fraction of cells in each quadrant for each sample. This procedure was repeated 10,000 times, resulting in four non-Gaussian distributions of axis ratios (one for the adult temporal-nasal axis, another for adult ventral-dorsal axis, and same for P14). We identified the confidence intervals based on the upper and lower 2.5% boundaries of these distributions. A similar procedure was used to calculate the error bars in Figure 2.6D. Here the samples were drawn only from the adult distribution, resulting in Gaussian distributions of possible values for each cardinal direction, centered at the actual value. The standard deviations of these distributions were used as the error bars for each direction.

Acknowledgement

Chapter 2, in full, is a reprint of the material as it appears in Elstrott, J; Anishchenko, A; Greschner, M; Sher, A; Litke, AM; Chichilnisky, EJ; Feller, MB. Direction selectivity in the retina is established independent of visual experience and Cholinergic Retinal Waves. Neuron, vol. 58, 2008 with permission from all authors. The dissertation author was the primary author of this paper.
References


Fig. 2.1  Frequency doubling analysis.  

A and C. Peristimulus time histograms (PSTHs) show the responses of two different cells to motion in their respective preferred directions, averaged across five repetitions. Nine complete stimulus periods were presented during each repetition; note that 18 peaks are evident in the cell’s firing rate in A compared to the 9 peaks for the cell in C. Bin size is 100 ms.  

B and D. Power spectra of the PSTHs in A and C, respectively. The robust On-Off responses of the cell shown in A are reflected in the large peak at f2 in B.
Fig. 2.2 Responses of On-Off DSGCs to moving gratings reveal strong direction selectivity in the adult and P14 dark reared mouse. A and C. Polar-plot of mean spike rate response to motion in 16 directions across five repetitions. Spike traces for all directions are shown for one period of the moving grating. The tuning curve was obtained using the mean firing rate in response to each direction (see Methods). The arrow indicates mean preferred direction of the example cell. Adult: n = 31 cells; P14 dark reared: n = 15 cells. B and D. The preferred directions of all On-Off DSGCs within one adult preparation (B) fall clearly into four groups along the cardinal directions. The cardinal directions were more variable at P14 (see also Figure 2.6). The preferred direction of each cell is shown as the normalized vector sum of the response, so that the length of each line indicates the normalized response magnitude.
Fig. 2.3  Development of On and Off responses. Scatterplots show the peak on and off responses for each cell in response to full-field steps of light across ages. The stimulus consisted of 50 repetitions of the following pattern: 2s gray - 4s white - 4s gray - 4s black - 2s gray. For some experiments, the pattern was 0.5s gray - 1s white - 1s gray - 1s black – 0.5s gray. The peak on or off response was defined as the maximum value in the PSTH (bin size 20ms) in the 0.5s window following the white or black step respectively. The baseline firing rate, defined as the maximum value of the PSTH during the gray screen presentation, was subtracted from the peak on and off responses. Note the different scales for each plot.
Fig. 2.4  Comparison of null direction and preferred direction firing rates for Adult and P14 WT DSGCs. Firing rates in the preferred and null direction for each cell in the Adult and P14 groups. P14 cells had significantly lower firing in their preferred directions compared to adults ($p < 0.01$, rank-sum test; P14: $n = 75$ cells from 11 mice, median = 7.5 Hz; adult $n = 90$ cells from 11 mice, median = 9.5 Hz); but similar firing rates in the null directions ($p = 0.84$, rank-sum test; P14 median = 0.54 Hz; adult median = 0.57 Hz).
Fig. 2.5  Population tuning widths change during development. A. Histogram shows the distribution of tuning widths (full width at half height of a von-Mises fit, see methods) of all adult WT cells (90 cells from 11 mice). Arrowhead shows mean value. Insets show the average responses (dots) and the von-Mises fits (lines) for two cells. The arrow indicates the preferred direction for each cell. B. A comparison of tuning widths across groups (adult n’s same as above, P14 WT: n = 75 cells from 11 mice; P14 dark reared: n = 50 cells from 4 mice; P14 β2 -/-: n = 13 cells from 4 mice). The adult WT and P14 β2 -/- mice showed significantly narrower tuning than both the P14 WT and P14 dark reared mice (p < .0001; one-way ANOVA with Tukey post-hoc test). Data are presented as mean ± s.d. C. An example On-Off DSGC from a P14 β2 -/- mouse.
Fig. 2.6 Distributions of preferred directions for On-Off DSGCs in adult and P14 mice. A and B. Scatter plot shows the preferred directions and normalized magnitudes of the responses of adult and P14 cells pooled across experiments (adult: n = 90 cells from 11 mice; P14: n = 125 cells from 15 mice). Each color represents data from a single experiment. At P14, squares are dark-reared mice; circles are normal mice. C and D. The fraction of cells belonging to each cardinal direction was calculated by grouping the data shown in A and B into 90 degree bins. C. The symmetry of preferred directions along a given axis (temporal-nasal or ventral-dorsal) was tested by calculating the fraction of cells falling in one quadrant along the axis (e.g. temporal) versus the fraction of cells in the opposite quadrant (e.g. nasal). A perfectly symmetric axis would have 50% of cells in opposite quadrants. The thick black bars shows the actual distribution of cells along the axes for adult and P14. The shaded gray regions show the 95% confidence interval around each value based on resampling of the adult and P14 distributions (see Methods). D. A comparison of adult and P14 distribution of preferred directions on a quadrant-by-quadrant basis. In case of a uniform distribution, each quadrant would encompass 25% of cells. The error bars show ±1 s.d. (inner ticks) and ± 2 s.d. (outer ticks) based on resampling the adult distribution (see Methods). Directions whose error bars lie beyond the dashed line have P14 values significantly different from adult values.
III. Genetic identification of an On-Off direction selective retinal ganglion cell subtype reveals a layer specific subcortical map of posterior motion

Abstract

Motion detection is an essential component of visual processing. On-Off direction selective retinal ganglion cells (On-Off DSGCs) detect objects moving along specific axes of the visual field due to their precise retinal circuitry. The brain circuitry of On-Off DSGCs, however, is largely unknown. We report a mouse with GFP expressed selectively by the On-Off DSGCs that detect posterior motion (On-Off pDSGCs), allowing two-photon targeted recordings of their light responses and delineation of their complete map of central connections. On-Off pDSGCs project exclusively to the dorsal lateral geniculate nucleus and superior colliculus and in both targets form synaptic lamina that are separate from a lamina corresponding to non-DSGCs. Thus, individual On-Off DSGC subtypes are molecularly distinct and establish circuits that map specific qualities of directional motion to dedicated subcortical areas. This suggests each RGC subtype represents a unique parallel pathway whose synaptic specificity in the retina is recapitulated in central targets.

Introduction

Direction selectivity - the preferential response of a neuron to stimuli moving in one direction of the visual field - is a hallmark feature of the mammalian visual system that is critical for perception and behavior. Direction selective retinal neurons were first identified nearly a half-century ago (Barlow and Hill, 1963) by recordings from rabbit
retinal ganglion cells (RGCs). Direction selectivity has also been described for neurons in retinorecipient nuclei (Simpson, 1984) and in visual cortex (Hubel and Wiesel, 1962). Understanding the circuitry that underlies direction selectivity at each stage of visual processing—retinal, subcortical and cortical—remains an intensive focus, both for understanding visual perception and as a general model for exploring how neural circuits encode specific features of the sensory environment.

There are three categories of direction selective ganglion cells (DSGCs): On DSGCs respond to global motion in one of three directions: upward, downward, or anterior (Oyster, 1968; Sun et al., 2006). Off DSGCs respond to upward motion (Kim et al., 2008). The third category, On-Off DSGCs, includes four subtypes, each responding to motion in one cardinal direction: anterior, posterior, superior, or inferior (Oyster, 1968; Weng et al., 2005). On-Off DSGCs are the most numerous DSGCs and are specialized for detecting object motion (Vaney et al., 2000). The current model is that the unique directional tuning of each On-Off DSGC subtype arises from an asymmetric pattern of inhibitory inputs that are different for each subtype (Fried and Masland, 2007; Demb, 2007). Remarkably, this subtype-specific connectivity is established independent of visual experience (Chan and Chiao, 2008; Chen et al., 2008; Elstrott et al., 2008). Together, these features suggest that each On-Off DSGC subtype is molecularly distinct. However, evidence of a unique molecular signature for individual On-Off DSGC subtypes has not been provided.

Despite the importance of On-Off DSGCs for detecting object motion, very little is known about their patterns of central projections (reviewed in: Berson, 2008). There is evidence in rabbits that On-Off DSGCs project to the SC (Vaney et al., 1981) and dLGN
(Stewart et al., 1971), but those may not be their exclusive targets (Pu and Amthor, 1990) and it is unclear if those results apply to other species. Moreover, how the axonal connections of individual On-Off DSGC subtypes are arranged within their targets, is completely unknown. Thus, how object motion detected by the retina is represented in the brain and contributes to higher order visual processing remains unclear. Here we report a transgenic mouse with GFP selectively expressed by On-Off DSGCs that respond to posterior motion, allowing us to resolve the complete pattern of axonal connections unique to this On-Off DSGC subtype.

**Results**

During a screen to identify mice with GFP selectively expressed by individual RGC subtypes, we examined retinas from transgenic mice with GFP expressed under the control of the dopamine receptor 4 promoter (DRD4-EGFP) (Gong et al., 2003). In these mice, GFP+ cells are present throughout the ganglion cell layer (Figure 3.1A, B) but not in other retinal layers (Figure 3.1C). Close examination revealed two distinct bands of GFP+ dendrites in the inner plexiform layer (IPL) (Figure 3.1C) suggesting the GFP+ cells were bistratified. Dendritic bistratification is a defining characteristic of On-Off DSGCs (Vaney et al., 2000; Berson, 2008). We therefore conducted a series of experiments to determine whether the GFP+ cells were in fact On-Off DSGCs and if so, whether they belonged to a single subtype.

First, all the GFP+ cells were RGCs because every GFP+ cell had an axon (Figure 3.1B,C,I), and all GFP+ cells disappeared following optic nerve transection (data not shown). Second, the GFP+ dendrites co-stratified with the processes of starburst amacrine
cells (SBACs) in the IPL (Figure 3.1E-H) - the anatomical feature most closely associated with the On-Off DSGC classification (Figure 3.1D) (Demb, 2007; Fried and Masland, 2007).

Next we filled individual $GFP^+$ RGCs with biocytin, then immunostained the retina for ChAT, which labels SBAC somas and processes. In every case (n=13 cells), the On and Off dendritic arbors of the filled $GFP^+$ RGC co-stratified with the processes of SBACs (Figure 3.1I-L). Also, the dendritic fields of the RGCs were ovoid-shaped and exhibited “looping” arborizations (Figure 3.1I-K) - additional characteristics of mouse DSGCs (Weng et al., 2005; Sun et al., 2006). Some $GFP^+$ RGCs exhibited asymmetric arbors (e.g., Figure 3.1I) but this varied across cells. Previous work established there is no relationship between dendritic asymmetry and preferred direction of an On-Off DSGC (Chan and Chiao, 2008; Yang and Masland, 1994).

The above data strongly suggested that all $GFP^+$ cells in the retinas of $DRD4$-$GFP$ mice were On-Off DSGCs. The somas of RGCs belonging to the same subtype are non-randomly distributed (Wässle, 2004). We therefore analyzed the spatial distribution of $GFP^+$ somas and discovered they form a regular mosaic (Figure 3.1M) (regularity index=2.46; P<0.0001 vs. a random distribution; n=205), with ~40 µm between $GFP^+$ somas (Figure 3.1N). The average density of $GFP^+$ RGCs was 275(+/-79) cells/mm² (n=20 fields; n=5 mice), which is 5-9% of the total RGC population (Jeon and Masland, 1998; Lin et al., 2004), which is within the range of other mouse RGC subtypes (Sun et al., 2002). Collectively, our findings support the hypothesis that in $DRD4$-$GFP$ mice, $GFP$ is expressed by one subtype of On-Off DSGCs.
To confirm that the $GFP^+$ RGCs represented a single subtype of On-Off DSGCs, we recorded their light responses. Under conventional fluorescent optics, exposure of the retina to the excitation wavelength for $GFP$ would significantly bleach the photoreceptors. Therefore, we performed two-photon-targeted patch clamp recordings (Margrie et al., 2003), in which an infrared femtosecond laser, tuned to a wavelength (920 nm) outside the absorption band of mouse photoreceptors, was used to localize a patch pipette filled with a fluorescent dye and the $GFP^+$ cells (Figure 3.2). Once the pipette and a $GFP^+$ cell were localized to the same field of view, infrared optics was used to obtain loose-patch recordings.

In response to drifting square-wave gratings, nearly all $GFP^+$ cells (18/21) had a strong directional response (Figure 3.3A,B). Remarkably, all the direction selective $GFP^+$ cells exhibited a strong preference for motion toward the nasal pole of the retina, which in visual coordinates corresponds to posterior motion (Figure 3.3C). Most $GFP^+$ cells exhibited both ON and OFF responses to flashes of a white spot centered on the soma (Figure 3.3B2), although the amplitudes of the ON and OFF responses varied between cells. Furthermore, both the brightest and less bright $GFP^+$ RGCs exhibited On-Off responses with strong posterior direction tuning (Figure 3.4). Hence, the $GFP^+$ cells in the retinas of $DRD4$-$GFP$ mice are the posterior-motion preferring subtype of On-Off DSGCs (On-Off pDSGCs).

In $DRD4$-$GFP$ mice, $GFP$ is present in the soma, dendrites and axons of On-Off pDSGCs (Figure 3.1). Moreover, there are no $GFP^+$ neurons within or projecting to retinorecipient nuclei. This raised the unprecedented opportunity to delineate all the brain
regions that receive synaptic input from On-Off pDSGCs and to reveal the subtype-specific patterns of connections made within their targets.

First, we labeled all RGC axons by injecting CTb-594 into both eyes. Then, to determine the connections specifically made by On-Off pDSGCs, we examined each retinorecipient area for axons that were both CTb-594+ and GFP+. No GFP+ axons were observed in the hypothalamus, vLGN, IGL, pretectum, or accessory optic system (Figure 3.5). GFP+ axons were, however, visible in the optic nerves, chiasm and tracts (Figures 3.5-7) and in the synaptic neuropil of the dLGN and SC (Figures 3.6-7). Thus, in mice, On-off pDSGCs project exclusively to the dLGN and SC.

What are the specific connections made by On-Off pDSGCs in their targets? Whereas CTb+ axons terminated throughout the dLGN (Figure 3.6B,D,E), the axons of On-Off pDSGCs were limited to a distinct lamina along the lateral dLGN (Figure 3.6 C,D,F). This was obvious at all dorsal-ventral and rostral-caudal positions in the dLGN (i.e., the entire retinotopic map), indicating that every GFP+ On-Off pDSGC that projects to this target faithfully restricts its axonal arbor to a specific laminar depth (Figure 3.6H). All GFP+ axons disappeared from the contralateral dLGN following unilateral eye removal (Figure 3.6I-L). The GFP+ lamina in the dLGN of DRD-GFP mice therefore represents On-Off pDSGC axons from the contralateral eye.

In CB2-GFP mice, transient Off-alpha (tOff-α) RGCs express GFP and project to a specific lamina in the dLGN (Huberman et al., 2008). Interestingly, the lamina formed by tOff-αRGCs and the lamina formed by On-Off pDSGCs were separated from one another along the width of the dLGN (Figure 3.6D-H). Thus, despite a lack of cellular lamination, the mouse dLGN contains multiple, highly organized, laminar-specific parallel pathways.
On-Off pDSGC projections to the SC were also laminar specific. CTb\(^+\) axons entered the SC through the stratum opticum (SO) and arborized throughout the overlying stratum griseum superficialis (SGS) (Figure 3.7A,B,D). By contrast, the axons of On-Off pDSGCs terminated exclusively in the upper half of the SGS (uSGS), forming a distinct \(GFP^+\) lamina (Figure 3.7C-H, O). Laminar specificity of On-Off pDSGC projections to the uSGS was clearly evident in both the sagittal (Figure 3.7A-H) and coronal (J-L) view of the SC (Figure 3.7J-L’), and thus the entire retinotopic map. Dye injections into the SC retrogradely labeled every \(GFP^+\) cell in the contralateral retina but no \(GFP^+\) cells in the ipsilateral retina (data not shown). Together, these findings indicate that i) the \(GFP^+\) axons in the dLGN are collaterals of \(GFP^+\) axons that also target the SC and, ii) all On-Off pDSGCs projections to the SC arise from the contralateral eye, are laminar specific and targeted to the uSGS.

In the SC, the projections of On-Off pDSGCs were spatially distinct from the projections of tOff-\(\alpha\)RGCs, which targeted the lSGS (Figure 3.7I,M-O) (Huberman et al., 2008). Indeed, the lamina for these two RGC subtypes, were complementary throughout the SC (Figure 3.7H-O). Thus, tOFF-\(\alpha\)RGCs and On-Off pDSGCs that reside next to each other, project their axons to the same topographic location in the contralateral SC. At that topographic location, however, On-Off pDSGCs ensure they position their synapses dorsal to those of tOFF-\(\alpha\)RGCs. Interestingly, whereas tOff-\(\alpha\)RGCs axons form patches (columns) in the SC (Figure 3.7I, M, N) (Huberman et al., 2008), columns of On-Off pDSGC axon arbors were never observed; instead they established a uniform map of posterior motion.
Discussion

Since their discovery nearly fifty years ago (Barlow and Hill, 1963), On-Off DSGCs have been the focus of intense study. However, because of an inability to selectively and completely label On-Off DSGCs, their brain circuitry remained poorly understood. Our data provide a complete map of central projections for one On-Off DSGC subtype and thereby reveal a highly precise laminar map of posterior object motion. These findings have several implications for understanding how different parallel pathways exiting the retina are translated into specific patterns of circuitry in the brain.

First, On-Off pDSGCs project to the dLGN and SC but not to any other retinorecipient areas (Figures 3.5-7), including the accessory optic nuclei. The accessory optic nuclei are the targets of On DSGCs, which respond to global movement of the visual scene and thereby contribute to image stabilization (Simpson, 1984). Thus, the outputs of retinal circuits that locally detect posterior motion are segregated into entirely different targets from the outputs of retinal circuits that detect global visual motion, and visa versa. Knowledge of where On DSGCs project in the brain led to understanding of their specific contributions to visual processing and behavior (Simpson, 1984; Yonehara et al., 2009). The genetically identified central projection map of On-Off pDSGCs provided here, can now be used to explore how DGSCs that detect local object motion, contribute to visual perception and behavior.

Second, the dLGN and SC neurons that receive input from On-Off pDSGCs should be influenced by posterior motion, and in the case of dLGN neurons, relay that information to visual cortex. Mouse dLGN receptive fields have been described as center-surround, with linear spatial summation and exclusively On-center or Off-center
responses (Grubb and Thompson, 2003). Posterior direction-selective dLGN or SC neurons have not been reported but it is unclear if recordings have ever been made from neurons directly postsynaptic to On-Off pDSGCs. Another possibility is that dLGN and SC receptive fields are built from the inputs of more than one RGC subtype, and thus are more broadly tuned. Here we provide a genetically labeled spatial map of On-Off pDSGC central connections that can be used to guide exploration of how this highly specific visual processing stream is combined with other retinal outputs and local circuit connections in the dLGN and SC.

Third, each RGC subtype is known to encode a unique aspect of the visual world due to the subtype-specific pattern of synaptic input it receives in the IPL (Roska and Werblin, 2001). Our results, combined with other recent findings (Huberman et al., 2008; Kim et al., 2008; Yonehara et al., 2009) now indicate that the axonal connections of each RGC subtype are also subtype-specific, thereby distributing unique visual information to highly specific locations in the brain. Those axonal connections can be laminar specific, as shown here, or they may rely on precise subcellular targeting. A prime example of subcellular specificity occurs in the retina where all four On-Off DSGC subtypes project their dendrites to the same two IPL sublamina and yet, each On-Off DSGC subtype responds to motion along a different axis of the visual field, due to the unique pattern of synaptic input it receives within those sublamina (Demb, 2007). To achieve such a remarkable degree of wiring specificity, apparently identical RGCs may in fact be molecularly distinct. Here we provide evidence that one of the On-Off DSGC subtypes indeed has a unique molecular signature, indicating that functional and molecular identity
rather than laminar specificity per se, dictates the wiring diagram between the eye and the brain.

**Methods**

*DRD4-GFP mice*

Mice were obtained from MMRRC (http://www.mmrre.org/strains/231/0231.html) (see: Gong et al., 2003) and crossed to C57/Bl6 in our laboratory. Physiology was carried out on C57/Bl6 mice.

**Retinal and brain histology**

Immunostaining and CTb protocols as in Huberman et al., (2008).

**Quantification of axonal projections**

Using a rectangular window, we measured *GFP* pixel intensity along the width of the retinorecipient dLGN and depth of the SC. We normalized the width and depth (SC) values to be a fraction of the total width/depth in that section and normalized the raw fluorescence signal (F\text{raw}) at each position as follows:

\[
F_{\text{norm}} = \frac{F_{\text{raw}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}}
\]

where F\text{min} was the minimum fluorescence value and F\text{max} was the maximum fluorescence value in a given section. Normalized fluorescence values were binned at 5% intervals and averaged across animals.
Quantification of \( \text{GFP}^+ \) RGC mosaics and density

The X-Y coordinates and density of \( \text{GFP}^+ \) RGCs were determined from 4 fields, 0.25 mm\(^2\) each (n=5 retinas; 20 fields total). For all cells in a given field, the distance to its nearest neighbor (NN) was calculated by dividing the mean NN distance by the standard deviation. Regularity indices were compared to random distributions of the same number of cells (Cook, 1996). Density was calculated across fields taken from different retinal quadrants and eccentricity. Autocorrelograms (ACGs) were prepared for each field and a density recovery profile (DRP) was constructed (Rodieck, 1991). (See Supplemental Experimental Protocols).

Electrophysiology

All retinas were P26-34 in age. Mice were anesthetized with isofluorane and decapitated. Retinas (4 right eye, 1 left eye; n=5 mice) were isolated from pigment epithelium in oxygenated Ames’ medium (Sigma) under IR illumination, cut into dorsal and ventral halves, and mounted photoreceptors down over a hole of 1-1.5 mm\(^2\) on filter paper (Millipore). Retinas were kept dark at room temperature in Ames’ medium bubbled with 95\% O\(_2\)/5\% CO\(_2\) until use (0 – 7 hr). All animal procedures approved by the UC Berkeley Institutional Animal Care and Use Committee and conformed to the NIH Guide for the Care and Use of Laboratory Animals, the Public Health Service Policy, and the SFN Policy on the Use of Animals in Neuroscience Research.

Two photon targeted recording from \( \text{GFP-labeled retinal ganglion cells} \): Filter paper mounted retinas were placed under the microscope and superfused with warmed (\(~32^\circ\)C)
Ames’ medium. An image of the GCL was formed by IR illumination (> 850 nm) through the condenser, acquired by a CCD camera, and displayed on a TV monitor. Glass microelectrodes filled with Ames medium and 20 uM Alexa 568 were placed right above the inner limiting membrane (ILM). GFP and Alexa 568 fluorescence were detected with a custom-built two photon microscope based on a Fluoview laser-scanning system and a Ti: Sapphire laser (Coherent) running at 920 nm to avoid bleaching of photoreceptors. GFP cells were identified by their relative position to the Alexa-filled electrodes, and mapped to the transmitted light image on the TV monitor. The ILM above the target GFP cell was then carefully dissected with the glass electrode. Loose-patch voltage-clamp recordings (holding voltage set to “OFF”) were performed with a new glass electrode (3-5 MΩ) filled with Ames’ medium and 20 uM Alexa 568. Data was acquired through an Axopatch 200B (Molecular Devices), and digitized at a sampling rate of 10 kHz. The Alexa-filled electrode and its attached GFP cell was imaged again with the two photon microscope at the end of the recording to confirm correct targeting and the integrity of the plasma membrane of the recorded cell (Figure 3.2).

*Visual Stimulation:* Visual stimuli were generated using an Intel core duo computer with a Windows XP OS running a white, monochromatic organic light-emitting display (OLED-XL, eMagin, Bellevue, WA, 800x600 pixel resolution, 85 Hz refresh rate). Custom stimuli were developed using Matlab and the Psychophysics Toolbox (Brainard, 1997; Pelli, 1997). The display image was projected through the same 60x water-immersion objective used for targeting cells (LUMPlanFl/IR, NA=0.9) via the side port of the microscope (Figure 3.2), centered on the soma of the recorded cell, and focused on
the photoreceptor layer. The diameter of the entire display on the retina was 225 μm across, with a gray screen intensity of \( \sim 1.2 \times 10^{13} \) 514nm equivalent photons/s/cm\(^2\). We used 10 repetitions of a 100 μm white spot (2s black, 2s white, 2s black) to test on and off responses. To test directional responses, we presented 5 repetitions of drifting square-wave gratings (spatial frequency = 225 μm/cycle, temporal frequency 4 cycles/second, \( \sim 30 \) degrees/sec in 12 pseudorandomly chosen directions spaced at 30 degree intervals, with each presentation lasting 3 seconds followed by 500 ms of gray screen.

Data Analysis: We used custom Matlab software to bandpass filter the data between 80 and 2000 Hz and extract spike times. The preferred direction of each cell and the direction selective index (DSI) were computed as described (Elstrott et al., 2008; Supplemental Protocols). Occasionally, cells would show high tonic firing independent of light stimulation. Therefore, we excluded cells if background firing before the spot stimulus (defined as maximal firing during the initial 2s of black screen) was greater than 40% of the maximum stimulus evoked response.

Intracellular filling and immunostaining filled cells: Retinas mounted on filter papers were placed under the microscope and superfused with warmed (\( \sim 32^\circ \) C) and oxygenated artificial CSF (ACSF) (in mM: 119 NaCl, 2.5 KCl, 1.3 MgCl\(_2\), 1.0 K2HPO\(_4\), 2.5 CaCl\(_2\), 26.2 NaHCO\(_3\), and 11 D-glucose). GFP-expressing cells were identified by epifluorescence. Glass microelectrode (3-5 MΩ) filled with an internal solution (containing 98.3 mM potassium-gluconate, 1.7 mM KCl, 0.6 mM EGTA, 5 mM MgCl\(_2\), 2 mM Na\(_2\)-ATP, 0.3 mM GTP, and 40 mM HEPES, pH 7.25 with KOH, 2 uM Alexa Fluor
60

488, and 3 mg/ml biocytin (Sigma)) was used to deliver biocytin into GFP-expressing cells in the whole-cell patch clamp configuration for 10 – 20 min. Alexas do not cross gap junctions between RGCs. The electrodes were then carefully withdrawn, the retina fixed with 4% PFA for 15 minutes and then processed for visualization of biocytin and ChAT (see Supplemental Methods).

Supplemental Methods

Electrophysiology Data Analysis

The preferred direction of each cell was determined by first normalizing the average spike count in each stimulus direction by the total number of spikes for all directions. The vector sum of these normalized responses yields a vector whose direction is the preferred direction of the cell, and whose magnitude gives the strength of tuning (between 0 and 1). The direction selective index (DSI) for each cell was calculated as:

$$DSI = \frac{(pref - null)}{(pref + null)}$$

where $pref$ is the average response in the preferred direction, defined as the stimulus direction with the maximum average response, and $null$ is the stimulus direction 180 degrees opposite $pref$ (Elstrott et al., 2008; Weng et al., 2005).

Intracellular dye loading and immunostaining of loaded GFP cells

Fixed retinas from DRD4-GFP mice in which GFP+ RGCs were filled with neurobiotin were processed for immunofluorescence using the following methods. After electrodes were withdrawn from the tissue, the retinas were immediately fixed with 4%
PFA at room temperature for 15 minutes, followed by three 15-minute washes in 0.01 M PBS. The retinas were then incubated in blocking solution (1% bovine serum albumin + 0.2% Triton-X in 0.01 M PBS) for 1 h at room temperature. Goat anti-ChAT antibody (Invitrogen) was diluted 1:200 in blocking solution and added to the retina for incubation overnight at 37°C. The retinas were then washed in blocking solution 3 times, for 20 minutes each, and incubated in secondary antibodies: donkey anti-goat IgG –Alexa Fluor 568 (1: 500) and Alexa Fluor 488 conjugated Streptavidin (1:1000) diluted in blocking solution for 2 hours at 37°C. Afterwards retinas were washed in blocking solution three times for 20 minutes each, rinsed with 0.01 M PBS, and then mounted onto glass slides with Vectashield (Vector, CA, USA). Three-dimensional image stacks containing 80-110 optical sections at the z-axis were collected using a confocal microscope and Fluoview software. Each optical section was resampled three times with 1 µm between sections. Images were analyzed using Image J and Metamorph.

Density recovery profile (Rodieck, 1991)

The DRP describes the average density of cells within an annulus of the ACG at a given distance from the soma and was calculated as:

\[ d_i = \frac{n_i}{N \Delta A_i} \]

where \( d_i \) is the density measure of the DRP for annulus \( i \) that contains \( n_i \) cells with an area \( \Delta A_i \) from a retinal field containing \( N \) total cells. The effective radius (\( r_e \)) was calculated as follows:
\[ r_e = \frac{V_e}{\sqrt{\pi D}} \]

where \( D \) is the density of cells in the retinal field and \( V_e \) corresponds to the volume of the DRP that falls below \( D \) and is calculated as follows:

\[ V_e = \frac{1}{N} \sum_{i=1}^{\text{until } n \leq \lambda_i} (\lambda_i - n_i) \]

where \( \lambda_i \) is the expected number of cells in annulus \( i \) and is calculated as follows:

\[ \lambda_i = N D \Delta A_i \]

**Acknowledgement**

Chapter 3, in full, is a reprint of the material as it appears in Huberman, AD; Wei, W; Elstrott, J; Stafford, BK; Feller, MB; Barres, BA. Genetic identification of On-Off direction selective retinal ganglion cells reveals a layer specific subcortical map of posterior motion. Neuron, vol. 62, 2009 with permission from all authors. The dissertation author was co-first author of this paper in addition to Drs. Andrew Huberman and Wei Wei.
References


Fig. 3.1 A mosaic of GFP+ On-Off RGCs in DRD4-GFP mice. A. Fluorescence image of whole-mount DRD4-GFP retina. T, temporal; N, nasal; D, dorsal; V, ventral. Scale = 500µm. B. Red-framed region in (A). Scale = 200µm. C. Blue-framed region in (A). Downward arrow, axis perpendicular to retinal layers. GFP+ dendrites (yellow arrows) in the inner plexiform layer (IPL). INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Arrowhead, GFP+ axon. Scale = 50µm. D. Starburst amacrine cell (SBAC) somas (red) and dendrites (pale red) in IPL sublamina 2 (S2) and S4. On-Off DGSCs (green) project dendrites to S2 and S4. Labels as in (C) plus NFL, nerve fiber layer and OS, outer segments. E-H. DRD4-GFP retinas stained for GFP (E), VACHT (F) and merged (G-H). Asterisks, GFP+ axons. Dashed line, IPL. Solid line, GCL. (E-G) Scale = 100µm. H. Scale = 50µm. (I-L). Biocytin filled GFP+ RGC and ChAT+ SBACs. (I) Complete view of filled RGC. White arrow, axon. Yellow arrows, "looping" arborizations. (J) The portion of the RGC in GCL-S4 and, (K) in S2. (L) Side view of the filled RGC (green) and ChAT+ SBACs (red) and their overlap (yellow). Arrow, axon. Asterisk, amacrine cell. Gray bars, depths of view for I-K. Scale = 50µm (I-K) and 25µm (L). M. Density recovery profile (Rodieck, 1991). Gray bar, average cell density. Dashed line, effective radius, within which it was rare to observe another GFP+ soma. N. Distribution of the distances between nearest-neighbor GFP+ RGC.
Two photon targeted recording from GFP+ retinal ganglion cells. LEFT: Schematic of the two-photon microscope in the configuration for targeted recording. INSET: A glass microelectrodes containing Alexa 568 (20μM in Ames medium) was visualized simultaneously with GFP using a two-photon confocal microscope. Subsequent to localizing the electrode above the GFP+ cell using two-photon fluorescence, transmitted IR light detected with a CCD camera was used to obtain a loose patch recording. Subsequent to the recording, a two-photon image of the electrode and cell was again obtained to confirm correct targeting and the integrity of the plasma membrane of the recorded cell. RIGHT: Schematic of the two-photon microscope in the configuration for light stimulation. Visual stimuli were generated using a white, monochromatic organic light-emitting display (OLED). The display image was projected through the same 60x water-immersion objective used for targeting cells via a side epifluorescent port of the microscope. To test directional responses, we presented drifting square-wave gratings (see Methods for details).
Fig. 3.3  2-photon targeted recordings of GFP+ cells reveal a preference for posterior motion. A. A GFP+ RGC’s responses to drifting gratings. Black tuning curve shows the mean response (spike count during 3s of gratings), colored curves show the responses for each repetition (red, green, blue, cyan, magenta). Black arrow indicates the vector sum of responses. Traces show the data for the first 500 ms of gratings stimuli (2 periods). Frequent presentation of edges in the gratings stimulus makes resolving the on and off responses difficult. B. Light responses for all recorded cells. B1. Responses to drifting gratings. Black curve shows the mean response for each cell. Red line shows the vector sum of the responses (see Supplemental Procedures). Gray box indicates the cell shown in A. Cells with DSI lower than 0.5 are shown in the last row. B2. Spike density histograms of corresponding On and Off spike responses to a 100 μm spot shown centered on the soma (10 repetitions, 50 ms bins). Yellow bar represents the time of the spot stimuli. C. Vector sums of all recorded cells (N = 21 cells from 5 retinas, 5 mice). Each color indicates a different retinal preparation. The black line shows the mean vector sum of all the cells.
Fig. 3.4 Both bright (right) and less bright (left) GFP+ RGCs show the same directional tuning profile. TOP: Polar plots of DS tuning (top) for two nearby GFP+ On-Off pDSGCs. Black tuning curves are the mean response to stimuli presented in 12 different directions. The red line indicates the vector sum of responses. S = superior, P = posterior, A = anterior, I = inferior. These directions refer to the direction of motion of drifting gratings in visual coordinates. BOTTOM: Live fluorescence images acquired with a two-photon confocal microscope. White arrow points to GFP+ soma of the less-bright RGC. Scale = 50μm.
Fig. 3.5  GFP$^+$ On-Off pDSGCs do not target any retinorecipient nuclei other than the dLGN and SC. A. CTb and GFP$^+$ axons in the optic nerve. B. CTb$^+$ RGC axons terminate in the retinohypothalamic nuclei, the suprachiasmatic nucleus (SCN) and supraoptic nucleus (SON). GFP$^+$ RGC axons do not terminate in these retinorecipient nuclei. Arrows point to a few GFP$^+$ near the SC. C. There are GFP$^+$ axons in the OT (arrows) adjacent to the SCN and SO, indicating the lack of GFP labeling within those nuclei is not a failure to detect On-Off pDGSC axons. D. CTb labeled RGC axons are seen in the peri-habenular region, but no GFP$^+$ axons are evident in this target. E. CTb labeled RGC axons in the pretectal complex that includes the medial pretectum (MPT), Posterior pretectum (PPT), Nucleus of the optic tract (NOT), Anterior pretectum (APT). Arrows indicate GFP$^+$ axons in the OT adjacent to the pretectum, indicating that the lack of GFP labeling in pretectal nuclei is not a failure to detect GFP$^+$ On-Off pDGSC axons.

F-I. Accessory optic nuclei. These are the main targets of the On-DSGCs (Simpson et al., 1988; Yonehara et al., 2009). CTb labeled RGC axons target these three structures. By contrast, no GFP$^+$ On-Off pDGSCs target these nuclei. (F) The medial terminal nucleus (MTN); (G) the lateral terminal nucleus (LTN); (H) Dorsal terminal nucleus (LTN). (I) Schematic of the position of the accessory terminal nuclei shown in H, G, F. (A-I) the background in the GFP images for this figure is intentionally high to reveal there are no sparse GFP$^+$ On-Off pDGSC axons). Scales=200µm.
Fig. 3.6  On-Off pDSGCs form laminar-specific connections in the dLGN.
A. Schematic of region shown in (B-L) and RGC axons entering the dLGN (arrows). Red, RGC axons from both eyes labeled with CTb-594; OT, optic tract; vLGN, ventral lateral geniculate nucleus; IGL, intergeniculate leaflet; dLGN, dorsal lateral geniculate nucleus. D, dorsal; M, medial. B. CTb⁺ RGC axons at the level depicted in (A). C. GFP⁺ On-Off pDSGC axons at the level depicted in (A). (B, C) Scale=400µm. D-F. Area framed in (B and C). Dashed line, lateral dLGN; solid line, medial dLGN. D. Merge of CTb⁺ and GFP⁺ RGC axons. E. CTb⁺ RGC axons. F. GFP⁺ On-Off pDSGC axons. G. GFP⁺ tOff-αRGCs axons. (Huberman et al., 2008). (D-G) Scale=200µm. H. Fluorescence intensity across the dLGN width in DRD4-GFP and CB2-GFP mice. (n=2 CB2 mice and n=3 DRD4 mice; +/-SEMs). I-L. , CTb⁺ (I,K) and GFP⁺ (J,L) axons in the dLGN contralateral (I,J) and ipsilateral (K,L) to the intact eye of a DRD-GFP mouse with one eye removed. (J) arrows, GFP⁺ On-Off DSGC lamina. (L) No GFP⁺ axons are present. Scale=100µm.
Fig. 3.7  On-Off pDSGCs form laminar-specific connections in the SC. A. Schematic of regions shown in (B-I) and sagittal view of RGC axons entering the SC (red arrows). SC, superior colliculus; SO, stratum opticum; Brackets denote the uSGS, (upper stratum griseum superficilis) and lSGS (lower stratum griseum superficilis). R, rostral; C, caudal; D, dorsal. B. CTb⁺ axons in the SC. Pt, pretectum; MTN, medial terminal nucleus. C. GFP⁺ On-Off pDSGC axons in the SC. (B, C) Scale=500µm. D-F. Framed area in (B, C). D. CTb⁺ axons in the SO and SGS. Brackets denote uSGS and lSGS as in (A). E. GFP⁺ On-Off pDSGC axons in the SO and uSGS. F. Merge of D and E. Scale=200µm. G. Merged view of CTb⁺ and GFP⁺ On-Off pDSGC axons in the SC and (H) GFP⁺ axons viewed alone. I. GFP⁺ tOff-αRGC axons targeting the ISGS, wherein they form columns. (Huberman et al., 2008). (G-I) Scale=200µm. J. Schematic of the region in (K-N) and coronal view of RGC axons entering the SC. L, lateral. K-L’. GFP⁺ On-Off pDSGC axons (K) and merged with CTb⁺ axons (L) in the SC. L’. Boxed region in L. Brackets denote lSGS and uSGS. The thinner bracket (arrow) straddles the stratum zonale (SZ), a thin retinorecipient lamina above the uSGS. M-N’. GFP⁺ tOff-αRGC axons (M) and merged with CTb⁺ axons (N) in the SC. N’. Boxed region in (N). Conventions as in (L’). (K-N) Scale=200µm. (L’, N’ Scale=50µm. O. Fluorescence intensity along the SC depth in DRD4-GFP and CB2-GFP mice. (n=5 CB2 mice and n=5 DRD4 mice; +/-SEMs).
IV. The interaction of direction selective cells and retinal waves

Abstract

Direction of motion is computed in the retina by a circuit in which there are asymmetric connections between inhibitory interneurons and direction selective ganglion cells (DSGCs). DSGCs have robust null-side inhibition at eye opening, suggesting that this precise wiring is established early in development. Prior to eye opening, spontaneous correlated activity known as retinal waves provides strong directional input to the retina. To test whether retinal waves drive directional circuitry before light responses emerge, we use calcium imaging and cell-attached recordings from transgenic mice expressing GFP in the subtype of On-Off DSGCs preferring posterior motion (pDSGCs). We find that pDSGCs participate in retinal waves propagating in all directions. These findings indicate that retinal waves may play a role in the establishment of directional circuits.

Introduction

A striking feature of neural circuits is the specificity of contacts between synaptic partners. In the retina, the direction selective circuit exemplifies this precision. The four subtypes of On-Off DSGCs prefer motion in one of four cardinal directions (inferior, superior, posterior, and anterior), and are suppressed by motion in the opposite, or null, direction (Oyster and Barlow, 1967). The current model for the direction selective circuit relies upon synapse formation between DSGCs and inhibitory starburst amacrine cells.
(SBACs) that are located on the null side (for review see: Taylor and Vaney, 2003). Though the On-Off DSGCs’ dendrites co-fasciculate extensively with the processes of SBACs, functional GABAergic synapses are formed preferentially with SBACs whose somas are located on the null side (Fried et al., 2002). Thus, each subtype of On-Off DSGC must choose appropriate synaptic partners from a dense plexus of possibilities.

When are these asymmetric GABAergic synapses first formed? Recent work has shown that functional On-Off DSGCs are present at eye-opening (Chan and Chiao, 2008; Chen et al., 2009; Elstrott et al., 2008) and in adult dark-reared animals (Chan and Chiao, 2008), suggesting that visual experience plays a minor role. Since these studies relied upon visual stimuli to identify DSGCs and assay direction selectivity, the lower bound for collecting physiology data was at the emergence of light responses (~P11 in mice). However, DSGC and SBAC processes co-fasciculate as early as P3 (Stacy and Wong, 2003; Yonehara et al., 2008), suggesting the synapses between these neurons may be present much earlier than P11.

Recording from specific RGC cell-types prior to light responses requires genetic markers. Recently, a number of mouse lines have been described where a promoter drives GFP expression in a single RGC type including: the Off Alpha cells (Huberman et al., 2008), the superior-preferring On DSGCs (Yonehara et al., 2009; Yonehara et al., 2008), a previously undescribed asymmetric RGC preferring superior motion (Kim et al., 2008), and the On-Off DSGCs preferring posterior motion (pDSGCs) (Huberman et al., 2009). Here, we use these transgenic lines to examine which classes of DSGCs participate in retinal waves.
Determining whether DSGCs participate in retinal waves is interesting for two reasons. First, if DSGCs were less active in waves moving in the null versus the preferred direction, this would suggest that the asymmetric inhibition was already present. Retinal waves propagate at roughly 5 degrees/second in the first postnatal week of mice (Demas et al., 2006), within the velocity tuning range for both On DSGCs (Sun et al., 2006) and On-Off DSGCs (Weng et al., 2005) in the mouse. Thus, retinal waves could serve as a “read-out” to determine when the directional circuitry is initially formed. Second, retinal waves may play a role in the establishment or maintenance of the nascent GABA$_A$ synapses underlying the null response of developing DSGCs. In other parts of the brain, there is increasing evidence that activation of GABA$_A$ receptors is required for the formation of stable GABAergic synapses (Huang et al., 2007).

Here we use calcium imaging and cell-attached recordings to examine the participation of identified RGC types in retinal waves. Specifically, we will test the hypothesis that pDSGCs respond more strongly to waves moving in their preferred direction (posterior 	extit{in vivo}, nasal 	extit{in vitro}) than to waves moving in the null direction (temporal 	extit{in vitro}). Below we present preliminary data from both WT mice and mice expressing GFP in the pDSGCs.

Results

To measure pDSGC participation in retinal waves, we used the multi-cell bolus loading of the calcium dye Oregon Green BAPTA-1-AM (OGB-1 AM) (Stosiek et al., 2003) (Blankenship et al., 2009) to label cells in the ganglion cell layer of a P11 transgenic mouse expressing GFP+ in the pDSGCs (Figure 4.1A). Retinal waves were
reliably detected as increases in intracellular calcium in the somas of neurons in the ganglion cell layer (Figure 4.1A,B) and the delay between calcium transients was used to determine propagation direction. A GFP- cell (non p-DSGC, blue square in Figure 4.1A) showed no difference in peak fractional change in fluorescence (ΔF/F) as a function of wave direction (Figure 4.2A) (p = 0.20; 1-way ANOVA) indicating that the depolarization that occurred during waves was similar for waves propagating in different directions.

We next examined whether pDSGCs participated in retinal waves, and if that participation depended upon wave direction. Though there is a significant overlap between the excitation and emission spectra of GFP and OGB-1 AM, we were able to distinguish GFP positive cells (see Methods). In a preliminary experiment, we found that a pDSGC showed robust ΔF/F deflections when a wave passed through the region of the retina surrounding the neuron (Figure 4.2B). As in the GFP- neuron (Figure 4.2A) there was no significant difference in peak ΔF/F signal across wave directions (Figure 4.2B) (p = 0.37, 1-way ANOVA).

The data above suggests that the activity of the pDSGC was not significantly suppressed by waves propagating in the null (temporal) direction. However, another possibility is that our calcium imaging was not sensitive enough to detect the variations in the action potential firing in pDSGCs associated with waves propagating in the null direction. To establish a relationship between action potential firing and the magnitude of the fluorescence change, we conducted simultaneous cell-attached recordings and calcium imaging (Figure 4.3A).
To quantify the relationship between spike count and ΔF/F in our experiments, we performed a linear regression between spike count during a wave and either the peak or the integrated ΔF/F during that wave (Figure 4.3B,C). Though there is a significant linear relation between spike count and these ΔF/F measures (p < 0.001), the regression accounts for only ~50% of the variance in the ΔF/F signals. In previous studies, it was established in the developing retina (Blankenship et al., 2009; Feller et al., 1996), as well in other parts of the brain (Ikegaya et al., 2005), that the rising phase of the calcium transient was correlated with depolarization of the cell. However, under our experimental conditions, the loading of OGB-1 AM in RGCs was weak and therefore the dominant contribution to the detected calcium transients was from the inner-plexiform layers or amacrine cell layer, which contain the somas and processes of cells also exhibiting increases in intracellular calcium during retinal waves (see Discussion). Hence, the variability not explained by the linear regression is due to the large contribution of the ΔF/F signal from the surrounding tissue.

Based on the observation that under our experimental conditions, calcium imaging does not accurately represent the action potential activity in individual RGCs, we used combined cell-attached recording and calcium imaging to characterize both non-pDSGC and pDSGC participation in retinal waves (Figure 4.4). Simultaneous calcium imaging and cell-attached recording revealed bursts of action potentials in a non-pDSGC that were correlated with increases in intracellular calcium (Figure 4.4B). In this example, we detected 36 waves and the RGC had an overall firing rate of 0.13 Hz with mean inter-wave interval of 105±37 seconds (Figure 4.5A,B), consistent with previously reported ranges (Blankenship et al., 2009; Demas et al., 2006). By plotting the firing rate
as a function of wave direction, we found that waves moving in the temporal direction elicited significantly more spikes from the non-pDSGCs compared to waves moving in the nasal and dorsal directions (Figure 4.6) (p < 0.05, 1-way ANOVA with Tukey-Kramer post-hoc test). Since we do not know the identity of these cells, we cannot determine whether this bias in spiking is due to biases in the strength of depolarization for waves propagating in the temporal direction or whether it was due to asymmetric inhibitory responses.

We repeated this experiment targeting GFP+ cells to determine whether an identified DSGC had a biased response to waves propagating in the preferred or null directions. If there were substantial inhibition of pDSGCs during waves moving in the null (temporal) direction, we would expect fewer spikes for temporal waves. Instead, we observed that the one recorded temporal wave elicited a spike count similar to the mean nasal spike count (Figure 4.7). In these preliminary experiments, there was no difference in pDSGC spiking across directions. More experiments will determine whether there are significant differences in pDSGC spike count as a function of wave propagation direction.

Discussion

Our hypothesis is that DSGCs will fire fewer action potentials during retinal waves propagating in the null direction. Using simultaneous calcium imaging and cell-attached recordings from transgenic mice expressing GFP in specific cell classes, we have demonstrated in preliminary experiments that pDSGCs participate in retinal waves, though we cannot yet say whether this participation is asymmetric.
Calcium imaging offers the significant advantage of simultaneously measuring the activity of many cells, allowing for direct comparisons between pDSGCs and other cell types. A current limitation of our imaging data is the large variability not explained by the cell’s spiking (Figures 4.3). A group studying the odor responses in the zebrafish olfactory bulb using a 2-photon microscope found a tight, linear correspondence between a neuron’s spiking and its $\Delta F/F$ trace (Yaksi and Friedrich, 2006). Their results suggest that using two-photon calcium imaging would reduce the variability due to fluorescence outside the focal plane. However, in addition to measuring the $\Delta F/F$ from our cell, we must also image enough of the surrounding area to determine the propagation direction of the waves as well as image long enough to gather reasonable wave statistics. Thus, 2-photon microscopy is a promising, but not certain, solution for improving our image data.

The age of the recordings is another important experimental factor due to GABA’s changing role in development. GABA changes from an excitatory to an inhibitory neurotransmitter as the chloride transporter KCC2 is upregulated during development (Rivera et al., 1999). This reversal in GABA’s effects occurs around P8 in the mouse retina (Leitch et al., 2005; Zhang et al., 2006). Thus, if asymmetric GABAergic inputs onto DSGCs are present before P8, they may cause DSGCs to respond more strongly for waves moving in the null direction early in development. Alternatively, the presence of a large chloride conductance could act as a shunt that reduces the probability of spiking even though the net effect is to depolarize the cell. We will repeat these experiments in GABA$_A$ receptor antagonists to determine what effects early GABAergic signaling has on any asymmetric response to waves we detect.
We observed non-DSCGs had a stronger response to temporal waves (Figure 4.6). An important observation is that in these recordings, there were strong biases in the distribution of wave directions. For example, the significantly higher spike count for temporal waves in Figure 4.6 was recorded from a retina that had a strong bias for wave propagating in the temporal direction. Thus, the high spike count for temporal waves may be correlated with frequent temporal waves that depolarized the cell more strongly than the infrequent nasal waves. Therefore, an interesting hypothesis is that a consistent wave bias might lead to a strengthening of synapses for activity in that direction regardless of cell type. Additional experiments will reveal whether these biases in wave directions are systematic across retinas, and whether there is a corresponding bias in the amount of spiking during a wave. Another caveat is that the identity of the non-pDSGCs was not known. To reduce the variability of our non-pDSGC data, we plan to record from mice in which other cell populations, such as the Off Alpha cells (Huberman et al., 2008), express GFP.

Methods

Animals

C57Bl/6 mice were used for all WT recordings. Drd4-EGFP mice expressing GFP in the pDSGCs (Huberman et al., 2009) were obtained from MMRRC (http://www.mmrcc.org/strains/231/0231.html). All animal procedures were approved by the University of California, Berkeley Animal Care and Use Committees and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals,
the Public Health Service Policy, and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

**Whole-Mount Retinal Preparation**

P9–P11 mice were anesthetized with isoflurane and decapitated. After enucleation, the eye was transferred to buffered artificial cerebrospinal fluid (ACSF) (in mM: 119.0 NaCl, 26.2 NaHCO₃, 11 glucose, 2.5 KCl, 1.0 K₂HPO₄, 2.5 CaCl₂, 1.3 MgCl₂). Next, the eye was hemisected posterior to the ora serrata, and the cornea, lens, and vitreous were removed with forceps. Blood vessel landmarks visible through the retina on the choroid were used to mark the nasal pole of the eyecup prior to removing the pigment epithelium with forceps. The isolated retina was then hemisected along the nasal-temporal axis as indicated by the landmarks and mounted on the filter paper RGC side up at a consistent orientation over a 1mm x 2mm hole. Retinas were incubated at room temperature in bubbled (95% O₂ / 5% CO₂) ACSF until they were transferred to the recording chamber, where they were constantly superfused with bubbled ACSF (32-34°C).

**Electrophysiology**

Cell-attached recordings were obtained from whole-mount retinas. Retinas were visualized with differential interference contrast optics on a Olympus BX61W1 microscope with an Achroplan 40x water immersion objective. The inner limiting membrane of the retina was removed with a glass recording pipette and RGCs were targeted using a micromanipulator (MP-225; Sutter Instruments). GFP+ cells were
detected in retinas loaded with OGB-1 AM based on the biased spectral selectivity of CFP cubes for GFP over OGB-1 AM. Recording pipettes (Garner Glass or Sutter Instruments; PP-830 Narishige puller) had a tip resistance of 3-5 MΩ and were filled 150 mM NaCl. Data was acquired with pCLAMP 9 recording software and a Multiclamp 700A amplifier (Molecular Devices), sampling at 10 kHz. Custom Matlab software (Mathworks) was used to bandpass filter the data between 80 and 2000 Hz with an 8th-order Butterworth filter and to extract spike times.

**Calcium imaging**

Retinas were bulk loaded with the calcium indicator Oregon Green 488 BAPTA-1 AM (OGB-1 AM; Invitrogen) using the multicell bolous loading (MCBL) technique (Stosiek et al., 2003). OGB-1 AM was prepared at a concentration of 10mM in a solution of 2% pluronic acid in DMSO, which was then diluted 1/10 in a solution containing (in mM): 150 NaCl, 2.5 KCl, 10 Hepes, pH 7.4 (Stosiek et al., 2003). OGB-1 AM solution was pressure ejected into the retinas from a borosilicate glass micropipette (Garner Glass or Sutter Instruments) using a PV-820 Pneumatic PicoPump (World Precision Instruments) at a pressure of 20 psi. Dye was ejected in 2-3 locations in the RGC layer. Epifluorescent calcium imaging was performed on an Olympus BX61W1 microscope using a 40x water immersion objective (Zeiss Achromplan) and a Hamamatsu Orca-ER camera, with illumination provided by a Sutter Lambda LS and controlled by a Uniblitz shutter. Images were acquired at 2 Hz in MetaMorph (Universal Imaging Corporation).

**Image Analysis**
Waves were detected and wave direction was determined using a semi-automated procedure written in Matlab (Mathworks). First, an 8μm x 8μm region of interest (ROI) was centered on the cell to be analyzed. Next, 8 additional ROIs, also 8μm x 8μm, were placed at 45 degree intervals along a circle with radius 50 μm centered on our original ROI. For each of the 9 ROIs, the pixel values in the ROI on each frame were averaged to obtain an average fluorescence time series. The fractional change in fluorescence intensity ΔF/F of the time series for each ROI was computed as:

\[ \frac{\Delta F}{F} = \frac{F_{\text{orig}}}{F_{\text{filt}}} - 1 \]

where \( F_{\text{orig}} \) are the original values of the fluorescence time series, and \( F_{\text{filt}} \) is the time series filtered with an 8th-order Butterworth bandstop filter with cutoffs between 0.01 and 0.99 Hz to remove wave events. A 2.3 standard deviation threshold was applied to the ΔF/F time series of each ROI to detect possible waves (Figure 4.1B). Threshold crossings detected on at least 50% of the ROIs were flagged as candidate retinal waves for further inspection. The propagation direction of each candidate wave was determined by selecting the start and stop points of the wave front as it progressed across the field of view (Figure 4.1C). The start and stop points form a vector indicating the wave’s propagation direction. Approximately 10% of waves could not be reliably classified and were excluded from subsequent analysis. Finally, the direction vectors were sorted into 90 degree bins centered at 0, 90, 180, and 270 degrees and labeled as nasal, dorsal, temporal, and ventral.
For GFP+ cells loaded with OGB-1 AM, the fractional change in fluorescence intensity $\Delta F/F$ is:

$$\frac{\Delta F}{F} = \frac{\Delta F}{F_{GFP} + F_{OGB}}$$

where $F_{GFP}$ is the fluorescence due to GFP, and $F_{OGB}$ is the fluorescence due to OGB-1 AM. If $F_{GFP} >> F_{OGB}$, it would be difficult to measure any meaningful $\Delta F/F$ signal. In practice, $F_{GFP} < F_{OGB}$, allowing for the reliable detection of changes in calcium levels in GFP+ cells.

**Statistics**

For multiple comparisons, a repeated measures ANOVA with a Tukey-Kramer post hoc test was used. Alpha was always 0.05.

**Acknowledgement**

Chapter 4 is original work in preparation as Elstrott J and Feller MB. The interaction of direction selective cells and retinal waves and is included with permission from all the manuscript’s authors. The dissertation author was the primary author of this paper.
References


Fig. 4.1 Measuring pDSGCs and non-pDSGCs activity using calcium imaging. A. Fluorescence imaging of a retina loaded with OGB-1-AM in which the pDSGCs express GFP. The green square shows the region of interest for a GFP+ cell, the blue square for a GFP- cell. B. The ΔF/F trace averaged over the blue region. The red line shows the threshold for detecting waves. C. Mean-subtracted images showing a ventral-propagating wave.
Fig. 4.2  Peak ΔF/F as a function of wave direction for a P11 pDSGC indicate participation in waves in all directions with no apparent bias. A. Peak ΔF/F as function of wave direction for a nearby GFP- cell (blue square in Figure 4.1A). Red diamonds show the mean ± s.e.m. for each direction. nasal = 2 waves, dorsal = 7, temporal = 7, ventral = 12. B. Peak ΔF/F as function of wave direction for a pDSGC (green square in Figure 4.1B). nasal = 3 waves, dorsal = 5, temporal = 7, ventral = 10.
Fig. 4.3  Large variability associated with the amplitude of $\Delta F/F$ as a function of spike count during a wave. **A.** An example of spiking during retinal waves. Peak $\Delta F/F$ (B) and integrate $\Delta F/F$ (C) versus spike count for the same cell in Figures 4.4-5. $n = 30$ waves. Red line is the least-squares fit.
Simultaneous calcium imaging and cell-attached recording show that RGCs bursts during retinal waves. A. Fluorescence image of ganglion cell layer loaded with the calcium indicator Oregon Green BAPTA-1 AM. This image was taken from a P10 transgenic mouse in which GFP is expressed in starburst amacrine cells. The white lines indicate the location of the recording electrode. The identity of the recorded RGC was not known. B. Rasters comparing waves directions (top), wave times (middle) and spiking activity for this cell. Inset – expanded timescale of burst that occurred during a wave propagating in the ventral direction. Directions are given in retinal coordinates: N=nasal, D=dorsal, T=temporal, and V=ventral.
Fig. 4.5  Spiking properties of a RGC during retinal waves.  A. A PSTH of the cell in Figure 4.1 Bin size = 1 s. B. Distribution of inter-wave intervals for the same cell. Mean = 105±37 seconds, n = 36 waves.
Fig. 4.6 Non-pDSGCs show asymmetric spiking across wave directions. Red diamonds show the mean $\pm$ s.e.m. for each direction. nasal = 7 waves, dorsal = 16, temporal = 16, ventral = 7 from 2 cells.
Fig. 4.7  pDSGCs show no significant difference in spiking across wave directions. Red diamonds show the mean ± s.e.m. for each direction. nasal =11 waves, dorsal = 4, temporal = 1, ventral = 1 from 2 cells.
V. Conclusions

The work presented here examines the development of a class of retinal direction selective cells, the On-Off DSGCs. Despite extensive research detailing the circuitry underlying the asymmetric responses of mature DSGCs (for reviews see Demb, 2007; Fried and Masland, 2007; Taylor and Vaney, 2003), surprisingly little is known about how On-Off DSGCs develop (but see Daw and Wyatt, 1974; Masland, 1977). In cortex, visual experience is critical for the normal development of direction selective cells (Daw and Wyatt, 1976; Li et al., 2006; Li et al., 2008). Therefore, we tested the hypothesis that vision is required for the normal development of On-Off DSGCs. Surprisingly, we found that dark rearing had little effect on the development of DSGCs, which were present at eye-opening in mice. Our work, together with the results from other labs (Chan and Chiao, 2008; Chen et al., 2009), suggests that vision plays a minor role in the development of retinal DSGCs, in sharp contrast to the direction selective cells in V1.

The presence of functional On-Off DSGCs at eye-opening implies that the null-side inhibitory synapses between starburst amacrine cells (SBACs) and DSGCs form before the retina is light responsive. Thus, examining the early development of On-Off DSGC wiring requires a method for identifying DSGCs before eye opening. This motivated our search for a transgenic mouse in which a class of On-Off DSGCs expressed a fluorescent marker, resulting in the discovery a mouse expressing GFP in the posterior-preferring On-Off DSGCS (pDSGCs) (Huberman et al., 2009). Below I discuss how the discovery of a transgenic mouse with a labeled subtype of On-Off DSGCs
creates new possibilities for resolving long-standing questions about the function of On-Off DSGCs.

The function of On-Off DSGCs

Since their discovery over forty years ago, much progress has been made in understanding how On-Off DSGCs detect motion in the retina. However, surprisingly little is known about how the outputs of On-Off DSGCs are used by the brain. This is in sharp contrast to the On DSGCs, whose role in visual reflexes has been well described (for review see: Vaney et al., 2000). Indeed, the detailed understanding of On DSGC projections to the nuclei of the accessory optic system (AOS) has allowed physiologists to study the input and output properties of directional AOS neurons (Simpson et al., 1988; Soodak and Simpson, 1988).

The paucity of anatomical data for On-Off DSGC projections has not prevented speculation about their purpose. The observation that the four preferred directions of On-Off DSGCs align with the four rectus muscles of the eye led some to suggest that these cells are also involved in visual reflexes (Oyster and Barlow, 1967). However, unlike the On DSGCs whose large receptive fields and weak inhibitory surrounds are ideally suited for detecting retinal slip, the On-Off DSGCs seem poorly suited for mediating visual reflexes (Vaney et al., 2000). Instead, the high density and strong inhibitory surrounds of On-Off DSGCs suggest they may play a role in detecting local motion in the visual scene (Vaney et al., 2000).

The discovery of a promoter that drives GFP expression in the pDSGCs creates exciting opportunities for further understanding On-Off DSGC function by revealing the
anatomy of these cells, and allowing for their manipulation. First, we found no pDSGC projections in the AOS (Figure 3.5), the sole target of the On DSGCs (Buhl and Peichl, 1986). This suggests that pDSGCs, and perhaps On-Off DSGCs in general, do not provide direct inputs to the brain areas involved in visual reflexes. Instead, pDSGCs project to the dorsal LGN and superior colliculus (3.6-7). This projection pattern supports the “local motion detector” hypothesis, in which On-Off DSGCs detect small moving objects within the visual field. Combining behavioral experiments with conditional knockouts of pDSGCs could further describe the function of On-Off DSGCs. In addition, conditional knockout of the pDSGCs would provide a means of testing models for how post-synaptic neurons pool On-Off DSGC inputs. The presence of sharply tuned directional cells in LGN led to the hypothesis that the directional cells in this area collect antagonistic inputs from On-Off DSGCs with opposing preferred directions (e.g. posterior and anterior) (Levick et al., 1969). If this model is correct, then silencing pDSGCs should broaden the tuning of anterior preferring DS cells in the LGN by relieving inhibition during posterior motion, but should have little effect on LGN cells preferring superior or inferior motion.
References


