Gap Junctional Coupling between Retinal Amacrine and Ganglion Cells Underlies Coherent Spike Activity Integral to Global Object Perception

Submitted by

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Abstract

Coherent spike activity between neighboring cells is a ubiquitous phenomenon exhibited by ensembles of neurons throughout the central nervous system, including the retina. In addition to the robust coherent activity between neighboring retinal ganglion cells (RGCs), there is evidence that widely separated RGCs can also show correlated spiking. These long range correlations can be evoked by large, contiguous light stimuli, but not to smaller, discontinuous objects. It has thus been posited that long range spike correlations between distant RGCs encode information critical to global object perception. Since the wide separation of the RGCs precludes common excitatory drive from bipolar cells, the mechanism underlying long range concerted activity has remained elusive. Most RGCs show gap junctional coupling to polyaxonal amacrine cells (PACs), which maintain extensive axonal arbors that can extend >1mm across the retina. The electrical coupling between RGCs and PACs thereby form a plausible circuit that can underlie long range correlated activity in the retina. In the current study we tested this hypothesis by targeting and recording from pairs of widely separated ON α-RGCs in the mouse retina, which are coupled indirectly through intermediary PACs. Pharmacological blockade of gap junctions or genetic ablation of connexin36 (Cx36) subunits eliminated the long range correlated spiking between the α-RGCs. These data indicated that electrical coupling between RGCs and PACs was responsible for the long range spike activity. In contrast, I found that direct, serial RGC-RGC coupling was incapable of supporting long range spike correlations. Finally, behavioral experiments were performed to test whether blockade of retinal gap junctions or ablation of Cx36 attenuates the ability of mice to discriminate large, global objects from small, disjointed stimuli. I found that Cx36 knockout mice indeed have significantly reduced ability to discriminate global objects from smaller discontinuous stimuli as compared to wild type littermates. This reduced perception of global objects was not due to a general reduction in spatial acuity that measured in knockout animals was similar to that for wild type mice. Taken together, our results indicate that long
range concerted firing between RGCs, derived from electrical coupling with amacrine cells, encodes information critical to global object perception.
Dedication

I dedicate this thesis work to my family, research advisor and committee members.
Acknowledgements

First and foremost I would like to thank my research advisor Dr. Stewart Bloomfield for his constant support over the years for my research and my career. It has been an absolute pleasure to work under his supervision in his lab. He is an excellent mentor and in the last four years I have constantly evolved as a researcher under his able guidance. His constant encouragement and inputs had helped me to complete my PhD successfully. Besides his scientific guidance I would like to thank Dr. Bloomfield for all the wonderful conversations we had over the years.

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List of Abbreviations

AC  Amacrine cell
AMPA  \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BC  Bipolar cell
CaCl\(_2\)  Calcium chloride
cAMP  Cyclic adenosine monophosphate
CCP  Crosscorrelogram profiles
C\(_6\)H\(_{12}\)O\(_6\)  Glucose
CNS  Central nervous system
CsOH  Cesium hydroxide
Cy3  Cyanine 3
DLP  Digital light processing
EGTA  ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N\(^\prime\),N\(^\prime\)-tetraacetic acid
GABA  gamma-Aminobutyric acid
HC  Horizontal cell
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
INL  Inner nuclear layer
IPL  Inner plexiform layer
IR  Infrared
JAVA  Just another virtual accelerator
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>LGN</td>
<td>Lateral geniculate nucleus</td>
</tr>
<tr>
<td>MFA</td>
<td>Meclofenamic acid</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mGluR6</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Sodium phosphate dibasic</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Sodium phosphate monobasic</td>
</tr>
<tr>
<td>NB</td>
<td>Neurobiotin</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>PAC</td>
<td>Polyaxonal amacrine cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>SMI32</td>
<td>Anti-Neurofilament H Non-Phosphorylated Mouse mAb</td>
</tr>
<tr>
<td>TEA-Cl</td>
<td>Tetraethylammonium chloride</td>
</tr>
</tbody>
</table>
TTX  Tetrodotoxin

WFAC  Wide field amacrine cell

18β-GA  18-β-glycyrrhetinic acid
1. Introduction

1.1 Structure of the retina.

The retina is the light sensitive neural tissue layer that lines the inner surface of the back of the eye adjacent to the pigment epithelium. The retina can be clearly divided into discrete layers, which include: the layers of rod and cones, external limiting membrane, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL) and internal limiting membrane (Fig. 1). The retina consists of five major neuronal cell types: the photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells and the ganglion cells (Kolb, 1995; Masland, 2012a) distributed within the above-mentioned layers. The following table (Table 1) shows the distribution of the five different cell types within the different layers of the retina.

Table 1:

<table>
<thead>
<tr>
<th>Layer</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Retinal pigment epithelium</td>
<td>Single layer of cuboidal cells</td>
</tr>
<tr>
<td>2. Layer of rods and cones</td>
<td>Outer segment of rods and cones</td>
</tr>
<tr>
<td>3. External limiting membrane</td>
<td>Muller cells</td>
</tr>
<tr>
<td>4. Outer nuclear layer (ONL)</td>
<td>Soma of rods and cones</td>
</tr>
<tr>
<td>5. Outer plexiform layer (OPL)</td>
<td>Axons of rods and cones</td>
</tr>
<tr>
<td>6. Inner nuclear layer (INL)</td>
<td>Nuclei and cell bodies of amacrine cells, bipolar cells and horizontal cells.</td>
</tr>
<tr>
<td>7. Inner plexiform layer (IPL)</td>
<td>Synapses between axons of bipolar cell and dendrites of ganglion cells and amacrine cells.</td>
</tr>
<tr>
<td>8. Ganglion cell layer (GCL)</td>
<td>Cell bodies and axons of ganglion cells</td>
</tr>
<tr>
<td>9. Internal limiting membrane</td>
<td>Endfeet of Muller cells</td>
</tr>
</tbody>
</table>

Each of the five major neuronal cell types has distinct structure and function as discussed below.
Fig. 1. The retinal layers and the five major neuronal cell types in the retina are outlined in the schematic. Additionally, the types of connexin (Cx) expressed by different neurons are also listed. The abbreviations for the retinal cell types are as follows: cone (C), rod (R), horizontal cell (HC), rod bipolar cell (RB), cone bipolar cell (CB), amacrine cell (AC), AII amacrine cell (AII), ganglion cell (GC).

Figure adapted from Bloomfield and Völgyi (2009).
A. Photoreceptors

The rod and cone photoreceptor express light sensitive pigments or opsins that respond to relatively dim and bright light, respectively, as a hyperpolarization of the membrane (Masland, 2012a). Hence, they are responsible for scotopic and photopic vision respectively. As the name implies, the cones are conical-shaped structures and their cell bodies are arranged in a single row below the outer limiting membrane with their inner and outer segments protruding into the subretinal space towards the retinal pigment epithelium (Kolb, 1995). In primates, the cones are concentrated in the foveal region of the retina where they are responsible for high spatial acuity, and show a precipitous decrease with peripheral eccentricity. Additionally, the cone opsins have different spectral absorption capabilities that form the basis of color vision (Masland, 2012a).

In contrast, rod photoreceptors display a slim rod shaped structure that occupies the subretinal space between the cell bodies of the cones and thereby constitutes the remainder of the outer nuclear layer not covered by the cones (Kolb, 1995). Rod photoreceptors either feed onto the rod bipolar cells or use a secondary pathway where they form gap junctions with the cones to send signals downstream (Seelig et al., 2011). Additionally, a tertiary rod pathway had also been demonstrated in rodents whereby the rod photoreceptors bypass the rod bipolar cells altogether and make synaptic contact with the OFF cone bipolar cells that expresses AMPA glutamate receptors. Thus the rod photoreceptors excite the cone bipolar cells directly through a sign conserving ionotropic glutamate receptors (Hack et al., 1999).

B. Horizontal Cells

The horizontal cells (HC) are second order neurons that form a lateral pathway in the outer retina. Most mammals have two types of horizontal cell and some rodents have only one type. Broadly the HCs can be classified into two types, the axonless or
the A type HCs and the axon-bearing or the B type HCs (Boycott et al., 1987; Gallego, 1986). The A-type HCs has sparsely branched dendritic arbors with relatively few and stout primary dendrites and no axonal processes. In contrast, the B-type HCs have a finely branched dendritic tree and an extensive axonal system (Kolb, 1974; Linberg and Fisher, 1988). The HCs are electrically coupled to one another via gap junctions that results in large receptive fields which extend well beyond the dendritic or axonal arbor of individual cells (Masland, 2012a). A functional tracer coupling study in connexin 57 (Cx57) deficient mice showed less than 1% spread of Neurobiotin (NB) tracer through coupled HCs as compared to the wild type littermates (Hombach et al., 2004). This experiment suggested that gap junctions formed by Cx57 proteins are responsible for the extensive coupling observed between the HCs. Furthermore, ablation of Cx57 in the mouse retina dramatically reduces the receptive field size of HC (Shelley et al., 2006). The A-type HCs in the rabbit retina express Cx50 but the types of connexin protein expressed by the B-type HCs, equivalent to the only type of HC found in the mouse retina, are yet to be elucidated (Massey et al., 2003).

The HCs provide inhibitory feedback to the photoreceptors and are responsible for center surround organization of the cells in the inner retina as described in details in later sections (Baylor et al., 1971; Naka and Witkovsky, 1972)

C. Bipolar Cells

Bipolar cells (BC) are relay neurons that transmit signals from the photoreceptors to the retinal ganglion cells (RGCs) and amacrine cells (ACs). The BCs are the first neurons in the visual system that exhibit a center-surround receptive field organization: ON BCs depolarize in response to illumination of their center receptive fields and hyperpolarize when their surrounds are stimulated. In contrast, OFF BCs hyperpolarize in response to small spots of light and depolarize to surround illumination such as to an annulus of light (Werblin and Dowling, 1969). Photoreceptors respond to a light stimulus with a membrane hyperpolarization. However, center receptive
fields of downstream neurons like RGCs can respond to both onset and offset of light as depolarizations and hyperpolarizations. This segregation of the signal from the photoreceptors into the so-called ON and OFF pathways is made possible by the different types of glutamate receptors expressed by BCs (see below).

C.1 Center-surround organization of BC

The center-surround organization whereby cells in the retina show antagonistic center and surround responses to light had been long accepted to be the fundamental design of the vertebrate retina (Dowling, 1987; Werblin, 1991). Center-surround organization in the retina was first demonstrated in the RGCs of both mammalian (Kuffler, 1953) and non-mammalian (Barlow, 1953) retinas more than 50 years back. However, it was subsequently shown in the mudpuppy retina that BCs also exhibit center-surround organization (Werblin and Dowling, 1969). The center-surround organization of retinal neurons has been reported in species ranging from lower vertebrates (Kaneko, 1970; Schwartz, 1974; Wunk and Werblin, 1979) to primates (Dacey et al., 2000).

It has been widely accepted that the center-surround receptive fields of BCs originates, at least in part with a feedback pathway from HCs to cones (Burkhardt, 1993). The center component emerges due to the direct glutamatergic input from the cones on to the BCs, whereas the antagonistic surround is generated by the suppression of glutamate release from neighboring cone mediated by the HC feedback circuitry (Kamermans et al., 2001). Experiments conducted in the fish and rabbit retina showed that current injection in the HCs affected center-surround organization of the RGCs (Mangel, 1991; Naka and Witkovsky, 1972). These studies further indicated that HCs are responsible for generating the center-surround receptive field in the BCs which in turn forms the basic substrate for similar organization in the ganglion cells (Fahey and Burkhardt, 2003). The center-surround organization of the retina enables contrast coding necessary for signaling of spatial details of scenes and thus allows
efficient signal processing at an early stage of the visual system (Srinivasan et al., 1982).

C.2 Segregation of ON and OFF pathway in the retina

The segregation of the neural circuitry in the retina into parallel ON and OFF pathway is a fundamental property that enables efficient visual processing both at onset and offset of light respectively (reviewed by Wassle, 2004). Hartline (1938) first observed that RGCs in vertebrate show three different types of responses to light: ON, OFF and ON-OFF. Subsequent studies showed that the ON and OFF pathways are created at the very first synapse between photoreceptors and BCs (Schwartz, 1974; Werblin and Dowling, 1969).

The BCs can be broadly classified into ON and OFF cone BCs (CBC) and rod BCs (RBC), which all show ON center-receptive fields. Photoreceptors undergo hyperpolarization in response to light and thereby release glutamate maximally in the dark (Dowling, 1987). The OFF CBCs expresses sign conserving AMPA or kainate glutamate receptors (DeVries, 2000), which produce a hyperpolarization to center illumination mimicking the photoreceptor response. In contrast, the ON CBCs and the RBCs express mGluR6 glutamate receptors that invert the signal from the photoreceptors, thereby resulting in a depolarization to light (Masu et al., 1995; Nomura et al., 1994; Slaughter and Miller, 1983). The segregation of the ON and OFF pathway is maintained downstream in the RGCs and the ACs in the inner retina. This is achieved by the synaptic organization whereby the axons of OFF BCs and dendrites of OFF RGCs and ACs stratify in the more distal sublamina-a of the IPL and the axons of ON BCs and dendrites of ON RGCs and ACs stratify in the more proximal sublamina-b of the IPL in the retina (Famiglietti et al., 1977; Famiglietti and Kolb, 1976). This scheme ensures that RGCs and ACs receive appropriate excitatory inputs from the BCs, thereby maintaining the segregation of the ON and OFF pathways.
The ON and OFF pathways remain segregated in the lateral geniculate nucleus (LGN) of the thalamus and subsequently in the visual cortex (Knapp and Mistler, 1983; Schiller, 1982; Thurlow et al., 1993). The parallel ON and OFF pathways thus form an essential organizing feature of the visual system that is essential for contrast detection and rapid processing of signals encoding stimulus onset and offset (Schiller et al., 1986).

D. Amacrine Cells

The AC are mostly inhibitory interneurons that releases GABA or glycine as the neurotransmitter and are electrically coupled to BCs, RGCs or other ACs through gap junctions. It has been estimated that there are 24-60 subtypes of ACs in the mammalian retina (Masland, 2012b). The ACs can maintain wide, medium or narrow dendritic fields and are responsible for various functions, including lateral inhibition of RGCs and creation of complex receptive field properties such as direction selectivity (Masland, 2012a). For example ablation of starburst ACs abolishes responses of direction selective RGCs and the optokinetic eye response (Yoshida et al., 2001). Likewise, the AII ACs forms an integral component of the primary rod pathway. The AII ACs receives signals from the RBCs and transmits them to the ON CBC via sign-conserving gap junctions and to the OFF CBC via inhibitory glycinergic synapses (Seeliger et al., 2011).

The ACs forms the main inhibitory interneurons in the retina; it is now generally accepted that either GABA or glycine are released by nearly 100% of the ACs (Kolb, 1997; Marc, 1989). An immunohistochemical study in the human retina showed that 40% of the ACs express glycine while 55% of ACs express GABA (Crooks and Kolb, 1992). A number of ACs express other neurotransmitters such as acetylcholine, dopamine and serotonin as well as neuroactive peptides, but most of these also co-localize glycine or GABA (Brecha et al., 1988; Crooks and Kolb, 1992; Vaney, 1990). Immunohistochemistry (Marc et al., 1995) and autoradiography (Pourcho and Goebel,
1983) studies have shown that most ACs that release GABA are medium or wide-field cells whereas most glycinergic ACs have small dendritic fields (Marc et al., 1995).

### D.1 Polyaxonal Amacrine Cell

The polyaxonal amacrine cells (PACs) are a unique subtype of wide field ACs with somata, 10-15 µm in diameter that lay in the proximal region of the INL or displaced to the GCL. The PACs are characterized by long axonal processes that can extend millimeters across the retina as well as a more circumscribed dendritic arbor (Famiglietti, 1992a; Freed et al., 1996; Taylor, 1996; Völgyi et al., 2001) (Fig. 2).

**Fig. 2.** Photomicrograph showing polyaxonal amacrine cells with large processes in the rabbit retina. Scale = 25µm. Figure adapted from Völgyi et al. (2001).

Völgyi et al., 2001) (Fig. 2). The center receptive fields of PACs are comparable to the diameter of their dendritic processes and hence are significantly smaller than the extensive axonal arbors. Tracer-coupling studies of PACs revealed that they are coupled both homologously to one another and heterologously to other ACs and RGCs (Bloomfield and Völgyi, 2007; Davenport et al., 2007). The PACs have been identified in a wide variety of animals (Greschner et al., 2014; Stafford and Dacey, 1997; Völgyi et al., 2005; Völgyi et al., 2009; Völgyi et al., 2001; Wright and Vaney, 2004) and thus form a ubiquitous subtype of cell.

Physiological studies showed that PACs show either ON or ON-OFF receptive fields (Bloomfield and Völgyi, 2007; Davenport et al., 2007). The PACs show somatic and axonal sodium spikes, the latter which appear to propagate centrifugally along their extensive arbors (Bloomfield and Völgyi, 2007; Davenport et al., 2007; Gre-
schnet et al., 2014). The distinctive morphology of the PACs suggests that they are also involved in processing of visual information over a large area but specific functions are unclear. However, studies in the rabbit and salamander retinas suggest that PACs are involved in suppression of spurious signals introduced by eye movements (Baccus et al., 2008; Olveczky et al., 2003).

E. Retinal Ganglion Cells

The RGCs provide the final step of visual processing in the retina and send axonal projections to the higher order visual centers. In mouse, as many as 36 morphological subtypes of RGCs have been described (Baden et al., 2016). RGCs also demonstrate center surround receptive field organization relayed from the BCs upstream (discussed in details above). RGCs perform a variety of computations including but not limited to contrast adaptation (Demb, 2008), motion detection (Zhou and Lee, 2008), color sensitivity (Dacey et al., 2005). RGCs project to several brain centers but the thalamus forms the main target for image forming signals. With only approximately one million axons in the human eye, the RGCs create a bottleneck in the visual system that limits the transmission of information via the optic nerve (Gray, 1999).

E.1 Alpha retinal ganglion cells

Alpha retinal ganglion cells (α-RGCs) are found in most mammalian species and usually show transient responses that can be divided into two subtypes: ON and OFF (Boycott and Wassle, 1974; Peichl, 1991; Peichl et al., 1987a; Peichl et al., 1987b). They are characterized by large soma and vast network of processes, which enables easy identification (Fig. 3).

The ON α-RGCs have a soma size of approximately 18μm with stout and smooth dendritic tree spanning an area of 200μm approximately that stratifies in sublamina-\(b\) of the IPL. The dendritic processes branched at acute angles with almost
no overlap with one another (Völgyi et al., 2009). ON α-RGCs injected with Neurobiotin tracer that can permeate through the gap junctions shows heterologous coupling to PACs whose soma is displaced to the GCL (Hu and Bloomfield, 2003; Völgyi et al., 2009; Xin and Bloomfield, 1997). In the mouse ON α-RGCs show sustained depolarizations and spiking in response to light presentation (Pang et al., 2003).

In contrast, the OFF α-RGCs in the mouse retina maintain a somata ~18µm in diameter, with a smooth and stout dendritic arbor spanning ~185µm, which stratifies within sublamina-α of the IPL. Neurobiotin injections revealed that the OFF cells are coupled both to PACs as well as to other OFF α-RGCs and hence show both heterologous and homologous coupling (Hu and Bloomfield, 2003; Völgyi et al., 2009; Xin and Bloomfield, 1997). Physiological recordings from OFF α-RGCs show either transient or sustained responses to light. The transient cells show increased response only at the offset of light, but do not have any response under constant darkness. In contrast, the sustained OFF α-RGCs maintains a continuous spike activity in darkness (Pang et al., 2003).

1.2 Synaptic transmission in the nervous system.

Anatomical evaluation of the central nervous system first began more than a
hundred years ago. In the late 1800s, based on his studies using his silver staining technique, Camillo Golgi advocated the “reticular theory”, which posited that the nervous system was formed by a single, continuous network. However, this theory was later refuted by the Spanish neuroanatomist Santiago Ramon y Cajal who exploited Golgi’s staining technique. He proposed the “neuron doctrine” in which the nervous system was composed of discreet cells or neurons. He further proposed that the communications between neurons occurs via axo-somatic or axo-dendritic junctions. The neuronal doctrine proposed by Ramon y Cajal was later found to be the correct prediction and the reticular theory was eventually discredited. In the early 1900s, Charles Scott Sherrington advocated that there was no actual confluence between the cells in the nervous system, but there exists a physical separation between the two neurons, for which he coined the term “synapse” (Sherrington, 1906).

The mechanism by which signals were transmitted between separated cells remained elusive. However, the research of Elliot (Elliott, 1905) and Loewi (Loewi, 1924) proved the existence of chemical neurotransmitters that are transmitted as signals at least between neurons and muscle cells. Bernard Katz and colleagues further demonstrated that the release of the neurotransmitter acetylcholine is controlled by influx of Ca$^{2+}$ via voltage gated calcium channels and occurs on a millisecond time scale (Katz and Miledi, 1969). It was thus strongly advocated and generally accepted that synaptic transmission was chemically mediated.

Yet the studies by Potter and Frushpan in crayfish provided evidence that neuronal communication could also be mediated by direct transfer of electrical signals between cells (Furshpan and Potter, 1957, 1959). This was followed by several other studies in fish (Bennett et al., 1963; Robertson et al., 1963) that established communication via electrical synapses popularly known as gap junctions today. Gap junctions are specialized structures between cells through which the cells can transmit ions and small molecules and hence they serve as a mode of communication between neigh-
boring cells. It is now clear that across the central nervous system, including the retina, both electrical and chemical synapses coexists and together play important roles for proper signal encoding and propagation (Pereda, 2014).

While both chemical and electrical transmissions are equally important for proper functioning of the nervous system (Pereda, 2014); chemical transmission requires more sophisticated machinery for neurotransmission compared to electrical synapses because it needs to fulfill several steps in order to release the neurotransmitters. In the case of a chemical transmission, as the action potential reaches the axon terminals causing depolarization, the voltage gated calcium channels opens resulting in the influx of Ca\(^{2+}\). The rise in the level of cytosolic calcium triggers the process of release of neurotransmitters packed within vesicles. The release of neurotransmitters is achieved over several stages that include docking of the vesicles to the plasma membrane, fusion and the release of the neurotransmitters into the synaptic cleft. Finally, the vesicles are taken back through uptake transporters for future use. The complete cycle from docking to reuptake requires less than a minute. The neurotransmitters released from the cells can be both excitatory (e.g., acetylcholine, glutamate) or inhibitory (GABA, glycine) (Lodish, 2000).

In contrast, electrical transmission via gap junctions, which are direct contacts between neighboring cells, allows bidirectional propagation of molecules up to ~1000Da between the cells (Willecke et al., 2002). Bidirectional propagation of ions allows any member of the coupled network to stimulate its counterparts. Thus, current can flow in either direction depending on which member in the network is invaded by membrane polarization including the action potential. Thus, electrical transmission forms an efficient and fast mode of cell communication compared to chemical synapses (Purves, 2001).
1.3 Structure and regulation of gap junctions in the retina.

Gap junctions form the morphological substrate of electrical synapses. They are characterized by a 20Å gap between membranes of neighboring cells (Goode-nough and Revel, 1970) and permeate ions and small molecules of up to 1000 Da in size. Gap junctions are composed of a pair of hemichannels (or connexon), one of which is expressed by each neighboring cells, that are placed adjacent to one another linking an extracellular space of 2-4nm though which ions and small molecules can be transported intracellularly. Hemichannels are hexameric structures formed by connexin subunits. Connexins are transmembrane proteins with four transmembrane domains with two extracellular loops and an amino and a carboxyl terminal end intracellularly; ~ 22 different connexin subtypes have been discovered in humans and mice and are named based on their molecular weight (e.g., Cx36 = 36,000 Daltons). Hemichannels can be composed of all the same connexins (homomeric) or different connexin proteins (heteromeric). Additionally, a gap junction may be composed of homologous (homotypic) or heterologous (heterotypic) hemichannels (Sohl and Willecke, 2003; Willecke et al., 2002). In the vertebrate retina, all five major neuronal cell types express an array of gap junctions that express different subtypes of connexins (Sohl et al., 2005; Sohl and Willecke, 2003). Most connexins are phosphoproteins meaning they undergo post-translational modification by phosphorylation and this occurs in the carboxyl terminal end located in the cytoplasm. Phosphorylation in the amino terminal end also located in the cytoplasm had not been shown (Lampe and Lau, 2000).

The phosphorylation or dephosphorylation of connexin proteins determines the conductance and expression of gap junctions. Studies of retinal HCs have shown that dopamine can reduce the receptive field size of the HCs (Lasater and Dowling, 1985). This effect has been shown to be mediated by D1 dopamine receptor that elevates cAMP activity via adenylyl cyclase (Dowling et al., 1983; Piccolino et al., 1984; Van Buskirk and Dowling, 1981). It has also been demonstrated that reduced coupling in
the HCs resulted from the reduction in the open probability of gap junction channels rather than a change in the conductance (McMahon et al., 1989). In addition, studies have shown that the expression of Cx36, a predominant gap junction in the nervous system is modulated by phosphorylation driven by cAMP/PKA (Phosphokinase A) activity (Ouyang et al., 2005) or nitric oxide (Patel et al., 2006).

It is thus clear that gap junctions are highly dynamic structures, which is reflected in the plasticity of electrical synaptic transmission. For example, the electrical coupling in All ACs in the retina is regulated by ambient light. It had been shown that the coupling is low in dark and bright light, but is relatively high under low-intensity illumination (Bloomfield and Völgyi, 2004; Bloomfield et al., 1997). The change in coupling is mediated by modulation of dopamine release by ACs by light, which alters the activation of D1 dopamine receptors and subsequently cAMP activity by adenylyl cyclase (Mills and Massey, 1995).

Thus, it is evident from the few examples mentioned above that a variety of signaling pathways regulate the strength of electrical synapses by means of phosphorylation or dephosphorylation mechanism.

1.4 Expression and functions of gap junctions in the retina.

Relatively recent evidence had established that all five major neuronal cell types in the vertebrate retina express a wide variety of gap junctions (Fig. 4), which plays diverse roles in order to ensure proper functioning of the retina (Bloomfield and Völgyi, 2009; Sohl et al., 2005; Sohl and Willecke, 2003). The following table (Table 2) briefly summarizes the gap junctional protein expressed by various neuronal cell types in the retina and their respective functions.
Fig. 4. Structure and molecular organization of gap junctions. (a) Schematic shows the structure of gap junction. It depicts that gap junctions are composed of hemichannels made up of connexin proteins. Hemichannels of neighboring cells are aligned against each other that form a functional synapse. (b) Schematic of a connexon subunit. Six connexon units, together form a functional hemichannel. Figure adapted from Bloonfield and Völgyi (2009).
<table>
<thead>
<tr>
<th>Retinal neurons expressing the gap junction</th>
<th>Gap junctional protein expressed</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cone-Cone photoreceptors</td>
<td>Cx36 (Feigenspan et al., 2004; Lee et al., 2003)</td>
<td>Improves signal to noise ratio by summation of the correlated visual signals and attenuation of the asynchronous noise (DeVries et al., 2002).</td>
</tr>
<tr>
<td>Rod-Cone photoreceptors</td>
<td>Cx36 (Raviola and Gilula, 1973)</td>
<td>Composes the secondary rod pathway to transmit scotopic signal (Nelson, 1977; Schneeweis and Schnapf, 1995).</td>
</tr>
<tr>
<td>Rod-Rod photoreceptors</td>
<td>Not known (Völgyi et al., 2004)</td>
<td>Constitutes the rod vision during dawn and dusk. Aids in the tertiary rod pathway (Tsukamoto et al., 2001).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>HC-HC</strong></td>
<td>Cx50 or Cx57 (Hombach et al., 2004; Massey et al., 2003)</td>
<td>Increases the lateral spread of HC that is responsible for the formation of center-surround receptive fields of the BCs (Mangel and Miller, 1987; Naka and Nye, 1971; Naka and Witkovsky, 1972)</td>
</tr>
<tr>
<td><strong>All-All amacrine cells</strong></td>
<td>Cx36 (Deans et al., 2002; Feigenspan et al., 2004)</td>
<td>Maintains high sensitivity to the rod pathway by responding throughout the scotopic and mesopic range (Smith and Vardi, 1995).</td>
</tr>
<tr>
<td><strong>All amacrine cell- Cone BCs</strong></td>
<td>Cx36 (Deans et al., 2002)</td>
<td>Maintains the primary rod pathway where-by rod signals are fed into cone bipolar cells via All amacrine cells (Völgyi et al., 2004).</td>
</tr>
</tbody>
</table>
AC-GC or GC-GC

Generates coherent firing activity between neighboring GC directly or indirectly via ACs which helps in encoding visual signals that are sent to the thalamus for further processing (Brivanlou et al., 1998; DeVries, 1999; Hu and Bloomfield, 2003).

| AC-GC or GC-GC | Cx36/Cx45 (Völgyi et al., 2005; Völgyi et al., 2009; Völgyi et al., 2013) | Generates coherent firing activity between neighboring GC directly or indirectly via ACs which helps in encoding visual signals that are sent to the thalamus for further processing (Brivanlou et al., 1998; DeVries, 1999; Hu and Bloomfield, 2003). |

It is quite evident from the above table that gap junctions are widely expressed by all major neuronal cell type in the retina and also plays an important role in various stages of visual processing.

Among the plethora of gap junctions expressed in the retina, electrical coupling between RGCs and ACs deserves special mention due to the extensive amount of coupling between them. It had been shown that the vast majority of RGC subtypes are coupled to ACs, which most, if not all, appear to be PACs (Völgyi et al., 2009). However, the function(s) of RGC-AC coupling remains unclear. In one study, the indirect coupling between ON direction selective (ON DS) RGCs via PACs was found to be responsible for synchronized activity between neighboring ON DS cells that was important for encoding their direction selective responses (Ackert et al., 2006). This study demonstrated that indirect gap junctional coupling between ON DS cell via the PACs can play an important role in local processing of visual signals. However, as mentioned above PACs are a unique subtype of ACs characterized by the presence of long axonal arbors that span millimeters across the retina (Völgyi et al., 2001). This
unique morphology suggests that PACs are involved in long range interactions between RGCs. In a study performed in the cat retina it was indeed demonstrated that widely separated RGCs respond to global, contiguous light stimuli with synchronous firing, but fails to do so in response to discreet stimuli. It was further demonstrated that this signal originated in the retina and was transmitted to the LGN and cortex for further processing (Gray et al., 1989; Neuenschwander and Singer, 1996). A specific aim of my dissertation research was to determine whether RGC-PAC electrical coupling is responsible for the long-range coherent activity between distant RGCs in response to global object presentation.

1.5 Concerted activity between ganglion cells in the retina.

Coherent neuronal spiking, often in the form of oscillatory activity, is ubiquitous across the CNS, including all levels of the visual system (Singer, 1999; Singer and Gray, 1995; Steriade, 2006). In the retina, although neighboring RGCs usually fire to the same stimulus, it was assumed that each RGC provided an independent signal to higher visual centers. However, results of simultaneous recordings from multiple RGCs in goldfish (Arnett, 1978; Johnsen and Levine, 1983), rabbit (Arnett and Spraker, 1981; DeVries, 1999), cat (Mastronarde, 1983a, b, c) and salamander (Brivanlou et al., 1998; Meister et al., 1995) retina argued against this idea showing that RGCs show robust coherent activity. These studies demonstrated that neighboring RGCs often show light-independent correlations with temporal precision ranging from synchronous activity to relatively loose cross-correlation profiles spanning tens of milliseconds suggesting the involvement of chemical and electrical synaptic transmission (Brivanlou et al., 1998; DeVries, 1999; Mastronarde, 1983a, b, c; Meister et al., 1995). Correlated spontaneous activity exhibited by the RGCs can be classified into broad (40-100ms), medium (2-10ms) and narrow categories (<1ms). Although the timescale can vary between warm-blooded and cold-blooded animals, the classification
appears to be consistent across various species (Brivanlou et al., 1998; Mastronarde, 1983a, b, c). The broad correlations appear to be mediated by chemical synapses as application of neurotransmitter blockers abolishes them (Völgyi et al., 2013). However, the medium and narrow correlation persist after chemical synapse blockade, indicating that they are mediated via the electrical synapses in the retina (Brivanlou et al., 1998). In support of this idea, studies in the cat (Mastronarde, 1983a, b, c) salamander (Brivanlou et al., 1998; Meister et al., 1995), and mouse retinas (Völgyi et al., 2013) retina show that narrow width correlations occurs mostly between RGCs that are directly coupled to one another. Direct coupling between RGCs most often result in a bimodal peaked crosscorrelogram profile (CCP). The bimodal peak is thought to reflect reciprocal excitation between the electrically coupled cells with a set delay resulting in the latency between the peaks (Mastronarde, 1989; Meister et al., 1995). Alternatively, medium width correlations with a unimodal peak at time zero are believed to result when the RGCs receive shared common excitatory drive via indirect electrical coupling to intermediary ACs (Mastronarde, 1989; Meister et al., 1995).

The RGCs also display coherent activity strongly dependent on light stimulus parameters, including intensity, size, contrast, and movement (Hu et al., 2010; Ishikane et al., 1999; Neuenschwander and Singer, 1996). Yet, the role of coherent activity in the retina remains unclear. Studies show that concerted spiking of RGC neighbors provides additional information to the brain, up to 20% more in the primate, which is multiplexed with asynchronous activity from individual RGCs, thus overcoming the limited bandwidth of the optic nerve (Meister and Berry, 1999; Pillow et al., 2008). It has been posited that light-dependent correlated activity of visual neurons serves to segregate distributed features in an image, thereby defining stimulus structure important for binding and perception of global objects (Roelfsema et al., 2004; Roelfsema and Singer, 1998; Shadlen and Movshon, 1999). Conversely, spike correlations could be disadvantageous in some cases, in which, reflecting inefficient redundancy of signals inherent to massive interconnectivity of cells, they actually limit
coding of information (Mazurek and Shadlen, 2002; Nirenberg et al., 2001; Pillow et al., 2008; Shadlen and Newsome, 1998)

As described above, there have been numerous studies of the coherent spike activity between neighboring RGCs, however, there is evidence that long range coherence between widely separated RGCs can also occur.

The was first demonstrated in a classic experiment in the cat retina (Neuen-schwander and Singer, 1996). In this study, RGC pairs separated by as much as 20 degrees of visual angle showed coherent firing when evoked by a contiguous light stimulus that covered the receptive fields of both the cells and the area between them. In contrast, when a pair of separate light stimuli was placed over the receptive field of each cell, it failed to evoke any correlation. Analogous experiments between cells located in separate hemispheres of the LGN also yielded the same result. The same group also showed inter-columnar synchronization of neurons between spatially separated columns in the cat visual cortex (Gray et al., 1989). Based on these observations, it was posited that long range synchronization between RGCs in the cat retina is responsible for binding features of an object critical for global object perception. However, the underlying mechanism responsible for this long range correlation between RGCs was never elucidated. It is crucial to understand the mechanism underlying such a phenomenon and whether it is important for the perception of global objects as it might play a pivotal role in understanding how a visual scene is scrutinized.

The goal of my dissertation research is to evaluate the mechanism of long range correlated spike activity between widely separated RGCs and to determine its importance in the perception of global objects.
1.6 PACs-RGC electrical coupling forms a plausible circuit for long range coherent activity in the retina.

The retina will be the model for my study since it is an approachable part of the central nervous system (CNS) and the circuitry in the inner plexiform layer has been well characterized. Since widely separate RGCs cannot receive convergent excitatory drive from bipolar cells, I posit that the excitatory drive that creates coherent activity is derived from PACs that are electrically coupled to RGCs including but not limited to the ON and OFF α-RGCs (Schubert et al., 2005; Völgyi et al., 2009).

As mentioned in earlier sections that PACs are wide-field ACs with enormous processes that can spread millimeters across the retina and can propagate sodium spikes via these processes apart from releasing GABA as the inhibitory neurotransmitter. Hence, long range excitation propagated via the arbors of the PACs makes it the most appropriate candidate for mediating coherent spike activity between widely separated RGCs in the retina.

Furthermore, evidences by means of computational modeling had demonstrated that reduction of gap junctional coupling between RGCs and axon-bearing ACs by 25% significantly reduces the long range coherent activity between the RGCs. Additionally, weakening the coupling by 50% reduces coherent activity to a level below the background (Kenyon et al., 2003). This study further strengthens our hypothesis that gap junctional coupling between PACs and RGCs is responsible for long range coherent activity between widely separated RGCs.

In this thesis work I combined electrophysiological, morphological, and behavioral techniques to investigate the neural circuitry that underlies long range correlated spike activity between widely separated RGCs in the mouse retina, as demonstrated earlier in the cat retina (Neuenschwander and Singer, 1996). The overall hypothesis is that electrical coupling between PACs and RGCs forms the circuit responsible for long
rang correlated activity. In addition, I examined whether long range coherent activity between RGCs is crucial for perception and binding of global objects as speculated by earlier studies done in the retina, LGN (Neuenschwander and Singer, 1996) and the visual cortex (Gray et al., 1989).
2. Methods

2.1 Mouse lines.

All animal procedures were carried out in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at State University of New York, College of Optometry. Electrophysiological experiments were performed on adult Kcng4-cre;Thy1-stop-YFP line 1; (henceforth Kcng4-YFP), Connexin36 knockout (Cx36\(^{-/-}\)), CxWT and C57BL/6 mice lines. Behavioral experiments were performed on Cx36WT and Cx36\(^{-/-}\) mice line. All mice lines were bred at State University of New York, College of Optometry animal facility under a 12:12 hour light/dark cycle and fed ad libitum.

Kcng4-YFP mice were generated taking advantage of the cre-lox “knockin” system. Kcng4-cre positive mouse were bred with a Thy1-YFP mouse where a STOP cassette flanked by flox sequences was introduced between the Thy1 and yellow
fluorescent protein (YFP) gene which ensured cell specific expression of YFP (Buffelli et al., 2003). Thy1-YFP was constitutively expressed hence; the expression of YFP is only contingent upon the expression of cre. Thus, only cre positive cells that could cut the flox sequence and remove the STOP codon expressed YFP (Duan et al., 2014). In the ganglion cell layer of the retina the cells that expressed YFP were mostly α-RGCs (Fig. 5a-b) (Duan et al., 2015). Hence, to ensure the expression of YFP in the α-RGCs mice were genotyped for both Kcng4-cre and Thy1 and only double positive mice were used for experiments.

The Cx36/− mice were derived from F2 generations of C57BL/6-129SvEv mixed background litters (Deans et al., 2001; Deans et al., 2002). Littermates without the Cx36 gene deletion were designated CxWT and used in control experiments.

The C57BL/6 mice were originally obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and subsequently bred in the animal care facility at the College of Optometry. All the experiments were performed on adult mice of either sex.

The primers used for all the genotyping are given below (Table 3):

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcng4 wild type</td>
<td>GAGATGGCTGGAG-CAGCTCA</td>
<td>GGAGCCAAAGTGATGGTGTC-CAGGA</td>
</tr>
<tr>
<td>Kcng4-cre</td>
<td>GAGATGGCTGGAG-CAGCTCA</td>
<td>TCTGCACACAGACAG-GAGATCTT</td>
</tr>
<tr>
<td>Thy1</td>
<td>GGCTTTTCTCTGAGTGG-CAAAGGACCTTGG</td>
<td>GGCCGTTTCTGGTGGTAGAG-GATCGATGGC</td>
</tr>
<tr>
<td>Cx36 wild type</td>
<td>AGCGGAGGGAGCAAAC-GAGAAG</td>
<td>CTGCCGAATTGGGAACACTGAC</td>
</tr>
<tr>
<td>Cx36 knockout</td>
<td>TCCGGCCGCTTGGGTG-GAG</td>
<td>CAGGGTAGCCGGATCAAGCGTA-TGC</td>
</tr>
</tbody>
</table>
2.2 Whole mount retina preparation for electrophysiology.

On the day of the experiments mice were transferred to a CO$_2$ chamber and euthanized. The eyes were enucleated immediately and were transferred to the HEPES buffered Ringer’s solution aerated for at least 15-20 minutes with 95%O$_2$: 5% CO$_2$ before the dissection of the retina. The composition of the solution is given below (Table 4):

Table 4:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>137</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>2.5</td>
</tr>
<tr>
<td>HEPES-Na</td>
<td>10</td>
</tr>
<tr>
<td>Glucose (C$<em>6$H$</em>{12}$O$_6$)</td>
<td>28</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl$_2$)</td>
<td>1</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl$_2$)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Note: 1M of MgCl$_2$ and CaCl$_2$ solutions was initially prepared and the required amount was added to the final solution to obtain the desired concentration. Final pH was adjusted to 7.4.

The retina from each eye was removed and placed in HEPES buffered Ringer’s solution and on filter paper (Millipore, Billerica, MA, USA) either whole or in quarters. A little suction with a 1ml syringe was applied to attach the retina firmly on the filter paper. The filter papers turn transparent when wetted, allowing for unhindered illumination of the retina in the recording setup. If the retina was dissected into quarters, the other pieces not to be used immediately were mounted on separate filter papers and were left in a dark chamber bubbled with 95% O$_2$: 5% CO$_2$. All chemicals used in the HEPES buffered Ringer’s solution was obtained from Sigma (St. Louis, MO, USA).
2.3 Electrophysiology.

Isolated retinas were placed in a heated brain slice chamber (Warner Instruments, Hamden, CT, USA) on the stage of an Olympus multiphoton microscope (FV1200MPE, Olympus, Tokyo, Japan) equipped with an IR laser (Spectra-Physics, Santa Clara, CA, USA). Retinas were superfused with a bicarbonate Ringer’s solution (35-37 degree Celsius). The composition of the Ringer’s solution is given in the following table (Table 5):

Table 5:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>120</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>5</td>
</tr>
<tr>
<td>Sodium Bicarbonate (NaHCO₃)</td>
<td>25</td>
</tr>
<tr>
<td>Sodium Phosphate Dibasic (Na₂HPO₄)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sodium Phosphate Monobasic (NaH₂PO₄)</td>
<td>0.1</td>
</tr>
<tr>
<td>D-Glucose (C₆H₁₂O₆)</td>
<td>10</td>
</tr>
<tr>
<td>Bubble the solution with 95% O₂: 5% CO₂ for 5-10 minutes</td>
<td></td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.01</td>
</tr>
<tr>
<td>Magnesium Sulphate (MgSO₄)</td>
<td>1</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂)</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: The Ringer’s solution was heated to 35-37°C and bubbled with 95% O₂: 5% CO₂ for oxygenation to maintain a pH 7.4.

Kcnq4-YFP mice with YFP expressing α-RGCs were visualized and targeted using the IR laser photoreceptors at 920nm under a 25x water immersion objective (Olympus, Tokyo, Japan) to limit light adaptation of photoreceptors. Loose patch recordings of spike responses were made from pairs of ON or OFF α-RGCs that lay 300-600 um apart, corresponding to ~12-24° of visual angle. Electrodes were fabricated from glass tubing (1B120F-4, World Precision Instruments LLC, Sarasota, FL, USA) using a Brown-Flaming puller (Sutter Instrument, Novato, CA, USA) to an im-
pedance of 7-10 MΩ when filled with HEPES buffered Ringer’s solution. Recordings were collected with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and digitized online using an analog-to-digital board (Digidata 1550, Molecular Devices) at a sampling rate of 20 KHz using pClamp 10.5 software (Molecular Devices) in gap free mode.

Analogous electrophysiological experiments were performed in Cx36<sup>−/−</sup>, CxWT and C57BL/6 mice lines where cells with large soma, presumably α subtypes, were targeted for electrophysiology. Following physiological recordings the cells were labeled with Neurobiotin-Streptavidin staining followed by SMI32 colabeling to confirm the subtype as α (for detailed method see section 2.8).

2.4 Pharmacology.

In order to determine the role of gap junctions in global object perception analogous experiments as described in section 2.3 were performed where the control Ringer’s solution was substituted with gap junction blockers that included 100µM of Meclofenamic Acid (MFA) or 25µM of 18-β-Glycyrrhetinic Acid (18β-GA) (Sigma). The concentration of the drugs was chosen based on previously published studies (Ackert et al., 2006; Pan et al., 2007). After the drug was perfused for at least 5-7 minutes, light evoked activity were obtained from the same pair of α-RGCs that were used for recordings under control condition. Although the gap junction blockers gradually abolishes spiking activity from the cells we ensured that we recorded equal or more number of spikes from the pair of α-RGCs in order to match the control conditions. In an event where we failed to collect the required number of spikes, the corresponding pair of cells was not considered for further analysis.
2.5 Light stimulation.

Light stimuli to mimic local and global objects were represented via a pair of separate rectangles and a single large rectangle, respectively. The light stimulus was computer-generated and was created with JAVA (Sun Microsystems, Menlo Park, CA, USA) or Matlab (MathWorks, Natick, MA, USA). The paradigm for most experiments included an initial presentation of two rectangles of light, with sides ranging from 80-120 µm, of equal size and illuminance each placed over the soma of the pair of RGCs being recorded. The rectangles were presented individually to ensure that the center receptive fields of the two RGCs were non-overlapping. Then the rectangles were presented together up to 250 times with 500 msec duration and 1 sec duty cycle (Fig. 6a). The stimulus was coded such that the size and the gap between the rectangles could be manipulated contingent upon the distance between the two cells. Additionally, we could also manipulate the intensity and the interval of the light flashes. Following this the separate rectangles were fused to evoke the same pair of cells and the light evoked activity was recorded (Fig. 6b).

The stimuli were generated on a DLP projector (Samsung, Seoul, South Ko-

![Fig. 6. Schematic for electrophysiological recordings. (a) Dual loose patch was performed on ON or OFF α-RGCs separated by at least 12°-24° of visual angle in response to two separate rectangles representing the local stimulus. (b) Dual loose patch performed on ON or OFF α-RGCs and light evoked activity was measured in response to a contiguous rectangle mimicking global stimuli pattern.](image-url)
rea) and delivered to the camera port of the microscope and projected down to the retina through the objective. Neutral density filters were used to control stimulus intensity, which ranged from approximately $10^5$-10$^6$ photons-$\mu$m$^{-2}$-s$^{-1}$ without any filters. All the experiments were conducted at the highest intensity to ensure optimal firing of the cells.

2.6 Offline spike sorting and calculation of area under the curve to generate histograms.

Spike sorting and CCP were generated as described previously (Hu et al., 2010). Spike trains recorded from each pair of cells were exported to Offline Sorter (Offline Sorter, Plexon, Dallas, TX, USA) for analysis. Spikes were sorted and time stamped from digitized recordings using principle component analysis. Following this the sorted spike data was transferred to Neuroexplorer (Nex Technologies, Madison, AL, USA) for further analysis. CCPs were generated from the time stamped spike recordings of RGC pairs using Neuroexplorer software. In order to generate a CCP any

![Fig. 7. Representative data showing CCP before and after the subtraction of shift predictor. (a) Example of a raw CCP computed from light-evoked spikes recorded from a pair of ON $\alpha$-RGCs. Red line represents the 99% confidence for which correlations are above chance. (b) Same CCP as in (a) after subtraction of the time shuffling using a shift predictor paradigm.](image-url)
one of the cell is randomly designated as the reference and the other one is the target cell. The spike trains of both the cells are lined up. For each spike in the reference spike train, a 1msec time window known as bin was made. Now, the spike train of the target cell is examined for each of these windows and the increment of bins is made in which the target cell shows a spike. The significance of correlated spikes above chance was determined as those correlations exceeding the 99% confidence intervals (Fig. 7a).

To correct for spike correlations between cell pairs that were time locked to the stimulus presentation, the spike data were time shuffled using a shift predictor analysis with the aid of the Neuroexplorer software. Shift predictor shuffles all the spikes from both the target and the reference cell and generates a CCP solely based on spike rates of the cell in absence of any physical relation (e.g. synaptic connectivity or common input). Hence, when the shift predictor is subtracted from the original CCP the resultant CCP shows the correlation between the cells that are temporally independent of the light stimulus (Fig. 7b).

To determine the percent of correlated spikes between RGC pairs, area under the curve (AUC) measures were computed for profiles within the shift predictor CCP that exceeded the 99% confidence interval as a percentage of the entire profile in a ± 50 msec epoch. AUC from several pairs were averaged together to determine any significant changes in correlation for each experiments. Average AUC from several pairs were then used to generate the histograms in Origin (Origin, OriginLab Corp., Northampton, MA, USA).

2.7 Immunohistochemistry.

The α-RGCs are rich in the neurofilament epitope SMI32. Hence, we confirmed the YFP expressing cells in Kcng4-YFP mice line as the α-subtype via immunolabel-
ing with SMI32 antibody. The retinas were first isolated and fixed in 4% paraformaldehyde (PFA) for 30mins. Following this, the retinas were washed in 0.1M phosphate buffer saline (PBS) and blocked for 30mins in a PBS solution containing 5% Chemiblocker (Millipore, Billerica, MA), 0.5% Triton X-100, and 0.05% sodium azide (Sigma, St. Louis, MO, USA). The retinas were then incubated in SMI32 mouse monoclonal primary antibody (1:2000, SMI32R-100, Covance, Princeton, NJ, USA) for 48 hours at room temperature. After several thorough washes in 0.1 M PBS, retinas were incubated in Alexa Flour 488/568 secondary antibody (1:500, A21202/A10037, Life Technologies, Carlsbad, CA, USA) overnight at room temperature. Finally, after several washes with 0.1 M PBS, the retinas were mounted in Vectashield mounting medium (H-1000, Vector Labs, Burlingame, CA, USA). Mounted retinas were imaged on a Fluoview FV1200 Olympus confocal microscope and high-resolution images (1024 x 1024 pixels) were obtained from compiled Z-stack profiles with 2 µm steps.

2.8 Cell labeling with Neurobiotin.

Individual or pairs of ON and OFF α-RGCs were filled with the gap junction permeant tracer Neurobiotin (NB, SP-1120, Vector Labs, Burlingame, CA, USA) to determine their dendritic morphology and coupling patterns in all the mice lines used for electrophysiology. Neurobiotin being a small molecule can penetrate through gap junctions thus labeling the coupled cells and hence is widely used to determine the coupling pattern of a cell. For our purpose cells were patched with 5-7MΩ electrodes in whole cell patch clamp mode. The electrodes were filled with 4% Neurobiotin in an internal solution (composition in Table 6) which was allowed to diffuse into the cell for 10-15mins. Immediately after this; the retina was fixed in 4% PFA for 20mins. Then the retina was washed thoroughly in 0.1M PBS and co-labeled with Cy3 conjugated streptavidin (1:500, S6402, Sigma, St. Louis, MO, USA) overnight at room temperature. Finally, the retina was mounted with Vectashield and imaged on the FV1200
confocal microscope as described above.

Table 6:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cesium hydroxide (CsOH)</td>
<td>120</td>
</tr>
<tr>
<td>D-Gluconic Acid</td>
<td>120</td>
</tr>
<tr>
<td>HEPES-Na</td>
<td>10</td>
</tr>
<tr>
<td>EGTA</td>
<td>11</td>
</tr>
<tr>
<td>TEA-Cl</td>
<td>10</td>
</tr>
<tr>
<td>Calcium chloride (CaCl$_2$)</td>
<td>1</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl$_2$)</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: pH was adjusted to 7.4.

2.9 Retrograde labeling.

In order to determine the concentration of MFA that was effective in uncoupling ACs from RGCs, the RGCs were retrogradely labeled with Neurobiotin injected into the optic nerve. Mice were anesthetized with ketamine (70 mg/kg) and xylazine (7 mg/kg) and MFA (2µl of 500µM solution) was injected intravitreously with a 34-gauge needle connected to a microsyringe (Hamilton Company, Reno, NV, USA). Animals were then sacrificed 3, 5, 7 and 9 days after the injection. The eyes were immediately enucleated with intact optic nerve for retrograde labeling as described previously in Akopian et al (Akopian et al., 2014). The globes were submerged in bicarbonate Ringer’s solution (see section 2.3 for composition) continuously bubbled with 95% O$_2$: 5% CO$_2$. A drop of 4% Neurobiotin in 0.1M PBS was applied to the cut end of the optic nerve and left for 40mins in the aerated Ringer’s solution. After 40mins the eye was hemisected, the anterior optics discarded, and the resultant retina-eyecup was incubated in the Ringer’s solution aerated with 95% O$_2$: 5% CO$_2$ for 1 hour. Then the retina was isolated and fixed in 4% PFA for 30mins. Following few washes with 0.1M PBS the retina was incubated with streptavidin conjugated with Cy3 (1:500, Sigma,
St. Louis, MO, USA) overnight. Finally, the retina was washed in 0.1 M PBS and then mounted and imaged as described above.

2.10 Behavioral assay.

2.10a Behavioral assay for global object perception task.

Behavioral studies were performed in a commercially available 15X3X6 inches (length X width X height) mouse Y water maze (San Diego Instruments, San Diego, CA, USA). Cx36\(^{-/-}\) and CxWT littermates were trained for a two alternate forced choice (2AFC) task. Each arm of the Y maze faced a computer monitor calibrated for equal luminance that displayed the stimuli. The stimulus was custom made using Matlab (MathWorks, Natick, MA, USA) and Psychtoolbox (Brainard, 1997). In initial experi-

![a] and ![b] schematic showing the setup for the behavioral experiments. (a) Schematic of the Y water maze used in the behavioral experiments to determine changes in global object perception before and after blockade of gap junctions. (b) Schematic of the Y water maze used in the behavioral experiments to determine changes in spatial acuity due to pharmacological blockade or genetic ablation of gap junctions when compared to WT littermates.

**Fig. 8.** Schematic showing the setup for the behavioral experiments. (a) Schematic of the Y water maze used in the behavioral experiments to determine changes in global object perception before and after blockade of gap junctions. (b) Schematic of the Y water maze used in the behavioral experiments to determine changes in spatial acuity due to pharmacological blockade or genetic ablation of gap junctions when compared to WT littermates.
ments, animals were tasked with discriminating between two separate rectangles and a single contiguous rectangle (Fig 8a). The size of the single rectangle was manipulated so that it covered an area equal to the sum of those covered by the two separate rectangles. The computer monitors placed adjacent to each arm of the Y maze displayed either a positive (contiguous rectangle) or a negative stimulus (separate rectangles) in pseudorandom sequence (Gellermann, 1933). Mice were trained to swim towards the contiguous rectangle and a hidden platform was placed in the arm facing the contiguous rectangle as a reward. The size of the rectangles was optimized such that each mouse could perform the task comfortably. The distance between the separate rectangles were varied in steps of 20°, 10°, 7°, 5°, 2°, 1.5° and 1° of visual angle. The training continued until the performance of the mice improved from 50% to 80-90%. All trials were carried out at 100% contrast and under photopic condition with normal background room lighting since earlier evidences revealed that Cx36−/− mice have compromised scotopic vision (Deans et al., 2002).

On the day of the experiment, mice were transferred to a holding cage lined with paper towels and illuminated with a 100watt light bulb to prevent hypothermia. Each mouse performed at least 6 sessions per visual angle and each session had ten interleaved trials. The trials from all the sessions were concatenated together for statistical analysis. No more than 4 sessions were carried out in a single day. After each trial the mouse was transferred to the holding cage to rest for 2-3 minutes. However, the activity was modified based on signs of fatigue.

Additionally, an independent group of CxWT mice were injected intravitreally with MFA to uncouple the RGCs and the ACs. After recovery from the injection, the mice were made to perform the global object perception task for all the visual angle separations described above. We have shown that the efficacy of MFA lasts for approximately 7 days post injection (see Results section 3.7 and Fig. 20); hence all experiments for the MFA injected group were performed within 7 days post injection.
It is to be noted that the MFA injected mice were first tested for spatial acuity task (for details see section 2.10b) before they performed the global object perception task to ensure that the vision was not compromised due to the injection.

**2.10b Spatial acuity.**

Spatial acuity of CxWT, Cx36<sup>-/-</sup> mice and CxWT mice injected with MFA were tested following the protocol described in earlier studies (Prusky et al., 2000). Briefly, the 2AFC task required the mice to swim towards the monitor that displayed sinusoidal grating (positive stimulus) and ignore the grey screen (negative stimulus) in a Y water maze (Fig 8b). The protocol for mice training and the experimental set up for the Y water maze is similar to what is described in section 2.10a. After the training phase when the performance of the mice rose to 80%-90% they were used to test for spatial acuity at 0.1, 0.3 and 0.5 cycles per degree (cpd) because earlier studies had demonstrated that optimal spatial acuity for wild type mice is 0.56 cpd (Prusky et al., 2000). Animals performed 3-6 sessions with 10 interleaved trials per session.

**2.11 Statistical analysis.**

One-way ANOVA was performed for both electrophysiology and behavioral experiments to test for significance. This was followed by Tukey’s multiple comparison test to determine difference between the groups. The α-level for all experiments was 0.05. All histogram data with error bars are presented as mean s.e.m. No data points were excluded. All data analysis and plotting of graph was performed using Origin or Prism software (GraphPad Software, La Jolla, CA, USA). Details of the statistical test including P values and F statistics are provided in text and figure legends as appropriate.
3. Results

3.1 Paired RGC recording paradigm in the Kcng4-cre;Thy1-stop-YFP line 1 mouse.

In initial experiments, our study focused on the ON α-RGCs in the mouse retina, which are coupled indirectly via gap junctions made with two or more subtypes of PACs (Völgyi et al., 2009; Völgyi et al., 2001; Xin and Bloomfield, 1997). We used the Kcng4-cre;Thy1-stop-YFP line 1 (Kcng4-YFP) mouse line in which the ON and OFF α-RGC subtypes uniquely express yellow fluorescent protein (YFP) in the GCL and thereby can be visualized and targeted for electrophysiological recordings (Duan et al., 2014; Duan et al., 2015) (Fig. 9a). Immunolabeling for SMI32, a marker for α-RGC cell bodies and axons (Duan et al., 2015; Meller et al., 1994), confirmed the identity of the YFP-expressing cells in the GCL of Kcng4-YFP mice as α-RGCs (Fig. 9a). We initially targeted pairs of sustained ON α-RGCs with non-overlapping center receptive fields for loose patch recordings (Fig. 9b-d). Cell somata were typically

![Image](image_url)
separated by ~300-600 μm, corresponding to 12-24° of visual angle, were visualized and recorded. We stimulated cell pairs with either two discreet rectangles of light placed over each cell soma or a large, single rectangular bar of light that extended over and covered both center receptive fields, thus mimicking local vs. global objects, respectively (for detailed description of the stimulus see section 2.5). To determine the coherent spike activity of cell pairs we generated CCPs for the light-evoked responses, which revealed correlated activity exceeding chance as histogram peaks above the 99% confidence level (red line; Fig. 7a). To demonstrate spike correlations between α-RGC pairs that were not time-locked to the light stimulus, data were time shuffled using a shift-predictor protocol, which was then subtracted from the original CCP (Perkel et al., 1967) (Fig. 7b).

3.2 Long range coherent RGC activity is dependent on gap junction coupling.

Studies in the cat retina described long-range excitatory interactions between
the RGCs that depended on the spatial and temporal properties of the stimulus (Neuenschwander et al., 1999; Neuenschwander and Singer, 1996). Earlier studies from our lab and others had demonstrated that α-RGCs are electrically coupled to PACs in the mouse retina (Völgyi et al., 2005; Völgyi et al., 2009) particularly via gap junctional protein Cx36 (Schubert et al., 2005; Völgyi et al., 2013). Additionally, the PACs have processes that spread millimeters across the retina and can transmit sodium mediated spikes via their processes (Bloomfield and Völgyi, 2007). Hence, we postulated that long-range interaction between α-RGCs could be mediated by PACs via Cx36.

Widely separated ON α-RGC pairs were first stimulated with small (sides ranging from 80µm-120µm) rectangular lights placed over their YFP-expressing somata to confirm that their center receptive fields were non-overlapping (Fig. 10c insert). Cells were injected with NB to determine their dendritic field morphology and tracer coupling with PACs, by post hoc histology, to confirm their α-cell identity (Fig. 10a). The rectangles of light were then presented simultaneously to reveal any coherent
A raw CCP generated from the spike data indicates an upregulated coherent activity both in response to two separate (Fig. 10b) and a contiguous rectangle (Fig. 10d). But, the correlated spike activity is greatly enhanced in response to the contiguous rectangle when compared to the two separate rectangular light stimuli.

However, on subtraction of the shift predictor from the raw CCPs, which were generated in response to the separate rectangles of light, all coherent activity was obliterated (Fig. 10c). In contrast, we observed that the coherent activity between the ON α-RGC pairs sustained even after the subtraction of the shift predictor when the two rectangles were merged into a single contiguous rectangle that spanned the receptive fields of the RGC pairs, (Fig. 10e), confirming findings in cat retina (Neuen-schwander and Singer, 1996). The shift predictor CCP profile showed a single peak at 0 sec indicating a prominent spike synchrony with a bandwidth of approximately ±
Interestingly, this type of CCP profile is believed to reflect indirect electrical coupling of RGCs via intermediary ACs, consistent with the coupling pattern of ON α-RGCs (Brivanlou et al., 1998; Völgyi et al., 2013).

**Fig. 11.** Different patterns of contiguous light stimuli can evoke long-range coherent activity between ON α-RGCs. (a) Shift predictor CCP generated from light-evoked responses of a pair of ON α-RGCs to three separate rectangular stimuli shows no long-range spike correlations. (b) CCP from the same pair of ON α-RGCs as in (a) in response to a contiguous object with complex structure shows long-range coherent activity. (c) CCP from the same pair of ON α-RGCs as in (a) in response to a contiguous object shows long-range coherent activity. (d-f) CCPs generated from the light-evoked activity recorded from a pair of ON α-RGC in response to an ovoid light stimulus of increasing size shows long-range coherent activity only when the stimulus covers, at least in part, the receptive fields of the RGCs. The relative size of the stimulus was: d, 0.5; e, 0.7; f, 1.00.
We presented a number of different stimulus shapes and contours and evaluated how these affected long-range synchronous activity between RGC pairs. Presentation of discontinuous rectangles divided into 3 segments also did not produce coherent activity between ON α-RGC pairs (Fig. 11a). In contrast, presentation of large stimuli of various shapes and sizes invariably produced long-range correlated activity between distant ON α-RGC pairs, with the proviso that they covered the center receptive fields of the individual cells and spanned the intermediate regions contiguously (Fig. 11b-f).

To determine if electrical coupling was critical to long-range coherent activity
Fig. 12. Long-range correlated activity between ON α-RGCs is found in different wild type mouse strains. (a) Representative micrograph of an ON α-RGC in the C57BL/6 mouse strain filled with NB following electrophysiological recordings shows that the cell is coupled to ACs (arrows). Scale bar = 100 µm. (b) CCP computed from the light evoked responses of a pair of ON α-RGCs in the C57BL/6 mouse strain shows no long-range coherence with presentation of two discreet rectangular stimuli centered over each RGC soma. (c) CCP generated from the responses of the same cell pair as in (b) shows long-range coherent activity when the separate rectangles of light are fused. (d) After application of MFA to block retinal gap junctions, the coherent activity of the same cell pair shown in (c) is largely abolished. (e) Representative micrograph of an ON α-RGC in the C57BL/6:129SvEv (CxWT) mouse strain filled with NB following electrophysiological recordings shows that the cell is coupled to ACs (arrows). Scale bar = 100 µm. (f) CCP computed from the light evoked responses of a pair of ON α-RGCs in the CxWT mouse strain shows no long-range coherence with presentation of two discreet rectangular stimuli centered over each RGC soma. (g) CCP generated from the responses of the same cell pair as in (f) shows long-range coherent activity when the separate rectangles of light are fused. (h) After application of MFA to block retinal gap junctions, the coherent activity of the same cell pair shown in (g) is abolished.

between ON α-RGC pairs, retinas were superfused with the nonselective gap junction blocker MFA prior to the presentation of a contiguous light stimulus. Raw CCP generated after the application of MFA indicated some coherent activity (Fig. 10f), however, all coherence was completely abolished on subtraction of the shift predictor (Fig. 10g), indicating that gap junctional coupling was necessary for coherent spiking between distant RGC pairs.

We also recorded from pairs of ON α-RGCs in C57BL/6 and CxWT mouse strains by targeting cells in the GCL with large somata and confirming their identities with NB labeling and post hoc histology (Fig. 12a,e). Similar to the findings in Kcng4-YFP mice, pairs of distant ON α-RGCs in both C57BL/6 and CxWT animals showed no coherent activity in response to separate rectangles of light, but displayed significant spike synchrony when the rectangular stimuli were fused into a single contiguous object (n = 3 pairs of cells; Fig. 12b,c,f,g). Further, blockade of gap junctions with
MFA effectively abolished the long-range correlations between ON α-RGCs in both wild type mouse strains \((n = 3\) pairs of cells; **Fig. 12d,h**). In separate experiments, we found that application of another gap junction blocker, 18β-GA \((25\mu\text{M})\) (Ackert et al., 2006), also abolished all long-range synchronous activity between ON α-RGCs in the Kcng4-YFP mouse retina when evoked with a large, contiguous stimulus \((n = 3\) cell pairs; **Fig. 13**).

Previous studies have shown that the gap junctional coupling of ON α-RGC to PACS express connexin36 (Cx36) subunits (Pan et al., 2010; Sohl et al., 2005; Völgyi et al., 2005; Völgyi et al., 2009; Völgyi et al., 2013). We therefore tested whether genetic ablation of Cx36 in the \(\text{Cx36}^{-/-}\) mouse strain disrupted long-range coherent spike activity between ON α-RGCs.

**Fig. 13.** Blockade of GJs with 18β-GA abolishes long-range correlated spike activity between ON α-RGCs. (a) CCP generated from the spike activity of a pair of distant ON α-RGCs to two discreet rectangles of light shows no coherent activity. (b) CCP generated from the responses of the same pair of cells in (a) when the separate rectangles are fused shows long-range coherent activity. (c) Application of the gap junction blocker 18β-GA abolishes the coherent activity shown in (b).

PACS express connexin36 (Cx36) subunits (Pan et al., 2010; Sohl et al., 2005; Völgyi et al., 2005; Völgyi et al., 2009; Völgyi et al., 2013). We therefore tested whether genetic ablation of Cx36 in the \(\text{Cx36}^{-/-}\) mouse strain disrupted long-range coherent
spiking between ON α-RGCs. Injection of NB into single ON α-RGCs retinas failed to label ACs, confirming that the interconnecting gap junctions were ablated in the Cx36⁻/⁻ mouse (Fig. 14a). Moreover, we found that neither separate rectangles of light covering the receptive fields of widely separated ON α-RGC pairs nor presentation of a fused contiguous rectangle evoked coherent firing (n = 5 pairs of cells; Fig. 14b,c).

Taken together, these data support the idea that functional gap junctions between RGCs and PACs are essential for generation of synchronous activity between distant ON α-RGCs in response to global object presentation.

Fig. 14. Long-range correlated activity of ON α-RGC pairs in response to a large, contiguous stimulus is abolished by genetic ablation of Cx36. (a) Single ON α-cell filled with NB in the Cx36⁻/⁻ mouse retina shows loss of coupling to ACs. Retina was immunolabeled with SMI-32 to confirm that the labeled cell is a α-RGC. (b) CCP computed from a pair of ON α-RGC in a Cx36⁻/⁻ mouse shows no correlation activity in response to separate rectangles. (c) CCP computed from the same pair of cells in (b) shows no increase in coherent activity when the separate rectangular stimuli were fused into a single global rectangle.

ON α-RGCs in response to global object presentation.

3.3 Serial direct coupling between RGCs does not support long range coherent activity.

Next, we extended experiments to pairs of distant OFF α-RGCs in the Kcng4-YFP mouse retina, which, like ON α-RGCs, are coupled to PACs, but are also coupled homologously to one another (Pan et al., 2010; Völgyi et al., 2005; Völgyi et
al., 2009) (**Fig. 15a**). Presentation of separate rectangular stimuli centered over the somata of widely separated (300-600 μm) OFF α-RGCs failed to evoke coherent activity as assayed by the shift predictor CCPs (*n* = 3 cell pairs; **Fig. 15b,c**). In contrast, when the rectangles of light were extended and fused into a single stimulus, the OFF α-RGCs showed significant synchronous activity (**Fig. 15d**), similar to that shown for ON α-RGC pairs. Blockade of gap junctions with MFA largely abolished coherent activity, although a small residual synchrony was often detected near the 99th per-

**Fig. 15.** Global stimulus can evoke correlated spike activity between OFF α-RGCs coupled both homologously and heterologously. (a) Micrograph of an OFF α-RGC filled with NB in the Kcng4-YFP mouse retina shows coupling to both ACs (arrowheads) and neighboring OFF α-RGCs (arrows). Scale bar = 100 μm. (b) Responses of a pair of OFF α-RGCs to separate rectangles of light restricted to the receptive field of each cell. (c) Shift predictor CCP computed from pairs of OFF α-RGCs in response to separate rectangles shows no coherent firing. (d) CCP from the same pairs of OFF α-RGCs in (c) in response to a fused rectangle shows a significant increase is correlated activity. (e) Application of MFA largely abolished the long-range synchronous activity between the same pair of OFF α-RGCs as in (d) in response to a contiguous rectangular stimulus.
centile significance level in the CCPs (Fig. 15e). This suggested that direct coupling between OFF α-RGCs may have contributed to the long-range coherent activity. We therefore examined whether direct RGC-RGC coupling was sufficient to produce long-range spike synchrony.

Earlier studies reported that OFF α-RGCs in the Cx36<sup>-/-</sup> mouse show only direct homologous coupling, as gap junctions made with ACs are ablated (Pan et al., 2010; Völgyi et al., 2005). We therefore targeted pairs of RGCs with large somata in Cx36<sup>-/-</sup> mice that showed OFF-center receptive field physiology. Cells were injected with NB for post hoc histological identification and retinas were immunolabeled for SMI32 to confirm that cell pairs were OFF α-RGCs (Fig. 16a). Recordings from pairs of distant OFF α-RGCs showed that presentation of separate rectangular stimuli to their individual receptive fields produces no coherent activity (n = 4 cell pairs; Fig. 16b), similar to that found for ON and OFF α-RGCs in control Kcng4-YFP and CxWT mice (Fig. 10d, Fig. 15c and Fig. 12f). However, in contrast to findings in control animals, presentation of a large, contiguous rectangle failed to produce synchronous

![Fig. 16. Serial direct coupling between RGCs does not evoke long-range correlated spike activity between OFF α-RGCs in Cx36<sup>-/-</sup> mouse. (a) An OFF α-RGC in the Cx36<sup>-/-</sup> mouse retina filled with NB shows loss of coupling to ACs, but homologous coupling between OFF α-RGCs (arrows) is preserved. Scale bar = 100µm. (b) CCP between a pair of OFF α-RGC in the Cx36<sup>-/-</sup> mouse in response to two separate light stimuli shows no coherent activity. (c) The same pair of OFF α-RGCs as in (b) still shows no correlated activity when the separate rectangles of light were fused into a single object.](image-url)
activity between OFF α-RGC pairs in Cx36⁻/⁻ mice (Fig. 16c). Thus, direct serial coupling between OFF α-RGCs was insufficient to produce long-range coherent activity.

3.4 Pairs of different RGC subtypes, which are not coupled, do not show long range coherent activity.

To further examine whether gap junctional coupling is necessary for long-range coherent activity between RGCs, we extended experiments to RGC pairs formed by different subtypes. Previous studies suggest that different subtypes of RGCs do not form gap junctions with one another or to a common cohort of ACs (Völgyi et al., 2009; Xin and Bloomfield, 1997). In these experiments, recordings were made from a YFP-expressing α-RGC in the Kcng4-YFP retina and a second RGC (cells with somata of >15 μm diameter were targeted to avoid displaced ACs) with the same ON/OFF receptive field polarity, but devoid of YFP expression, indicating a different subtype identity.

Fig. 17. Uncoupled RGCs shows no long-range correlated firing in response to a contiguous light stimulus. (a) CCP computed from the responses of an ON α-RGC in the Kcng4-YFP mouse retina and a distant ON RGC that did not express YFP and was therefore of another subtype. These cells showed no coherent activity to separate rectangles of light each placed over their respective receptive fields. (b) CCP generated from the same pair of RGCs as in (a) in response to a fused rectangle again showed no coherent spike activity.
Separate rectangles of light were presented over the individual somata to confirm that the cells had non-overlapping center receptive fields. Recordings made from dissimilar pairs of RGCs did not show coherent activity to either separate or fused rectangular stimuli ($n = 5$ cell pairs; Fig. 17).

3.5 Incremental fusing of separate stimuli reveals a threshold property of the long range coherent activity between RGCs.

Our results clearly showed that while presentation of separate stimuli covering the respective receptive fields of distant RGCs did not produce coherent activity, fusing of the stimuli into a single global object induced significant long-range spike synchrony. We next examined the process by which spike synchrony emerged between ON α-RGC pairs as the separate rectangular stimuli were incrementally fused. For these experiments, the two rectangular stimuli were first centered over the somata of the distant ON α-RGCs and were subsequently elongated symmetrically and incrementally to fusion (Fig. 18a-e). Interestingly, we found that long-range synchronous activity did not emerge in a linear fashion related to stimulus separation, but rather showed a clear threshold characteristic. Stimuli could be separated by as little as 20 μm (the smallest separation possible using our stimulus software) without evoking a significant increase in spike synchrony between pairs of ON α-RGCs. In contrast, fusion of the rectangles in a single, large contiguous object spanning the receptive fields of cell pairs produce a dramatic increase in synchronous activity (Fig. 18f).

Apart from the threshold property exhibited by the ON α-RGCs, our results also suggest that the spatial acuity of α-RGCs is at least up to 20μm approximately, which is less than one degree of visual angle computed from measurements performed by earlier studies which showed that in an adult mouse (P30-39) 26.12μm equates to 1 degree of visual angle (Koehler et al., 2011).
Fig. 18. Incremental fusing of separate light stimuli reveals a threshold characteristic of coherent activity between RGC pairs in terms of stimuli separation. (a-e) Light-evoked activity were recorded from a pair of ON α-RGC in the Kcng4-YFP mouse in response to two separate rectangular light stimuli, which were incrementally fused in discreet steps (a, >300 μm; b, 100 μm; c, 50 μm; d, 20 μm; e, 0 μm). The shift predictor CCP computed from the light-evoked spikes showed that significant correlated activity occurred only when the stimuli were completely fused. (f) Cumulative histogram from 6 pairs of ON α-RGCs shows that the percent of correlated spike activity did not increase significantly until the rectangles were fused into one single object. One-way ANOVA followed by Tukey’s multiple comparison test at α = 0.05; **P < 0.01. Data presented as mean ± s.e.m.
3.6 *Stimulus presentation to the intermediary zone between distant RGCs does not evoke long range coherent activity.*

Our results suggested that activity of PACs coupled to RGCs produces the long-range coherent spiking to global stimuli. Therefore, in the next series of experiments we examined whether stimulation of these ACs alone with a small stimulus could “fool” the RGCs by evoking the same long-range coherent activity as produced by a global stimulus. Although coupled homologously to each other, individual PACs show relatively small center receptive fields suggesting inputs restrictive to the most proximal processes (Davenport et al., 2007; Greschner et al., 2014; Völgyi et al., 2001). We therefore presented a single rectangle to the intermediary zone between
and outside the receptive fields of a pair of widely separated ON α-RGCs, with the presumption that this configuration would directly stimulate PACS and not the RGCs to which they were coupled. Interestingly, light presentation to intermediary zone between two distant ON α-RGCs failed to produce correlated activity (Fig. 19a,b). Coherent activity was not observed until the light stimulus was extended to cover, at least in part, the receptive fields of the ON α-RGCs (Fig. 19c-e). An analysis of the correlated spikes indicated that a significant increase in long-range coherence was evoked only when the rectangular stimulus covered at least one-half of the dendritic arbor of each of the α-RGCs (Fig. 19f). These results suggest that stimulation of PACs alone was ineffective in producing long-range correlated spiking between RGCs. Similar results were found using stimuli with an ovoid configuration (Fig. 11d-f).

3.7 Genetic ablation or pharmacological blockade of gap junction reduces global object perception in mice.

A number of studies performed in the retina, LGN and primary visual cortex of the vertebrates showed synchronous firing between remotely located cells in response to a global object (Gray et al., 1989; Neuenschwander and Singer, 1996).
Based on these observations, it was proposed that such coherent firing is responsible for coding information that is critical for global object perception. Gray C.M., discusses and strongly advocates for the hypothesis in his review (Gray, 1999). However, whether coherent firing exhibited by long range RGCs in the retina in response to a contiguous stimulus is a crucial introductory step for global object perception remained speculative.

We therefore examined whether pharmacological blockade of gap junctions or deletion of Cx36, both of which we found eliminated long-range synchronous activity

![Fig. 20. Retrograde labeling shows that efficacy of MFA to block gap junctional coupling between RGCs and ACs lasts for 7 days. (a) Flat mount view of the proximal INL shows ACs labeled with NB derived from gap junctions made with RGCs; RGCs were initially labeled retrogradely by NB injection into the optic nerve. Scale bar = 100µm. (b-e) Flat mount view of the proximal INL at different time points after intravitreal injection of MFA and NB injection into the optic nerve (b, 3 days; c, 5 days; d, 7 days; e, 9 days). Data show that MFA eliminates coupling between RGCs and ACs for up to 7 days after intravitreal injection. (f) Number of ACs labeled with NB at different time points after MFA and NB application. Measures were made within the proximal INL from 600 x 600 µm areas in each of four retinal quadrants and averaged (n = 3 retinas). One-way ANOVA with Tukey’s multiple comparison test; ***P <0.001.](image-url)
between distant RGCs, attenuated an animal’s ability to discriminate a contiguous
global object from smaller discontinuous stimuli. In initial experiments we trained
CxWT mice on a Y water maze global task to discriminate a solid rectangle from one
in which the rectangle was divided into two parts separated by 1-20° of visual angle
(Fig. 8a). We then made bilateral intraocular injections of MFA into the mice and
retested them. In control experiments, we found that intraocular injections with MFA
uncoupled RGCs from ACs cells for up to 7 days and so animals were retested within
one week after the MFA injections (Fig. 20a-f). In complementary experiments, we
trained Cx36−/− on the Y maze test and then compared their discrimination ability to
that of CxWT littermates. We found that CxWT control animals could readily discrim-
inate contiguous rectangles from two rectangles that were separated by as little as 2°
of visual angle, but failed the discrimination test when the separation was reduced to

![Figure 21](image.png)

**Fig. 21.** Blockade of retinal gap junctions or genetic deletion of Cx36 reduc-
eses the global object perception of mice compared to their wild types

littermates. (a) Cumulative behavioral data from WT and Cx36−/− (n = 5 animals for
each group) and WT mice intravitreally injected with MFA (WT + MFA; n = 5 mice for
20°, 5°, 2°; n = 3 mice for 10°, 7°, 1.5°, 1°). The bar graph shows significant attenu-
ation of global object perception in CX36−/− and WT + MFA animals compared to WT
mice. Threshold was taken as 75% correct (dashed line). One-way ANOVA with
Tukey’s multiple comparison test; **P < 0.01; ***P < 0.001. (b) Spatial acuity tested for
the same mice as in (a) using sinusoidal gratings of 0.1, 0.3, and 0.5 cycles/degree
(cpnd) showed no attenuation in vision between WT, WT + MFA, and Cx36−/− mice.
One-way ANOVA with Tukey’s multiple comparison test. α = 0.05 for all analyses in
the figure. Data presented as mean ± s.e.m.
1.5° or 1.0° degrees (Fig. 21a). However, both MFA-injected CxWT mice and Cx36−/− mice did significantly worse than control mice on the Y water maze task as they were unable to reliably discriminate a contiguous rectangle from two rectangles separated by 7° or less (Fig. 21a). In a final set of experiments we examined whether the reduced discrimination ability of the MFA-injected CxWT and Cx36−/− mice compared to control animals may have reflected a general attenuation in spatial acuity. Animals were therefore trained on the Y water maze to discriminate sinusoidal gratings with different spatial frequencies from a solid grey stimulus (Prusky et al., 2000) (Fig. 8b). We found no difference between CxWT, MFA-injected CxWT, or Cx36−/− mice in their ability to discriminate spatial frequencies of 0.1-0.5 cycles/degree (cpd), which corresponded to the normal spatial acuity of C57BL/6 mice (Prusky et al., 2000) (Fig. 21b).
4. Discussion

The results of the present study add to the growing list of functional roles played by gap junctions and electrical synaptic transmission in the retina. Specifically, our data indicate that electrical coupling between RGCs and PACs, which express Cx36, form the circuitry responsible for long-range coherent activity between widely separated RGCs as first demonstrated in the cat retina and LGN (Neuenschwander and Singer, 1996). Further, the data from behavioral experiments provide strong evidence that long-range coherent activity of RGCs provide information to higher brain centers that is critical for the perception of global objects and thus forms an important part of signal processing in the visual system.

4.1 Role of electrical synapse in generating coherent activity in the central nervous system and the retina.

Coherent activity is a ubiquitous phenomenon exhibited by large neuronal ensembles in the nervous system. Studies spanning decades have elucidated that synchronous firing between neurons is integral to a number of high level brain functions, including perception, memory and object recognition (Connors, 2017; Hormuzdi et al., 2004). Neurons can be considered as biological oscillators (Stiefel and Ermentrout, 2016) and electrical coupling between these oscillators result in an interactive network that can generate simple or complex signals. Concerted neural activity forms the clearest function executed by electrical synapses (Bennett and Zukin, 2004; Connors and Long, 2004). Examples from all levels of vertebrate nervous system show that synchrony by means of electrical synapses is a universal phenomenon exhibited by all neurons in the CNS (Galarreta and Hestrin, 1999; Gibson et al., 1999) including the retina (Trenholm et al., 2014; Veruki and Hartveit, 2002). Numerous studies have demonstrated that synchronous activity in the nervous system convey signals that
are indispensible for efficient processing of the nervous system (Blatow et al., 2003; Draguhn et al., 1998; Hormuzdi et al., 2001; Neuenschwander and Singer, 1996; Ylinen et al., 1995a; Ylinen et al., 1995b).

The RGC neighbors form an excellent example of neurons that display extensive coherent activity that can be both dependent and independent of light stimulation (Brivanlou et al., 1998; DeVries, 1999; Hu et al., 2010; Meister et al., 1995). Concerted activity in the retina can account for up to one half of all the retinal spikes conveyed to the central targets, suggesting that correlated spikes form a mechanism for encoding visual information that is independent from and multiplexed with that encoded by uncorrelated activity (Castelo-Branco et al., 1998; Schnitzer and Meister, 2003). Still, the exact role(s) played by the correlated activity of RGCs has remained elusive due to the paucity of studies evaluating their role in shaping the responses of central target neurons.

In addition to the correlated activity of RGC neighbors, long-range coherence between widely separated RGCs was first demonstrated in an elegant study in the cat retina (Neuenschwander and Singer, 1996), from which it was speculated that the correlated signals from the retina are efficiently transmitted to the LGN and visual cortex and thereby encode information crucial for perception and binding of global objects (Gray et al., 1989; Neuenschwander and Singer, 1996). However, the neural circuitry responsible for such long range coherence remained undetermined over decades. There had been speculations that electrical synapses between RGCs and PACs could play a role in mediating long range inhibition or excitation between the RGCs (Demb et al., 1999; Kenyon et al., 2003), but clear direct evidence was lacking. The present results provide the first ever direct evidence that corroborates these claims. The experiments performed here strongly suggest that electrical coupling between RGCs and PACs is responsible for long-range coherent firing between the RGCs. As mentioned in the introduction section, synchronous activity of RGCs driven by a common
excitatory input results in a medium width CCP with a unimodal peak. Indeed the CCPs generated in the present study between pairs of widely separated α-RGCs were unimodal with a temporal width of 10ms, consistent with previous descriptions of medium width correlations (Brivanlou et al., 1998). The present data are thus in agreement with the idea that medium width CCPs reflect an excitatory drive from a common interneuron. In this case, the common interneuron is the PACs, because it is the only cell type in the retina that has extensive arbors spanning >1mm, and the excitatory drive is derived from the electrical synapses formed with the RGCs. Specifically, my findings show that gap junctions expressing Cx36, which couple RGCs and PACs (Pan et al., 2010; Schubert et al., 2005; Völgyi et al., 2005; Völgyi et al., 2013), form the portals by means of which excitation from intermediary PACs can synchronize activity between distant RGCs to which they couple. Thus, blockade of gap junctions or genetic ablation of Cx36 effectively abolished the long-range correlated spike activity between RGCs. Overall, this study together with previous studies in the brain, including the inferior olivary (Benardo and Foster, 1986; Crill, 1970; Llinas and Yarom, 1981; Mathy et al., 2009) and cerebral cortex (Butovas et al., 2006; Deans et al., 2001) depicts that electrical synapse plays a major role in shaping the synchronous activity of the neurons, which encode and transmit information indispensible for efficient processing of the nervous system.

4.2 Role of PACs in long range correlation between widely separated RGCs.

PACs are a unique subtype of WFACs with both dendritic and axonal systems and since they form electrical synapses with RGCs (Völgyi et al., 2009), they are the most plausible candidate for transmission of long range signals (Demb et al., 1999; Kenyon et al., 2003). Although we restricted our study only to the α-RGCs in the Kcng4-YFP mouse line, many other RGC subtypes across a variety of species are
tracer coupled to PACs (Greschner et al., 2014; Stafford and Dacey, 1997; Völgyi et al., 2005; Völgyi et al., 2009; Völgyi et al., 2001; Wright and Vaney, 2004). It is therefore likely that long range interactions between RGCs are a common facet of signal processing in the retina, including the primates.

In addition to excitatory transmission through electrical synapses, PACs are inhibitory interneurons that likely release GABA (Kalloniatis et al., 1996; Marc and Jones, 2002; Pang et al., 2013; Wassle et al., 1990). Hence, PACs may also play a role in long-range inhibitory interactions. Indeed, PACs have been implicated as role players in the peripheral or shift effects that extend beyond the classic center/surround receptive fields of RGCs (Demb, 2008; Derrington et al., 1979; McIlwain, 1964). Thus, PACs may play dual, opposing roles with chemical synaptic output providing inhibition and electrical synapses delivering direct excitation to distant RGCs. In contrast, a computational modeling study suggested that both the inhibition and excitation provided by PACs may play a complementary role in synchronizing the oscillatory activity of the RGCs (Kenyon et al., 2003). While the present results clearly show that excitatory outputs from PACs via gap junctions are critical for long-range synchrony of distant RGC activity, whether inhibitory outputs may play a role needs to be evaluated in future studies.

Anatomical and physiological studies have shown that different subtypes of PACS exist in rabbit (Famiglietti, 1992a, b, c; Völgyi et al., 2001), mouse (Lin and Masland, 2006) and primates (Davenport et al., 2007). Whether all or only a subset of the PAC subtypes plays a role in long-range coherent activity is presently unclear.

4.3 Emergence of long range coherence in the retina has a threshold in terms of stimuli separation.

We found that the emergence of coherence is not linearly related to the space
separation of the two rectangular light stimuli, but rather showed a clear threshold as
the rectangles were incrementally enlarged towards one another and ultimately fused.
These data are consistent with a computational model of PAC innervation of RGCs,
which predicted that synchronous activity would fall off sharply as a solid object span-
ning RGC pair receptive fields was separated (Kenyon et al., 2004). However, the un-
derlying mechanism for this threshold phenomenon is unclear. One plausible scenario
is that an inhibitory mechanism, possibly provided by PACs, prevents the correlated
activity, and there is a release from inhibition when a contiguous object is presented.
Computational models of retinal RGCs and the pyramidal cells of the visual cortex
have supported such a disinhibitory scheme (Bush and Sejnowski, 1996; Kenyon and
Marshak, 1998). Clearly, future studies are necessary to elucidate the mechanism
underlying the threshold characteristic of long-range coherent activity of RGCs.

Interestingly, we found that, presentation of stimuli to an intermediary zone
between the RGC pairs did not evoke long-range coherence; thus, the RGCs could
not be “fooled” to show coherence to a small, centered stimulus. Synchronous activi-
ty emerged only when the stimulus was extended to cover at least partially the recep-
tive field of the RGCs and the intermediate AC. Thus, combined activation of RGCs
together with stimulation of the coupled PACs was necessary to generate coherent
RGC firing. These data suggest that coherent activity between RGCs cannot occur
until the RGCs are “primed” by stimulation of their receptive fields together with ac-
tivation of the intermediary PACs. This priming likely leads to the activation of excit-
atory drive from independent cohorts of bipolar cells that is presynaptic to each of the
widely separated RGCs. This scenario suggest that a combination of excitatory drive
from chemical and electrical synapses play a role in long-range coherent activity, an
idea that has been advanced for coherent activity between RGC nearest neighbors
(Hu et al., 2010; Trenholm et al., 2014).
### 4.4 Serial coupling between RGCs is not sufficient for long range coherent activity.

Since many subtypes of RGCs are directly coupled to one another, we tested whether this type of circuit could also create long-range coherent activity. However, we found that distant OFF α-RGCs in Cx36\(^{-/-}\) mice, which are only coupled to each other homologously, did not show long-range coherence. Yet the OFF α-RGCs in wild type animals, which show direct coupling and coupling to PACs, showed clear increased correlated activity in response to a global stimulus. These results are consistent with tracer coupling studies, which showed that NB injected into single RGCs will diffuse only across first-tier gap junctions and thereby label only the nearest neighbor RGCs (Hu and Bloomfield, 2003; Hu et al., 2010; Völgyi et al., 2009; Xin and Bloomfield, 1997). The limited tracer movement is thought to reflect the relatively low conductance of neuronal gap junctions in the inner retina, which limits the lateral spread of visual signals that would otherwise result in unwanted blurring of the image. While direct RGC-RGC coupling does not play a role in long-range coherence, it is clear that coupling between RGC neighbors does provide for their coherent activity (Brivanlou et al., 1998; Hu and Bloomfield, 2003; Mastronarde, 1983a, b, c; Meister et al., 1995). These observations suggest that direct RGC-RGC coupling play a role in processing of local visual signals, possibly important for encoding features like spatial resolution or fine details within objects. Interestingly, it appears that Cx45, not Cx36, is the predominant connexin expressed by gap junctions that link RGC neighbors. Thus the electrical synapses formed between RGCs likely have different biophysical properties and are regulated by different molecular signals than those formed between RGCs and PACs.
4.5 Spatial acuity of RGCs appears to be superior to that of the animal.  

As described above, our results showed that emergence of coherent firing is not linear, but clearly has a threshold when separate rectangular light stimuli are extended towards each other in discreet steps until they fused. In these sets of experiments we initially placed a pair of rectangular light stimuli each on the soma of a RGC separated by 12°-24° of visual angle. We then gradually expanded the rectangles towards each other while maintaining a separation of 100µm, 50µm, 20µm and finally 0µm (fused). We found that the RGCs did not show any significant increase in correlated firing until a separation of 20µm (the smallest separation possible using our stimulus software) which corresponds to ~0.7 degree of visual angle. Correlated spike activity drastically increased when the rectangles were fused, indicative of a clear threshold in emergence of the correlation.

In contrast, in the behavioral experiments where mice discriminated between two separate rectangles from a single contiguous one, the WT mice could readily distinguish 2° of separation between the disconnected rectangles but failed to perform the task when the separation between the two rectangles were reduced to 1.5°. Surprisingly, a comparison between the results show that the RGCs could discriminate discontinuities in objects, as assayed from activity coherence, with better precision than the animals could on perceptual tasks. While this difference could reflect general issues related to animal’s performance on the Y water maze task (Prusky et al., 2000), as well as lack of anterior optics, we did find that spatial acuity measured from maze were comparable to values obtained electrophysiologically (Porciatti et al., 1999). These data are consistent with the hypothesis that, with the enormous sensory information generated, not all signals reach the brain, but rather bottlenecks are utilized to limit transmission of only the most important details (Gray, 1999).
4.6 Response of RGCs to a variety of complex stimuli patterns.

We used rectangular, ovoid and elliptical rainbow stimulus patterns to compare the coherent RGC activity in response to contiguous vs. disjointed objects. However, we found that widely separated RGCs responded similarly to all these stimulus patterns; they showed concerted activity as long as the stimulus was contiguous and covered the receptive fields of both the RGCs along with the intermediate PAC. It is important to note that in case of the rainbow stimulus, the PAC(s) in the intermediate zone directly in the plane between the RGC pairs that was directly coupled to them was likely not stimulated, yet the RGCs showed concerted firing. This likely reflects the fact that PACs show extensive coupling with one another (Davenport et al., 2007). Thus, while the rainbow stimulus illuminated PACs above or below the intermediate plane, their signals would be conveyed to coupled PACs within the plane through their interconnecting electrical synapses. Therefore, we presume that although the intermediate PAC is not stimulated directly, when other PACs coupled to it are stimulated, it is sufficient to evoke correlated activity.

Future studies should be performed to determine RGC responses to more complicated stimuli. Additionally, analogous experiments using rectangular dark bars on light background (e.g. black bars on white background) should be used to target the OFF RGCs particularly. The responses of OFF RGCs to flashing dark bars on a light background would allow us to evaluate if a contiguous dark bar similar to a light bar is also able to evoke correlated firing from widely separated OFF RGCs.

It has been reported that presentation of an overhead looming object over a mouse or other small animals results in a scurrying behavior whereby the mouse moves to a shelter within their cage as a mechanism of self-defense (Yilmaz and Meister, 2013). A looming object mimics a contiguous global stimulus, as it encompasses a large area in the visual field. Hence, it will be interesting to examine if widely separated RGCs also respond coherently to looming objects provided it covers their
entire receptive field and the area between them. In the event of a positive result, it would be interesting to conduct behavioral experiments in order to determine if eliminating this correlated activity also affects the scurrying behavior otherwise demonstrated by mouse. If so, this result would indicate that long-range synchronous activity between RGCs is also key to a self-defense mechanism in rodents.

It would also be interesting to extend this study to examine coherent activity of RGCs to natural scenes (Roska and Werblin, 2003). Natural scenes have more spatial frequencies and the performance of RGCs could be evaluated to determine the parameters of global objects that can evoke long-range concerted firing.

4.7 Behavioral data suggest that global object perception is reduced in mice following gap junction blockade or Cx36 ablation.

Two independent approaches were taken in the behavioral experiments using the Y-water maze to determine whether gap junction obstruction and the resulting loss of long-range correlated activity effectively abolished the perception of global objects. In the first approach, we used Cx36\(^{-/-}\) mice and evaluated their ability to discriminate contiguous global vs. disjointed objects and compared this to the performance of their wild type littermates. Indeed, we found that the performance of the Cx36\(^{-/-}\) mice was significantly poorer than that of the wild types. While the wild type mice could discriminate a contiguous rectangle from two rectangles separated by a little as 2° degrees, the Cx36\(^{-/-}\) failed the discrimination task at 5° degrees of separation. Since the Cx36\(^{-/-}\) mouse we used in our experiments was a global knock out and thus the results could reflect effects of Cx36-expressing gap junctions in central targets (Parenti et al., 2000; Teubner et al., 2000), we also performed intraocular MFA injections to localize the blockade of gap junctions to the retina. The results with MFA paralleled to that found for the Cx36\(^{-/-}\) mice in terms of the reduced ability to discriminate global from disjoint-
ed objects as compared to control animals. Thus, using the same stimulus paradigms and gap junction manipulation techniques, we found strong agreement between the electrophysiological and behavioral data. Taken together, these results provide strong support for the conclusion that long-range correlated activity of RGCs created by RGC-PAC electrical coupling plays a role in encoding information critical for global object perception. Still, MFA is a general gap junction blocker and so certainly other gap junctions, which can express different connexins in the retina were also affected and so the conclusion drawn must be considered with this caveat.

4.8 Decoding of signals in the cortex for perception of global objects after being encoded by the retina.

Overall, our results are consistent with the idea that synchronous activity in retina supports perceptual grouping at the cortical level as suggested by earlier studies of retina, LGN, and visual cortex (Gray, 1999; Gray et al., 1989; Gray and Singer, 1989; Neuenschwander and Singer, 1996; Singer, 1999; Singer and Gray, 1995). However, studies in which recordings were made from neurons in the visual cortex while monkeys performed a perceptual grouping task suggested that perceptual grouping is achieved by enhancement of the mean firing rate of neurons rather than synchrony (Gilad and Slovin, 2015; Roelfsema et al., 2004). Likewise, psychophysical experiments supported the idea that synchronous firing, at least at the cortical level, is not responsible for perceptual grouping (Kiper et al., 1996).

While these data would appear to conflict with the present findings, it is important to emphasize that the mechanism by which the retina encodes visual information may be different than the mechanism by which it is decoded in the cortex. Thus, while the present results indicate that synchronous spikes generated in the retina provide information to central targets critical to global object recognition, the decoding
process may give rise to a different type of neural code at the cortical level with differing temporal and rate properties (Ainsworth et al., 2012).
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6. Appendix

6.1 Codes for generating the electrophysiology stimulus: discreet vs. global object.

Box Move Main

/*
 * Decompiled with CFR 0_101.
 */

package hu.javaportal.boxmove;

import hu.javaportal.boxmove.DrawerBasic;
import java.awt.Color;
import java.awt.Dimension;
import java.awt.Graphics;
import java.awt.GraphicsDevice;
import java.awt.GraphicsEnvironment;
import java.awt.Point;
import java.awt.Rectangle;
import java.awt.Toolkit;
import java.awt.Window;
import java.awt.event.KeyAdapter;
import java.awt.event.KeyEvent;
import javax.swing.JFrame;

public class BoxMoveMain extends JFrame {
    private final DrawerBasic drawer;
    
    public BoxMoveMain() {
        drawer = new DrawerBasic();
        
        // Additional code...
    }

    public static void main(String[] args) {
        new BoxMoveMain();
    }

    // Override methods...
}


public BoxMoveMain(Dimension dim, String[] args) {
    this.drawer = new DrawerBasic(dim, this);
    this.addKeyListener(new KeyAdapter() {

        @Override
        public void keyPressed(KeyEvent e) {
            BoxMoveMain.this.drawer.keyboardHandler(e);
        }
    });

    }

    }

    @Override
    public void paint(Graphics g) {
        this.update(g);
    }

    }

    @Override
    public void update(Graphics g) {
        g.setColor(Color.BLACK);
        g.fillRect(0, 0, this.getWidth(), this.getHeight());
        this.drawer.paint(g);
    }

    public static void main(String[] args) {
        Toolkit tk = Toolkit.getDefaultToolkit();
        BoxMoveMain fr = new BoxMoveMain(tk.getScreenSize(), args);
        fr.setDefaultCloseOperation(3);
        fr.setBounds(new Rectangle(new Point(0, 0), tk.getScreenSize()));
        fr.setUndecorated(true);
        fr.setVisible(true);
    }
fr.setResizable(false);
if (!fr.isDisplayable()) {
    fr.setUndecorated(true);
}

GraphicsDevice gd = GraphicsEnvironment.getLocalGraphicsEnvironment().getDe-
defaultScreenDevice();
try {
    if (gd.isFullScreenSupported()) {
        gd.setFullScreenWindow(fr);
    }
}
finally {
    gd.setFullScreenWindow(null);
}

Column Drawer

/*
 * Decompiled with CFR 0_101.
 */
package hu.javaportal.boxmove;

import hu.javaportal.boxmove.Drawer;
import java.awt.BasicStroke;
import java.awt.Color;
import java.awt.Component;
import java.awt.Dimension;
import java.awt.Graphics;
import java.awt.Graphics2D;
import java.awt.Point;
import java.awt.RenderingHints;
import java.awt.Stroke;
import java.awt.event.KeyEvent;

public class ColumnDrawer
extends Drawer {
    public Point position = new Point(0, 0);
    public int width = 60;
    public Stroke stroke = null;
    public int angle = 0;
    private int lineSize;

    public Stroke getStroke() {
        this.stroke = new BasicStroke(this.width * 2);
        return this.stroke;
    }

    public ColumnDrawer(Dimension dim, Component component) {
        super(dim, component);
        this.lineSize = (int)Math.ceil(Math.sqrt(Math.pow(this.getDimension().width, 2.0) + Math.pow(this.getDimension().height, 2.0)));
        this.clear();
    }

    @Override
    public void handleKey(KeyEvent e) {
        super.handleKey(e);
        switch (e.getKeyCode()) {
            case 37: {
            
            } 
        }
    }
}
if (this.position.x - this.width * 2 < 0) break;
this.position.x-=this.width;
break;
}

case 39: {
    if (this.position.x + this.width * 2 > this.getDimension().width) break;
    this.position.x+=this.width;
    break;
}

case 38: {
    if (this.position.y - this.width * 2 < 0) break;
    this.position.y-=this.width;
    break;
}

case 40: {
    if (this.position.y + this.width * 2 > this.getDimension().height) break;
    this.position.y+=this.width;
    break;
}

case 80: {
    if (this.width >= this.getDimension().height / 2) break;
    this.width+=10;
    this.stroke = new BasicStroke(this.width);
    break;
}

case 79: {
    if (this.width <= 10) break;
    this.width-=10;
    this.stroke = new BasicStroke(this.width);
    break;
```java
    @Override
    public void paint(Graphics g) {
        Graphics2D g2 = (Graphics2D) g;
        g2.setRenderingHint(RenderingHints.KEY_ANTIALIASING, RenderingHints.VALUE_ANTIALIAS_OFF);
        g2.setStroke(this.getStroke());
        g2.setColor(this.getCurrentColor());
        int startX = this.position.x;
        int startY = this.position.y;
        double angleNormal = Math.toRadians(this.angle);
        int x1 = (int)((double)startX + (double)this.lineSize * Math.sin(angleNormal));
        int y1 = (int)((double)startY + (double)this.lineSize * Math.cos(angleNormal));
        int x2 = startX;
        int y2 = startY;
        g2.drawLine(x1, y1, x2, y2);
    }

    @Override
    public void clear() {
```
this.position.x = this.getDimension().width / 2;
this.position.y = 0;
}

public Point getPosition() {
    return this.position;
}

public void setPosition(Point position) {
    this.position = position;
}

public int getAngle() {
    return this.angle;
}

public void setAngle(int angle) {
    this.angle = angle;
}

public int getWidth() {
    return this.width;
}

public void setWidth(int width) {
    this.width = width;
}

public int getLineSize() {
    return this.lineSize;
public void setLineSize(int lineSize) {
    this.lineSize = lineSize;
}

public abstract class Drawer {
    private String intensity = "FF";
    private Color color;
    private final Dimension dimension;
    private Component component;
    private COLOR currentColor = COLOR.GREEN;

    public void handleKey(KeyEvent e) {
        switch (e.getKeyCode()) {
            case 57: {
                // Code continues here
            }

        }
    }
}
```javascript
this.currentColor = this.currentColor == COLOR.GREEN ? COLOR.WHITE : COLOR.GREEN;
    break;
}

case 48: {
    this.intensity = "00";
    break;
}

case 49: {
    this.intensity = "20";
    break;
}

case 50: {
    this.intensity = "40";
    break;
}

case 51: {
    this.intensity = "60";
    break;
}

case 52: {
    this.intensity = "80";
    break;
}

case 53: {
    this.intensity = "A0";
    break;
}

case 54: {
    this.intensity = "C0";
```
break;
}

case 55: {
    this.intensity = "E0";
    break;
}

case 56: {
    this.intensity = "FF";
}

if (this.currentColor == COLOR.GREEN) {
    this.setColor(Color.decode("0x00" + this.intensity + "00"));
} else {
    this.setColor(Color.decode("0x" + this.intensity + this.intensity + this.intensity + this.intensity));
}

}

public abstract void paint(Graphics var1);

public abstract void clear();

public Color getCurrentColor() {
    return this.color;
}

public void setColor(Color color) {
    this.color = color;
}

public Drawer(Dimension dim, Component component) {

this.dimension = dim;
this.component = component;
this.setColor(Color.decode("0x00FF00"));
}

public Dimension getDimension() {
    return this.dimension;
}

private static enum COLOR {
    GREEN,
    WHITE;

    private COLOR(String string2, int n2) {
    }
}

Drawer Basic

/*
 * Decompiled with CFR 0_101.
 */
package hu.javaportal.boxmove;

import hu.javaportal.boxmove.ColumnDrawer;
import hu.javaportal.boxmove.Drawer;
import hu.javaportal.boxmove.TwoBoxDrawer;
import java.awt.Component;
public class DrawerBasic
implements ActionListener {
    private Mode currentDrawer = Mode.COLUMN;
    private Map<Mode, Drawer> modes = new HashMap<Mode, Drawer>();
    private JFrame frame;
    private boolean currentStateShow = true;
    private int currentBlinkDelay = 500;
    private int currentBlinkWait = 1000;
    private boolean currentStateBefore = false;
    private final Timer blinkTimer;

    public void keyboardHandler(KeyEvent e) {
        switch (e.getKeyCode()) {
            case 32: {
                if (this.currentDrawer == Mode.COLUMN) {
                    this.currentDrawer = Mode.TWOBOX;
                } else {
                    TwoBoxDrawer box = (TwoBoxDrawer) this.modes.get((Object) Mode.TWOBOX);
                    double x = box.getBox2().x - box.getBox1().x;
                }
            }
        }
    }
}
double y = box.getBox2().y - box.getBox1().y;

Double beta = Math.toDegrees(1.5707963267948966 - Math.atan(y / x));

int width = box.getBiggestBoxWidth();

ColumnDrawer cbox = (ColumnDrawer)this.modes.get((Object)Mode.COLUMN);

    cbox.setAngle(beta.intValue());

    cbox.setPosition(new Point(box.getBox2().x, box.getBox2().y + width));

    cbox.setWidth(width);

    int lineSize = (int)Math.round(Math.sqrt(x * x + y * y));

    cbox.setLineSize(- lineSize);

    this.currentDrawer = Mode.COLUMN;

    if (!this.blinkTimer.isRunning()) break;

    this.blinkTimer.stop();

    this.currentStateShow = true;

    break;

    }

    case 27: {

    this.frame.setVisible(false);

    this.frame.dispose();

    break;

    }

    case 66: {

    if (this.blinkTimer.isRunning()) {

    this.currentStateShow = true;

    this.blinkTimer.stop();

    } else {

    this.currentBlinkDelay = 1000;

    this.currentBlinkWait = 500;

    this.blinkTimer.start();

    }

    }
this.frame.repaint();
break;
}
case 74: {
    if (this.currentBlinkDelay <= 100) break;
    this.currentBlinkDelay-=100;
    break;
}
case 75: {
    this.currentBlinkDelay+=100;
    break;
}
case 78: {
    if (this.currentBlinkWait <= 100) break;
    this.currentBlinkWait-=100;
    break;
}
case 77: {
    this.currentBlinkWait+=100;
    break;
}
case 77: {
    this.currentBlinkWait+=100;
    break;
}
default: {
    this.modes.get((Object)this.currentDrawer).handleKey(e);
}
}
if (!this.blinkTimer.isRunning()) {
    this.frame.repaint();
}
}
public void paint(Graphics g) {
    if (this.blinkTimer.isRunning() && this.currentStateShow != this.currentStateBefore) {
        if (this.currentStateShow) {
            this.modes.get((Object)this.currentDrawer).paint(g);
            this.blinkTimer.setDelay(this.currentBlinkDelay);
        } else {
            this.blinkTimer.setDelay(this.currentBlinkWait);
        }
        this.currentStateBefore = this.currentStateShow;
    } else {
        this.modes.get((Object)this.currentDrawer).paint(g);
    }
}

public DrawerBasic(Dimension dim, JFrame frame) {
    this.blinkTimer = new Timer(this.currentBlinkDelay, this);
    this.frame = frame;
    this.blinkTimer.setInitialDelay(0);
    this.modes.put(Mode.COLUMN, new ColumnDrawer(dim, frame));
    this.modes.put(Mode.TWOBOX, new TwoBoxDrawer(dim, frame));
}

@Override
public void actionPerformed(ActionEvent e) {
    this.frame.repaint();
    this.currentStateShow = !this.currentStateShow;
}

static enum Mode {
    COLUMN,
}
private Mode(String string2, int n2) {
}

Two Box Drawer

/*
 * Decompiled with CFR 0_101.
 */

package hu.javaportal.boxmove;

import hu.javaportal.boxmove.Drawer;
import java.awt.Color;
import java.awt.Component;
import java.awt.Dimension;
import java.awt.Graphics;
import java.awt.Graphics2D;
import java.awt.Point;
import java.awt.RenderingHints;
import java.awt.event.KeyEvent;

public class TwoBoxDrawer extends Drawer {
    private final Point box1 = new Point();
    private final Point box2 = new Point();
    private boolean box1Visible = true;
private boolean box2Visible = true;
private Dimension boxDimension1 = new Dimension(60, 60);
private Dimension boxDimension2 = new Dimension(60, 60);
private boolean firstBox = true;

public int getBiggestBoxWidth() {
    return Math.max(this.boxDimension1.width, this.boxDimension2.width);
}

public Point getBiggestBox() {
    if (this.boxDimension1.width >= this.boxDimension2.width) {
        return this.box1;
    }
    return this.box2;
}

public Point getSmallestBox() {
    if (this.boxDimension1.width >= this.boxDimension2.width) {
        return this.box2;
    }
    return this.box1;
}

public Dimension getBoxDimension() {
    if (this.firstBox) {
        return this.boxDimension1;
    }
    return this.boxDimension2;
}
public TwoBoxDrawer(Dimension dim, Component component) {
    super(dim, component);
    this.clear();
}

private Point getBox() {
    if (this.firstBox) {
        return this.box1;
    }
    return this.box2;
}

@Override
public void handleKey(KeyEvent e) {
    super.handleKey(e);
    switch (e.getKeyCode()) {
    case 88: {
        this.firstBox = !this.firstBox;
        break;
    }
    case 37: {
        if (this.getBox().x - this.getBoxDimension().width * 2 < 0) break;
        this.getBox().x-=this.getBoxDimension().width;
        break;
    }
    case 39: {
        if (this.getBox().x + this.getBoxDimension().width * 2 > this.getDimension().width)
            break;
        this.getBox().x+=this.getBoxDimension().width;
        break;
    }
    case 39: {
        if (this.getBox().x + this.getBoxDimension().width * 2 > this.getDimension().width)
            break;
        this.getBox().x+=this.getBoxDimension().width;
        break;
    }
case 38: {
    if (this.getBox().y - this.getBoxDimension().height < 0) break;
    this.getBox().y-=this.getBoxDimension().height;
    break;
}

case 40: {
    if (this.getBox().y + this.getBoxDimension().height * 2 > this.getDimension().height)
        break;
    this.getBox().y+=this.getBoxDimension().height;
    break;
}

case 80: {
    if (this.getBoxDimension().height >= this.getDimension().height / 2) break;
    this.getBoxDimension().width+=10;
    this.getBoxDimension().height+=10;
    break;
}

case 79: {
    if (this.getBoxDimension().height <= 10) break;
    this.getBoxDimension().width-=10;
    this.getBoxDimension().height-=10;
    break;
}

case 81: {
    this.box1Visible = !this.box1Visible;
    break;
}

case 87: {
    this.box2Visible = !this.box2Visible;
}
@Override
public void paint(Graphics g) {
    Graphics2D g2 = (Graphics2D)g;
    g2.setRenderingHint(RenderingHints.KEY_ANTIALIASING, RenderingHints.VALUE_ANTIALIAS_OFF);
    g2.setColor(this.getCurrentColor());
    if (this.box1Visible) {
        g2.fillRect(this.box1.x - this.boxDimension1.width, this.box1.y, this.boxDimension1.width * 2, this.boxDimension1.height * 2);
    }
    if (this.box2Visible) {
        g2.fillRect(this.box2.x - this.boxDimension2.width, this.box2.y, this.boxDimension2.width * 2, this.boxDimension2.height * 2);
    }
}

@Override
public void clear() {
    int width = this.getDimension().width / 4;
    int height = this.getDimension().height / 2;
    this.box1.setLocation(width, height);
    this.box2.setLocation(width * 3, height);
}

public Point getBox1() {
    return this.box1;
}
6.2 Codes for generating electrophysiology stimulus: ellipse.

```matlab
f1 = figure('units','Pixels','Position',[200 200 800 600]);
set(f1, 'menubar', 'none','NumberTitle','off');
% the flashing block on frequency
    t = timer;
    set(t, 'executionMode', 'fixedRate');
    freq = 2;
    period = 1/freq;
    set(t, 'Period', 1/freq);
    set(t, 'TimerFcn', 'show');
%Set axis clipping off to make sure the rectangles can be moved all around
%the window otherwise the rectangles will be clipped off by where the axes
%are
    axis ([0 10 0 10]);
    axis off
    ax = gca;
    ax.Clipping = 'off';
%set the x,y,w,h variables for all the three rects. units are set in inch
    set(0,'defaultuicontrolunits','inch');
    x1 = 3;
    x2 = 4;
    y1 = 4;
    y2 = 3;
    w1 = 3;
```
w2 = 1;

h1 = 6;

h2 = 6;

show1 = rectangle('Position',[x1 y1 w1 h1],'FaceColor','w','Edgecolor','k', ...
    'Curvature',[0.5 0.5], 'Visible','off');

hide1 = rectangle('Position',[x1 y1 w1 h1],'FaceColor','k','Edgecolor','k', ...
    'Curvature',[0.5 0.5], 'Visible','off');

show2 = rectangle('Position',[x2 y2 w2 h2],'FaceColor','k','Edgecolor','k', ...
    'Curvature',[1 0.5], 'Visible','off');

hide2 = rectangle('Position',[x2 y2 w2 h2],'FaceColor','k','Edgecolor','k', ...
    'Curvature',[1 0.5], 'Visible','off');

t = hgtransform('Parent',gca);

set(show1,'Parent',t);

set(show2,'Parent',t);

degree = pi/18;

Txy = makehgtform('zrotate',degree);  % define a transform matrix

set(t,'Matrix',Txy)

% set the background and axes to black

set (gcf, 'Color', [0 0 0]);

set (gca, 'Color', [0 0 0]);

ValidKeyEntry = 1;

while ValidKeyEntry == 1;

    pause

    PressedKey = get(f1,'CurrentCharacter');

    if PressedKey == 'f';

        ValidKeyEntry = 1;

    % use 'f' key to keep the ellipses flashing

    // Play with the “Visible” property to show/hide the rectangles.
set(show1,'Visible','on');
set(show2,'Visible','on');
pause(period)

set(show1,'Visible','off');
set(hide1,'Visible','on');
set(show2,'Visible','off');
set(hide2,'Visible','on');
drawnow

pause(period)

set(hide1,'Visible','off');
set(hide2,'Visible','off');

% use 'x' to make the ellipses visible
elseif PressedKey == 'x';
    ValidKeyEntry = 1;
    set(show1,'Visible','on');
    set(show2,'Visible','on');

% use 'u' to move up together; 'd' to move down together; 'l' to move left together and 'r' to move right together
elseif PressedKey == 'u';
    ValidKeyEntry = 1;
    y1 = y1+1;
    y2 = y2+1;
    set(show1,'Position',[x1 y1 w1 h1]);
    set(show2,'Position',[x2 y2 w2 h2]);
drawnow
elseif PressedKey == 'd';
    ValidKeyEntry = 1;
y1 = y1-1;
y2 = y2-1;
set(show1,'Position',[x1 y1 w1 h1]);
set(show2,'Position',[x2 y2 w2 h2]);
drawnow
elseif PressedKey == 'l';
    ValidKeyEntry = 1;
x1 = x1-1;
x2 = x2-1;
set(show1,'Position',[x1 y1 w1 h1]);
set(show2,'Position',[x2 y2 w2 h2]);
drawnow
elseif PressedKey == 'r';
    ValidKeyEntry = 1;
x1 = x1+1;
x2 = x2+1;
set(show1,'Position',[x1 y1 w1 h1]);
set(show2,'Position',[x2 y2 w2 h2]);
drawnow

%use '2' and '3' to increase or decrease the width of show1 only
elseif PressedKey == '2';
    ValidKeyEntry = 1;
w1 = w1+0.25;
set(show1,'Position',[x1 y1 w1 h1]);
drawnow
elseif PressedKey == '3';
    ValidKeyEntry = 1;
w1 = w1-0.25;
set(show1,'Position',[x1 y1 w1 h1]);
drawnow

%use '4' and '5' to move show1 left and right individually

elseif PressedKey == '4';
    ValidKeyEntry = 1;
    x1 = x1+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

elseif PressedKey == '5';
    ValidKeyEntry = 1;
    x1 = x1-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

%use '6' and '7' to increase and decrease the width of show2 only

elseif PressedKey == '6';
    ValidKeyEntry = 1;
    w2 = w2+0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

elseif PressedKey == '7';
    ValidKeyEntry = 1;
    w2 = w2-0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

%use '8' and '9' to move show2 left or right individually

elseif PressedKey == '8';
    ValidKeyEntry = 1;
    x2 = x2+0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

elseif PressedKey == '9';
ValidKeyEntry = 1;
x2 = x2-0.25;
set(show2,'Position',[x2 y2 w2 h2]);
drawnow

%use 'o' and 'p' to increase and decrease the width of show1 and show2
%together
elseif PressedKey == 'o';
    ValidKeyEntry = 1;
    w1 = w1+0.25;
    w2 = w2+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == 'p';
    ValidKeyEntry = 1;
    w1 = w1-0.25;
    w2 = w2-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

% use 'j' and 'k' to increase and decrease the height of show1 and show2
% together
elseif PressedKey == 'j';
    ValidKeyEntry = 1;
    h1 = h1+0.25;
    h2 = h2+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == 'k';
    ValidKeyEntry = 1;
    h1 = h1-0.25;
    h2 = h2-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
    % use 'c' and 'v' to rotate the ellipses left or right respectively
elseif PressedKey == 'c';
    ValidKeyEntry = 1;
    degree = degree+pi/18;
    t = hgtransform('Parent',gca);
    set(show1,'Parent',t);
    set(show2,'Parent',t);
    Txy = makehgtform('zrotate',degree);  % define a transform matrix
    set(t,'Matrix',Txy)
elseif PressedKey == 'v';
    ValidKeyEntry = 1;
    degree = degree-pi/18;
    t = hgtransform('Parent',gca);
    set(show1,'Parent',t);
    set(show2,'Parent',t);
    Txy = makehgtform('zrotate',degree);  % define a transform matrix
    set(t,'Matrix',Txy)
end
end

6.3 Codes for generating electrophysiology stimulus: ovoid.

f1 = figure('units','Pixels','Position',[200 200 1280 800]);
set(f1, 'menubar', 'none', 'NumberTitle', 'off');

% the flashing block on frequency

t = timer;
set(t, 'executionMode', 'fixedRate');

freq = 1.5;
period = 1/freq;
set(t, 'Period', 1/freq);
set(t, 'TimerFcn', 'show');

% Set axis clipping off to make sure the rectangles can be moved all around
% the window otherwise the rectangles will be clipped off by where the axes
% are

axis ([0 10 0 10]);
axis off
ax = gca;
ax.Clipping = 'off';

% set the x,y,w,h variables for all the three rects. units are set in inch

set(0, 'defaultuicontrolunits', 'inch');
x1 = 3;
y1 = 4;
w1 = 3;
h1 = 6;
show1 = rectangle('Position', [x1 y1 w1 h1], 'FaceColor', 'w', 'Edgecolor', 'k', ...
   'Curvature', [1 1], 'Visible', 'off');
hide1 = rectangle('Position', [x1 y1 w1 h1], 'FaceColor', 'k', 'Edgecolor', 'k', ...
   'Curvature', [1 1], 'Visible', 'off');

% set the background and axes to black

set(gcf, 'Color', [0 0 0]);
set(gca, 'Color', [0 0 0]);

ValidKeyEntry = 1;
while ValidKeyEntry == 1;
    pause
    PressedKey = get(f1,'CurrentCharacter');
    if PressedKey == 'f';
        ValidKeyEntry = 1;
    % use ‘f’ key to keep the ellipses flashing
    %// Play with the “Visible” property to show/hide the rectangles.
        set(show1,'Visible','on');

    pause(period)

    set(show1,'Visible','off');
    set(hide1,'Visible','on');
drawnow

    pause(period)

    set(hide1,'Visible','off');

    % use ‘x’ to make the ellipses visible
elseif PressedKey == 'x';
    ValidKeyEntry = 1;
    set(show1,'Visible','on');

    % use ‘u’ to move up together; ‘d’ to move down together; ‘l’ to move
    % left together and ‘r’ to move right together
elseif PressedKey == 'u';
    ValidKeyEntry = 1;
    y1 = y1+1;
    set(show1,'Position',[x1 y1 w1 h1]);
drawnow

elseif PressedKey == 'd';
    ValidKeyEntry = 1;
    y1 = y1-1;
    set(show1,'Position', [x1 y1 w1 h1]);
    drawnow

elseif PressedKey == 'l';
    ValidKeyEntry = 1;
    x1 = x1-1;
    set(show1,'Position', [x1 y1 w1 h1]);
    drawnow

elseif PressedKey == 'r';
    ValidKeyEntry = 1;
    x1 = x1+1;
    set(show1,'Position', [x1 y1 w1 h1]);
    drawnow

%use '2' and '3' to increase or decrease the width of show1 only
elseif PressedKey == '2';
    ValidKeyEntry = 1;
    w1 = w1+0.25;
    set(show1,'Position', [x1 y1 w1 h1]);
    drawnow

elseif PressedKey == '3';
    ValidKeyEntry = 1;
    w1 = w1-0.25;
    set(show1,'Position', [x1 y1 w1 h1]);
    drawnow

%use 'o' and 'p' to increase and decrease the width of show1 and show2
%together
elseif PressedKey == '4';
    ValidKeyEntry = 1;
    h1 = h1+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

elseif PressedKey == '5';
    ValidKeyEntry = 1;
    h1 = h1-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

elseif PressedKey == 'o';
    ValidKeyEntry = 1;
    w1 = w1/1.05;
    h1 = h1/1.05;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

elseif PressedKey == 'p';
    ValidKeyEntry = 1;
    w1 = w1*1.05;
    h1 = h1*1.05;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

end
end

6.4 Codes for generating electrophysiology stimulus: three separate rectangles.

f1 = figure('units','Pixels','Position',[200 200 1280 800]);
set(f1, 'menubar', 'none','NumberTitle','off');

% the flashing block on frequency

t = timer;
set(t, 'executionMode', 'fixedRate');

freq = 2;
period = 1/freq;
set(t, 'Period', 1/freq);
set(t, 'TimerFcn', 'show');

%Set axis clipping off to make sure the rectangles can be moved all around
%the window otherwise the rectangles will be clipped off by where the axes
%are

axis ([0 10 0 10]);

axis off

ax = gca;

ax.Clipping = 'off';

%set the x,y,w,h variables for all the three rects. units are set in inch

set(0,'defaultuicontrolunits','inch');

x1 = 1;
x2 = 4;
x3 = 8;
y1 = 5;
y2 = 5;
y3 = 5;
w1 = 2;
w2 = 2;
w3 = 2;
h1 = 2;
h2 = 2;
h3 = 2;

%Draw the rectangles both show and hide in order to use them for flashing
show1 = rectangle('Position',[x1 y1 w1 h1],'FaceColor','w','Edgecolor','k','Visible','off');
hide1 = rectangle('Position',[x1 y1 w1 h1],'FaceColor','k','Edgecolor','k','Visible','off');
show2 = rectangle('Position',[x2 y2 w2 h2],'FaceColor','w','Edgecolor','k','Visible','off');
hide2 = rectangle('Position',[x2 y2 w2 h2],'FaceColor','k','Edgecolor','k','Visible','off');
show3 = rectangle('Position',[x3 y3 w3 h3],'FaceColor','w','Edgecolor','k','Visible','off');
hide3 = rectangle('Position',[x3 y3 w3 h3],'FaceColor','k','Edgecolor','k','Visible','off');

% set the background and axes to black
set (gcf, 'Color', [0 0 0]);
set (gca, 'Color', [0 0 0]);

ValidKeyEntry = 1;

while ValidKeyEntry == 1;
    pause
    PressedKey = get(f1,'CurrentCharacter');
    if PressedKey == 'f';
        ValidKeyEntry = 1;
    end

    // Play with the “Visible” property to show/hide the rectangles.
    set(show1,'Visible','on');
    set(show2,'Visible','on');
    set(show3,'Visible','on');
    pause(period)
    set(show1,'Visible','off');
    set(hide1,'Visible','on');
    set(show2,'Visible','off')
set(hide2,'Visible','on');
set(show3,'Visible','off')
set(hide3,'Visible','on');
drawnow

pause(period)

set(hide1,'Visible','off');
set(hide2,'Visible','off');
set(hide3,'Visible','off');

elseif PressedKey == 'x';
    ValidKeyEntry = 1;
    set(show1,'Visible','on');
    set(show2,'Visible','on');
    set(show3,'Visible','on');
%Move Sq 1 up/down/left and right
elseif PressedKey == 'q';
    ValidKeyEntry = 1;
y1 = y1+1;
    set(show1,'Position',[x1 y1 w1 h1]);
elseif PressedKey == 'w';
    ValidKeyEntry = 1;
y1 = y1-1;
    set(show1,'Position',[x1 y1 w1 h1]);
drawnow
elseif PressedKey == 'e';
    ValidKeyEntry = 1;
x1 = x1+1;
    set(show1,'Position',[x1 y1 w1 h1]);

120
drawnow

elseif PressedKey == 'r';
    ValidKeyEntry = 1;
    x1 = x1-1;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

%Move sq 2 up/down/left and right
elseif PressedKey == 't';
    ValidKeyEntry = 1;
    y2 = y2+1;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == 'y';
    ValidKeyEntry = 1;
    y2 = y2-1;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == 'u';
    ValidKeyEntry = 1;
    x2 = x2+1;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == 'i';
    ValidKeyEntry = 1;
    x2 = x2-1;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

elseif PressedKey == 'o';
    ValidKeyEntry = 1;
y3 = y3+1;
set(show3,'Position',[x3 y3 w3 h3]);
drawnow
elseif PressedKey == 'p';
    ValidKeyEntry = 1;
    y3 = y3-1;
    set(show3,'Position',[x3 y3 w3 h3]);
drawnow
elseif PressedKey == 'a';
    ValidKeyEntry = 1;
    x3 = x3+1;
    set(show3,'Position',[x3 y3 w3 h3]);
drawnow
elseif PressedKey == 's';
    ValidKeyEntry = 1;
    x3 = x3-1;
    set(show3,'Position',[x3 y3 w3 h3]);
drawnow

% Change the width and height of each of the rects individually
% Use numbers for width and height only increasing. For decreasing height
% use 'b', 'n', 'm'.
elseif PressedKey == '2';
    ValidKeyEntry = 1;
    w1 = w1+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
drawnow
elseif PressedKey == '4';
    ValidKeyEntry = 1;
    w2 = w2+0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
elseif PressedKey == '6';
    ValidKeyEntry = 1;
    w3 = w3+0.25;
    set(show3,'Position',[x3 y3 w3 h3]);
    drawnow
elseif PressedKey == '3';
    ValidKeyEntry = 1;
    w1 = w1-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow
elseif PressedKey == '5';
    ValidKeyEntry = 1;
    w2 = w2-0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow
elseif PressedKey == '7';
    ValidKeyEntry = 1;
    w3 = w3-0.25;
    set(show3,'Position',[x3 y3 w3 h3]);
    drawnow
elseif PressedKey == '8';
    ValidKeyEntry = 1;
    h1 = h1+0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow
elseif PressedKey == '9';
    ValidKeyEntry = 1;
    h2 = h2+0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
drawnow

elseif PressedKey == '0';
    ValidKeyEntry = 1;
    h3 = h3+0.25;
    set(show3,'Position',[x3 y3 w3 h3]);
    drawnow

elseif PressedKey == 'b';
    ValidKeyEntry = 1;
    h1 = h1-0.25;
    set(show1,'Position',[x1 y1 w1 h1]);
    drawnow

elseif PressedKey == 'n';
    ValidKeyEntry = 1;
    h2 = h2-0.25;
    set(show2,'Position',[x2 y2 w2 h2]);
    drawnow

elseif PressedKey == 'm';
    ValidKeyEntry = 1;
    h3 = h3-0.25;
    set(show3,'Position',[x3 y3 w3 h3]);
    drawnow

else
    ValidKeyEntry = 0;
end
end

6.5 Codes for generating behavioral stimulus: global object perception task.

% Clear the workspace
close all;
clear all;

% Here we call some default settings for setting up Psychtoolbox
PsychDefaultSetup(2);

% Get the screen numbers
Screen('Preference', 'SkipSyncTests', 2);
screens = Screen('Screens');

% Draw to the external screen if available
screenNumber = max(screens);
PsychImaging('PrepareConfiguration');
PsychImaging('AddTask', 'FinalFormatting', 'DisplayColorCorrection', 'SimpleGamma');

% Define black and white
white = WhiteIndex(screenNumber);
black = BlackIndex(screenNumber);

% Open an on screen window
[window, windowRect] = PsychImaging('OpenWindow', screenNumber, black);

% Get the size of the on screen window
[screenXpixels, screenYpixels] = Screen('WindowSize', window);
PsychColorCorrection('SetEncodingGamma', window, 0.43, 'FinalFormatting');

% Query the frame duration
ifi = Screen('GetFlipInterval', window);

escapeKey = KbName('ESCAPE');
sizeupKey = KbName('UpArrow');
sizedownKey = KbName('DownArrow');
condownKey = KbName('LeftArrow');
conupKey = KbName('RightArrow');
moveupKey = KbName('U');
movedownKey = KbName('D');
moveleftKey = KbName('L');
moverightKey = KbName('R');

% Get the centre coordinate of the window
[xCenter, yCenter] = RectCenter(windowRect);

pixelsperpress = 5;

% Make a base Rect of 50 by 100 pixels
baseRect = [0 0 50 25];

% Screen X positions of our three rectangles
squareXpos = [screenXpixels * 0.07 screenXpixels * 0.36 screenXpixels * 0.62 screenXpixels * 0.75 screenXpixels * 0.8];

squareYpos = [screenYpixels * 0.13 screenYpixels * 0.13 screenYpixels * 0.16 screenYpixels * 0.16 screenYpixels * 0.16];

numSquares = length(squareXpos);

% Set the colors to all white
allColors = [0.25 0.25 0.25; 0.25 0.25 0.25; 0.25 0.25 0.25];

allColors = [1 1 1];

% Sync us and get a time stamp
vbl = Screen('Flip', window);

waitframes = 1;
% Make a multiplier to modulate the size of our squares
sizeChanger = [0.5 0.5 0.6 0.6 0.6];

% Make our rectangle coordinates
allRects = nan(4, 4);

rectmult=1.01;
conadd=.15; %[0 .25 .5 .75 1];

exitDemo=false;

% Loop the animation until the escape key is pressed
while exitDemo == false

% Check the keyboard to see if a button has been pressed
[keyIsDown,secs, keyCode] = KbCheck;

if keyCode(escapeKey)
    exitDemo = true;
elseif keyCode(sizeupKey)
    sizeChanger=sizeChanger.*rectmult;
    for i = 1:numSquares
        allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i),... squareXpos(i), squareYpos(i));
    end
elseif keyCode(sizedownKey)
    sizeChanger=sizeChanger./rectmult;
    for i = 1:numSquares
        allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i),...
elseif keyCode(conupKey)

    if max(max(allColors.*(1+conadd)))>1
        %allColors=ones(size(allColors));
        else
            allColors=allColors.*(1+conadd);
    end

elseif keyCode(condownKey)

    allColors=allColors.*(1-conadd) ;
end

if keyCode(moveleftKey)

    squareXpos = squareXpos - pixelsperpress;

    for i = 1:numSquares
        allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i),...
                                             squareXpos(i), squareYpos(i));
    end
else if keyCode(moverightKey)

    squareXpos = squareXpos + pixelsperpress;

    for i = 1:numSquares
        allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i),...
                                             squareXpos(i), squareYpos(i));
    end
else if keyCode(moveupKey)

    squareYpos = squareYpos - pixelsperpress;

    for i = 1:numSquares

allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i), ...
    squareXpos(i), squareYpos(i));
end
elseif keyCode(movedownKey)
    squareYpos = squareYpos + pixelsperpress;
    for i = 1:numSquares
        allRects(:, i) = CenterRectOnPointd(baseRect .* sizeChanger(i), ...
            squareXpos(i), squareYpos(i));
    end
end
end
% Draw the rect to the screen
Screen('FillRect', window, allColors, allRects);

% Flip to the screen
vbl  = Screen('Flip', window, vbl + (waitframes - 0.5) * ifi);
end

%
% This is the cue which determines whether we exit the demo
exitDemo = false;
% Loop the animation until the escape key is pressed
while exitDemo == false
    % Check the keyboard to see if a button has been pressed
    [keylsDown, secs, keyCode] = KbCheck;

    % Depending on the button press, either move the position of the square
% or exit the demo
if keyCode(escapeKey)
    exitDemo = true;
elseif keyCode(leftKey)
    squareX = squareX - pixelsPerPress;
elseif keyCode(rightKey)
    squareX = squareX + pixelsPerPress;
elseif keyCode(upKey)
    squareY = squareY - pixelsPerPress;
elseif keyCode(downKey)
    squareY = squareY + pixelsPerPress;
end

% We set bounds to make sure our square doesn’t go completely off of
% the screen
if squareX < 0
    squareX = 0;
elseif squareX > screenXpixels
    squareX = screenXpixels;
end

if squareY < 0
    squareY = 0;
elseif squareY > screenYpixels
    squareY = screenYpixels;
end

% Center the rectangle on the centre of the screen
centeredRect = CenterRectOnPointd(baseRect, squareX, squareY);
% Draw the rect to the screen
Screen('FillRect', window, rectColor, centeredRect);

% Flip to the screen
vbl = Screen('Flip', window, vbl + (waitframes - 0.5) * ifi);

end
%
% Clear the screen
sca;
close all;
clear all;

6.6 Codes for generating behavioral stimulus: spatial acuity task.

function grating1(angle, cyclespersecond, freq, gratingsize, internalRotation)

% Make sure this is running on OpenGL Psychtoolbox:
AssertOpenGL;

% Initial stimulus parameters for the grating patch:

if nargin < 5 || isempty(internalRotation)
    internalRotation = 0;
end

if internalRotation
    rotateMode = kPsychUseTextureMatrixForRotation;
else
    rotateMode = [];
end
if nargin < 4 || isempty(gratingsize)
    gratingsizex = 1920 ;
    gratingsizey = 1200 ;
end

% res is the total size of the patch in x- and y- direction, i.e., the
% width and height of the mathematical support:
res = [gratingsizex gratingsizey];

if nargin < 3 || isempty(freq)
    % Frequency of the grating in cycles per pixel: Here 0.01 cycles per pixel:
    freq = 1/300 ;
end

if nargin < 2 || isempty(cyclespersecond)
    cyclespersecond = 0;
end

if nargin < 1 || isempty(angle)
    % Tilt angle of the grating:
    angle = 0;
end

% Amplitude of the grating in units of absolute display intensity range: A
% setting of 0.5 means that the grating will extend over a range from -0.5
% up to 0.5, i.e., it will cover a total range of 1.0 == 100% of the total
% displayable range. As we select a background color and offset for the
% grating of 0.5 (== 50% nominal intensity == a nice neutral gray), this
% will extend the sinewaves values from 0 = total black in the minima of
% the sine wave up to 1 = maximum white in the maxima. Amplitudes of more
% than 0.5 don’t make sense, as parts of the grating would lie outside the
% displayable range for your computers displays:
amplitude = 0.5;

% Choose screen with maximum id - the secondary display on a dual-display
% setup for display:
Screen('Preference', 'SkipSyncTests', 2);
screenNumber = max(Screen('Screens'));

% Open a fullscreen onscreen window on that display, choose a background
% color of 128 = gray, i.e. 50% max intensity:

[win, windowRect] = PsychImaging('OpenWindow', screenNumber, 128);
%win = Screen('OpenWindow', screenid, 128);
[screenXpixels, screenYpixels] = Screen('WindowSize', win);
% Make sure the GLSL shading language is supported:
AssertGLSL;

% Retrieve video redraw interval for later control of our animation timing:
ifi = Screen('GetFlipInterval', win);
[xCenter, yCenter] = RectCenter(windowRect);

% Phase is the phase shift in degrees (0-360 etc.) applied to the sine grating:
phase = 0;

% Compute increment of phase shift per redraw:
phaseincrement = (cyclespersecond * 360) * ifi;
% Build a procedural sine grating texture for a grating with a support of
% res(1) x res(2) pixels and a RGB color offset of 0.5 -- a 50% gray.
[gratingtex, gratingRect] = CreateProceduralSineGrating(win, res(1), res(2), [0.5 0.5 0.5 0.0]);
squareXpos = screenXpixels * 0;
squareYpos = screenYpixels * 0;
dstRect = OffsetRect(gratingRect, squareXpos, squareYpos);
% Wait for release of all keys on keyboard, then sync us to retrace:
KbReleaseWait;
vbl = Screen('Flip', win);

% Animation loop: Repeats until keypress...
while ~KbCheck
    % Update some grating animation parameters:
    % Increment phase by 1 degree:
    phase = phase + phaseincrement;

    % Draw the grating, centered on the screen, with given rotation ‘angle’,
    % sine grating ‘phase’ shift and amplitude, rotating via set
    % ‘rotateMode’. Note that we pad the last argument with a 4th
    % component, which is 0. This is required, as this argument must be a
    % vector with a number of components that is an integral multiple of 4,
    % i.e. in our case it must have 4 components:
    Screen('DrawTexture', win, gratingtex, [], dstRect, angle, [], [], [], [], rotateMode, [phase, freq, 1, amplitude]);

    % Show it at next retrace:
    vbl = Screen('Flip', win, vbl + 0.5 * ifi);
end

% We’re done. Close the window. This will also release all other resources:
Screen('CloseAll');
return;