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Assembly and disassembly of a retinal cholinergic network

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Abstract

In the few weeks prior to the onset of vision, the retina undergoes a dramatic amount of development. Neurons migrate into position and target appropriate synaptic partners to assemble the circuits that mediate vision. During this period of development, the retina is not silent, but rather exhibits spontaneous correlated activity called retinal waves. The retina assembles and disassembles a series of transient circuits that use distinct mechanisms to generate waves. During the first postnatal week, this transient circuit is comprised of reciprocal cholinergic connections between starburst amacrine cells. A few days before the eyes open, these cholinergic connections are eliminated as glutamatergic circuits involved in processing visual information are formed. Here we discuss the assembly and disassembly of this transient cholinergic network and the role it plays in various aspects of retinal development.

The mammalian retina has long been a model system for study of development of neural circuits in the CNS. The development of the retina requires several steps. First, the 7 cell types that comprise the retina need to be generated in the right proportion. Second, postmitotic cells leave the ventricular zone and migrate to one of three cell layers. Third, they start to form synaptic connections with other retinal neurons. Finally, these groups of synaptically coupled cells evolve into the circuits contained in the adult retina.

Acetylcholine (ACh) signaling plays a key role throughout these developmental stages. Prior to synapse formation, paracrine action of ACh is essential for regulating early developmental events, such as regulation of the cell cycle and the growth of neurites. In addition, cholinergic synapses are among the earliest to mature and thereby constitute the earliest functional circuits in the retina. Here we review the maturation of the retinal cholinergic circuit, describing its role in mediating early spontaneous activity and the development of non-cholinergic circuits.

ACh in the retina is produced by a subtype of amacrine cell termed the starburst amacrine cell (SAC). Of the roughly 30 morphologically distinct amacrine cell types (MacNeil *et al.*, 1999), the starburst amacrine cell is perhaps the best characterized (Famiglietti, 1983; Tauchi & Masland, 1984; Vaney, 1984). So named for its radially symmetric processes, SACs are the only neurons to produce ACh in the adult retina (Hayden *et al.*, 1980). The population of SACs is divided into two sub-populations with somas located in the proximal inner nuclear layer (regular) or in the ganglion cell layer (displaced) (Famiglietti, 1983). In addition to releasing ACh, SACs also release GABA (O'Malley & Masland, 1989) and adenosine (Blazynski, 1989). In the mature retina, ACh release acts on both muscarinic and nicotinic receptors to modulate response properties of many different types of ganglion cells (Masland & Ames, 1976; Masland *et al.*, 1984; Schmidt *et al.*, 1987; Baldrige, 1996; Strang *et al.*, 2005). The SAC is of particular importance in a retinal circuit that detects motion (Fried *et al.*, 2002; reviewed in Taylor & Vaney, 2003), the topic of another review in this series.

In mature retina, ACh released from SACs acts exclusively on other cell types (Baldrige, 1996). However, during development cholinergic signaling occurs between SACs. Long

before eye opening, ACh is produced by SACs and functional cholinergic receptors exist on SACs as well as other cells of the developing retina. SACs are spontaneously active and can release ACh onto neighboring SACs and ganglion cells. We refer to this early, transient signaling between SACs as the cholinergic network.

A key developmental function for the cholinergic network is the generation of retinal waves. During an extended period of time prior to visual input, the developing retina exhibits propagating spontaneous activity, termed retinal waves (Meister *et al.*, 1991; Wong *et al.*, 1993; Feller *et al.*, 1996). The cholinergic network appears at birth in mice and mediates the initiation and propagation of waves until the second postnatal week, when waves are mediated by glutamatergic circuits (Bansal *et al.*, 2000; Wong *et al.*, 2000; Zhou & Zhao, 2000).

In this review, we first describe the assembly of the transient cholinergic circuit, which involves early expression of proteins associated with release and action of ACh. Second, we review the process by which SACs orchestrate the wave generating circuit. Third, we describe the transition from cholinergic waves to glutamatergic waves, which requires the disassembly of the cholinergic connections that mediate waves. Last, we review the evidence that SACs and waves play a role in the development of retinal circuits, including dendritic refinement and stratification. (The role of retinal waves in the targeting and refinement of retinofugal synapses has been reviewed elsewhere (Torborg & Feller, 2005; Huberman *et al.*, 2008a; Feldheim & O'Leary, 2010)).

Assembly of the cholinergic network

The proteins associated with cholinergic synapses appear early in development (Figure 1). SACs first begin to express choline-acetyltransferase (ChAT), the enzyme responsible for synthesis of acetylcholine, at embryonic day 17 (E17) in rats (Kim *et al.*, 2000). SAC cell bodies organize into two layers on opposite sides of the nascent inner plexiform layer (IPL) shortly before birth. By postnatal day 3 (P3) two distinct bands are apparent within the IPL, representing regular and displaced populations of SACs (Bansal *et al.*, 2000; Kim *et al.*, 2000). Expression of the vesicular transporter for ACh (VAChT) follows a similar developmental profile, with punctate expression found at birth (Stacy & Wong, 2003). Evidence for presynaptic release machinery, including synaptic vesicle protein 2C (SV2C, (Wang *et al.*, 2003)), synaptophysin (Dhingra *et al.*, 1997), and SNAP-25 (West Greenlee *et al.*, 1998) are present at birth as well.

The broad distribution of nicotinic and muscarinic receptors at birth indicates that ACh released from SACs is poised to act on several cell types. mRNA expression of nicotinic acetylcholine receptors (nAChRs) subunits alpha 3–4 and beta 2–4 is present in the embryonic and early postnatal retina (Hoover & Goldman, 1992). Dividing cells in the ventricular zone have M1 muscarinic receptor dependent calcium increases in response to carbachol application, whereas differentiated amacrine and ganglion cells have voltage-gated channel dependent calcium increases in response to nicotine (Wong, 1995). Among the responsive amacrine cells are SACs themselves, which are depolarized at this early age by ACh acting on nicotinic receptors (Zheng *et al.*, 2004). The presence nAChRs on SACs indicates that they have the potential to form a recurrent excitatory network at this early stage of development.

Despite the abundance of pre- and postsynaptic components for cholinergic signaling, there is little ultrastructural evidence of conventional synapses at this early stage of development. Conventional synapses between amacrine and ganglion cells begin to form after P3 in mice (Fisher, 1979) and ChAT expression precedes synapse formation by at least 2 days in chick

retina (Spira *et al.*, 1987). However, despite the lack of structural evidence for synapses, there is physiological evidence for cholinergic synaptic transmission between pairs of SACs at this early age. Paired recordings between SACs from embryonic rabbit showed that SACs can indeed release both ACh and GABA to excite neighboring SACs in a reciprocal fashion (Zheng *et al.*, 2004) (Figure 2D). Postsynaptic nAChR mediated currents are slow, appearing to reflect an asynchronous and/or diffuse release of ACh from the presynaptic SAC. Paired recordings of SACs in early postnatal mice reveal similar slow currents (Ford *et al.*, 2010).

SACs also form GABAergic synapses with other SACs and with subpopulations of ganglion cells. Unlike most GABAergic neurons in the retina, SACs express the GABA synthesizing enzyme GAD-67 rather than the GAD-65 isoform (Brandon & Criswell, 1995). GAD-67 expression is present early in development (Famiglietti & Sundquist, 2010). SACs can release GABA onto neighboring SACs during the first postnatal week in rabbit (Zheng *et al.*, 2004). Direction selective ganglion cells also form GABAergic synapses with SACs well before eye opening (Lee *et al.*, 2010; Wei *et al.*, 2011).

A distinguishing feature of the SAC network is that the somas of SACs form a mosaic -- a term used to describe the regular arrangement of somas with a minimal spacing between them (reviewed in Vaney, 1990; Cook & Chalupa, 2000; Novelli *et al.*, 2005). SAC mosaics arise very early in development. SAC somas are initially positioned randomly with respect to each other as they migrate from the ventricular zone toward the developing ganglion cell layer. By birth in mice, SAC somas have distributed in a mosaic fashion so that there is a minimum distance observed between somas (Galli-Resta *et al.*, 1997; Galli-Resta *et al.*, 2000). The formation of the soma mosaic correlates with the complete coverage of the IPL by SAC processes.

Homotypic interactions between SAC processes are thought to instruct the initial formation of the mosaic (Reese & Galli-Resta, 2002). The developmental window during which these interactions establish the mosaic is finite, as disruption of microtubules (Galli-Resta *et al.*, 2002) or ablation of subsets of SACs by excitotoxicity (Farajian *et al.*, 2004) a few days after birth fails to disrupt the established mosaic. At this point, the mechanisms by which interactions between SAC processes mediate the development of mosaics are unknown.

The role of starburst amacrine cells in generation of retinal waves

The most well studied function of the early cholinergic network in the retina is the generation of retinal waves (Figure 2). Retinal waves consist of bursts of action potentials in retinal ganglion cells that propagate in a wave front across the plane of the retina. Retinal waves occur in many species including turtle (Sernagor *et al.*, 2003), chick (Catsicas *et al.*, 1998; Wong *et al.*, 1998; Sernagor *et al.*, 2000), mice (Mooney *et al.*, 1996; Bansal *et al.*, 2000), rabbit (Zhou, 1998), ferret (Meister *et al.*, 1991; Wong *et al.*, 1993; Feller *et al.*, 1996), and primate (Warland *et al.*, 2006). Across all species, waves share similar characteristic features. Waves initiate at random positions and propagate at speeds ranging from ~100µm/s (mouse, (Singer *et al.*, 2001)) up to 400µm/s (chick, (Sernagor *et al.*, 2000)). Individual waves stop at discrete but shifting borders that reflect recently active regions of retina (Feller *et al.*, 1996). This refractory period following a wave lasts for approximately one minute (Feller *et al.*, 1996; Feller *et al.*, 1997), shortly after which an additional wave can be initiated and spread over the same area (Figure 2A). In addition, this refractory period dictates the frequency with which a local region of the retina experiences a wave.

The circuitry underlying the generation of retinal waves progresses through three stages that reflect the maturation of retinal circuits (Blankenship & Feller, 2010) (Figure 3A). Before

formation of the IPL (E17 in mice (Kim *et al.*, 2000), E25 in rabbit (Sharma & Ehinger, 1997)), retinal waves propagate through the developing ganglion cell layer. These early stage waves are blocked by gap-junction antagonists (Syed *et al.*, 2004b). With the initial extension of SAC processes to form the IPL, waves transition from propagation via gap-junctions to propagation via activation of nAChRs (Feller *et al.*, 1996). Finally, a third stage of waves develop several days before the eyes open and co-exist with light responses for a short period of time (Demas *et al.*, 2003). These waves are blocked by ionotropic glutamate receptors (Wong *et al.*, 2000; Zhou & Zhao, 2000; Blankenship *et al.*, 2009).

The cholinergic network plays an exclusive role in generating waves during an extended period of development. Neighboring ganglion cells and SACs receive simultaneous slow nAChR and GABA-A mediated currents that give rise to correlated action potential firing (Feller *et al.*, 1996; Zhou, 1998). In perinatal mice, ferrets, and rabbits, waves monitored by calcium imaging are blocked by nAChR antagonists (Feller *et al.*, 1996; Penn *et al.*, 1998; Bansal *et al.*, 2000; Zhou & Zhao, 2000), but not by ionotropic or metabotropic GABA or glutamate receptor antagonists (Zhou & Zhao, 2000). Gap-junction signaling seems to play a role in the generation of these waves in chick (Catsicas *et al.*, 1998), but is involved to a lesser extent in mammalian retina (Bansal *et al.*, 2000; Syed *et al.*, 2004b).

The features of the developing cholinergic network that give rise to waves are now beginning to be understood. The generation of waves requires a source of depolarization for wave initiation, a network of excitatory interactions for propagation and a source of inhibition that limits the spatial extent of waves and dictates the minimum interval between waves. We discuss the properties of the cholinergic network that give rise to these features below.

Initiation

SACs themselves are the likely source of wave initiation. In order for a wave to start, a cell or group of cells must spontaneously excite neighboring cells to initiate the spread of depolarization. Waves initiate at random locations (Feller *et al.*, 1996), suggesting that the cells responsible for the spontaneous excitation that drives wave initiation are present uniformly across the retina. In the presence of neurotransmitter antagonists, SACs from rabbit retinas exhibit spontaneous depolarizations that could be measured with cell attached, whole cell recordings, and calcium imaging (Zheng *et al.*, 2006) (Figure 2B). Spontaneous depolarizations occur fairly regularly with a frequency of about once per 20s in cell attached and current clamp recordings, though spontaneous calcium transients were less regular. These spontaneous depolarizations presumably lead to release of ACh onto neighboring SACs, which may trigger the start of a wave. Indeed, we find that injecting current to depolarize a single SAC can robustly initiate a wave in mouse retina (Ford *et al.*, 2010).

The ion channels that underlie spontaneous depolarizations in SACs are unknown. Activation of TTX sensitive sodium channels, which are present in developing SACs (Zhou & Fain, 1996), are unlikely to be the cause of spontaneous depolarization, as TTX does not block retinal waves (Stellwagen *et al.*, 1999). Spontaneous opening of calcium channels may provide a depolarizing drive. Adult SACs have N-, P/Q-, and R-type calcium channels (Cohen, 2001; Kaneda *et al.*, 2007), but these channels are activated at depolarized potentials. Some L-type voltage gated calcium channels are activated at hyperpolarized potentials (Koschak *et al.*, 2001) and have been implicated in pacemaking neurons (Putzier *et al.*, 2009). Retinal waves are blocked by the L-type channel antagonist nifedipine (Singer *et al.*, 2001), suggesting that these channels may play a role in either the spontaneous depolarization or propagation of waves.

Propagation

Waves propagate through a cholinergic circuit that depends on activation of a specific subtype of nAChRs. Studies of adult rabbit retina have demonstrated strong expression of alpha7 homomeric channels (Dmitrieva *et al.*, 2007) as well as alpha3/beta2 containing heteromeric channels (Keyser *et al.*, 2000) throughout the IPL. Expression of alpha3 and alpha8 containing receptors during the period of cholinergic waves has been shown in chick retina (Hamassaki-Britto *et al.*, 1994). Waves are blocked by α -conotoxin-MII, a toxin that is most specific for alpha3/beta2 containing nAChRs, but not α -bungarotoxin (targeted to homomeric alpha7 nAChRs) or α -conotoxin-AU1B (targeted to alpha3/beta4 containing receptors) (Penn *et al.*, 1998; Bansal *et al.*, 2000). Further evidence for a unique set of receptors mediating waves came from studies using knockout mice -- mice lacking either alpha3 or beta2 subunits lacked cholinergic retinal waves (Bansal *et al.*, 2000; Sun *et al.*, 2008; Stafford *et al.*, 2009). Hence, wave propagation depends upon activation of specific heteromeric alpha3/beta2 nAChRs even though other nAChRs exist in the retina.

In addition to ACh, SACs and ganglion cells receive GABAergic input through GABA-A receptors during waves (Feller *et al.*, 1996; Zhou, 1998). At this developmental age GABA-A mediated currents are depolarizing due to the accumulation of intracellular chloride (Zhang *et al.*, 2006b; Barkis *et al.*, 2010). GABA-A receptor activation is not required for the propagation of waves in rabbit or mice (Syed *et al.*, 2004b; Wang *et al.*, 2007), but does influence the structure of the firing patterns (Wang *et al.*, 2007). Not all studies have reached this conclusion – early waves in both turtle (Sernagor *et al.*, 2003) and ferret (Fischer *et al.*, 1998; but see Stellwagen *et al.*, 1999) depend on GABA-A receptor activation.

A possible explanation for the widespread effects of ACh during waves is that ACh is released by volume transmission – the diffuse release of neurotransmitter in the absence of pre- and postsynaptic specializations (for a comparison of direct vs. volume ACh transmission see (Sarter *et al.*, 2009)). Several lines of evidence support this. First, retinal ganglion cells with dendritic arbors at opposite ends of the IPL are synchronized during waves, despite the fine stratification of SAC processes (Wong & Oakley, 1996). Second, slow synaptic currents recorded between pairs of SACs (Zheng *et al.*, 2004) (Figure 2D) indicate that ACh acts diffusely. Third, cholinergic retinal waves are present prior to the formation of conventional synapses, as identified in electron microscopy studies (Fisher, 1979; Greiner & Weidman, 1981). Finally, in rabbit retina, calcium waves propagate through the ventricular zone that are correlated with waves in the ganglion cells (Syed *et al.*, 2004a). The ventricular zone waves are blocked by muscarinic receptor antagonists as well as nAChR antagonists, implying that ACh released during waves in the ganglion cell layer diffuses tens of microns to the ventricular zone where it activates muscarinic receptors.

Refractory period

The frequency of retinal waves is limited by a refractory period following waves. Over a period of about one minute, waves initiated at random points appear to tile the retina, avoiding regions active recently (Feller *et al.*, 1996)(Figure 2A). Depolarization by focal application of high potassium solution could also cause a refractory period within the affected region. This refractory period is thought to be imparted by a slow after-hyperpolarization (sAHP) following depolarization in SACs (Zheng *et al.*, 2006) (Figure 2C). The sAHP is blocked by cadmium, suggesting that it is activated by calcium entry through voltage-gated calcium channels. Interestingly, the channel underlying the sAHP is inhibited by elevating levels of cAMP, similar to the calcium-activated potassium channel which is thought to underlie sAHPs in various other regions of the brain (Alger & Nicoll, 1980; Lancaster & Adams, 1986; Sah & Isaacson, 1995; Vogalis *et al.*, 2001), the molecular

identity of which is unknown. Elevating cAMP increases the frequency of retinal waves (Stellwagen *et al.*, 1999; Hanganu *et al.*, 2006), consistent with the notion that the sAHP limits the frequency of waves.

Recent studies have incorporated the measured features of the cholinergic network into computational models. Modeling studies are of use in identifying the relevant features of a circuit that give rise to the physiological output. For instance, two modeling studies have shown that in order to achieve propagating waves that have finite borders, i.e. do not encompass the entire retina, there must be local variability in the refractory state of the retina (Godfrey & Swindale, 2007; Hennig *et al.*, 2009). This variability is generated by random spontaneous depolarizations of SACs followed by sAHPs that occur in between waves. While spontaneous depolarization of SACs between waves has been observed in rabbit (asterisk Figure 2C) (Zheng *et al.*, 2006), we have not observed these in mouse (Ford *et al.*, 2010). As the details of the SAC network are worked out for different species, there will likely be different, and perhaps disparate, solutions that generate very similar waves.

Role of cholinergic signaling in establishing non-cholinergic retinal circuits

Influence on later progenitor cells

Cholinergic retinal waves occur during a period of rapid retinal development. Progenitor cells in the ventricular zone are dividing and differentiating into each of the 7 types of retinal cells (Figure 1). These newly differentiated cells must then migrate to the proper position within the retina and extend processes to form the neuropil layers. The onset of cholinergic waves correlates with the formation of the IPL, where amacrine and ganglion cell processes stratify into 10 discrete layers (in mice) that process visual information in parallel (Masland, 2001; Roska & Werblin, 2001; Wässle, 2004). Once stratified in these layers, bipolar, amacrine, and ganglion cells must form synapses with their proper partners to form functional circuits that process visual information. Before the onset of vision, these discrete synaptic layers are visible (Kim *et al.*, 2000; Drenhaus *et al.*, 2003; Famiglietti & Sundquist, 2010) and some functional circuits are already established (Elstrott *et al.*, 2008; Wei *et al.*, 2011). To what extent does the cholinergic network shape the outcome of these developmental events?

Rhythmic increases in intracellular calcium play an important role in cell proliferation and differentiation (reviewed in Martins & Pearson, 2008) for retinal neurons born during cholinergic waves. ACh drives increases in intracellular calcium of progenitor cells in the ventricular zone through activation of M1 muscarinic receptors (Wong, 1995), which can alter their progression in the cell cycle (Pearson *et al.*, 2002). Blocking muscarinic receptors in chick eyes leads to an increase in the size of the eye (Pearson *et al.*, 2002). A probable source for ACh acting on muscarinic receptors is from cholinergic waves, which drive correlated calcium waves in the ventricular zone (Syed *et al.*, 2004a). In other parts of the nervous system, elevation of intracellular calcium can also influence neuronal migration (reviewed in Komuro & Kumada, 2005) and neurotransmitter selection (reviewed in Spitzer *et al.*, 2004). It remains to be seen whether calcium increases caused by retinal waves have similar effects.

IPL stratification

The retina is a highly laminar structure. Cell bodies reside in three vertically oriented layers composed of the distinct cell classes. The IPL comprises axons from bipolar cells, processes from amacrine cells, and dendrites of ganglion cells. These processes are separated into the On and Off layers corresponding to the functional responses to light of the corresponding cells. Within On and Off layers, processes are further segregated into at least 10 different

strata where distinct neural computations are performed. The structural layering and corresponding functional separation has made the retina an ideal model system for studying synapse specificity – i.e. the process by which pre- and postsynaptic neurons wire to their appropriate partners.

The development of lamina in the IPL occurs in several steps. First, amacrine cells extend processes into the IPL. The processes of different amacrine cells identified by cell-specific antibody staining form discrete layers as soon as they first appear (Famiglietti & Sundquist, 2010). Second, the dendrites of ganglion cells extend into the IPL. While some ganglion cells initially stratify within their appropriate mature lamina, a large portion of ganglion cells stratify diffusely throughout future On and Off layers. Next, On and Off bipolar cell axons extend into the IPL where they stratify into the appropriate layers (Johnson *et al.*, 2003). Finally, ganglion cells restrict their dendrites to their mature lamina (reviewed in (Xu & Tian, 2004)). Below we discuss how SACs could play a role in these developmental steps.

A growing body of literature from zebrafish studies points to a critical role for amacrine cells in patterning the IPL. The processes of amacrine cells have directed growth within their target layers without exuberant growth in other layers (Godinho *et al.*, 2005). While ganglion cells are the first-born cells in the retina, they play a lesser role in formation of layers in the IPL. A genetic model in which ganglion cells are never born have delayed IPL formation, although this is partially corrected and normal lamination of the IPL occurs throughout most of the retina (Kay *et al.*, 2004). Hence, interactions between amacrine cells are sufficient to give rise to the layering within the IPL. Interestingly, bipolar cell axons target correctly to the normal IPL in these mutants, but fail to innervate regions of abnormal IPL (Kay *et al.*, 2004), suggesting that amacrine cells provide the laminar cues for bipolar cell axon targeting. Finally, ganglion cells exhibit directed outgrowth of their processes within established layers of amacrine cell processes (Mumm *et al.*, 2006), implying that amacrine cells can provide lamination cues for ganglion cells. A zebrafish homologue of the SAC has not been conclusively identified (though see (Kay *et al.*, 2004)), so it is unclear what role SACs in particular play in these developmental events.

In mammals, SACs are among the first cells to extend processes within the IPL (Kim *et al.*, 2000) and could potentially serve as a foundation for the layering of subsequent strata within the IPL. Lamination signals, such as members of the immunoglobulin superfamily (Yamagata & Sanes, 2008) or semaphorins (Matsuoka *et al.*, 2011), expressed on SAC processes could instruct the processes of other amacrine, ganglion, and bipolar cells to stratify above, below or between the two cholinergic strata. Despite this early genesis, it is unlikely that SACs provide lamination cues for other amacrine and bipolar cells. Retinal deletion of the transcription factor islet-1 results in a near complete loss of ChAT positive amacrine cells (Elshatory *et al.*, 2007). Despite this loss of differentiated SACs, several markers for other amacrine cell types still form layers within the IPL. Similarly, a genetic model of retinal blastoma lacks several markers of mature SACs but stratification of other amacrine and bipolar cell types are not altered (Chen *et al.*, 2007). Furthermore, SACs ablated by immuno-toxin (Gunhan *et al.*, 2002) or by excitotoxicity with L-glutamate (Reese *et al.*, 2001) at two to three days after birth show no defects in the stratification of bipolar cells, suggesting that SAC processes do not provide an instructive role for stratification of these cell types.

While SACs are unlikely to guide the lamination of other amacrine and bipolar cells, they may instruct the localization of some types of ganglion cell dendrites. Using random dye labeling, a certain class of bi-stratified ganglion cell, mostly likely corresponding to direction selective ganglion cells, was found to co-stratify with the cholinergic processes shortly after birth (Stacy & Wong, 2003), although other mono-stratified ganglion cells

never stratified with the cholinergic bands. Recently, genetically labeled mouse lines of functionally identical ganglion cell classes have been developed that allow for consistent characterization across development (Huberman *et al.*, 2008b; Kim *et al.*, 2008; Huberman *et al.*, 2009). The dendrites of genetically identified direction selective ganglion cells co-stratify with SAC processes shortly after birth (Kim 2010, Wei 2011), supporting the hypothesis that SACs provide an instructive role for lamination of certain ganglion cell types.

The dendrites of most retinal ganglion cells initially arborize diffusely within the IPL before restricting their dendrites to distinct lamina (Bodnarenko *et al.*, 1999; Bansal *et al.*, 2000; Sernagor *et al.*, 2001; Xu & Tian, 2004; Coombs *et al.*, 2007; Kim *et al.*, 2010). Some studies have implicated a role for activity in bipolar cells in the segregation of ganglion cell dendrites. Hyperpolarizing ON bipolar cells during the period of glutamatergic retinal waves using the drug APB prevents the segregation into ON and OFF layers (Bodnarenko & Chalupa, 1993; Bodnarenko *et al.*, 1995). Additionally, mice that lack the MHCI receptor CD3zeta have altered glutamatergic retinal waves. The ganglion cells of these mice have reduced dendritic motility and have more diffuse dendrites within the IPL (Xu *et al.*, 2010). However, not all manipulations of activity during development alter dendritic stratification. Preventing synaptic release of glutamate from ON bipolar cells by expression of tetanus toxin fails to prevent the stratification of ganglion cell dendrites, although synapse formation onto ON bipolar cells is reduced (Kerschensteiner *et al.*, 2009).

Is there a role for cholinergic waves in the stratification process? Blocking nAChRs during the period of cholinergic waves reduces the motility of filipodia on the dendrites of ganglion cells (Wong & Wong, 2001), demonstrating that waves can drive structural changes in dendrites. Studies in turtle have demonstrated that blocking cholinergic waves with nAChR antagonists inhibits dendritic growth (Mehta & Sernagor, 2006) and reduces receptive field sizes (Sernagor & Grzywacz, 1996). Additionally, mice lacking the beta2 subunit of the nAChR had a delay in, though did not prevent, the fine stratification of ganglion cell dendrites (Bansal *et al.*, 2000). These findings indicate that cholinergic waves do influence the outgrowth of ganglion cell dendrites but are not the primary factor dictating their final organization.

One intriguing hypothesis for a role of retinal waves is in the development of direction selective circuits. Retinal waves have directional information both in their propagation and in the observation that there is a propagation bias, with more waves traveling toward the nasal direction than the other cardinal axes (Stafford *et al.*, 2009; Elstrott & Feller, 2010). However, direction selective ganglion cells participate equally in all waves irrespective of propagation direction (Elstrott & Feller, 2010) and complete blockade of retinal activity using intraocular muscimol injections did not prevent the development of direction selectivity (Wei *et al.*, 2011). These findings indicate that waves are not likely to provide the instructive signal for the establishment of asymmetric direction selective circuits. However, whether retinal waves play a role in the refinement of DS circuits remains to be determined.

Is there a role for cholinergic waves in the transitions between wave-generating circuits?

Another role of cholinergic waves in development of the retina circuitry may be to terminate early stage gap-junction mediated waves (Figure 3). Knock-out animals that lack nAChR receptor subunits still exhibit wave like activity under certain recording conditions, such as elevated temperature (alpha3(-/-): (Bansal *et al.*, 2000), beta2(-/-) (Sun *et al.*, 2008; Stafford *et al.*, 2009)). This activity is likely mediated by gap-junctions (Sun *et al.*, 2008), as in earlier stage waves. Similarly, a study using a genetic model which eliminates ChAT in a large portion of the retina found normal cholinergic waves in the spared region, but compensatory waves in the region lacking ChAT (Stacy *et al.*, 2005). These studies point to

a sequential maturation of the retinal circuitry that relies on checkpoints to make transitions from one stage (gap-junction mediated waves) to the next (cholinergic transmission mediated waves). This sort of checkpoint model of neuronal development (Ben-Ari & Spitzer, 2010) is further evidenced in the disassembly of the cholinergic network to make way for glutamatergic signaling (Blankenship & Feller, 2010).

Disassembly of the cholinergic network

Shortly before eye opening, retinal waves transition from being mediated by ACh to glutamate. Bipolar cell axons invade the IPL and begin to release glutamate at the end of the first week after birth in rodents (Johnson *et al.*, 2003). Shortly thereafter, waves change their pharmacological profile and spatiotemporal properties. Waves are no longer blocked by nAChR antagonists but rather are sensitive to a combination of AMPA and NMDA antagonists (Wong *et al.*, 1998; Zhou & Zhao, 2000; Blankenship *et al.*, 2009). These glutamatergic waves occur as periodic clusters of activity followed by periods of silence (Kerschensteiner & Wong, 2008; Blankenship *et al.*, 2009). Waves exist simultaneously with light responses and disappear a few days after eye opening in a manner that is independent of visual experience (Demas *et al.*, 2003).

With the onset of glutamatergic waves, the SAC network loses the features necessary for propagation of cholinergic waves. First, mature SACs are no longer depolarized by application of nicotine (Baldridge, 1996). This is the result of a rapid decline in nAChR activation that coincides with the start of glutamatergic waves (Zheng *et al.*, 2004). Second, acetylcholinesterase, the enzyme that degrades ACh, is increased during this time window (Hutchins *et al.*, 1995), which would decrease the spread of ACh. Third, the action of GABA becomes hyperpolarizing a few days before the onset of glutamatergic waves (Zhang *et al.*, 2006b; Barkis *et al.*, 2010). Intracellular chloride is reduced as the expression of the potassium-chloride transporter KCC2 is increased in ganglion cells (Zhang *et al.*, 2006a). Mature SAC processes have a graded expression of KCC2 that increases along the length of the process (Gavrikov *et al.*, 2006). When this graded distribution of KCC2 occurs is unknown. Fourth, SACs become less excitable because maturation of processes increases the electronic space constant. The long and skinny processes of SACs likely prevent the spread of depolarization between processes, which would serve to electrically isolate the soma from the processes. A further decrease in the excitability of SACs is imparted by the loss of voltage gated sodium channels (Zhou & Fain, 1996) and an increase in expression of the voltage gated potassium channel Kv3.1 (Ozaita *et al.*, 2004; Zheng *et al.*, 2006).

At this same time, other circuits involving SACs are rapidly maturing. GABAergic connections between SACs are maintained during this period (Zheng *et al.*, 2004) and cholinergic and GABAergic synapses onto direction-selective ganglion cells develop (Lee *et al.*, 2010; Wei *et al.*, 2011). SACs greatly extend their processes as much as 7 fold and develop varicosities at the distal portions that are presumed to be the sites of GABA release (Wong & Collin, 1989).

The nature of cholinergic transmission in the adult retina is not well understood. Many ganglion cells with dendrites distal to the cholinergic strata within the IPL still respond to ACh released from SACs (Schmidt *et al.*, 1987). Paired recordings between SACs and direction selective ganglion cells reveal symmetrical sites of ACh release (Lee *et al.*, 2010), however ultrastructural reconstruction of synaptic contacts onto direction selective ganglion cells failed to find evidence for these cholinergic synapses (Briggman *et al.*, 2011). Furthermore, expression of SNAP-25, a SNARE protein involved in evoked release of neurotransmitter, is transiently highly expressed in SAC processes during the period of

cholinergic waves (West Greenlee *et al.*, 1998), suggesting that the presynaptic machinery for neurotransmitter release might change during development.

The onset of glutamatergic waves may play an active role in the disassembly of cholinergic circuits, similar to the transition from gap-junction to cholinergic waves (Blankenship & Feller, 2010) (Figure 3). Mice lacking the vesicular glutamate transporter VGLUT1 lack glutamate release from bipolar cells. These mice still have retinal waves at the period of time when littermate control animals have glutamatergic waves. Interestingly, these later waves in the VGLUT1 knockout mice are unaffected by glutamate receptor antagonists but are blocked by nAChR antagonists (Blankenship *et al.*, 2009). Glutamate signaling from bipolar cells is therefore required to dismantle the cholinergic network.

The mechanisms underlying this transition are not understood. SACs have functional kainate, AMPA, and NMDA receptors shortly after birth (Acosta *et al.*, 2007) suggesting that they can respond to glutamate when it is first released by bipolar cells. In addition to ionotropic receptors, SACs also express the metabotropic glutamate receptor 2 starting shortly after birth in rodents (Koulen *et al.*, 1996). Action of glutamate on either ionotropic or metabotropic receptors on SACs could result in down-regulation of functional nAChRs and perhaps the regulation of other proteins that are necessary for cholinergic waves. The mechanisms involved in this transition will likely be of general importance, as several other developing neural circuits such as the spinal cord, hind brain, cochlea, and hippocampus transition through different stages of spontaneous activity before reaching their mature state (Blankenship & Feller, 2010).

Conclusions

Here we have reviewed the assembly, disassembly and function of cholinergic circuits in retinal development. Starburst amacrine cells release acetylcholine and nicotinic and muscarinic acetylcholine receptors appear early in development to provide signaling that influences events prior to synapse formation. The processes of starburst amacrine cells define the early strata of the developing inner plexiform layer and therefore may play a role in organizing processes of other cells. For a brief period of development, starburst amacrine cells can mutually excite each other. These recurrent excitatory connections provide the substrate for the initiation and propagation of retinal waves. Future work will continue to elucidate how other transient features of developing starburst cells and the nature of their synaptic connections influences the propagation properties of retinal waves.

One outstanding question that remains is how early signaling in the retina influences the formation of retinal circuits. The assembly of this cholinergic circuit is critical for driving the transition from an earlier, gap junction-mediated wave generating circuit. Similarly, maturation of the glutamatergic wave generating circuit drives the disassembly of the cholinergic network. Hence, each circuit is critical for eliminating the circuit before it. In addition, retinal waves drive correlated depolarizations of many synaptic partners (e.g. starburst amacrine cells and direction selective ganglion cells). Whether these early signaling events are critical for the maturation of these functional circuits remains an open question. Answers to these questions require a deeper understanding of the interplay between activity and other cellular mechanisms that underlie the wiring up of functional circuits.

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Developmental Timeline

Figure 1. Timeline showing major developmental events in mouse retina

Starburst cells become postmitotic between E11 and E17. ChAT expression is first seen at E17 and increases during the first week after birth. The IPL first appears at E17 and SAC processes form two discrete bands by P3. Conventional synapses first appear between amacrine and ganglion cells in the IPL at P3. Retinal waves transition through three stages during development: gap-junction, cholinergic, and glutamatergic. Bipolar cells first innervate the IPL at P7. Eyes open around P12. Ganglion cell dendrites stratify into lamina for an extended time beginning around P9 and continuing through the first month. The action of GABA switches from depolarizing to hyperpolarizing around P8. SACs are initially responsive to ACh action on nAChRs, but lose this responsiveness with the corresponding transition to glutamatergic waves.

References: (1) (Voinescu *et al.*, 2009) (2) (Kim *et al.*, 2000) (3) (Hinds & Hinds, 1983) (4) (Fisher, 1979) (5) (Bansal *et al.*, 2000) (6) (Johnson *et al.*, 2003) (7) (Xu & Tian, 2004) (8) (Barkis *et al.*, 2010) (9) (Zheng *et al.*, 2004) (approximate age from rabbit data)

Figure 2. Properties of cholinergic waves are generated by the SAC network

A. Spatial and temporal properties of waves assessed with calcium imaging.

TOP: Low power calcium imaging of Fura-2AM labeled neonatal ferret retinas show decreases in fluorescence following calcium increases associated with waves.

BOTTOM: Spatial propagation of 30 waves during four consecutive minutes. Each panel shows the propagation of waves that occurred during a one-minute period. Cholinergic retinal waves initiate at random locations and propagate over finite regions. Waves occur at intervals between waves of around one minute for a given region (for example see asterisk). Adapted from (Feller *et al.*, 1996).

B. Cell-autonomous spontaneous depolarization of SACs may initiate retinal waves.

SACs (red regions and traces) from perinatal rabbit retinas loaded with Fura-2AM show spontaneous depolarizations in the presence of antagonists to ionotropic and metabotropic glutamate, GABA, glycine, and ACh receptors, whereas retinal ganglion cells do not (blue regions and traces). Adapted from (Zheng *et al.*, 2006).

C. The inter-wave refractory period may be due to a slow after-hyperpolarization in SACs. Current clamp recording from a SAC in rabbit retina showing wave evoked and spontaneous (asterisk) depolarizations followed by slow after-hyperpolarizations. Adapted from (Zheng *et al.*, 2006).

D. Waves may propagate via reciprocal connections between SACs.

Voltage clamp recordings from pairs of neighboring SACs show both fast GABAergic postsynaptic currents and slow cholinergic postsynaptic currents. Top: Schematic of voltage command of presynaptic SAC (-70mV to 0mV). Bottom: Evoked PSCs at positive and negative holding potentials to isolate cholinergic and GABAergic currents, respectively. Adapted from (Zheng *et al.*, 2004).

Figure 3. Wave circuits transition through check-points

A. Schematics of changing circuits that mediate waves. Left: Prior to birth waves are thought to propagate via gap-junctions between ganglion cells. Middle: Postnatal day 1–10, waves are propagated via SAC release of acetylcholine onto other SACs (black box). Acetylcholine also depolarizes ganglion cells. During this period of development, the gap-junction signaling between ganglion cells is reduced (red box).

Right: P10–P15 bipolar cells release glutamate to propagate waves in a mechanism that is thought to involve spillover of glutamate to excite neighboring bipolar cells (black box). Cholinergic signaling between SACs is reduced (red box). Adapted from (Blankenship & Feller, 2010).

B. Summary timeline of how genetic disruption of cholinergic or glutamatergic waves result in an extended action of the previous wave generating circuit

In wild type mice gap-junction mediated waves (gray) are followed by cholinergic waves (blue) starting at P0, then glutamatergic waves (green) at P10. In mice lacking the Beta2 subunit of the nicotinic acetylcholine receptor, gap-junction mediated waves persist until ~P8. In mice lacking vesicular glutamate transporter VGLUT1, cholinergic waves persist through the second postnatal week.