Calcium-dependent mechanisms in the chicken retina

Merve Tekmen
Louisiana State University and Agricultural and Mechanical College

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CALCIUM-DEPENDENT MECHANISMS IN THE CHICKEN RETINA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Merve Tekmen
B.Sc., Hacettepe University, 2005
August, 2011
Dedication

For my mom Hatice Tekmen, my dad Hikmet Tekmen
and my brother Dr. Mohac Tekmen
for believing in and encouraging me for what I can do.
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AC      Adenylate cyclase
AC1/8   Adenylate cyclase 1/8
BAPTA   1,2-bis-(o-aminophenonxy)ethane-N,N,N',N'-tetraacetic acid
BC      Bipolar cell
CaM     Calmodulin
cAMP    Cyclic AMP
CB      Chicken brain
CDI     Ca$^{2+}$/calmodulin-dependent inactivation
CICR    Ca$^{2+}$-induced Ca$^{2+}$ release
CMZ     Calmidazolium chloride
CR      Chicken retina
CsA     Cyclosporin A
DAF-FM  4-amino-5-methylamino-2’,7’-difluorofluorescein
DSGC    Direction selective ganglion cells
EGTA    Ethylene glycol-bis(2-aminoethyl-ether)-N,N,N’,N’-tetraacetic acid
eNOS    Endothelial nitric oxide synthase
FCCP    Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
GABA    Gamma amino butyric acid
GC      Ganglion cell
GCL     Ganglion cell layer
GS      Glutamine synthatase
H89     N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl
HEPES   4-(2-hydroxyethyl)-1-piperazzineethanesulfonic acid
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>HC</td>
<td>Horizontal cell</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>MB</td>
<td>Mouse brain</td>
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<tr>
<td>MCU</td>
<td>Mitochondrial Ca$^{2+}$ uptake</td>
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<tr>
<td>MGC</td>
<td>Müller glial cell</td>
</tr>
<tr>
<td>8-M-IBMX</td>
<td>8-methoxymethyl-1-methyl-3-(2-methylpropyl)xanthine</td>
</tr>
<tr>
<td>NADPH-d</td>
<td>Nicotinamide adenine dinucleotide phosphate-diaphorase</td>
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<tr>
<td>NFL</td>
<td>Nerve fiber layer</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDE1</td>
<td>Phosphodiesterase 1</td>
</tr>
<tr>
<td>PDZ domain</td>
<td>PSD-95 discs large/ZO-1 homology domain</td>
</tr>
<tr>
<td>PR</td>
<td>Photoreceptor</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post synaptic density 95</td>
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<td>RyR</td>
<td>Ryanodine receptor</td>
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</table>
sGC  Soluble guanylate cyclase
SNAP  S-nitroso-D-acetylpenicillamine
TAC  Target amacrine cell
TEACl  Tetra-ethyl ammonium chloride
TTX  Tetrodotoxin
Abstract

Multiple classes of retinal amacrine cells use L-type Ca\(^{2+}\) channels to mediate synaptic transmission. I have used whole cell voltage clamp recordings from primary cultures of amacrine cells to investigate the regulation of these channels. In this study, I show that inhibiting mitochondrial calcium uptake (MCU) caused a reversible reduction in the Ca\(^{2+}\) current amplitude. Replacing external Ca\(^{2+}\) with Ba\(^{2+}\) minimized the effects of blocking MCU indicating that the Ca\(^{2+}\) influx is the primary source of the inhibition. With 1,2-bis-(o-aminophenonxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) in the recording pipette, MCU inhibition caused an increase in the current amplitude indicating that the fast buffering capability of BAPTA minimizing the Ca\(^{2+}\)-dependent inactivation of the channels, revealing a Ca\(^{2+}\)-dependent enhancement, possibly through protein kinase A (PKA) activity. The effect of a PKA inhibitor was consistent with this possibility. Inhibiting the calcium-induced calcium release (CICR) also decreased the Ca\(^{2+}\) current amplitude. These results indicate that MCU, PKA and CICR are critical to maintain the availability of L-type Ca\(^{2+}\) channels for depolarization-dependent signaling in amacrine cells.

Production of nitric oxide (NO) by neuronal and endothelial nitric oxide synthases (nNOS; eNOS) is another Ca\(^{2+}\)-dependent mechanism in the retina. In the chicken retina, I demonstrate the cell-autonomous nature of the NO signal by comparing the pattern of NO production to the expression of NOS. The NO indicator fluorescence dye, DAF was used to detect the pattern of NO production. A NOS inhibitor L-NAME suppressed the DAF signal suggesting that the source of DAF-signal was due to NOS activity. I also demonstrate the presence of NOS-immunoreactivity in the chicken retina. Neuronal NOS and eNOS antibodies labeled photoreceptors, amacrine cells and cells in the ganglion cell layer (GCL). Anti-e NOS also labeled horizontal cells, a small subset of bipolar cells and Müller cells. Different subsets of
amacrine cells were labeled in dorsal and ventral retina with anti-nNOS. Endothelial NOS labeling did not show difference in dorsal and ventral retina but expression was more wide spread than nNOS. These results suggest that the potential for NO production is wide spread in the avian retina.
Chapter 1

Introduction
Photoreceptors are the first site where photons are transduced into the electrical signal that passes through multiple layers in the retina before being sent to the higher visual centers in the brain. The vertebrate retina is relatively accessible and part of the central nervous system, which makes the retina a good working model for studying and understanding the complex signaling in the brain and nervous system. As a sensory system, the retina also has the technical advantage of a well-defined input, patterns of photons. This feature makes studies of information processing more tractable than in other parts of the central nervous system.

The vertebrate retina consists of 6 layers: the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer (GCL) and the nerve fiber layer (NFL) (Fig. 1.1). The ONL includes rod and cone photoreceptor cells, the INL contains horizontal cells, bipolar cells, and amacrine cells, and the GCL includes ganglion cells and “displaced” amacrine cells. The OPL and IPL are the synaptic layers. The OPL contains the synaptic connections of photoreceptor terminals, horizontal cell processes and bipolar cell dendrites whereas the IPL contains the processes and synapses of bipolar cells, amacrine cells and ganglion cells. The NFL contains the axons of ganglion cells and efferent fibers originating from isthmo optic nucleus. Muller glial cells extend through all of the layers in the retina.

Light enters the vertebrate retina at the GCL and passes through all the layers before reaching the photoreceptors where it is transduced into an electrical signal. This signal then is carried back through the layers of the retina by vertical and horizontal interactions. The vertical pathway involves photoreceptors, bipolar cells, and ganglion cells. The horizontal pathway consists of photoreceptors, horizontal cells, and amacrine cells (Cook and McReynolds 1998). Once the signal processing is completed in the retina, the neural message is sent to the higher visual centers in the brain for further processing.
Figure 1.1: A model of a vertebrate retina. The outer nuclear layer (ONL) contains the rods (R) and cones (C) photoreceptors. The inner nuclear layer (INL) contains horizontal cells (HC), bipolar cells (BC), amacrine cells (AC) and target amacrine cells (TAC). The ganglion cell layer (GCL) contains ganglion cells (GC). The synaptic layers, the outer plexiform layer (OPL) and the inner plexiform layer (IPL) contain the synapses between retinal cell types. The nerve fiber layer (NFL) contains the axons of ganglion cells and efferent synapses originating from isthmo optic nucleus. Modified from the artwork by Marcia Duggin.

Compared to photoreceptor cells, bipolar cells and ganglion cells, amacrine cells are poorly understood. Amacrine cells sit in the middle of the retina making them difficult to record from in a whole mount retina. These cells can also be difficult to study in a retinal slice because of the extensive lateral extensions of their processes. Amacrine cells in the retina participate in large numbers of complex synaptic interactions (Marc and Liu, 2000) making studies of their cellular and synaptic physiology particularly challenging. In our laboratory, we focus on amacrine cells from the chicken retina. We use cells from the chicken retina because chicken amacrine cells form functional mature synapses with each other in culture. This makes them a good model for studying the synaptic functions of amacrine cells in the retina. A chemical
synaptic transmission between pairs of neurons contains pre- and post-synaptic neurons, voltage- and ligand-gated ion channels, neurotransmitters and neurotransmitter receptors, synaptic vesicles, synaptic proteins and second messengers.

$\text{Ca}^{2+}$ is a second messenger and is involved in many cellular mechanisms such as neurotransmitter release. In chapter 2, my dissertation will focus on the regulation of voltage-gated L-type channels that are permeable for $\text{Ca}^{2+}$ and are involved to control neurotransmitter release in cultured retinal amacrine cells. I will show evidence that the different types of mechanisms that are regulating the function of these channels are $\text{Ca}^{2+}$-dependent. One of other roles of $\text{Ca}^{2+}$ is that it can alter enzyme activity, and it activates the enzyme that synthesizes an unconventional neurotransmitter nitric oxide (NO), nitric oxide synthase (NOS). In chapter 3, my dissertation will focus on the distribution pattern of NO by looking at the actual production of NO using a fluorescence indicator of NO and by looking at the potential production of NO using antibodies against the NOS enzymes to identify the cells that express these enzymes in the chicken retina. My work provides evidence for the importance of specific $\text{Ca}^{2+}$-dependent mechanisms and the outcomes of their activation in the function of the amacrine cells.

**Retinal Amacrine Cells**

The name “amacrine” was given to these cells by Ramon y Cajal (1892) because they typically lack axons. The majority of amacrine cells are located in the INL and sometimes in the GCL as displaced amacrine cells. Amacrine cells project their processes into IPL to make synapses with bipolar cells, ganglion cells and other amacrine cells (Dowling and Boycott, 1985; Dubin 1970). There are many subsets of amacrine cells and they are characterized by their physiology, diversity of stratification in the IPL, and their neurotransmitters. Based on morphology and function, the most well known amacrine cells are the AII (Kolb 1979; Kolb and Famiglietti, 1974), the A17 (Kolb et al 1981; Nelson and Kolb 1985), the starbust (Famiglietti

Amacrine cells are known to use GABA, glycine, acetylcholine or dopamine as conventional neurotransmitters and nitric oxide (NO) as an unconventional neurotransmitter. Cells post-synaptic to amacrine cells will have specific receptors for the conventional neurotransmitters. Judging from the large number of distinct neurotransmitter and synaptic connections of amacrine cells to other interneurons, it is predicted that they have many and important functions in the retina. One example is that the glycinergic AII amacrine cells are mainly responsible for mediating the rod-driven retinal pathway in dim-light (scotopic) conditions (Dacheux and Raviola 1986; Kolb and Famiglietti 1975) and GABAergic A17 amacrine cells have influence on this scotopic pathway (Dong and Hare 2002).

Amacrine cells are known to shape the response properties of ganglion cells, which are the output neurons of the retina. For example, ganglion cells that show directional selectivity are post-synaptic to starburst amacrine cells. These direction-selective ganglion cells (DSGC) (Barlow and Lewick 1965; for review see, Demb 2007) respond to stimuli that move in one (preferred) direction and have a minimal response to stimuli moving in the opposite (null) direction. Starburst amacrine cells produce and release both acetylcholine and GABA as their
neurotransmitters (Brecha et al. 1988; O’Malley and Masland 1989; Vaney and Young 1988). DSGCs receive inhibitory input from starbust amacrine cells and this is mediated by calcium transients in the dendrites of starbust amacrine cells (Euler et al. 2002). Knowing how these amacrine cells function will help us to understand their role in visual signaling.

The Cell Culture System

The retina is a complex network with multiple cell types making it technically challenging to investigate the physiology of a specific cell type. The cultured chick amacrine cell system has allows access to details of the cellular physiology of amacrine cells that would not be possible in the intact retina. After 6 days in culture, amacrine cells express the same voltage- and ligand-gated ion channels expressed in the intact adult chicken retina (Huba and Hofmann 1990). Importantly, Gleason et al. (1983; 1993) showed that amacrine cells form functional GABAergic synapses with each other starting at about 6 days in culture. Although subsets of amacrine cells express different neurotransmitters, more than 90% of amacrine cells can be GABAergic (Marc and Liu 2000). GABA is a major transmitter not only in the retina but elsewhere in the brain. Therefore, it is especially important to understand the mechanisms and regulation of GABAergic amacrine cell signaling in the retina. Several classes of amacrine cells in the retina employ L-type voltage-gated Ca\(^{2+}\) channels at their synapses to control their neurotransmitter release (Bieda and Copenhagen 2004; Gleason et al 1994; Habermann et al 2003; Vigh and Lasater 2004). Thus, these cells in culture are a useful model system for studying the primary form of amacrine cell signaling in the retina.

L-Type Calcium Channels

The pore-forming region of L-type Ca\(^{2+}\) channels is encoded by genes Ca\(_{v}\)1.1-Ca\(_{v}\)1.4. Ca\(_{v}\)1.1 is expressed in skeletal muscle, Ca\(_{v}\)1.2-Ca\(_{v}\)1.4 are expressed mainly in the nervous system (Hell et al. 1993; Strom et al. 1998). Skeletal muscle L-type channels and neuronal L-
type channels have similar subunit composition as both have $\alpha_1$, $\alpha_2$, $\beta$, and $\delta$ subunits (Ahlijanian et al. 1990; Campbell et al. 1988; Catterall et al. 1988; Takashi et al. 1987; Takashi and Catterall 1987). In addition to these, skeletal muscle L-type channels also have a $\gamma$ subunit.

L-type Ca$^{2+}$ channels are voltage-gated and regulated by the changes in the membrane voltage and are sensitive to intracellular Ca$^{2+}$ concentration. L-type Ca$^{2+}$ channels are inactivated by the Ca$^{2+}$/calmodulin (Ca$^{2+}$/CaM) complex. This negative feedback is called Ca$^{2+}$/CaM-dependent inactivation (CDI) (for review see, Halling et al. 2005) and the calcium sensing role of CaM has previously been studied (Dick et al. 2008; Erickson et al. 2001; Pitt et al. 2001; Tadross et al. 2008). Mitochondrial calcium uptake (MCU) has been shown to affect synaptic transmission in amacrine cells, potentially through regulation of L-type Ca$^{2+}$ channels in cultured chick amacrine cells (Medler and Gleason 2004). A regulatory effect of MCU on L-type Ca$^{2+}$ channel CDI has been shown in chromaffin cells (Hernandez-Guijo et al. 2001). Unlike CDI, channel phosphorylation by protein kinase A (PKA) enhances the channel activation by increasing the open time of the channels (Bean et al. 1984; Yue et al. 1990b). In Chapter 2, I investigate the multiple Ca$^{2+}$-dependent mechanisms regulating L-type Ca$^{2+}$ channels in cultured retinal amacrine cells (Tekmen and Gleason 2010).

**Nitric Oxide Synthases and Nitric Oxide in the Retina**

Nitric oxide (NO) is produced during an enzymatic reaction that converts arginine into citrulline and NO. The enzyme that catalyzes this reaction is called nitric oxide synthase (NOS). Nitric oxide is an unconventional neurotransmitter in that it is not produced in and released from synaptic vesicles. It is produced upon physiological demand and it does not have a membrane bound receptor that it binds and activates. Nitric oxide initiates the “classical” pathway by stimulating the soluble guanylate cyclase (sGC) activity to produce cGMP (Arnold et al. 1977). Nitric oxide can also have direct effects on protein function via nitrosylation at cysteine residues.
(Davis et al. 2001; Stamler et al. 1997). Nitrosylation is known to alter protein function and has some parallels with protein phosphorylation such as reversibility.

Nitric oxide-dependent effects have been identified for most cell types in the vertebrate retina. Nitric oxide has been shown to affect synaptic transmission at photoreceptor synapses due to modulation of calcium and cyclic-nucleotide-gated channels (Rieke and Schwartz 1994; Savchenko et al. 1997). Nitric oxide can also reduce the gap junction coupling between interneurons in the rabbit retina (Mills and Massey 1995; Xin and Bloomfield 1999). Previous work from our laboratory has demonstrated that NO can switch inhibitory amacrine cell synapses into excitatory synapses by altering cytosolic Cl⁻ levels (Hoffpauir et al. 2006). Unfortunately, we are still lacking a cohesive picture of NO production in the retina and the overall outcome of its generation.

There are three forms of NOS: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). Neuronal NOS and eNOS are activated by cytosolic calcium elevations and are expressed in central nervous system. Nitric oxide synthase has been detected in subsets of amacrine cells and cells in the ganglion cell layer by NADPH-diaphorase-method and nNOS immunolabeling in the vertebrate retina (Cobcraft et al 1989; Fischer and Stell 1999; Kim et al 1999; Koistinaho et al 1993; Neufeld et al, 2000; Oh et al 1999; Osborne et al 1993; Perez et al 1995; Provis and Mitrofanis 1990; Rios et al 2000; Sagar 1986; Sandell 1985; Vaccaro et al 1991; Vaney and Young 1988; Wassle et al 1987; Wilson et al. 2011). The NADPH-diaphorase method allows researchers to detect NOS because NOS can produce NADPH-diaphorase reaction in the processes of converting arginine into citrulline and NO (Hope et al. 1991). Amacrine cells that are NADPH-diaphorase-positive have been shown in the chicken retina (Fischer and Stell 1999; Rios et al. 2000; Wilson et al. 2011). Fischer and Stell and Wilson and colleagues have also shown NOS-immunolabeling in the chicken retina.
Neuronal NOS-immunolabeling was demonstrated in subsets of amacrine cells (Eldred and Blute 2005). Goureau et al. (1997) was first to show anti-eNOS labeling in the chicken retina, followed by Haerkamp and colleagues in 1999. These studies; however, focused primarily on expression in the central retina. Here, we examine the entire retina along the dorsal-ventral axis and observe regional differences in nNOS expression which have not been previously reported.

My research described in Chapter 3 indicates that NO can be produced by both nNOS and eNOS expressing neurons. Based on the binding of an eNOS antibody, this enzyme appears to be broadly expressed in the chicken retina and unlike nNOS; the pattern of eNOS-labeled cells was not differently distributed within dorsal and ventral retina in chicken. This evidence confirms the broad distribution of the NOS-expressing cells that are potentially capable of generating NO signals in the chicken retina.

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Chapter 2

Multiple Ca^{2+}-Dependent Mechanisms Regulate L-Type Ca^{2+} Current in Retinal Amacrine Cells*

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**Introduction**

Amacrine cells are retinal interneurons that signal extensively in the inner plexiform layer of the retina. The functions of amacrine cells are diverse but include shaping the response properties of ganglion cells, the output cells of the retina (Baccus 2007; Demb 2007; Zhou and Lee 2008). These key players in retinal signal processing often participate in serial (Dowling and Boycott 1966; Dubin 1970; Guiloff et al. 1988; Pollard and Eldred 1990; Zhang et al. 1997) and reciprocal synapses (Hartveit 1999; Shields and Lukasiewicz 2003; Vigh and von Gersdorff 2005), implying that local synaptic environments might be regulated independently of one another. It has been established for several classes of amacrine cells that L-type Ca\(^{2+}\) channels are employed at their synapses to control neurotransmitter release (Bieda and Copenhagen 2004; Gleason et al. 1994; Habermann et al. 2003; Vigh and Lasater 2004). Thus the regulation of these channels can play a central role in visual signal processing.

The pore-forming region of the L-type channel is encoded by one of four genes: Ca\(_V\)1.1–1.4. Ca\(_V\)1.1 is expressed in skeletal muscle, Ca\(_V\)1.2 and -1.3 are the dominant L-type channels in the brain (Hell et al. 1993), and Ca\(_V\)1.4 is expressed predominately at ribbon synapses in the retina (Strom et al. 1998). L-type Ca\(^{2+}\) channels are distinctive in that they can support fairly sustained levels of Ca\(^{2+}\) influx. This Ca\(^{2+}\) influx can have a variety of effects including regulation of the channel itself via Ca\(^{2+}\)/calmodulin (CaM)-dependent inactivation (CDI) that occurs for most Ca\(_V\)1 and -2 (non-L-type) Ca\(^{2+}\) channels (for review see, Halling et al. 2005).

The molecular players and details of this inactivation have been described by an elegant set of experiments on Ca\(_V\)1/2 channels (Dick et al. 2008; Tadross et al. 2008). The efficiency of the inactivation process is optimized by the preassociation of CaM to the channel (Erickson et al. 2001; Pitt et al. 2001). Ca\(^{2+}\) entering through the channel binds CaM and inactivation is initiated. In Ca\(_V\)1.2/1.3 channels, the CaM sensors detect both local and global Ca\(^{2+}\) (Dick et al. 2008).
The local concentration of Ca\(^{2+}\) eliciting this response is on the order of 100 µM, which only exists within hundreds of angstroms of the channel pore (Neher 1998; Sherman et al. 1990).

Another known regulator of L-type Ca\(^{2+}\) channels is protein kinase A (PKA). Phosphorylation of L-type channels by PKA enhances the whole cell current amplitude by increasing the open time of the channels (Bean et al. 1984; Yue et al. 1990b). The level of PKA activity can be regulated by cell surface receptors linked to G proteins that either stimulate (\(G_s\)) or inhibit (\(G_i\)) adenylate cyclase (AC). There are nine membrane bound isoforms of AC, all of which can be stimulated by activated \(G_s\) (for review, see Willoughby and Cooper 2007).

Alternatively, AC1 and AC8 can be directly activated by the Ca\(^{2+}\)/CaM complex with AC1 being about five times more sensitive to Ca\(^{2+}\)/CaM (Kds \(\sim\)100 nM, AC1; \(\sim\)500 nM, AC8) (Fagan et al. 1996; Wu et al. 1993). We have previously reported that metabotropic glutamate receptor 5- and phospholipase C-dependent activation of PKA enhances the amplitude of L-type Ca\(^{2+}\) currents in retinal amacrine cells, possibly via an AC1-dependent mechanism (Sosa and Gleason 2004).

If these two Ca\(^{2+}\)-dependent Ca\(^{2+}\) channel regulators (CDI and AC1/8) coexist in amacrine cells, then we would predict that mechanisms regulating cytosolic Ca\(^{2+}\) will influence the outcome of L-type Ca\(^{2+}\) channel regulation. It has been previously shown that synaptic transmission between retinal amacrine cells is affected by mitochondrial Ca\(^{2+}\) uptake (MCU) (Medler and Gleason 2002). This work led us to hypothesize that at least part of the impact of MCU on synaptic transmission was in maintaining the L-type Ca\(^{2+}\) channels in a relatively noninactivated state. Here we test this hypothesis by examining the effects of disrupting MCU on L-type Ca\(^{2+}\) channel function.

Entry of Ca\(^{2+}\) through L-type channels is known to elicit Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) in amacrine cells (Mitra and Slaughter 2002; Warrier et al. 2005). Because this
amplification of the Ca²⁺ signal has the potential to affect other Ca²⁺-dependent processes, we also test the role of ryanodine receptor (RyR) activity in the Ca²⁺-dependent regulation of these channels.

Using a primary cell culture system consisting of identified GABAergic amacrine cells (Gleason et al. 1993), we have begun to clarify the physiological relationships between L-type Ca²⁺ channel inactivation, MCU, PKA activity, and CICR. Although the molecular details of CDI and PKA-dependent current enhancement have been worked out, most of this work has been done in expression systems or in cardiac myocytes. It remains to be determined how these factors interact in the native environment of retinal amacrine cells; an interneuron critical to shaping the output of the retina. Given the dependence of synaptic transmission on L-type Ca²⁺ channel function in these cells (Gleason et al. 1994), our aim is to investigate the balance of Ca²⁺-dependent mechanisms regulating L-type Ca²⁺ channel functions in retinal amacrine cells.

**Methods**

**Cell Culture**

Primary cell cultures of chick retinal amacrine cells were used in our experiments. The chicken embryos (Gallus gallus, Animal Science Department, Louisiana State University, Baton Rouge, LA) were dissected on embryonic day 8, and retinal cells were dissociated and cultured as previously described (Hoffpauir and Gleason 2002). Cell cultures were maintained at 37°C under 5% CO₂ atmosphere until they were ready for experiments, 8-14 days after plating. For electrophysiology experiments, amacrine cells were identified based on their morphology. Cells with large somas (10-15 µm) with two to five primary processes have been previously identified as amacrine cells based on immunocytochemical and physiological criteria (Gleason et al. 1993; Huba and Hofmann 1990, 1991; Huba et al. 1992).
Solutions

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). External solutions consist of the following (in mM): 116.7 NaCl, 20.0 TEACl, 3.0 CaCl₂, 0.4 MgCl₂, 5.6 glucose, and 10.0 HEPES. Voltage clamp experiments performed in the perforated-patch configuration employed the following internal solution (in mM): 135.00 CsAc, 10.0 CsCl, 1.0 NaCl, 2.0 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10.0 HEPES, and 200 µg/ml amphotericin B. Voltage clamp experiments performed in the ruptured-patch configuration used the following internal solution (in mM): 100.00 CsAc, 10.0 CsCl, 2.0 MgCl₂, 0.1 CaCl₂, 10.0 HEPES, 3.0 ATP (dipotassium), 1.0 ATP (disodium), 20.0 phosphocreatine, 2.0 GTP, and 50 U/ml creatine phosphokinase. Solutions were adjusted to pH 7.4 with NaOH for external solutions and with CsOH for internal solutions. Two different Ca²⁺ buffers were also included in internal solutions in ruptured-patch recordings: Ethylene glycol-bis (2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA, 1.1 or 14 mM) and 1,2-bis-(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA, 10 mM, Enzo Life Sciences, Plymouth Meeting, PA).

A pressurized gravity flow perfusion system (1.5-2 ml/min) was used to deliver the external solutions (Automate Scientific, Berkeley, CA). Unless otherwise indicated, the following reagents were purchased from Enzo Life Sciences. Reagents added via the bath included the protonophore carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP, 1 µM), a PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide· 2HCl (H89, 1 µM), and adenylate cyclase (AC) inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine, (SQ 22,536, 200 µM) a general phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 100 µM), a calcineurin inhibitor cyclosporine A (CsA, 1 µM), and a selective inhibitor of Ca²⁺/CaM-dependent phosphodiesterase (PDE 1) 8-methoxymethyl-1-methyl-3-(2-methylpropyl) xanthine (8-M-IBMX, 100 µM), inhibitors of the RyR, ryanodine (14 µM) and
dantrolene (20 µM, Sigma). CaM inhibitor calmidazolium chloride (CMZ, 10 µM) was added to the pipette solution. In all electrophysiology experiments, (-)-bicuculline methobromide (10 µM, tocris Bioscience, Ellisville, MO) was included in external solutions to block GABA\textsubscript{A} receptor-mediated autaptic currents (Gleason et al. 1993). Tetrodotoxin (TTX, 300 µM, Alomone Labs, Jerusalem, Israel) was included in external solutions to block voltage gated Na\textsuperscript{+} currents.

**Electrophysiology**

Cell culture dishes were mounted on the stage of an Olympus IX70 inverted microscope. A reference Ag/AgCl pellet served to ground the bath. Patch electrodes were pulled from thick-walled borosilicate glass with a filament (1.5 mm OD, 0.86 mm ID; Sutter Instrument, Novato, CA) using a Flaming-brown Micropipette Puller (Sutter Instruments). For electrophysiology experiments, either ruptured- or perforated-patch whole cell recording was performed. For perforated-patch recordings, only cells with stable resistance (changes of <5 MΩ) were used in the experiments. Recordings were made using Axopatch 1D-patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Data were recorded using Clampfit 9.2 ad 10.0 software (Molecular Devices). Electrode resistance values were monitored and ranged from 3 to 8 MΩ. Junction potential corrections (-8 mV) were made for the data in Fig. 2, D and E.

Because we were concerned that some experimental manipulations might produce long term changes in the cells, experimental and control recordings were often done in separate groups of cells. We also used separate groups of cells for experiments with different internal solutions. When this sort of protocol was used, the different treatments (or internal solutions) were alternated from cell to cell. As such, comparisons were made between cells from the same culture and often from the same culture dish.
Western Blots

Chicken and mouse brains were homogenized in nondenaturing lysis buffer containing a cocktail of protease inhibitors [PMSF (1 mM), leupeptin (5 µg/ml) aprotinin (2.5 µg/ml), 1,10 ortho-phenantroline (0.2 µg/ml), and pepstatin (0.7 µg/ml)]. Samples were spun at 4,000 rpm for 20 min at 4°C. Protein content was determined using the BCA protein assay kit from Pierce (Rockford, IL). Proteins (300 µg) were separated on a 7.5% SDS gel along with 10 µl Pageruler molecular weight markers (Fermata, Glenburnie, MD). Proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 4% milk, 0.1% Tween 20 in Tris buffered saline. The polyclonal antibody raised against human AC1 (Abcam, Cambridge, MA) was diluted 1:500 in PBS with 1% BSA. Goat anti-rabbit secondary antibody conjugated on horseradish peroxidase (Pierce) was diluted to 1:1,000 in PBS with 1% milk. Membranes were incubated in primary and secondary antibodies for 1.5 h each, at room temperature. Proteins were visualized using the Supersignal Western Pico Reagent (Pierce).

Immunocytochemistry

Adult White Leghorn chickens were killed by intraperitoneal injection of sodium pentobarbital (500 mg/kg, Sigma-Aldrich) followed by decapitation. These methods were approved by the Institutional Animal Care and Use Committee, Louisiana State University. The eyes were enucleated and hemisected. After removing the vitreous, 4% paraformaldehyde was added to the eye cups and kept at 4°C for 1 h. Following fixation, eye cups were washed in PBS + 1% glycine. Retinae were then dissected from eye cups and incubated in 15% sucrose for 30 min, 20% sucrose for 1 h, and 30% sucrose solution overnight at 4°C. Retinae were embedded in OCT compound (Sakura Finetek, Torrence, CA) by freezing in dry ice and isopentane. Sections (12-16 µm) were cut on a Leica CM1850 cryostat (Wetzlar, Germany) and mounted on presubbed slides (Southern Biotech, Birmingham, AL).
Cells grown on glass coverslips were fixed in 2% paraformaldehyde for 30 min at 4°C after 8 days in culture. Fixed cells and retinal sections were preincubated for 1 h at room temperature in a blocking solutions consisting of dilution solution (see following text) with 10% normal goat serum. Primary polyclonal antibodies raised against human AC1 were diluted at 1:100 in dilution solution (PBS, 1% bovine serum albumin, 0.5% saponin) and applied to either retinal sections or cells for 1 h at room temperature then washed in PBS. Secondary goat-anti-rabbit antibodies conjugated to Cy3 were obtained from Millipore (Temecula, CA) and were diluted 1:1,000 in dilution solution. Cells were incubated in secondary antibodies for 1 h at room temperature. After washing, coverslips and slides were mounted in a medium containing 70% glycerol, 28% PBS and 2% n-propyl gallate. Cells and retinal sections were viewed on an Olympus IX70 microscope equipped with epifluorescence and images were captured using Slidebook software and hardware (Intelligent Imaging Innovations, Denver, CO).

**Data Analysis**

The Origin 7.5 and 8.0 (OriginLab, Northampton, MA) software package was used to analyze and plot the data. Images in Fig. 8 were adjusted for brightness and contrast in Adobe Photoshop (San Jose, CA). Equivalent adjustments were made for experimental and control images. Figures were assembled in Adobe Illustrator. Statistical analyses were done using the t-test and data are presented as mean ± SE. Maximum P value for significance was 0.05.

**Results**

**Ca²⁺ Current Amplitude Increases with Repeated Depolarizations**

Whole cell recordings were made from single isolated amacrine cells in the perforated-patch configuration. It has been previously established that these cells express L-type but not N or P-type Ca²⁺ channels (Gleason et al. 1993). To examine the normal variability in current amplitude over time, Ca²⁺ currents were elicited by depolarizing amacrine cells from -70 to
0 mV either for 1,000 ms, every 60 s, or for 100 ms every 30 s (Fig. 1, A and C, respectively). It is evident, especially for the longer voltage steps (Fig. 1A), that inactivation of the current occurs over the duration of the step. It was also observed that the time course and degree of inactivation could vary from cell to cell. The physiological basis for these differences are not known but could feasibly be due to different expression levels of calmodulin or other effectors (see DISCUSSION). The tail currents after the voltage step (observable in Fig. 1C as well as in subsequent figures) are primarily due to the activity of the plasma membrane Na/Ca exchanger transporting Ca$^{2+}$ back out of the cell. Our ability to identify and to measure electrogenic Na/Ca exchange activity has been firmly established for these cells (Gleason et al. 1994, 1995; Hurtado et al. 2002; Medler and Gleason 2002).

Although inactivation occurred during the voltage step, the peak current amplitudes tended to increase over time, implying that multiple levels of regulation are occurring. Figure 1, B and D, shows the peak current amplitude for steps (1 s and 100 ms) delivered every 60 and 30 s (respectively) from separate populations of amacrine cells. A progressive increase in the current amplitude was typically observed with both protocols. This increase is not due to changes in series resistance because only cells with stable series resistances (see METHODS) were included in the analysis. The rate of the increase in current amplitude was inherently variable among cells as indicated by the substantial error bars that tended to increase over the duration of the recording. Because the only “treatment” that the cells received in these recordings was the history of voltage steps and the resulting Ca$^{2+}$ influx, we predicted that the primary sources of enhancement were Ca$^{2+}$ dependent. Perhaps then, variability in current amplitude enhancement was related to the Ca$^{2+}$ current density in each amacrine cell. To determine whether the rate of increase in current amplitude was linked to Ca$^{2+}$ current density, these quantities were plotted for
**Figure 2.1: The Ca\(^{2+}\) current amplitude increases over time in control conditions.**

A and C: perforated-patch recordings are shown from 2 different amacrine cells. Cells were depolarized from -70 to 0 mV for 1 s, every 60 s (A and B); or for 100 ms, every 30 s (C and D). Current traces are shown from the time points indicated in B and D. Pairs of traces were selected to show that currents recorded a minute apart are only slightly different in amplitude (A and C, left). These traces were also selected for comparison because traces from similar time points are depicted in subsequent figures. Traces collected further apart in time show that the differences in amplitude are augmented over a longer time frame (A and C, right).

B and D: each data point is the mean normalized peak current amplitude elicited by the 2 protocols and plotted over time (30 s \(n = 6\); 60 s \(n = 4\)).

E: current density (pA/pF) for the current elicited by the 1\(^{st}\) voltage step is plotted against its rate of current amplitude increase for each cell. The rate was estimated by drawing a line (fit by eye) through the data at the 1\(^{st}\) 3 time points for each cell, then calculating the slopes for those lines. The measurements from all cells depolarized from -70 to 0 mV for 100 ms, every 30 s were included in this analysis. Regression analysis does not reveal a correlation between these 2 quantities (\(R^2 = 0.02\)).
a population of cells that had been recorded under the same conditions (step to 0 mV for 100 ms, every 30 s, Fig. 1E). Regression analysis revealed an $R^2$ value of 0.02 (Fig. 1E) and does not indicate a dependence on current density. This implied that the regulation under these stimulus protocols is not a simple transform based on the amount of Ca\textsuperscript{2+} influx. Instead it suggested that the regulation of these channels has multiple Ca\textsuperscript{2+}-dependent components that vary intrinsically among cells. This suggestion is borne out by much of the data presented in subsequent sections.

**Disruption of MCU Inhibits the Ca\textsuperscript{2+} Current**

To test the hypothesis that L-type Ca\textsuperscript{2+} channel inactivation in amacrine cells is regulated by MCU (Medler and Gleason 2002), we used the protonophore FCCP to temporarily collapse the proton gradient across the inner mitochondrial membrane and disrupt MCU via the Ca\textsuperscript{2+} uniporter (Herrington et al. 1996; Werth and Thayer 1994; White and Reynolds 1997). Whole cell current recordings were made in the perforated patch configuration. Amacrine cells were depolarized from -70 to 0 mV for 1 s, every 60 s. Disrupting MCU had two effects: an increase in the inward current amplitude recorded at -70 mV (before and after the voltage step to 0 mV) and a decrease in the Ca\textsuperscript{2+} current amplitude recorded during the step to 0 mV. We have previously shown that the relatively time-invariant increase in inward current at -70 mV (Fig. 2A, arrow) is due to a persistent FCCP-dependent increase in cytosolic Ca\textsuperscript{2+} that activates the electrogenic plasma membrane Na/Ca exchanger (Medler and Gleason 2002). Furthermore, it has been established that the FCCP-dependent Ca\textsuperscript{2+} increase driving this exchanger activity is due to the RyR-dependent leakage of Ca\textsuperscript{2+} from stores that is normally sequestered by mitochondria via the uniporter (Sen et al. 2007). Importantly, we have also previously demonstrated that Na/Ca exchange activity is negligible at 0 mV and thus does not contribute significantly to the current recorded during the voltage step to 0 mV (Gleason et al. 1995). To simplify the appearance of the data, the FCCP-dependent Na/Ca exchange current at -70 mV has
been subtracted from subsequent data (as in Fig. 2B). However, we show this current in insets (Figs. 5 and 6) to confirm that this FCCP-dependent Ca\(^{2+}\) elevation and exchanger activity persists under some key experimental conditions. More directly relating to our hypothesis, disrupting MCU also caused a reversible decrease in the Ca\(^{2+}\) current amplitude (Fig. 2, A and B, gray trace). Under these conditions, disrupting MCU significantly reduced the Ca\(^{2+}\) current amplitude by 35 ± 7% \((n = 7, P = 0.03, \text{Fig. 2C})\). A series of control experiments previously established that the effects of FCCP on depolarization-induced Ca\(^{2+}\) elevations in these cells are not due to ATP depletion (Medler and Gleason 2002) (also see DISCUSSION) or changes in pH (Sen et al. 2007). Another possibility was that the decrease in current amplitude was due to an FCCP-dependent shift in the activation range of the channels. To address this, currents were elicited by steps over a range of voltages from -80 to -10 mV in 5 mV increments to reveal the voltage of activation. These experiments were done in ruptured-patch because the voltage of activation can be better resolved with the relatively low series resistance recordings achieved in this configuration. These recordings were made with two different internal Ca\(^{2+}\) buffering conditions (1.1 mM EGTA, 10 mM BAPTA). Although we observed a difference in the effects of FCCP on Ca\(^{2+}\) current amplitude in 10 mM BAPTA (see following text), no FCCP-dependent shift in activation range was observed under either Ca\(^{2+}\) buffering condition (Fig. 2, D and E, respectively). These data are consistent with a role for MCU in limiting the degree of Ca\(^{2+}\)/CaM-dependent inactivation for these channels.

**Effects of Disrupting MCU Are Dependent On the Duration and Frequency of the Voltage Step**

To examine whether increases in Ca\(^{2+}\) influx enhanced the effects of disrupting MCU, we first altered the duration of the voltage steps. Ca\(^{2+}\) currents were recorded in response to voltage steps lasting either 50 ms or 3 s (Fig. 3, A and B, respectively). Currents from 50 ms steps were reduced by 35 ± 4% \((n = 7)\), whereas currents from 3 s steps were reduced by 60 ± 5% in the
Figure 2.2: Inhibition of mitochondrial Ca\(^{2+}\) uptake (MCU) reduces the Ca\(^{2+}\) current amplitude without altering the voltage of activation. A and B: perforated-patch recordings from an amacrine cells in the absence (black trace) and presence of carboxylsynaide-4-((trifluoromethoxy)-phenylhydrazone (FCCP, gray trace). Amacrine cells were depolarized from -70 to 0 mV for 1 s every 60 s. Representative current traces are from the 3\(^{rd}\) and 4\(^{th}\) voltage steps. The sustained FCCP-dependent inward current at -70 mV (A, arrow) is due to Na/Ca exchanger activity and has been subtracted from subsequent data. This current is negligible at 0 mV so no subtraction was done for the data collected during the voltage step (B). C: normalized mean peak current amplitude is plotted over time (n = 7). D and E: activation range of the channels was revealed by eliciting currents by steps over a range of voltages (-80 to +10 mV, in 5 mV increments). Two different internal Ca\(^{2+}\) buffering conditions were used (1.1 mM EGTA n = 5, or 10 mM BAPTA n = 4) in ruptured-patch configuration. No FCCP-dependent shift in activation range was observed under either condition in any of the cells.

presence of FCCP (n = 6, C). The significantly (P = 0.002) larger effect of FCCP for longer voltage steps supports the possibility that Ca\(^{2+}\)-dependent inactivation was being altered by disrupting MCU. If the level of FCCP-dependent inhibition of L-type channel current increased with prolonged voltage steps, and this was due to the greater Ca\(^{2+}\) influx during longer steps, we
would also predict that increasing the frequency of depolarization would intensify the effects of inhibiting MCU. To test this, single amacrine cells were depolarized from -70 to 0 mV, for 100 ms, and voltage steps were delivered either every 60 s (Fig. 3D) or every 5 s (E). Disrupting MCU caused a reduction in the Ca\(^{2+}\) current amplitude under both recording condition; however, the FCCP-dependent reduction in current amplitude was significantly larger for higher frequency depolarizations [61 ± 4% reduction for every 5 s (n = 6), and 35 ± 4% reduction for every 60 s (n = 7), P = 0.001, Fig. 3F]. These results further support the possibility that inhibition of the Ca\(^{2+}\) current amplitude is dependent on cytosolic Ca\(^{2+}\) levels.

**Effects of MCU Are Not Dependent On Calcineurin**

The established role of PKA-dependent channel phosphorylation in L-type current enhancement raises the possibility that the effect of inhibiting MCU is to reduce channel phosphorylation. The most likely candidate for such an activity under these conditions would be the Ca\(^{2+}/CaM\) dependent phosphatase, calcineurin. To test for the involvement of calcineurin, we asked whether the potent (IC\(_{50}\) = 7 nM) (Fruman et al. 1992) calcineurin inhibitor CsA (for review, see Kunz and Hall 1993) would block the effects of FCCP. Recordings were made using 100 ms steps from -70 to 0 mV, every 30 s. Cells were preincubated for 10–20 min in CsA (1 µM) before FCCP was applied. The inhibitory effect of FCCP persisted in the presence of CsA (Fig. 4, A and B), and CsA alone had no consistent effect on the current (C). Negative results require cautious interpretation but it is important to note that with similar exposure times (10–20 min) and concentrations (0.1–1 µM), CsA has been demonstrated to be effective in inhibiting calcineurin in intact neurons (Xu and Krukoff 2007) and smooth muscle cells (Schuhmann et al. 1997). These data therefore suggest that calcineurin activity is not mediating the FCCP-dependent inhibition of the current and may not be a major regulator of L-type Ca\(^{2+}\) channel activity in these amacrine cells.
Figure 2.3: The effect of inhibiting MCU is dependent upon the duration and frequency of depolarization. A and B: perforated-patch recordings from representative amacrine cells depolarized from -70 mV to 0 mV for either 50 ms (A) or 3 s (B). Steps were delivered every 60 s. Recordings were made in the absence (black traces) and presence (gray traces) of FCCP. C: mean normalized peak current amplitudes are plotted against step number for both step durations (50 ms, n = 7; 3 s, n = 6). D and E: perforated-patch recordings from individual amacrine cells stepped from -70 mV to 0 mV for 100 ms. Voltage steps were delivered either every 60 s (D) or 5 s. (E). Currents were recorded in the absence (black traces) and presence (gray traces) of FCCP. F: normalized mean peak current amplitude plotted over time for each depolarization frequency (5 s, n=6; 60 s, n = 7).

**Effects of MCU Are Ca\textsuperscript{2+} Influx-Dependent**

Were the effects of disrupting MCU on the current amplitude due to an excess of Ca\textsuperscript{2+} originating from channel entry or were they due to the FCCP-dependent elevation in cytosolic Ca\textsuperscript{2+} as revealed by the increase in the activity of the Na/Ca exchanger (Fig. 2A)? To distinguish between these two possibilities, we replaced external Ca\textsuperscript{2+} with Ba\textsuperscript{2+}. Ba\textsuperscript{2+} is known to carry the current through L-type channels but is a poor substitute for Ca\textsuperscript{2+} with respect to inactivation
Figure 2.4: Calcineurin is not a major regulator of the Ca\textsuperscript{2+} current. A: a representative recording from an amacrine cell showing the Ca\textsuperscript{2+} current recorded in cyclosporin A (CsA, 1 \( \mu \text{M} \)) just prior to the addition of FCCP (black trace) and after 1 min of FCCP exposure (gray trace). Cells were depolarized from -70 mV to 0 mV for 100 ms once every 30 s. B: the time course of normalized Ca\textsuperscript{2+} current amplitude is plotted for 6 cells treated with CsA. C: under the same recording conditions as in A and B: CsA alone had no consistent effect on the Ca\textsuperscript{2+} current (n = 5).

(Brehm and Eckert 1978; Chad and Eckert 1986; Tillotson 1979; Yue et al. 1990a; Zuhlke et al. 1999). Perforated-patch recordings were made in amacrine cells bathed with either normal external (3 mM Ca\textsuperscript{2+}, Fig. 5A) or external solution with equimolar Ba\textsuperscript{2+} replacing Ca\textsuperscript{2+} (B). Cells were stepped from -70 to 0 mV for 1 s. The currents recorded in Ba\textsuperscript{2+} inactivated relatively little over the course of the 1 s depolarization consistent with minimal Ca\textsuperscript{2+}-dependent inactivation. With Ba\textsuperscript{2+} as the charge carrier, the effect of disrupting MCU was significantly reduced (18 ± 3% reduction with Ba\textsuperscript{2+}, n = 7; 44 ± 7% reduction with Ca\textsuperscript{2+}, n = 7; \( P = 0.007 \)) indicating that the primary source of the inhibition was from Ca\textsuperscript{2+} crossing the plasma membrane (Fig. 5C). The relatively small inhibitory effect of FCCP on Ba\textsuperscript{2+} current amplitude that was observed may be due to either the low level of CaM activation known to occur with Ba\textsuperscript{2+} (Dick et al. 2008) and/or the FCCP-dependent Ca\textsuperscript{2+} elevation (Medler and Gleason 2002; Set et al. 2007). The persistence of the FCCP-dependent Ca\textsuperscript{2+} elevation in external Ba\textsuperscript{2+} is demonstrated by the presence of the FCCP-dependent Na/Ca exchange current shown in Fig. 5B, *inset*. The relatively small effect of
inhibiting MCU on Ba\(^{2+}\) current amplitude is consistent with the hypothesis that MCU normally sequesters Ca\(^{2+}\) entering through L-type Ca\(^{2+}\) channels.

Figure 2.5: The effects of inhibiting MCU are dependent upon Ca\(^{2+}\) influx. A and B: voltage steps were delivered from –70 mV to 0 mV for 1 s, every 60 s in perforated-patch recordings. Recordings were made in external solutions containing either 3 mM Ca\(^{2+}\) or 3 mM Ba\(^{2+}\). Currents are shown from the 3\(^{\text{rd}}\) (black traces) and 4\(^{\text{th}}\) voltage steps (in FCCP, gray traces). B, inset: unsubtracted current at -70 mV just prior to the voltage step to 0 mV reveals that the FCCP-dependent Na/Ca exchange current (and thus the FCCP-dependent Ca\(^{2+}\) elevation) persists in external Ba\(^{2+}\). Inset: scale bar is 20 pA. C: mean normalized peak current amplitudes are plotted over time for data collected in both Ca\(^{2+}\) and Ba\(^{2+}\) (n=7 for each).

**Increasing Cytosolic Ca\(^{2+}\) Buffering Alters the Effects of Inhibiting MCU**

Different levels of internal Ca\(^{2+}\) buffering are known to alter CDI (Brehm and Eckert 1978; Dick et al. 2008; Kalman et al. 1988; Kohr and Mody 1991; Tadross et al. 2008). To further explore the role of CDI in the effects of blocking MCU, experiments were repeated under different Ca\(^{2+}\) buffering conditions. Either EGTA (1.1 or 14 mM) or the faster buffer BAPTA
(10 mM) (Adler et al. 1991) was included in recording pipette. For these experiments, Ca\(^{2+}\) currents were recorded in the ruptured-patch configuration. Currents were recorded before (Figs. 6, A–C, black traces) and during application of FCCP (gray traces). First, it should be noted that the different buffering environments differentially affect the two components of the Na/Ca exchange current. The FCCP-dependent component of the exchange current occur (-35 pA in each cell) under all three Ca\(^{2+}\) buffering conditions (Fig. 6, A–C, insets), indicating that Ca\(^{2+}\) elevations can persist under these buffering conditions. The Ca\(^{2+}\) current-dependent component of the Na/Ca exchange current (visible as tail currents after the voltage step) is more sensitive to Ca\(^{2+}\) buffering conditions with the current nearly eliminated in 10 mM BAPTA.

With 1.1 mM EGTA, the effect of FCCP was not significantly different from perforated-patch recordings (perforated 45 ± 4% reduction, n = 6; 1.1 mM EGTA 36 ± 6% reduction, n = 6, P = 0.23, Fig. 6, A, D, and E), suggesting that this level of artificial Ca\(^{2+}\) buffering approximates that found in intact amacrine cells. However, with 14 mM EGTA, the effects of disrupting MCU were significantly suppressed [13 ± 6% reduction (vs. 36 ± 6%), n = 6, P = 0.02] and delayed (no Ca\(^{2+}\) current reduction observed until a minute of FCCP exposure) when compared with 1.1 mM EGTA (Fig. 6, B, D, and E). Hence more EGTA minimized but did not eliminate the effect of MCU on the Ca\(^{2+}\) current amplitude, consistent with the idea that stronger buffering reduces CDI. Overall, blocking MCU in the presence of 10 mM BAPTA produced a 12 ± 5% (n = 22) enhancement in the Ca\(^{2+}\) current that was significantly different from the results in 1.1 mM EGTA (P = 0.0002, Fig. 6E). The sign of the effect varied from cell to cell with current enhancement in 12/22 cells (31 ± 6% enhancement) or small reductions in the current in 10 /22 cells (0.7 ± 2%, Fig. 6, C, D, and E). These data indicated that strong Ca\(^{2+}\) buffering reduced the impact of MCU on channel inactivation. We hypothesize that in BAPTA, CDI is reduced. In some cells, this reveals another Ca\(^{2+}\)-dependent process that enhances current amplitude.
Figure 2.6: Increasing cytosolic Ca\(^{2+}\) buffering alters the effects of inhibiting MCU. A-C: representative Ca\(^{2+}\) currents recorded in the ruptured-patch configuration with internal solution containing either 1.1 mM EGTA (A), 14 mM EGTA (B), or 10 mM bis-(\(\sigma\)-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA, C). Dashed lines (A-C) define the 0 current level and the end of the voltage step so that tail currents can be compared under the 3 buffering conditions. Currents were elicited by 100 ms voltage steps from -70 mV to 0 mV delivered every 30 s. Currents shown are from the 2\(^{nd}\) voltage step (black traces) or the 4\(^{th}\) voltage step (in FCCP, gray traces). A-C, insets: the unsubtracted current recorded at -70 mV (Na/Ca exchange current) demonstrating the presence of the FCCP-dependent Ca\(^{2+}\) elevation under all 3 Ca\(^{2+}\) buffering conditions. Inset scale bar is 20 pA. D: mean normalized peak current amplitude is plotted over time for the 3 buffering conditions. E: FCCP-dependent changes in current amplitude (comparing data points 2 and 4) are plotted for each buffering condition. Single asterisk, \(P < 0.05\); triple asterisks, \(P < 0.001\).

**Inhibition of PKA and AC Decreases the Ca\(^{2+}\) Current Amplitude**

It is well known that PKA-dependent phosphorylation can enhance L-type Ca\(^{2+}\) currents by increasing the open time of the channels (Bean et al. 1984; Yue et al. 1990b). In amacrine cells, we have previously demonstrated that metabotropic glutamate receptor 5 activation leads
to a PKA-dependent enhancement of the L-type current that does not result from changes in voltage sensitivity (Sosa and Gleason 2004). To test for the involvement of PKA in the FCCP-dependent enhancement of the Ca$^{2+}$ current in BAPTA, we examined the effects of the PKA inhibitor, H89 (1 µM) with BAPTA (10 mM) internal. H89 has been shown to be specific for PKA when used at concentrations <10 µM (Chijiwa et al. 1990). For these experiments, different cells were used for the different treatments to avoid complications due to previous drug exposures (see METHODS). On average, BAPTA-loaded cells tested with FCCP showed enhancement of the Ca$^{2+}$ current (12 ± 5%, $n = 22$, Fig. 6E). Inhibition of PKA with H89 reduced the Ca$^{2+}$ current amplitude in all cells tested indicating basal PKA activity (41 ± 5%, $n = 4$, Fig. 7, A and C). When used in combination (H89 + FCCP), the effect on the Ca$^{2+}$ current was generally larger but the difference was not statistically significant (58 ± 8% reduction, $n = 6$, $P = 0.17$, Fig. 7, B and C) than with either reagent alone indicating that with reduced PKA activity, inhibition of MCU still contributes to inactivation. Our interpretation of the target of H89 (PKA) is consistent with our previous observation that 8-bromo cAMP enhances the Ca$^{2+}$ current in these cells (Sosa and Gleason 2004). PKA is activated by cAMP which is generated by the enzymatic activity of AC. To confirm the involvement of this classical pathway, we asked whether inhibition of AC would have the same effects on the Ca$^{2+}$ current as inhibition of PKA. The general AC inhibitor SQ 22,536 (200 µM) was used to inhibit AC (Fabbri et al. 1991). In all cells tested (84 ± 4% reduction, $n = 7$), SQ 22,536 exposure reduced the amplitude of the Ca$^{2+}$ current (Fig. 7, D and E), consistent with basal AC activity producing cAMP and driving basal PKA activity.

**Expression of AC1**

Because the PKA activity appeared to be independent of cell surface receptor activation in these experiments, we postulated that the enzyme is stimulated by cAMP that has been
generated through the activity of the Ca$^{2+}$/CaM-dependent adenylate cyclase AC1. The Kd of AC1 for Ca$^{2+}$ is 100 nM (Fagan et al. 1996; Wu et al. 1993) is near resting cytosolic Ca$^{2+}$ levels in these cells (50–100 nM, Hurtado et al. 2002) making this enzyme a good candidate for mediating both basal PKA activity as well as enhanced activity due to Ca$^{2+}$ influx via L-type V-gated Ca$^{2+}$ channels. We have previously demonstrated AC1-like immunoreactivity in cultured amacrine cells (Sosa and Gleason 2004). Here we further examine the expression of AC1 using a
different, and more fully characterized, polyclonal antibody raised against human AC1. The specificity of this antibody was confirmed in Western blots using homogenates of both chicken and mouse brain (Fig. 8A). Single bands near the predicted molecular weight of AC1 (130 kDa) were detected for both chicken and mouse brain homogenate, indicating that the antibody recognizes the avian form of AC1. On sections of chicken retina, the anti-AC1 antibody labeled photoreceptors most strongly (Fig. 8C), but labeling was also strong in cell bodies in the ganglion cell layer. Cells at the inner border of the inner nuclear layer (most likely amacrine cells) were also labeled in a distinctly punctate pattern. Processes could be observed extending from these cells down into the inner plexiform layer where amacrine cell synapses form (Fig. 8D). In culture, cone photoreceptors were usually the most strongly labeled cells. The intensity of anti-AC1 labeling was variable among amacrine cells but all amacrine cells showed some level of AC1 expression (Fig. 8F). Punctate AC1 expression was detected both in cell bodies and processes of amacrine cells in culture (Fig. 8G). It is important to note that the AC inhibitor SQ 22,536 (Fig. 7) has been demonstrated to be an effective inhibitor of AC1 in neurons at the concentration used in our experiments (Liauw et al. 2005). These results support the hypothesis that AC1 can be involved in L-type Ca\(^{2+}\) channel regulation in retinal amacrine cells.

**Inhibition of CaM Primarily Affects Inactivation**

From the results presented thus far, a scenario emerges where at least two Ca\(^{2+}\)/CaM-dependent mechanisms might collaborate to regulate the function of L-type Ca\(^{2+}\) channels in retinal amacrine cells: first, the Ca\(^{2+}\)/CaM-dependent inactivation that is sensitive to Ca\(^{2+}\) from 5 to 100 µM (Tadross et al. 2008) and second, the Ca\(^{2+}\)/CaM-dependent activity of AC1 that is more Ca\(^{2+}\) -sensitive (K\(_d\) = 100 nM). If these suggestions are valid, then we would predict that inhibition of CaM activity would be least effective in blocking the effects of AC1 activity. To test this, we looked at the effects of the calmodulin inhibitor calmidazolium (CMZ, 10 µM)
Figure 2.8: AC1 is expressed in the retina and in cultured retinal cells. A: image of a Western blot showing that the anti-AC1 antibody binds a band at the predicted molecular weight (130 kDa) in both chicken and mouse brain tissue. B-D: fluorescent images of frozen sections of chicken retina treated with Cy3-conjugated secondary antibodies only (B) and the anti-AC1 primary antibody plus the secondary antibody (C and D). The secondary-only controls show low non-specific binding of the secondary antibody. In C, strong AC1 immunoreactivity is observed in the photoreceptor layer. Also labeled are cells at the inner border of the inner nuclear layer (INL) and in the ganglion cell layer. The labeling pattern is punctate and is most prominent in cell bodies. D: higher magnification view of 2 cells at the inner border of the INL shows that labeling is found on processes extending down into the inner plexiform layer (arrowheads). E-G: fluorescent images of retinal neurons in culture. E, A field of cells labeled with the Cy3-conjugated secondary antibody only shows low non-specific binding of the secondary antibody. F: a field of retinal neurons is shown, most of which are amacrine cells (arrows, for example). A strongly labeled cone photoreceptor is also shown (arrowhead). G: a higher magnification image of three amacrine cells. As in retinal sections, the labeling pattern of the anti-AC1 antibodies is punctate and appears in cell bodies and processes. All scale bars are 10 µm.

(Weiss et al. 1982) on the FCCP-dependent alterations in current amplitude. Ruptured-patch recordings were made with either EGTA (1.1 mM) and CMZ in the pipette (Fig. 9A) or BAPTA (10 mM) and CMZ in the pipette (Fig. 9B) and then tested the effects of blocking MCU with
FCCP. With inhibition of CaM, suppression of MCU produced an enhancement of the Ca$^{2+}$ current under either buffering condition (EGTA + CMZ 36 ± 12% enhancement, $n = 6$, $P = 0.0003$; BAPTA + CMZ 19 ± 9% enhancement, $n = 6$, $P = 0.5$; Fig. 9, C and D). Recall that in the absence of CMZ, inhibition of MCU caused a decrease in current amplitude (presumably due to increased CDI) with 1.1 mM EGTA internally (EGTA 36 ± 6% reduction, $n = 6$, Fig. 6A). The switch in the sign of the response to FCCP when calmodulin is partially inhibited is consistent with a shift in the balance toward AC1 activation. The enhancement tended to be larger in EGTA than BAPTA however this difference was not statistically significant.

**Phosphodiesterases Play a Role in Regulating Ca$^{2+}$ Current Amplitude**

If the enhancement is due to the generation of cAMP via AC1 and subsequent activation of PKA, then phosphodiesterase (PDE) activity could affect the current amplitude. To test the involvement of PDEs, we employed the general PDE inhibitor IBMX (100 µM) (Beavo et al. 1970). Ca$^{2+}$ current recordings were made in the perforated-patch configuration. At the outset, we reasoned that if we boosted cAMP levels by inhibiting its degradative enzyme, then the current amplitude should be enhanced. Our results, however, did not conform to expectations in that the effect of IBMX on the current amplitude varied from cell to cell. Twenty six percent (Fig. 10, A and B) of amacrine cells tested ($n = 23$) responded with the expected increase in current amplitude (75 ± 29% enhancement $P = 0.04$). However, IBMX produced a decrease in the current amplitude in 26% of cells (Fig. 10, C and D, 13 ± 2% reduction, $P = 0.00002$) and no change (less than ± 5%) in 48% of cells (E and F). Some of the variability in responses could be due to the diversity of PDEs that can be inhibited by IBMX. To address this possibility we used 8-M-IBMX (100 µM), an inhibitor that is specific for PDE1 a Ca$^{2+}$/CaM-dependent phosphodiesterase (Fig. 10G) (Wells and Miller 1988). All cells ($n = 12$) tested responded to 8-M-IBMX with small, consistent decrease in the current amplitude (4 ± 1% reduction, $P = 0.004$, 38
Fig. 10H). These results imply that relatively high levels of cAMP generated in an environment with reduced PDE activity can have an inhibitory effect on the L-type channels (Ishikawa et al. 1993).

**Figure 2.9: The effects of inhibition of calmodulin depend on the Ca$^{2+}$ buffering environment.** A and B: representative ruptured-patch recordings from amacrine cells loaded with calmidazolium chloride (CMZ) via the patch pipette. Recordings were made with either 1.1 mM EGTA + CMZ (A), or 10 mM BAPTA + CMZ (B) in the pipette. Current are shown from the 3rd voltage step (black trace) and the 5th voltage step (in FCCP, gray trace). C: mean normalized peak current amplitudes are plotted over time for both recording conditions. D: percent change in Ca$^{2+}$ current amplitude was determined by comparing the current from the voltage step delivered just before FCCP to that elicited by the voltage step delivered 1 min later (in FCCP). CMZ-free data are re-plotted from Fig 2.6E for comparison purposes. Triple asterisks, $P < 0.001$. Other pair-wise comparisons were not statistically different.
Figure 2.10: Phosphodiesterase activity can regulate the Ca\textsuperscript{2+} current amplitude. A, C, E and G: representative perforated-patch recordings from 100 ms voltage steps from -70 to 0 mV delivered every 30 s. Both pairs of recordings in each panel (A, C, E and G) were made from the same amacrine cell. Control traces on the left were obtained from the 1st (black) and 2nd voltage steps (gray). Right: the control current (black) was recorded just prior to the application of 3-isobutyl-1-methylxanthine (IBMX, 2nd voltage step, A, C, and E) or 8-methoxymethyl-1-methyl-3-(2-methylpropyl) xanthine (8-M-IBMX, G). IBMX or 8-M-IBMX traces (gray) were recorded after 30 s of exposure to the reagent (3rd voltage step). Cells were separated into 3 groups based on the effect of IBMX on the Ca\textsuperscript{2+} current amplitude: cells exhibiting current amplitude increases (A and B, n = 6), cells exhibiting current amplitude decreases (C and D, n = 6) and cells the Ca\textsuperscript{2+} current of which was unaffected (± <5% change) by IBMX (E and F, n = 11). G: representative recordings from a single amacrine cell using the same protocol as for A, C and E but with the PDE1 selective inhibitor 8-M-IBMX. 8-M-IBMX produced a small inhibition in all cells examined (n = 12, G and H). B, D, F and H: mean percent changes in Ca\textsuperscript{2+} current amplitude are plotted for each group of cells (comparing time points depicted in A, C, E and G). Single asterisk $P < 0.05$, double asterisks $P < 0.01$ and triple $P < 0.001$. 
Ca²⁺-induced Ca²⁺ release functions to enhance the L-type calcium channel current.
**Ca\textsuperscript{2+}-Induced Ca\textsuperscript{2+} Release Functions to Enhance the L-Type Calcium Channel Current**

Thus far our data suggest that influx of Ca\textsuperscript{2+} can regulate L-type channels in at least two ways: by activating Ca\textsuperscript{2+} /CaM-dependent inactivation and by activating AC1 and ultimately PKA. Ca\textsuperscript{2+} released from internal stores might also have regulatory effects on L-type channels. It is established that in amacrine cells, activation of L-type Ca\textsuperscript{2+} channels leads to CICR (Mitra and Slaughter 2002; Warrier et al. 2005). To examine the role of CICR, we looked at the effects of inhibiting the RyRs using a blocking concentration of ryanodine (14 µM) (Meissner 1986) and the RyR inhibitor dantrolene (20 µM) (Nelson et al. 1996). If CICR normally contributes to channel inactivation, then blocking CICR should enhance the current. If CICR normally contributes to activation of AC1 and ultimately stimulation of PKA, then inhibition of RyRs would cause a decrease in current amplitude. We found that inhibition of the RyRs with either ryanodine (Fig. 11A) or dantrolene (B) consistently produced suppression of the Ca\textsuperscript{2+} current (ryanodine 25 ± 4%, \(n = 3\), \(P = 0.002\), Fig. 11C; dantrolene 11 ± 3%, \(n = 7\), \(P = 0.0002\), D).

These results were consistent with the hypothesis that CICR normally functions to enhance the L-type Ca\textsuperscript{2+} current, possibly by increasing the activation of AC1. Interestingly, 8-M-IBMX tended to block the effects of these RYRs inhibitors although this effect was not statistically significant for dantrolene (ryanodine + 8-M-IBMX \(P = 0.002\); dantrolene + 8-M-IBMX \(P = 0.2\)). This observation implied that in the absence of PDE activity, increased cAMP levels can compensate for the lower level of AC1 activation during CICR suppression.

**Discussion**

We find that in retinal amacrine cells, L-type Ca\textsuperscript{2+} channels are regulated by multiple Ca\textsuperscript{2+} -dependent processes (Fig. 12). Under control conditions, Ca\textsuperscript{2+} currents tend to increase in current amplitude over time under our recording conditions. This increase in amplitude may represent the balance of PKA-dependent enhancement via Ca\textsuperscript{2+} /CaM-dependent AC1 activity.
Figure 2.11: Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) normally contributes to Ca\textsuperscript{2+} current enhancement. A and B: the perforated-patch configuration was used to record Ca\textsuperscript{2+} current from individual amacrine cells with a similar voltage protocol as in Fig. 2.10. The three pairs of traces in each panel (A and B) are all from the same amacrine cell. Control traces were recorded 30 s apart (A and B, left). Either ryanodine (14 µM, A, middle) or dantrolene (20 µM, B, middle) was used to inhibit activation of the ryanodine receptor. Co-application ryanodine and 8-M-IBMX (A, right) and dantrolene and 8-M-IBMX (B, right) are also shown. C and D: mean percent change in current amplitude (comparing time points depicted in A and B) is plotted for both inhibitors (ryanodine n=3, dantrolene n=5). Single asterisk, $P < 0.05$; triple asterisks, $P < 0.001$.

and CDI, which is largely mediated by Ca\textsuperscript{2+} entering through the Ca\textsuperscript{2+} channels. Interestingly, we observe an enhanced rate of Ba\textsuperscript{2+} current amplitude increase before FCCP application for cells recorded in external Ba\textsuperscript{2+} (Fig. 5C, 1st 3 data points). This enhanced rate of increase might
represent the smaller contribution from CDI relative to basal AC1 activity. Disruption of MCU reduces the Ca\textsuperscript{2+} current amplitude in a Ca\textsuperscript{2+}-dependent manner, suggesting that under normal conditions, mitochondria function to limit CDI and thus maximize the availability of L-type Ca\textsuperscript{2+} channels for signaling. We provide evidence that the reduction of the inhibitory effects of blocking MCU in BAPTA reveals the enhancing effect of PKA activity that is most likely due to the Ca\textsuperscript{2+}/CaM-dependent activation of AC1. Inhibition of RyRs reduces the Ca\textsuperscript{2+} current amplitude suggesting that internal Ca\textsuperscript{2+} stores normally contribute to Ca\textsuperscript{2+} current enhancement. We propose that the link between the PKA-dependent enhancement of the Ca\textsuperscript{2+} current and the enhancing effects of CICR might also be the activity of AC1. Together, these studies indicate that L-type Ca\textsuperscript{2+} channels in amacrine cells are regulated by Ca\textsuperscript{2+} via complex and interacting mechanisms.

**Variability in Cultured GABAergic Amacrine Cells**

Throughout this work there is variability observable in our data. One example appears in Fig. 1, B and D. Under control conditions the current amplitude increases over time, but as indicated by the large error bars, different levels of this effect in different cells becomes apparent over time. The origin of this variability is not known, but if the enhancement is due to AC1 activity (as we have suggested), it is perhaps relevant that our AC1 antibody labeling intensity varies among amacrine cells in culture implying differing AC1 expression levels among these cells (Fig. 8F). Another example of variability would be in the time course of inactivation during the voltage steps. We have not yet investigated the source of this variability except to confirm that cells that are different in this regard to not have distinctive response properties in our experiments. These examples of variability (and others) raise the question of whether some of the variability is the representation of the multiple amacrine cell types known to exist in the vertebrate retina. It is important to consider, however, that our culture conditions have apparently
narrowed the range of possible amacrine cells fates. In the retina, different classes of amacrine cells can release GABA, glycine, acetylcholine, or dopamine at their synapses. In our cultures, however, we have only observed GABAergic synaptic transmission from these cells. We have viewed this as a benefit of the system that allows us to study a specific subset of amacrine cells that represent a substantial fraction of amacrine cells in the retina. Amacrine cells in the vertebrate retina can be further categorized by their morphology including their lamination
pattern in the inner plexiform layer (MacNeil and Masland 1998). In culture, however, this information is lost. There appear to be a few morphological groups of amacrine cells identifiable in the two dimensions of the culture dish, but we have not made a systematic attempt to relate these morphological groups to those found in the intact retina. We considered the possibility that different levels of AC1 expression might correspond to different morphological types of amacrine cell in culture. On examination, this seemed unlikely because it was clear that cells with similar morphologies had different levels of AC1 antibody labeling.

L-Type Ca\(^{2+}\) Channels Expressed by Amacrine Cells

The molecular identity of the L-type Ca\(^{2+}\) channels expressed by amacrine cells is not fully determined. However, it has been established that Ca\(_V\)1.3 mRNA is expressed by AII amacrine cells in the mouse retina (Habermann et al. 2003). In the chicken retina, an immunohistochemistry study demonstrated amacrine cell expression of Ca\(_V\)1.3 and possibly Ca\(_V\)1.4 but not Ca\(_V\)1.2 (Firth et al. 2001). In cultures of chick retinal neurons enriched for photoreceptors, other neuronal cells (presumably including amacrine cells) are reported to also express Ca\(_V\)1.3 (Ko et al. 2007). Interestingly the characteristics of CDI differ between Ca\(_V\)1.3 and Ca\(_V\)1.4 in that Ca\(_V\)1.3 contains sequence bestowing local as well as global Ca\(^{2+}\) sensing, whereas Ca\(_V\)1.4 should only sense global Ca\(^{2+}\) levels (Dick et al. 2008). This combination would be consistent with our data showing that 10 mM BAPTA removes a variable fraction of CDI but not all of it.

Effects of FCCP in Amacrine Cells

Our interpretation of the results in FCCP are based on the assumption that the primary effect of FCCP in amacrine cells is to reduce the proton gradient across the inner mitochondrial membrane and thus inhibit Ca\(^{2+}\) uptake via the uniporter. Several pieces of evidence from our previous work indicate that this is a fair assumption. It is well known that in the absence of a
proton gradient, the ATP synthase can consume ATP. We have previously established, however, that at the same FCCP concentration (1 µM) and time frame of exposure used here (1 min), the interruption of ATP synthesis does not alter the amplitude or time course of depolarization-dependent Ca^{2+} elevations (Medler and Gleason 2002). This is consistent with reports from other neuronal cell types indicating that the glycolytic pathway can maintain ATP levels for ten’s of minutes (Kauppinen and Nicholls 1986; Peng 1998; Werth and Thayer 1994; White and Reynolds 1995). Another potential complication would be if FCCP (as a protonophore) altered cytosolic pH. However, using SNARF-1 pH imaging we have established that under similar conditions, cytosolic pH is unperturbed by FCCP in amacrine cells (Sen et al. 2007). Furthermore, we have demonstrated that the effects of FCCP on cytosolic Ca^{2+} in amacrine cells are localized to within ~10 µm of a mitochondrion indicating that the effects of FCCP are mitochondria-associated (Sen et al. 2007).

**Mitochondrial Ca^{2+} Uptake Regulates Ca^{2+} -Dependent Inactivation**

We know that the inhibition of uniporter activity causes an increase in basal (un-stimulated) cytosolic Ca^{2+} levels. This Ca^{2+} elevation is independent of extracellular Ca^{2+} (Medler and Gleason 2002) but is dependent on internal Ca^{2+} stores (Sen et al. 2007). As such, we cannot rule out the possibility that some fraction of the effects we see in FCCP are due to the increase in basal Ca^{2+} levels. Nonetheless we propose that when the uniporter is inhibited, the primary effect is that normally sequestered Ca^{2+} now initiates additional CDI. What is our evidence that CDI is being enhanced when the uniporter is inhibited? The activity dependence of the effect of FCCP is consistent with a Ca^{2+} -dependent inhibition of the current such as CDI in that both increasing the duration of the voltage steps and the frequency of the voltage steps intensified the effects of FCCP. Furthermore, replacement of extracellular Ca^{2+} with Ba^{2+} reduces the effects of FCCP and Ba^{2+} is known to substitute poorly for Ca^{2+} in calmodulin
binding and activation (Dick et al. 2008). Interestingly, the sign of the effect of FCCP on the 
Ca\(^{2+}\) current amplitude is often inverted when BAPTA is present internally. Because BAPTA is 
estimated to bind Ca\(^{2+}\) ~400 times faster than EGTA (Adler et al. 1991), the differential effects 
of the two Ca\(^{2+}\) buffers can provide information about the spatial relationships between sources 
of Ca\(^{2+}\) and Ca\(^{2+}\) targets. The greater effectiveness of BAPTA over EGTA in reducing MCU-
sensitive CDI suggests that mitochondria are in close proximity to the L-type Ca\(^{2+}\) channels, on 
the order of tens of nanometers (Burrone et al. 2002). L-type Ca\(^{2+}\) channel CDI has also been 
shown to be regulated by MCU in chromaffin cells (Hernandez-Guijo et al. 2001). In these cells, 
however, 14 mM EGTA internally eliminated the inhibitory effect of disrupting MCU on CDI. In 
amacrine cells, FCCP-dependent effects on CDI were clearly observed in 14 mM EGTA 
suggesting a more intimate association between mitochondria and L-type Ca\(^{2+}\) channels in 
amacrine cells than in chromaffin cells. Ca\(^{2+}\) -dependent inactivation has also been demonstrated 
for store operated CRAC channels (Parekh 1998; Zweifach and Lewis 1995), and this 
inactivation is also minimized by MCU in T lymphocytes and basophilic leukemia cells (Gilabert 
and Parekh 2000; Hoth et al. 2000; for review, see Gilabert and Parekh 2000). These related 
observations in a neuron, a secretory cell, and cells of the immune system suggests that a widely 
expressed function of mitochondria is their ability to maintain the availability of Ca\(^{2+}\) influx 
pathways.

**PKA-Dependent Regulation of L-Type Ca\(^{2+}\) Channels**

PKA is known to phosphorylate L-type Ca\(^{2+}\) channels and to increase their open time 
(Bean et al. 1984; Yue et al. 1990b). PKA and the Ca\(^{2+}\) /CaM-activated phosphatase calcineurin 
are known to be localized to Ca\(_V\)1.2 channels by the anchoring protein AKAP79/150 in neurons 
(Oliveria et al. 2007; for review, see Dai et al. 2009). Our evidence that calcineurin is not a major 
regulator of L-type Ca\(^{2+}\) current in amacrine cells is consistent with the report that amacrine cells
do not express Ca\textsubscript{V}1.2 (Firth et al. 2001). Comparatively little is known about the regulation of Ca\textsubscript{V}1.3, but consistent with our observations, it has been demonstrated that PKA-dependent phosphorylation of Ca\textsubscript{V}1.3 leads to current enhancement (Liang and Tavalin 2007; Qu et al. 2005). Current enhancement for Ca\textsubscript{V}1.4 is apparently voltage dependent rather than phosphorylation dependent (Kourennyi and Barnes 2000).

**AC1 in the Retina**

Intriguingly, expression of AC1 mRNA is enriched in the retina in comparison to brain and spinal cord (Xia et al. 1993). In the developing mouse retina, an in situ hybridization study demonstrated AC1 mRNA in photoreceptors and ganglion cells (Nicol et al. 2006). An immunocytochemistry study in the mouse retina found AC1 expression in the inner nuclear layer. In the chicken retina, we find AC1 protein strongly expressed in photoreceptors, in nearly all cells in the ganglion cell layer, and in amacrine cells. This labeling pattern is consistent with what we find in culture where AC1 expression was detected in amacrine cells and quite strongly in cone photoreceptors. AC1 expression was not determined for cultured ganglion cells because they do not persist under our culture conditions (Hyndman and Adler 1982).

**Ca\textsuperscript{2+} -Dependent AC Activation**

It has been established that AC1 and AC8 can be activated by Ca\textsuperscript{2+} entering the cell across the plasma membrane. The strongest evidence for influx-dependent AC1/8 activation is for capacitative Ca\textsuperscript{2+} entry (CCE), but it is clear that entry via voltage-gated Ca\textsuperscript{2+} channels is effective as well (for review, see Willoughby and Cooper 2007). In rat cerebellar granule cells, depolarization-dependent Ca\textsuperscript{2+} influx was shown to promote cAMP accumulation (Cooper et al. 1998). An expression study with AC8 in a pituitary cell line has demonstrated that both CCE and voltage-gated Ca\textsuperscript{2+} channel activity were effective in stimulating the enzyme (Fagan et al. 2000). Interestingly, although release of Ca\textsuperscript{2+} from stores was substantial in this preparation, it was not
effective in stimulating AC8 activity. In immortalized gonadotropin–releasing hormone neurons derived from the rat hypothalamus, it was also demonstrated that AC1 was activated by voltage-dependent Ca\(^{2+}\) influx but not by release of Ca\(^{2+}\) from stores (Krsmanovic et al. 2001). Here we provide evidence that in amacrine cells, both Ca\(^{2+}\) influx and release from stores enhances the L-type Ca\(^{2+}\) current amplitude, and we propose that the effect of store release could be due to further stimulation of AC1. It might be that the structure and organization of amacrine cells differ from the cells discussed in the preceding text such that release of Ca\(^{2+}\) from stores has an impact on AC1 activity. The spatial relationship between RyRs, L-type Ca\(^{2+}\) channels and AC1 is not known. The resistance of AC1 activity to 10 mM BAPTA could imply that AC1 is intimately associated with both channel types. The importance of localization is also made relevant in light of our previous work showing that the metabotropic glutamate receptor 5 (coupled to the IP3 pathway)-dependent enhancement of this same Ca\(^{2+}\) current can be suppressed by 5 mM BAPTA internally. This receptor-mediated enhancement of the current is also thought to involve AC1, but the source of the activating Ca\(^{2+}\) here is presumably IP3 receptors rather than RyRs (Sosa and Gleason 2004). An additional consideration is the high affinity of AC1 for Ca\(^{2+}\) that is about five times that of BAPTA [100 nM AC1 (Fagan et al. 1996; Wu et al. 1993) vs. 500 nM BAPTA (Adler et al. 1991)]. It has been shown in amacrine cells that RyR-mediated CICR can activate Ca\(^{2+}\)-sensitive K\(^+\) currents (Mitra and Slaughter 2002). A previous study on cultured amacrines demonstrated a role of CICR in enhancing synaptic GABA release, but IP3 receptors rather than RyRs were involved (Warrier et al. 2005). Although RyRs and IP3 receptors are expressed throughout the cell bodies and dendrites of cultured amacrine cells (Warrier et al. 2005; Sen et al. 2007), they might be differentially regulated in the two regions. If this is case, then nonsynaptic L-type Ca\(^{2+}\) channels might be the primary target of the regulatory mechanism involving CICR and RyR demonstrated here. In the retina, bipolar
cells can support sustained release of glutamate (for review, see Heidelberger et al. 2005), and amacrine cells are one of their postsynaptic targets. Although some amacrine cells are known to feedback onto bipolar cells, limiting the duration of excitation (Chavez et al. 2006; Dong and Hare 2003; Hartveit 1999; Singer and Diamond 2003), others may be subjected to prolonged depolarization and potentially significant elevations in cytosolic Ca^{2+}. On the whole, the mechanisms described here (MCU, PKA activity, and CICR-dependent enhancement) tend to promote and preserve the activity of the L-type channels in amacrine cells. This may be important in maintaining signaling capabilities of amacrine cells at their cell bodies or at their synapses or, in some cases, both.

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Chapter 3

The Pattern of Nitric Oxide Production in the Chicken Retina
Introduction

The initial processing of the visual signal involves the transfer of information at an unknown number of synapses as the signal is transmitted through the retina. It is well established that retinal neurons express a variety of traditional neurotransmitters that are loaded into synaptic vesicles. These neurotransmitters are released when a pre-synaptic Ca\textsuperscript{2+} signal initiates fusion of synaptic vesicles with the pre-synaptic membrane. Less traditional intercellular signaling molecules are also found in the retina such as endocannabinoids (for review see, Yazulla 2008), sphingolipids (Crousillac et al. 2009), and nitric oxide (NO; Blute et al. 2000). These compounds can be synthesized on physiological demand and are not dependent upon exocytosis for their release. The functions of these non-traditional signaling molecules are poorly understood. Nitric oxide is produced by an enzymatic reaction converting arginine into citrulline and NO by the activity of nitric oxide synthase (NOS). Nitric oxide initiates the “classical” pathway by stimulating soluble guanylate cyclase (sGC) activity to produce cGMP (Arnold et al. 1977). Nitric oxide can also nitrosylate proteins at cysteine residues (Davis et al. 2001; Stamler et al. 1997). Nitrosylation is analogous to phosphorylation in that it is a reversible chemical modification that alters protein function (Lane et al. 2001). Potential target proteins include receptors, ion channels, transporters, enzymes, G-proteins, and transcription factors (Stamler et al. 1997).

Nitric oxide-dependent effects have been found in most cell types in the vertebrate retina. Nitric oxide may modulate both calcium and cyclic-nucleotide-gated channels at the photoreceptor synaptic terminals, and this has been shown to alter synaptic transmission due to the role of these channels in exocytosis (Rieke and Schwartz 1994; Savchenko et al. 1997). In the rabbit retina, the NO donor, S-nitrozo-N-acetyl-DL-penicillamine (SNAP), has been shown to reduce the gap junction conductance between the bipolar cells and AII amacrine cells (Mills and
Massey 1995; Xin and Bloomfield 1999). Previous work from our laboratory has shown that NO can switch amacrine cell inhibitory synapses into excitatory synapses by moving the equilibrium potential of chloride to more positive values due to elevation in cytosolic chloride (Hoffpauir et al. 2006). Release of endogenous NO has been shown to enhance the effect of visual stimulation in amacrine cells of the rabbit retina (Koistinaho et al. 1995). Examining the production pattern of NO may form the basis for physiological experiments to understand the details and cell-specific roles of NO.

There are three forms of NOS: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). Neuronal NOS and eNOS are activated by cytosolic calcium elevations and are expressed in the central nervous system. Nitric oxide synthase was detected in subsets of amacrine cells and cells in the ganglion cell layer by the NADPH-diaphorase method and nNOS immunolabeling in the vertebrate retina (Cobcraft et al 1989; Kim et al 1999; Koistinaho et al 1993; Neufeld et al, 2000; Oh et al 1999; Osborne et al 1993; Perez et al 1995; Provis and Mitrofanis 1990; Rios et al 2000; Sagar 1986; Sandell 1985; Vaccaro et al 1991; Vaney and Young 1988; Wassle et al 1987). Nitric oxide synthase can produce a NADPH-diaphorase reaction in the processes of converting arginine into citrulline and NO (Hope et al. 1991). Perez and colleagues (1995) were the first to identify the nNOS-immunolabeling in the rabbit retina. They have also shown NADPH-diaphorase-positive photoreceptors and horizontal cells in the rabbit retina, whereas Kim and colleagues (1999) identified it only in photoreceptors. Fischer and Stell (1999) identified four subsets of amacrine cells and efferent target cell synapses that are NADPH-diaphorase- and nNOS-positive in the chick retina. Rios and colleagues (2000) have also shown subsets of NADPH-diaphorase-positive amacrine cells in chicken central retina that appear to be similar to the amacrine cell subtypes described in Fischer and Stell (1999). A recent study by Wilson and colleagues (2011)
identified fifteen types of neurons comprising amacrine cells, ganglion cells and a centrifugal midbrain neuron in chicken whole mount retina by using NADPH-diaphorase method and nNOS-immunolabeling. They also performed neurobiotin injections to identify the detailed morphology of neurons. Neuronal NOS immunolabeling was demonstrated in subsets of amacrine cells (Eldred and Blute 2005) and ultrastructural localization of nNOS-immunolabeling has been shown in horizontal cell membranes and processes of bipolar and horizontal cells in the turtle retina (Haverkamp and Eldred, 1998). Goureau and colleagues (1997) were first to show anti-eNOS labeling in chick retina, followed by Haverkamp and colleagues in 1999. Endothelial NOS-immunolabeling was shown in Müller glia end feet of in the retinae of fish, frog, salamander, turtle, chicken, rat, ground squirrel, monkey, and in horizontal cells in the retinae of turtle and fish. These studies have mostly done on the central retina; however, here, we focus on the dorsal and ventral retina.

In the following, we provide supporting evidence that NO can be produced by both nNOS and eNOS-expressing cells. Based on the binding of a polyclonal eNOS antibody we used, we demonstrate that eNOS is broadly expressed in the chicken retina. Unlike eNOS, the nNOS labeling pattern appears to be different in dorsal and ventral retina. These results indicate a broad distribution of the NOS-expressing cells that are potentially capable of generating NO signals to modulate the visual signaling at all levels of the chicken retina.

**Methods**

All the methods including use of animals are approved by the Institutional Animal Care and Use Committee, Louisiana State University (Assurance # A3612-01). Adult White Leghorn chickens (4-8 weeks old) were sacrificed by intraperitoneal injection of sodium pentobarbital (500 mg/kg, Sigma-Aldrich) followed by decapitation. The eyes were enucleated and hemisected.
After removing the vitreous, the retinas were dissected from the eye cup and protocols were applied as follows in sections Western Blots, Immunohistochemistry and Daf Loading.

**Western Blots**

Chicken and mouse brains (a gift from Dr. Jackie Stephens) and chicken retina were homogenized in lysis buffer containing a cocktail of protease inhibitors [PMSF (1 mM), leupeptin (5 µg/ml), aprotinin (2.5 µg/ml), 1,10 ortho-phenantroline (0.2 µg/ml), and pepstatin (0.7 µg/ml)]. Samples were centrifuged at 4,000 rpm for 20 min at 4°C. Protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (300 µg) were separated on a 7.5% SDS gel along with 10 µl Pageruler molecular weight markers (Fermata, Glenburnie, MD). Proteins were transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in 4% milk, 0.1% Tween 20 in Tris buffered saline. A polyclonal antibody raised against rat nNOS (Sigma-Aldrich, St.Louis, MO) was diluted 1:5,000 in PBS with 1% BSA. A polyclonal antibody raised against bovine eNOS (Sigma-Aldrich) was diluted 1:20,000 in PBS with 1% BSA. Goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Pierce-Thermo Scientific, Rockford, IL) was diluted to 1: 5,000 (for anti-nNOS) and 1:10,000 (for anti-eNOS) in PBS with 1% milk. Membranes were incubated in primary and secondary antibodies for 1.5 h each, at room temperature. Proteins were visualized using the Supersignal Western Pico Reagent (Pierce).

**Immunohistochemistry**

Chickens at 4-8 weeks of age were sacrificed as it is described above. After removing the vitreous, the retinas were dissected from the eye cup and separated into dorsal and ventral parts followed by fixing the tissue with 4% paraformaldehyde for 30 min at room temperature. Following fixation, retinas were washed in PBS + 1% glycine. Retinas were then incubated in a series of sucrose solutions: 15% sucrose for 30 min, 20% sucrose for 1 h, and 30% sucrose
solution overnight at 4°C. Retinae were embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Torrence, CA) by freezing on a slurry of dry ice and isopentane. Sections (12-16 µm) were cut on a Leica CM1850 cryostat (Wetzlar, Germany) and mounted on gelatin-subbed slides (Southern Biotech, Birmingham, AL).

Frozen retinal sections were pre-incubated for 1 h at room temperature in a blocking solution consisting of dilution solution (PBS, 1% bovine serum albumin, 0.5% saponin) with 10% normal goat serum. General antibody information is reported in Table 3.1. Polyclonal antibodies raised against rat brain nNOS were diluted at 1:1,000 in dilution solution and applied to retinal sections for 1 h at room temperature, then washed in PBS. Polyclonal antibodies raised against bovine eNOS were diluted at 1:2,000 in dilution solution and unless otherwise indicated, applied to retinal sections for 72 hrs at 4°C followed by washing in PBS. Monoclonal antibodies nNOS (1:500, Sigma-Aldrich), calretinin (1:100, Millipore, Billerica, MA), Protein Kinase C (PKC, 1:100, Abcam, Cambridge, MA) were diluted in dilution solution and applied to retinal sections for 72 hrs at 4°C and anti-syntaxin (HPC-1, 1:100, Sigma-Aldrich) and glutamine synthetase (GS, 1:500, Millipore) were applied to retinal sections for 1 hr at room temperature followed by washing in PBS. Sections were incubated in secondary antibodies (goat anti-rabbit Cy3, 1:3,000 and goat anti-mouse Dylight 488, 1:100) for 1 h at room temperature. After the incubation with the secondary antibodies, sections were washed in PBS, and mounted in a medium containing 70% glycerol, 28% PBS and 2% n-propyl gallate.

For the double-labeling experiments with polyclonal eNOS and monoclonal calretinin, PKC, and nNOS antibodies, frozen retinal sections were co-incubated for 72 hrs at 4°C with the primary antibodies and 1 h at room temperature with the secondary antibodies (Fig. 3.9, 3.10, 3.12, and 3.14). For the double-labeling experiments with polyclonal eNOS and monoclonal HPC-1 and GS antibodies, frozen retinal sections were sequentially incubated for 72 hrs at 4°C.
with anti-eNOS and 1 h at room temperature with the anti-HPC-1 and anti-GS, and 1 h at room temperature with the secondary antibodies (Fig. 3.9, 3.11 and 3.13).

For the antigen peptide (AP) and eNOS antibody double-labeling experiments (Fig. 3.2), frozen retinal tissue sections were treated with eNOS antibody (1:2,000) and a cocktail of anti-eNOS and the AP (designed by Protein Facility, LSU Ag Center, Baton Rouge, LA; anti-eNOS:AP, 1:10) for overnight incubation at room temperature. Tissue sections were then washed in PBS and incubated in Cy3-conjugated goat-anti-rabbit secondary antibody (1:3,000) for 1 h at room temperature followed by washing in PBS.

**DAF Loading**

Chickens at 4-8 weeks of age were sacrificed and retinas were dissected as described above. The dissected retinas were loaded in nitric oxide indicator fluorescence dye, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate, 20 µM, Invitrogen, Carlsbad, CA) in the presence or absence of nitric oxide synthase (NOS) inhibitor L-NAME (200 µM, Plymouth Meeting, PA) at room temperature for 30 min. The DAF-loading solution contained L-arginine (1 mM), glycine (10 µM) and NMDA (250 µM) in addition to DAF. Wash solution also contained L-arginine, glycine, NMDA, and ± L-NAME. After washing (3 times, 5 min each) the retinas were fixed in 4% paraformaldehyde for 30-40 min at room temperature. Retinas were kept in the dark (vials covered with foil) except during dissections and while changing solutions. Solution changes were done in dim light conditions. DAF-loaded and fixed retinas were then prepared for frozen sectioning as discussed above. Sections were imaged immediately to avoid fading of the DAF signal that we have observed to occur.

**Image Processing and Analysis**

DAF-loaded, fixed and frozen tissue sections were imaged on either a Zeiss (Thornwood, NY) Axiovert 200M inverted microscope equipped with epifluorescence and a Zeiss Apotome
or a Leica TCS SP2 wide-field confocal fluorescence microscopy (Buffalo Grove, IL). Images were processed with Axiovision 4.6 software and Imaris software (Fig. 3.1A and B) or Leica TCS SP2 confocal microscopy software. Images were adjusted for their brightness and contrast in Adobe Photoshop (Adobe, San Jose, CA).

<table>
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<th>Antibody</th>
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<th>Catalog No.</th>
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<th>Working Dilution</th>
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<td>Syntaxin, Clone HPC-1</td>
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<td>Synaptosomal plasma-membrane fraction from adult rat hippocampus</td>
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Table 3.1: General antibody information. Unless otherwise indicated, primary antibody incubations were performed for 72 hrs at 4°C for monoclonal eNOS, calretinin, and PKC antibodies; overnight at room temperature for monoclonal nNOS; 1 h at room temperature for monoclonal HPC-1 and GS and polyclonal nNOS antibodies.

Results

Specificity of NOS Antibodies

We have previously shown nNOS-immunolabeling in cultured chicken retinal amacrine cells (Crousillac et al. 2003). Here, we further examine the expression of nNOS and also eNOS.
Specificity of these polyclonal antibodies was confirmed by Western blot analysis using homogenates of chicken retina, chicken brain and mouse brain (Fig. 3.1). Polyclonal eNOS antibodies labeled proteins as indicated by the appearance of the band near the expected molecular weight for eNOS (135 kDa) both in mouse and chicken brain homogenates (Fig. 3.1A). The eNOS antibodies also labeled a second band near the molecular weight of 115 kDa. This binding suggests that this antibody is binding to non-eNOS type elements in the chicken brain. Polyclonal nNOS antibodies also labeled a band near the expected molecular weight for nNOS (150-160 kDa) both in the chicken brain and retina (Fig. 3.1B). These results suggest that these antibodies recognize the avian form of nNOS and eNOS.

Figure 3.1: Endothelial NOS and nNOS enzyme proteins are expressed in the chicken retina. A and B: Western blots of protein (300 µg/lane) from chicken (CB), mouse brains (MB) and chicken retinas (CR). A: eNOS antibody (1:20,000) detects protein near the appropriate molecular weight for eNOS (135 kDa) in both MB and CB. B: nNOS antibody (1:5,000) detects protein near the appropriate molecular weight for nNOS (150-160 kDa) in both CB and CR.

To examine the specificity of these NOS antibodies on tissue by blocking peptide, we have used the antigen peptide (AP) designed specifically for the eNOS antibody (see METHODS). Figure 3.2A shows the eNOS-immunolabeling after overnight incubation. Co-incubation with the
AP suppressed the labeling by the eNOS antibody (Fig. 3.2B). Endothelial NOS expression has previously been shown only in Müller glia end feet in vertebrate retina by using a monoclonal eNOS antibody obtained from Transduction Labs (Haverkamp et al. 1998). Using a polyclonal eNOS antibody from Sigma-Aldrich, we observe a distinct labeling pattern different from that observed by Haverkamp and colleagues (1998). The Sigma-Aldrich eNOS antibody labels another band around a molecular weight of 115kDa in the chicken brain and the synthesized antigen peptide of the same antibody suppressed but did not completely block the eNOS antibody labeling on the chicken retinal tissue. We believed that these polyclonal eNOS antibodies can recognize avian form of eNOS enzyme and might bind to other non-eNOS elements on the tissue, as such; we will refer to this labeling as eNOS-immunolabeling.

Due to unforeseen difficulties in synthesizing the antigen peptide for nNOS antibody sequence (personal communication with the Protein Facility, LSU Ag Center), we have not yet been able to do co-incubation experiments with an AP for nNOS antibody. In an alternative strategy, we have performed double-labeling experiment with polyclonal and monoclonal nNOS antibodies to see whether their labeling patterns overlap in chicken retina (Fig. 3.3, McMains, unpublished data). Both antibodies co-label within the majority of the retina section (Fig. 3.3 A-
C). Different from polyclonal nNOS, monoclonal nNOS labeled some short, horizontal elements near the border of INL and IPL (Fig. 3.3A, arrows), and unlike monoclonal nNOS, the polyclonal nNOS labeled the photoreceptor terminals in the outer retina (Fig. 3.3B, arrows).

Although we detected a strong band near the expected molecular weight for nNOS (Western blot analysis, Fig. 3.1B), a possible explanation for the few differences in the labeling pattern between monoclonal and polyclonal nNOS antibodies is that different cells of the retina might express distinct isoforms of the nNOS enzyme, and these antibodies might recognize the different isoforms of nNOS (Eliasson et al. 1997; Silvagno et al. 1996; Huang et al. 1993; Brenman et al. 1996; Giove et al. 2009; for review see Alderton et al. 2001).

**Figure 3.3: Monoclonal and polyclonal nNOS antibodies have similar labeling patterns.** A (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of monoclonal (A) and polyclonal (B) nNOS double-labeling. Photoreceptors, Müller glia, amacrine cells and their processes, synaptic bands in the IPL, cells in the GCL, and processes and/or nerve fibers in the nerve fiber layer (NFL) were labeled with both antibodies (C). The monoclonal antibody also labeled elements on the border of the INL and IPL (A, arrows), and the polyclonal antibody also labeled the photoreceptor terminals in the OPL (B, arrows). Scale bar is 50 µm.

**Nitric Oxide Is Produced Endogenously in the Chicken Retina**

Previous studies have shown nitric oxide production in retinal slice of turtle and tiger salamander by using DAF-2 and DAF-FM (Blute et al. 2000, and Eldred and Blute 2005). To investigate the production pattern of nitric oxide in the chicken retina, DAF-loaded intact retinal tissue was subsequently fixed and processed for frozen sectioning. Multiple cell types appeared to be DAF-positive in the chicken retina (Fig. 3.4A). The intensity of DAF fluorescence was
strong in photoreceptors, horizontal cell bodies and processes (arrow heads), efferent synapses (arrows), nerve fibers consisting of ganglion cell axons and efferent axons from isthmo optic nucleus. We also observed weak DAF fluorescence in some somata in the GCL. To investigate the role of nitric oxide synthases in generation of the DAF signal, we used a general nitric oxide synthase inhibitor, L-NAME. L-NAME (200 µM) was included in the external solution (before, during and after the DAF-loading). L-NAME reduced the DAF signal in the chicken retina (Fig. 3.4B) confirming the hypothesis that the DAF signal represents NO generated by NOS activity. The DAF signal most resistant to NOS inhibition was in the photoreceptors and some elements in the nerve fiber layer.

![Figure 3.4: NO production in the chicken retina. A and B: images of DAF-loaded, fixed and frozen tissue sections in the absence and presence of the NOS inhibitor L-NAME. Arrowheads indicate DAF-positive horizontal cells (A). Arrows show efferent processes and their synapses onto target amacrine cells labeled with DAF (A). Scale bar is 30 µm.](image)
To compare the DAF signal with the expression of Ca\(^{2+}\)-activated NOS enzymes (nNOS and eNOS), we used polyclonal antibodies raised in rabbit against rat nNOS and bovine eNOS. Figure 3.5 shows the secondary antibody-only images and shows that non-specific binding of the secondary antibodies did not occur in these DAF-loaded-fixed sections when labeled with antibodies. DAF-positive efferent synapses were labeled with nNOS antibody (Fig. 3.6A-C, arrows). Neuronal NOS antibody also labeled the associated target amacrine cell somata in the INL (Fig. 3.6B). Arrowheads (A-C) indicate some of the DAF-positive processes labeled with anti-nNOS. Some of the DAF-positive horizontal cells exhibited eNOS immunolabeling (Fig. 3.6D-F, vertical arrows). A DAF-positive efferent synapse was also positive for eNOS antibody (D-F, diagonal arrow). This observation was relatively rare as efferent synapses much more commonly express nNOS. Arrowheads in E indicate the frequently observed band labeling near the IPL and right above the GCL with eNOS antibody.

Figure 3.5: Secondary antibody-only control. A, B, and C: confocal images of secondary antibody-only control for goat-anti-rabbit Cy3 applied on DAF-loaded and fixed tissue. Scale bar is 50 µm.

The Distribution of nNOS and eNOS

Amacrine cell type differences between dorsal and ventral retina have been previously demonstrated for the chicken retina (Lindstrom et al. 2009). To examine whether these differences extended to patterns of NOS expression, we explored the expression pattern of nNOS and eNOS in dorsal and ventral samples of retinal tissue (Fig. 3.7 and 3.8). The nNOS antibody
Figure 3.6: Nitric oxide synthase (NOS) expressing cells and NO production. A-F (left): transmitted light images of the antibody-treated sections marked for retinal layers. A-C: confocal images of DAF labeling along with anti-nNOS. Efferent synapses are both DAF- and nNOS-positive (arrows), and anti-nNOS also labels target cell somata (B). Arrowheads indicate some processes that are labeled with DAF and anti-nNOS. D-F: confocal images of DAF labeling along with anti-eNOS. Vertical arrows indicate DAF- and anti-eNOS-positive horizontal cells. Diagonal arrow shows an efferent synapse positive for anti-eNOS and DAF. Arrowheads in E indicate a band (near the border of IPL and GCL) that is labeled typically with eNOS antibody. Scale bar is 50 µm.
typically labels photoreceptors, photoreceptor terminals, target amacrine cells, efferent synapses, non-target amacrine cells and processes, bands in the IPL, and some cell somata in the GCL in chicken retina. This basic expression pattern is the same for dorsal and ventral retina (Fig. 3.7A-F). However, there are also differences in the labeling pattern in dorsal and ventral retina. Anti-nNOS labels different subtypes amacrine cells in dorsal than it does in ventral retina. Neuronal NOS-positive amacrine cells in dorsal retina have smaller somata (< 7 µm vs. >10 µm in diameter) and also have vertical processes. These cell types might be similar to the ones identified by Fischer and Stell (1999) and Wilson and colleagues (2011); but until I confirm that I will refer to them as “the stick-man” amacrine cells. Figure 3.7B demonstrates an amacrine cell body (arrow) and two of its processes extending from each side of the soma along the IPL (vertical arrowheads) and a vertical process projecting into the IPL (lateral arrowhead). This type of cell does not look similar to any of the cells previously identified by Fischer and Stell (1999) or Wilson and colleagues (2011). Until I confirm that, I will refer to this cell type as “the long-arm” amacrine cell. In the ventral retina, we observe nNOS-immunolabeling mostly in target amacrine cells and efferent synapses (Fig.3.7D, arrows).

In the ventral retina, we occasionally observe cells that have large (~17 µm), round and flat somata with thick processes stretching on the sides of the somata (Fig. 3.7F, arrows). I will refer to these cells as “the octopus” amacrine cells. Photoreceptor terminals were also strongly labeled with anti-nNOS in the ventral retina (Fig. 3.7D). Contrary to the nNOS antibody, we did not observe differences in the labeling pattern in dorsal vs. ventral retina with the eNOS antibody (Fig. 3.8A and B). However, we occasionally observed eNOS-immunoreactivity at efferent synapses in the ventral retina (Fig. 3.8C, arrows). The reason that we did not observe anti-nNOS-positive photoreceptor terminal labeling in every ventral retina section and anti-eNOS-positive horizontal cell labeling is likely due to differences in the angle at which the sections w
Figure 3.7: Neuronal NOS antibodies have differences in the labeling pattern in dorsal and ventral retina. A-F (left): transmitted light images of the antibody-labeled sections marked for retinal layers. Confocal images of dorsal and ventral retina labeled with the polyclonal nNOS antibodies (A-C, dorsal; D-F, ventral). IPL synaptic bands labeled with nNOS are visible both in dorsal and ventral retina. Cells in the GCL are labeled with anti-nNOS both in both regions of the retina; however, more cells are labeled in dorsal retina. There is a weak signal in the OPL and photoreceptor labeling in both dorsal and ventral retina. Dorsal retina contains more numerous amacrine cells that have smaller somata (< 7 µm) and vertical processes (“the stick-man” cells; A-C) than found in ventral retina. B: a representative amacrine cell (“the long-arm” cell; soma indicated by an arrow) that has two long processes coming out of the soma extending laterally on each side in the IPL (vertical arrowheads). The same cell also projects another process into the IPL (lateral arrowhead). Target amacrine cells and their efferent synapses are confined to the ventral retina (Lindstrom et al. 2009) (D, arrows). Ventral retina also contains amacrine cells with larger (~17 µm in diameter) and round somata labeled with the nNOS antibody (F, arrows) which are absent in dorsal retina. Scale bar is 50 µm.
Endothelial NOS Antibodies Label Multiple Cell Types in the Chicken Retina

To investigate which cell types were positive for eNOS-immunolabeling (Sigma-Aldrich), we performed double-labeling experiments with the eNOS antibody and four cell marker antibodies previously established for the chicken retina (Fig. 3.10-3.14; Caminos et al. 1999; Ellis et al. 1991; Fischer et al. 2007; Gleason et al. 1993; Koulen et al. 1997; Linser et al. 1979; Norenberg et al. 1980). Figure 3.9 indicates the secondary antibody-only images and shows that non-specific binding of the secondary antibodies did not occur in these double labeling experiments.

Calretinin is a calcium-binding protein that is expressed in most horizontal cells and subsets of bipolar cells, amacrine cells and ganglion cells (Ellis et al. 1991; Fischer et al. 2007). In the present study we used a monoclonal calretinin antibody to identify horizontal cells. The calretinin antibody labeled horizontal cell bodies near the outer border of the INL and their processes in the OPL (indicated by arrowheads, and arrows in Fig. 3.10A). We also observed weaker labeling with the antibody at some cell bodies in the INL other than horizontal cell bodies and ganglion cells in the GCL. Co-incubation with eNOS antibodies revealed an overlap with horizontal cell bodies (Fig. 3.10B-D, arrowheads) as well as on some processes (arrows). A few amacrine cell bodies in the INL and ganglion cells in the GCL were also labeled with both eNOS and calretinin antibodies.

To confirm that eNOS antibodies label amacrine cells in the INL of the chicken retina, we used a monoclonal HPC-1 antibody as an amacrine cell marker. HPC-1 antibody labels the syntaxin 1 protein primarily in amacrine cells (Barnstable et al. 1985; Sherry et al. 200) and in multiple species including chicken (Gleason et al. 1993). HPC-1-labeling was strong in the INL and the IPL indicating the amacrine cells and processes, respectively. HPC-1 labeling was also observed in the OPL (Fig. 3.11A).
Figure 3.8: Endothelial NOS expression is similar in dorsal and ventral retina. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of dorsal and ventral retina labeled with the polyclonal eNOS (A, dorsal; B and C, ventral). Labeling pattern of anti-eNOS does not differ in dorsal and ventral retina except that we infrequently observe target cell efferent synapses in the ventral INL labeled with anti-eNOS (C, arrows). Scale bar is 50 µm.
All the amacrine cells that showed HPC-1 immunolabeling were also labeled with the eNOS antibody, suggesting that eNOS is expressed in amacrine cells in the INL (Fig. 3.11A-C, i.e. arrows). We also noticed weak co-labeling in the OPL (Fig. 3.11C). The monoclonal antibody raised against PKC recognizes the alpha and beta isoforms of the protein kinase C and has been established as a marker for two populations of bipolar cells in the chicken (Caminos et al. 1999; Koulen et al. 1997). In order to examine the expression of eNOS by bipolar cells, we performed double-labeling experiment with eNOS and PKC antibodies (Fig. 3.12). The PKC antibody labeled a population of bipolar cell bodies in the INL, bipolar cell dentrites in the OPL and processes extending from INL into the IPL. We also observed weak labeling in photoreceptors, IPL synaptic bands and cells in the GCL. Co-incubation with the eNOS antibodies demonstrated that relatively few bipolar cells express eNOS (Figs.3.12A-C, arrow heads). This is consistent with the lack of DAF labeled bipolar cells. PKC also labeled the band that we frequently see with the eNOS antibody near the border of the IPL and the GCL (Fig. 3.12A-C, arrows).

Figure 3.9: Secondary antibody-only control images for double-labeling experiments. A and B: confocal images of the double-labeling of goat anti-rabbit Cy3 and goat anti-mouse Dylight 488. A: is an example for co-incubation of secondary antibodies. B: shows an example for sequential-incubation of secondary antibodies. Scale bar is 50 µm.
Figure 3.10: Double-labeling experiment with eNOS and calretinin antibodies. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. A-C: confocal images of calretinin and eNOS antibodies double-labeling. Some horizontal cell somata (arrowheads) and processes (arrows) were co-labeled with both antibodies. Scale bar is 50 µm.
Figure 3.11: Double-labeling experiment with eNOS and HPC-1 antibodies. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of HPC-1 (A) and eNOS (B) antibody labelings. The merged image (C) indicates the co-labeled amacrine cells in the INL (i.e arrows). Scale bar is 75 µm.
Figure 3.12: Double-labeling experiment with eNOS and PKC antibodies. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of PKC antibody labeling (A), eNOS antibodies labeling (B). The merged image is shown in C. Anti-eNOS labels very few bipolar cells in the chicken retina. Arrowhead shows an example of a bipolar cell body labeled with both antibodies. Vertical arrows indicate the band at the border of IPL and GCL labeled with both antibodies. Scale bar is 50 µm.
Endothelial NOS-immunolabeling suggests possibility for Müller glia labeling on the chicken retinal tissue. Müller glia spans through all the layers in the retina and wraps around the cell bodies in the INL. In order to confirm eNOS-immunolabeling on the Müller glia, we employed a monoclonal antibody raised against the enzyme glutamine synthetase (GS). The GS enzyme is expressed in the Müller glial cells in the retina and used as a glial cell marker in the chicken retina (Linser et al. 1979; Norenberg et al. 1980). The GS antibody labeled all the Müller glia spanning the retinal tissue (Fig. 3.13A). The merged image confirmed the eNOS-immunolabeling for Müller glia in the chicken retina (Fig. 3.13C, arrow heads). The arrows indicate some co-labeled elements around the cell bodies in the GCL. These elements might be parts of Müller glia that is wrapped around the cells.

Both nNOS and eNOS are expressed in the retina. Do the cells express both NOS enzymes? In order to test whether nNOS and eNOS enzymes are expressed in the same cells, we performed an experiment with co-incubation of the polyclonal eNOS and monoclonal nNOS antibodies (Fig. 3.14). We did not observe a large similarity between the labeling of NOS antibodies (Fig. 3.14A-C). The low level of overlap in nNOS and eNOS immunolabeling suggests that retinal neurons do not typically express both enzymes. Arrows and arrowheads indicate the examples of co-labeled amacrine cell bodies in the INL and cells in the GCL, respectively (Fig. 3.14A-C).

These results confirm the broad expression of eNOS-immunolabeling of different cell types in the chicken retina. The generous expression of eNOS (based on the labeling of this antibody) suggests that most retinal neurons in the chicken retina are capable of producing NO.

Discussion

We find that in the chicken retina, there is a NOS-dependent endogenous production of nitric oxide. Endothelial NOS is broadly expressed in chicken retina and it appears that eNOS is
Figure 3.13: Double-labeling experiment with eNOS and glutamine synthetase (GS) antibodies. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of GS antibody labeling (A) and eNOS antibody labeling (B). The merged image is shown in C. Arrowheads indicate glia labeled with both antibodies. We also see some double-labeled elements by GCL (arrows). Scale bar is 50 µm.
Figure 3.14: Immunolabeling of nNOS and eNOS antibodies is not identical. A-C (left): transmitted light images of the antibody-treated sections marked for retinal layers. Confocal images of monoclonal nNOS (A) and polyclonal eNOS (B) antibody labeling. The merge image is shown in C. Examples of co-labeling of amacrine cells in the INL and cells in the GCL were indicated by arrows and arrowheads, respectively. Scale bar is 50 µm.
expressed by multiple cell types in the retina (Fig. 3.15). Neuronal NOS expression is less wide spread than eNOS expression; however, the distribution of nNOS differs in dorsal and ventral retina. Our results imply that the ability to generate nitric oxide is broadly distributed among most cell types in the chicken retina. If this is true, it raises the question of how circuit-specific NO signals could be generated. A possible explanation lies in the subcellular localization of NOS enzymes. Neuronal NOS has been reported to be bound to the plasmalemmal NMDA receptors; where as eNOS binds to plasmalemmal caveolin proteins.

In our DAF-experiments, inhibition of NOS activity suppressed the DAF signal. This is consistent with the NOS-dependent production of NO. The DAF signal in the INL, IPL and GCL was strongly suppressed; however, the signal in the photoreceptors persisted in the presence of L-NAME although at reduced levels. DAF labeling was observed to be strong in photoreceptors and NOS expression was also strong. It is possible that the DAF signal in the photoreceptors was not fully suppressed because the concentration of L-NAME was insufficient to inhibit the activity of NOS enzymes in photoreceptors. Another possible scenario is that there is an alternative nitric oxide production pathway in photoreceptors. Keeping these possible scenarios in mind, we can conclude that the DAF-signal that was suppressed was due to the activity of NOS enzymes.

**Subcellular Localization of nNOS**

Unlike eNOS and iNOS, the N-terminus of nNOS contains a PDZ-domain containing PSD-95 at the N-terminal of its amino acid structure. Receptors and signaling enzymes form clusters known as post synaptic density (PSD) beneath the plasma membrane at the synapses. PSD-95 is one of the PSD proteins, and is a membrane-associated guanylate kinase (MAGUK) containing PDZ domains (for review see, Tomita et al. 2001). The PDZ domain of nNOS links to the similar PDZ-motif of the PSD-95. PSD-95 can also interact with the C-terminus of NMDA
Figure 3.15: The map of the expression pattern of nNOS and eNOS in the chicken retina.
There are fewer amounts of white colored retinal cells and their extensions in panel eNOS
compared to nNOS, suggesting a broad expression for eNOS.

receptors. PSD-95 physically and most probably functionally links nNOS to NMDA receptors
(Aoki et al. 1997; Aoki et al. 1998; Christopherson et al. 1999). An increase in the rate of NO
production due to the presence of PSD-95 has been also shown in CHO1 (Chinese Hamster
Ovary) cells expressing NMDA receptor subunit and nNOS enzyme (Ishii et al. 2006). Ishii and
colleagues were first to measure the Ca^{2+} concentration beneath the plasma membrane nearby the
NMDA receptors ([Ca^{2+}]_{NR}) and found to be 5.4 µM, which is higher than the cytosolic Ca^{2+}
concentration ([Ca^{2+}]_{C} = 0.6 µM) upon NMDA stimulation. Neuronal NOS immunolabeling and
NO production have previously been shown to increase by NMDA stimulation of PC12 cells
expressing PSD-95 (Arundine et al. 2003). The proximate localization of nNOS to the NMDA
receptors not only favors the NO production stimulated by the Ca^{2+} influx through the NMDA
receptor channels, but also favors the down-regulation of these channels by the NO that is
produced (Lipton et al. 1994). NMDA with a PDZ-motif actually induces the localization of
nNOS towards the plasma membrane by the NMDA receptors for its down-regulation. So, why is the location of nNOS by the NMDA receptors important? This enzyme is activated by Ca\textsuperscript{2+} and NMDA receptors are Ca\textsuperscript{2+} permeable. Being bound to the NMDA receptor channels may provide a source for Ca\textsuperscript{2+} and favor rapid activation of nNOS.

**NMDA Receptors in the Retina**

NMDA receptors are Ca\textsuperscript{2+} permeable and blocked by Mg\textsuperscript{2+}, and are voltage-dependent. There are different NMDA receptor subunits: NR1, NR2A-NR2D (Ozawa et al. 1998), and the recently cloned NR3A and NR3B (Cull-Candy et al. 2001; Prybylowski et al. 2004; Sucher et al. 2003). The receptor-channel requires binding of glycine for its activation. NR1 is the glycine binding subunit of the receptor channel, and NR2 has the glutamate binding site (McBain et al. 1994). In the present study, we demonstrate NOS-dependent DAF signal and nNOS-immunolabeling in photoreceptors, amacrine cells and processes, and cells in the ganglion cell layer. We observed DAF signal (but not nNOS-immunolabeling) in horizontal cells as well. These results and the previous studies that have reported an association of nNOS-NMDA receptors raise the question of whether all the retinal cell types expressing nNOS also express NMDA receptor subunits.

NMDA receptor subunits have previously been shown in different cell types in vertebrate retina. Based on the lack of physiological and immunohistochemical evidence, it has been thought that NMDA receptors are not expressed in the photoreceptors (Eliasof and Werbin 1993; Vanderbranden et al. 2000). However, one recent study examined the localization of some NMDA receptor subunits and demonstrated the immunoreactivity for NR1C2 subunit at the photoreceptor terminals in the rat and the rabbit retinae (Fletcher et al. 2000; Kalloniatis et al. 2004). Goebel and colleagues (1998) demonstrated immunoreactivity for NR2A and NR2B subunits at the photoreceptor outer segments suggesting an alternative pathway to the Ca\textsuperscript{2+} influx.
that is responsible for neurotransmitter release. The majority of amacrine cells receive glutamatergic input from bipolar cells. Early physiological studies have reported subtypes of amacrine cells capable of depolarizing in the presence of NMDA, thus suggesting the existence for NMDA receptors in these cells (Massey and Miller 1990; Slaughter and Miller 1983). A slice preparation of the tiger salamander retina has revealed that amacrine cells use both NMDA and non-NMDA receptors in response to light stimuli (Dixon and Copenhagen 1992). In the mammalian retina, all the GABAergic amacrine cells and displaced amacrine cells have shown to express NR2A subunit (cat retina, Wenzel et al. 1997), suggesting a role for NMDA receptors in inhibitory GABAergic pathway. Like amacrine cells, ganglion cells are known to express NR1 and NR2A-C subunits (Brandsätter et al. 1998; Hartveit et al. 1994; Vandenbranden et al. 2000). These findings establish that multiple cell types in the retina express different subunits of NMDA receptors. It will be interesting to determine whether nNOS and NMDA receptors are co-localized in the chicken retina. Immunocytochemical double-labeling experiments would be a useful way to address this question. Interestingly, a study done by Lee-Rivera and colleagues (2003) reported that chicken retina lacks PDZ-domain on the NR1 subunit of the NMDA receptors (Lee-Rivera et al. 2003) and this suggests an existence of other kinds of domains that are involved in localization of nNOS. Based on this finding, the possibility for the presence of other domains should be kept in mind while performing the studies suggested above.

**Subcellular Localization of eNOS**

Endothelial NOS is known to co-localize to plasmalemmal caveolae (Garcia-Cardena et al. 1996; Shaul et al. 1996). Unlike nNOS and iNOS, eNOS is acylated on glycine (by myristolation) and cysteine (by palmitoylation) residues of the N-terminus of its structure (Michel 1999; for review see Alderton et al. 2001). Endothelial NOS needs to be acylated for its localization to the caveolae in the endothelial plasma membrane (Garcia-Cardena et al. 1996;
Shaul et al. 1996). Caveolins are integral membrane proteins and are part of caveolae (plasmalemmal invaginations). Caveolin proteins are important for specific localization of cellular proteins. Endothelial NOS in endothelial cells binds to caveolin-1 and eNOS in cardiac myocytes binds to caveolin-3 (Feron et al. 1996). Caveolin proteins inhibit the activity of eNOS, and this inhibition is reversed by the Ca$^{2+}$/CaM complex (Ju et al. 1997; Michel et al. 1997). Previous studies done in vertebrates have shown the expression of caveolin proteins 1-3 in the retina (Berta et al. 2007; Kachi et al. 2000; Kim et al. 2006). Different retinal layers and cells were labeled with antibodies against the three isoforms of caveolin proteins. Berta and colleagues (2007) demonstrated the caveolin 1 localization within the cell membranes in the ONL and INL, and diffuse staining in the GCL, OPL and IPL in the lemur retina. Caveolin 2 antibody labeling was limited in the lemur retina. Weak signal in the GCL, and strong labeling around the blood vessels were observed with anti-caveolin 2. Caveolin 3 was expressed in all layers of the lemur retina. Kim and colleagues (2006) examined the expression of caveolin proteins 1 and 2 in the rat retina. Caveolin 1 was observed in some neurons identified as amacrine cells and bipolar cells in the INL, as a weak signal in photoreceptor cellular membranes and inner segments in the ONL, in some glial cells, and vascular endothelial cells. Caveolin 2 was expressed diffusely to the IPL, OPL, and in Müller glia. Kachi and colleagues (2000) showed caveolin 1 staining in photoreceptor synaptic ribbons in mouse retina. The expression of these proteins and their roles in the avian retina remains unknown. Endothelial NOS is activated by Ca$^{2+}$. A possible explanation for eNOS-caveolin association could be that caveolin proteins may localize eNOS enzymes in the close vicinity of a Ca$^{2+}$ source such as a Ca$^{2+}$-permeable plasma membrane channel. An immunocytochemical study examining the co-relation between eNOS and caveolin protein subtypes in the the chicken retina will provide evidence to support the hypothesis that chicken retinal eNOS is associated with caveolin proteins. In order to test the
hypothesis that caveolin proteins are localized next to the Ca2+-permeable plasma membrane channels, immunocytochemical double-labeling experiments could be performed. These studies will provide further understanding of the functions and therefore roles of these enzymes and the retinal cells that are expressing these enzymes in the visual signaling of chicken retina.

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Chapter 4

Conclusions
The retina is both a sensory detector and a signal processor. The photoreceptors serve as the detectors of the visual stimuli and this process is fairly well understood. Signal processing starts at the first synapse in the retina between cone photoreceptor synaptic terminals and bipolar cell dendrites. Here, light stimuli are segregated into the ON and OFF pathways that are carried forward all the way to the visual cortex. The circuitry of the inner retina somehow decodes other features of visual stimuli such as motion, luminance, contrast, ambient light conditions and directional selectivity. With the notable exception of directional selectivity, the circuitry and mechanisms underlying most of these capabilities remain obscure. We do know that most of the synapses in the IPL are amacrine cell synapses (Marc and Liu 2000). Furthermore, it is becoming clear that synaptic signaling in the amacrine cell processes does not involve spatial integration but instead, synaptic sites can operate autonomously (Grimes et al. 2010). There is also anatomical (Dowling and Boycott 1966) and physiological (Chavez et al. 2006; Hartveit 1999; Shields and Lukasiewicz 2003; Vigh and von Gersdorff 2005) evidence that pre- and post-synaptic sites on amacrine processes can be immediately adjacent and function to regulate synaptic activity in a highly localized way. In chapter 2, I describe work that is relevant to the local nature of synaptic transmission in amacrine cells.

In chapter 2, I demonstrated different Ca\(^{2+}\)-dependent mechanisms regulating function of L-type Ca\(^{2+}\) channels. Cultured GABAergic amacrine cells use L-type Ca\(^{2+}\) channels to regulate synaptic transmission (Gleason et al. 1994) and the same has been demonstrated for other amacrine cell types in salamander and mammalian retina (Bieda and Copenhagen 2004; Habermann et al. 2003; Vigh and Lasater 2004). As well as being voltage-gated, L-type channels are inactivated by the Ca\(^{2+}\)/calmodulin complex. During Ca\(^{2+}\) influx through these channels, the local elevated cytosolic Ca\(^{2+}\) levels causes L-type channels to inactivate via Ca\(^{2+}\)-dependent inactivation (CDI). Calcium is taken up into the mitochondria through mitochondrial uniporter.
due to the steep electrical gradient for \( \text{Ca}^{2+} \) across the membrane, and it is released back to the
cytosol by mitochondrial \( \text{Na}^+ / \text{Ca}^{2+} \) exchanger (mNCX). It has previously been shown in EM
studies that mitochondria can be located within the 1 \( \mu \)m of pre-synaptic active zones of the
amacrine cells (Dowling and Boycott 1966; Dubin 1970; Famiglietti and Kolb 1975; Guilhoff et
al 1988; Kolb and Nelson 1993). In addition to this, a previous study done by our lab has
reported that mitochondrial calcium uptake (MCU) enhances synaptic transmission between
cultured retinal amacrine cells (Medler and Gleason 2002) and that the effects of MCU are
spatially restricted to about 10 \( \mu \)m (Sen et al. 2007). These studies led us to hypothesize that
mitochondria locally buffer \( \text{Ca}^{2+} \) thus limiting CDI. In chapter 2, I reported that MCU normally
regulates L-type channel function by sequestering local cytosolic \( \text{Ca}^{2+} \) thus limiting CDI. In a
previous study from our lab Sen et al. (2007) demonstrated that ryanodine receptors on the
endoplasmic reticulum can sense the \( \text{Ca}^{2+} \) release from mitochondrial to the cytosol through
mNCX, and release more \( \text{Ca}^{2+} \) from the endoplasmic reticulum via calcium-induced calcium
release (CICR). In chapter 2, I also demonstrate the involvement of CICR in regulation of
function of L-type \( \text{Ca}^{2+} \) channels. Together, these studies build a picture of a tightly regulated
presynaptic \( \text{Ca}^{2+} \) environment where the localization of organelles to synaptic terminals may
contribute to the localized synaptic interactions known to occur in amacrine cells.

To extend our understanding of the impact of these localized mechanisms on synaptic
transmission, a useful first step would be to make double patch recordings on pair of cells in
culture and test the effects of altering the cytosolic mechanisms described here on post-synaptic
responses. These experiments would confirm that the regulatory mechanisms I have elucidated in
whole cell recordings are localized to synapses. Similar experiments in retinal slices would be
much more challenging but might suggest the role these mechanisms play in retinal signal
processing.
Along with the hard-wired circuitry of the retina, the retina also contains a wealth of neuromodulatory substances. Traditional neurotransmitters can interact with the metabotropic versions of their receptors, there are a large number of peptide neurotransmitters expressed that also interact with metabotropic receptors and there are compounds such as NO that interact with less well defined targets. In chapter 3, I examine the pattern of NO production and define the populations of cells capable of generating NO. While past research has focused on the expression of nNOS almost exclusively, my work provides evidence that eNOS is expressed as well. The widespread expression of eNOS suggested by my research implies that NO production is more widespread than formerly recognized. Endothelial NOS expression appears especially strong in photoreceptors. Because cone photoreceptors can be unambiguously identified in chick retinal cultures (by the presence of the oil droplets), this would be an ideal system for examining the stimulus conditions required for NO production via eNOS. Imaging of DAF-loaded cone cells would allow us to investigate how activation of voltage-gated Ca\(^{2+}\) channels, or glutamate receptors or even release of Ca\(^{2+}\) from internal store might affect the production of NO.

Furthermore, it would be interesting to compare the conditions promoting production of NO in cultured GABAergic amacrine cells (all of which express nNOS) to the conditions promoting production of NO in cultured cone photoreceptors (pre-dominantly eNOS expressing). Differences in the regulation of NO production might reveal why two different NOS enzymes would be expressed in the retina.

In chapter 3, I demonstrated the difference in the distribution pattern of nNOS in dorsal and ventral retina. Part of this difference is due to the presence of a special retina-brain circuit only found in a subset of vertebrate species. There is circuitry that loops from the retina to the brain and back to the retina called centrifugal visual system (CVS). CVS consists of ganglion cells that are mostly in the dorsal retina that project to the optic tectum. The optic tectum provides
inputs to the ipsilateral isthmo-optic-nucleus (ION) of the midbrain (Cowan et al. 1961; Cowan and Powell 1963). The ION consists of the ION neurons. These ION neurons send out large numbers of (~8000) myelinated efferent fibers to the contralateral retina in the ground feeding birds where they terminate on the cell body of target amacrine cells to form one-to-one calyx synapse in the ventral retina (Cowan and Powell 1963; Fritzsch et al. 1990; Hayes and Holden 1983; Lindstrom et al. 2009; Nickla et al. 1994; for review see, Wilson and Lindstrom et al. 2011). A recent study in the chicken retina demonstrates that efferent synapses and their target amacrine cells are localized to the ventral retina (Lindstrom et al. 2009).

The ION and CVS are highly developed in mostly ground feeding birds such as chickens and are designed for fast conduction by using myelinated fibers. In ground feeding birds, two things are critical to be aware of: a moving shadow on the ground, and the owner of the shadow, a predator in the sky. The ganglion cells (mostly direction selective ganglion cells, DSGC) in the dorsal retina detect the moving shadow and transfer the information to the ION. ION most probably decodes this information of moving shadow as a “threat” and produces output to the efferent synapses projecting to the ventral retina to make synapses with the target amacrine cells for further processing with other types of amacrine cells in the inner retina and finally acting on the “threat” (for review see, Wilson and Lindstrom 2011).

Previous studies have shown that the efferent synapses and target amacrine cells that are involved in this circuitry are NOS immunoreactive (Callerino et al 2000; Fischer and Stell 1999; Goureau et al. 1997; Lindstrom et al. 2009; Morgan et al. 1994; Posada and Clarke 1999; Rios et al. 2000). In chapter 3, I provided evidence confirming that these efferent synapses and target amacrine cells are expressing nNOS or in few cases, eNOS. Furthermore, it is not just the absence of target cells in the dorsal retina that creates the difference; instead, there are certain morphological types of amacrine cells that are primarily found in the dorsal retina. For example,
a distinctive population of amacrine cells with small somata and vertical processes (the stick-
man cells) primarily reside in the dorsal retina.

The different patterns of nNOS expression in the dorsal and ventral retina suggest that
NO differentially affects signal processing in these two regions of the retina. In order to test the
hypothesis that effects of NO on light response of ganglion cells would be different in dorsal and
ventral retina, whole cell recordings of ganglion cells in dorsal and ventral regions of the whole-
mount retina from adult chickens could be performed to start to understand what the nNOS
system is doing differently in the two regions. Another feasible approach would be to test the
idea that synaptic input for dorsal vs. ventral DAF-positive amacrine cells may be different.
Performing whole cell recordings of DAF-positive amacrine cells in dorsal vs. ventral slices of
adult chicken retina could provide further information for understanding the role of nNOS
system through amacrine cells. Filling the amacrine cells that are recorded from would also
provide additional information on the morphology of the cell types. This way, we could combine
electrophysiological recordings of amacrine cells in dorsal vs. ventral regions of the retina with
the detailed morphology of the same cells to learn the relationship between the function of the
cells and the subtype.

These studies together with cellular localization of organelles that determine the synaptic
capabilities of amacrine cells and localization of the amacrine cells in the retina that might
determine the function and role of these cells in the retina will help us understand more about
visual processing is achieved in the inner retina.

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Merve Tekmen was born to Hatice and Hikmet Tekmen in Ankara, Turkey, in February, 1983. She received her primary, high school and college educations in Ankara. She attended high school at the TED Ankara College Foundation Private High School graduating in June 2001. She then enrolled in Hacettepe University in Ankara in 2001 and was graduated with Bachelor of Science degree in biology in June 2005. She got accepted into and started her doctorate program at Department of Biological Sciences at Louisiana State University in August 2005 under the guidance of Dr. Evanna Gleason and will complete the requirements for the Doctor of Philosophy degree in August 2011.