EFFECTS OF GAP JUNCTION BLOCKERS ON CIRCADIAN REGULATION OF GENE EXPRESSION IN EMBRYONIC RETINAL EXPLANT CULTURES

By

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To my beloved parents who always provide the strongest support, the greatest encouragement, and the most sincere advice.
To all the kind people who have guided, taught, and assisted me through twenty-five years of education
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The suprachiasmatic nucleus (SCN) is comprised of autonomous, single-cell oscillators that work in concert to generate coordinated circadian rhythms. Substantial evidence demonstrates that intercellular gap junction communication plays a role in the coordination of circadian rhythms in SCN. Chicken retina also contains functional circadian oscillators that drive coordinated transcript rhythms of several photoreceptor-specific genes, including iodopsin and Arylalkylamine N-acetyltransferase (AANAT), tryptophan hydroxylase. Based on the studies in SCN, we hypothesized that gap junction communication also plays a role in coordinating transcript rhythms of circadian-regulated genes in retina.

To test this hypothesis, we first established a chicken embryonic retinal explant culture system in which robust, self-sustaining, and light-entrainable iodopsin transcript rhythms were observed under different lighting conditions. Although iodopsin transcription in retinas of chicken embryos is primarily driven by light, the functional
characteristics of circadian oscillators driving iodopsin transcript rhythms in culture are similar to those found in post-hatch chicken retina, an observation that supports use of the explant cultures in our study.

The role that gap junctions play in coordinating rhythms in retinas was examined using two reversible gap junction blockers, 18α-glycyrrhetnic acid-3-hemisuccinate (ACO) and 18β-glycyrrhetnic acid (18β-GA), which were applied to the explant cultures maintained under different lighting conditions. Both gap junction blockers produced a rapid and persistent reduction of iodopsin and AANAT transcript levels. Following removal of the blockers, the transcript rhythms of both genes reappeared within a 24 hr period. Our data suggest that the change in iodopsin mRNA levels was not due to disruption of the function or the phase of the circadian oscillators driving the iodopsin rhythms. These blockers may either directly uncouple the circadian oscillators from driving transcription of these genes or alter the stability of these transcripts in the photoreceptors cells.
CHAPTER 1
INTRODUCTION

General Features of Circadian Rhythms

Basic Properties of Circadian Rhythms

Circadian rhythms are self-sustained cyclic changes in physiological processes or behavioral functions that have a period of approximately 24 hours (Chang and Reppert 2001). A circadian system is comprised of three components: a core circadian oscillator that acts like a ticking clock to produce self-sustained rhythmic changes, output pathways through which the oscillators regulate physiological and behavioral functions, and input pathways through which the oscillators are synchronized or entrained to environmental time cues (Dunlap 1999). Circadian rhythms are virtually ubiquitous, controlling a myriad of physiological processes in organisms ranging from spore production in fungi, leaf movement in plants, eclosion in insects, rest:activity cycles in animals, and sleep:wake cycles in humans. Although the physiological processes regulated by circadian clocks may vary between organisms, these rhythms share three basic properties. First, the rhythms are self-sustaining and persist or free-run under constant conditions. Second, the rhythms can be entrained to environmental stimuli, light being the dominant and most potent entraining stimulus. Finally, the rhythms are temperature compensated in the sense that the period of the rhythm stays constant over a range of ambient temperatures (Reppert and Weaver 2001).
Molecular Bases of Circadian Clocks

The molecular mechanism driving core circadian oscillators consists of interlocking transcription-translation feedback loops. This mechanism is best understood in the fruit fly, *Drosophila melanogaster*, and in the mouse suprachiasmatic nuclei (SCN). In *Drosophila*, seven genes that encode the proteins involved in these molecular feedback loops have been identified, *period (per)*, *timeless (tim)*, *Drosophila clock (dClk)*, *cycle (cyc)*, *double-time (dbt)*, *shaggy (sgg)*, and *vrille (vri)* (Blau and Young 1999; Martinek et al. 2001; Young 2000). In one transcription-translation feedback loop, the basic helix-loop-helix (bHLH)–PER-ARNT-SIM (PAS) domain-containing transcription factors, dCLK and CYC, form heterodimers that target E-box regulatory *cis* elements (CACGTG) located in the promoters of the *per* and *tim* genes. Binding of the dCLK and CYC heterodimers to the promoters drives the rhythmic transcription of the *per* and *tim* genes (Glossop et al. 1999). As the *per* and *tim* mRNAs are translated into PER and TIM proteins, TIM and PER proteins begin to accumulate; however, this process is somewhat slowed by the binding of PER proteins to the constitutively expressed kinase DBT, an interaction that leads to the phosphorylation and degradation of PER. PER is stabilized when TIM protein accumulates to levels sufficient to bind the PER/DBT heterodimers. The formation of the TIM-PER-DBT trimeric protein complex allows it to enter the cell nucleus (Price et al. 1998). Contrary to the effect of DBT, which retards the accumulation of PER and the entry of PER and TIM into the nucleus, the phosphorylation of TIM by the glycogen synthase kinase-3, SGG, accelerates PER/TIM heterodimerization and subsequent translocation into the nucleus (Martinek et al. 2001). Once in the nucleus, the PER/TIM/DBT protein complex interacts with dCLK-CYC heterodimers to reduce the activation of *per* and *tim* transcription (Darlington et al. 1998). In the other transcription-
translation feedback loop, dCLK and CYC heterodimers repress \(dClk\) transcription either directly or via intermediate factors. The binding of PER-TIM-DBT heterodimers to dCLK-CYC heterodimers releases dCLK-CYC dependent repression of \(dClk\) transcription, thereby allowing separate activator(s) to activate transcription of the \(dClk\) gene (Glossop et al. 1999).

In mouse SCN, eight clock genes contribute to the autoregulatory feedback loops that define the core oscillator. One transcription-translation feedback loop involves the dynamic regulation of three \textit{period} genes, designated \textit{mper1–3}, and two cryptochrome genes, known as designated \textit{mcry1} and \textit{mcry2}. Rhythmic transcription of the \textit{mper} and \textit{mcry} genes is driven by mCLOCK and mouse mBMAL1 heterodimers. Mouse BMAL1 is a homolog of Drosophila CYC. As the mPER and mCRY proteins are translated, they form multimeric complexes that are translocated to the nucleus. In the nucleus, the mCRY component of the multimer acts as a negative regulator by directly interacting with the mCLOCK: BMAL1 heterodimers and inhibiting the transcription of \textit{mper} and \textit{mcry} genes (Reppert and Weaver 2001). Mouse PERs, unlike their counterpart in Drosophila, do not play a critical role in transcriptional inhibition. Instead, mPER1 affects the function of the clock at the post-transcriptional level, presumably through protein-protein interactions that affect the stability and nuclear entry of other clock proteins (Bae et al. 2001). mPER2 drives the rhythmic transcription of \textit{mbmal1}, which exhibits a phase opposite to that of \textit{mper} and \textit{mcry}, forming a positive transcriptional loop. Increased availability of BMAL1 presumably promotes the formation of the CLOCK:BMAL1 heterodimers that are required to restart the \textit{mper} and \textit{mcry}
transcription cycle (Shearman et al. 2000). At this point, it appears that mPER3 protein is not essential for the maintenance of circadian rhythmicity (Bae et al. 2001).

**Light Entrainment of Circadian Clocks**

Many of the circadian clocks that drive rhythmic processes and behaviors are synchronized (entrained) to the daily changes that occur in the levels of ambient light that occur between dawn and dusk. The imposition of an artificial 12 hour light:12 hour dark (12L:12D) cycle on these clocks forces them to run with a period near 24 hours that is in phase with the light/dark cycle. The phase of the clock is defined by the cyclic changes that occur in the levels of the various proteins that comprise the clock over the course of a single 24-hour light/dark cycle. Light entrainment is a dynamic process. A change in the light/dark cycle causes changes in the concentrations of the clock proteins, which in turn produce a shift in the phase of the clock so that it is properly synchronized to the new light/dark cycle. The direction and amplitude of the phase shift of the clock are determined by the magnitude of the change in the concentration of the clock proteins that is induced by the new light cycle (Devlin and Kay 2001). For example, in *Drosophila*, levels of TIM protein can be directly modulated by light as a result of TIM’s interaction with the flavoprotein CRYPTOCHROME (CRY). Light pulses delivered during the dark period trigger the degradation of TIM and reset the phase of the clock to a point in the cycle where the concentration of TIM would normally be low (Young 2000). In mouse SCN, the transcription of *mper1* and/or *mper2* genes has been shown to be rapidly induced by light pulses delivered during the subjective night, results that suggest that mPER1 and/or mPER2 protein may be involved in mediating light entrainment of the mammalian clock (Albrecht et al. 1997; Okamura et al. 1999; Shearman et al. 1997). The response of the circadian clock to the changes of light stimuli varies over the course of
the day. In general, light pulses delivered in late afternoon or early night delay the phase of the clock, light pulses administered in late night or early morning result in phase advances, and light pulses delivered during the middle of the day are relatively ineffective in inducing changes in the phase of the clock (Rosenwasser and Dwyer 2001).

Circadian Clocks in the Central Nervous System (CNS)

In mammals, the master circadian "clock" that controls physiological and behavioral rhythms resides in the suprachiasmatic nuclei (SCN) that are located within the anterior hypothalamus. The clock in the SCN is composed of multiple, autonomous, single-cell circadian oscillators that receive information about ambient light levels directly from the eyes via the retinohypothalamic tracts (Green 1998). Synchronization of these oscillators by light permits the SCN to generate a coordinated circadian output that is capable of regulating overt rhythms (Reppert and Weaver 2001). One of the more important rhythms regulated by the SCN is the production of melatonin by the pineal gland. Two types of regulatory signals are transmitted to the pineal gland through efferents from SCN. One is the permissive signal that originates from the circadian clock and restricts melatonin production to the night. The other is the inhibitory signal that is induced by inappropriate light stimuli at night and acutely suppresses nocturnal melatonin production (Gillette and McArthur 1996). Thus, in mammals, the eye, the SCN and the pineal body function in sequence as photoreceptor, master circadian oscillator, and melatonin output organ for maintaining circadian rhythms at the organism level.

In avian species, the eye, the pineal gland, and deep brain structures including the hypothalamus and SCN have all been implicated in the regulation of behavioral rhythms, although diversity among avian species is great. In the House sparrow and the Java sparrow, pinealectomy abolishes free-running circadian rhythms under constant
conditions, observations that indicate that the pineal gland is the site of an essential circadian clock in sparrows (Gaston and Menaker 1968). But in Japanese quails, pinealectomy does not have a significant effect on circadian locomotor activity rhythms (Underwood 1994). The eyes (Underwood 1994) and the hypothalamus (Simpsom and Follett 1981) of the Japanese quail have been suggested to be the locations of the major circadian oscillators in this species. The eyes, pineal, and SCN must all be removed to abolish circadian locomotor rhythms in pigeons, a result that indicates that all of these organs are necessary for maintaining circadian rhythmicity in pigeon (Ebihara et al. 1984). Based on these observations, it has been suggested that the circadian system in the avian CNS contains multiple oscillators comprised of the pineal gland, the eyes, and deep brain structures that include the SCN (Oishi et al. 2001).

**Circadian Rhythms In Retina**

**Retinal Functions Regulated by Circadian Clocks**

There is now substantial evidence that vertebrate retinas contain circadian clocks and that these clocks play an important role in maintaining the function and health of the retina (Moog 1995). These functions include the synthesis and release of neuromodulators such as melatonin (Cahill et al. 1991; Tosini 2000) and dopamine (Besharse and Iuvone 1992), photoreceptor disc shedding and phagocytosis by the retinal pigment epithelium (RPE) (Nguyen-Legros and Hicks 2000), retinomotor movement (Burnside 2001), gene expression (Pierce et al. 1993; Green and Besharse 1994; Green et al. 1996; Larkin et al. 1999; Chong et al. 2000), and visual sensitivity (Li and Dowling 2000).
Cellular Location of Circadian Clocks Within Retina

Currently, the most direct evidence for the presence of functional circadian clocks in a specific retinal cell type comes from studies of melatonin synthesis in reduced *Xenopus* retina cultures. Melatonin in the retina is produced by the retinal photoreceptor cells and is regulated by circadian clocks located in the retina. By monitoring melatonin release from the photoreceptor cultures under constant dark conditions, Cahill and Besharse (Cahill and Besharse 1992;1993) were able to show that the circadian oscillators controlling melatonin rhythms in *Xenopus* retina are localized to the photoreceptor cells. The observations that the activities of tryptophan hydroxylase (TPH) and serotonin N-acetyltransferase (AA-NAT), two key enzymes in the melatonin biosynthesis pathway, are both expressed in *Xenopus* photoreceptors and are also under the control of a circadian oscillator are consistent with the localization of circadian clocks to these cells (Besharse and Iuvone 1983;Green and Besharse 1994;Green et al. 1995). Indirect support for the presence of circadian oscillators in photoreceptor cells comes from studies of low-density cultures of avian retina. Iodopsin is a red-sensitive pigment that is expressed in cone photoreceptors in the retinas of birds (Yoshizawa and Kuwata 1991). The transcription of the iodopsin gene in chicken retina has been shown to be regulated by a circadian clock (Pierce et al. 1993;Larkin et al. 1999). The observations that transcription of the iodopsin gene exhibits a circadian rhythm in low density cultures of both quail (Pierce 1999) and chicken retina (Pierce et al. 1993) suggest that the circadian clocks driving expression of this gene are located in the cone photoreceptor cells.

If circadian clocks are present in photoreceptor cells, then the clock genes should be expressed in these cells. In mouse, transcripts encoding mPER1 (Shearman et al.
1997; Sun et al. 1997; Sun et al. 1997; Shearman et al. 1997), mPER2 (Shearman et al.
1997), mPER3 (Zylka et al. 1998), mTIM (Zylka et al. 1998), mCLOCK (Gekakis et al.
1998) and BMAL1 (Gekakis et al. 1998) have been isolated from the retina. In situ
hybridization analyses of mouse retina have revealed that mclock, mper1 and mbmal1 are
coop-expressed in retinal photoreceptors, cells within the inner nuclear layer and in the
ganglion cell layer (Gekakis et al. 1998). In Xenopus, XClock (Zhu et al. 2000), Xper2
(Zhuang et al. 2000), and three cryptochromes genes (Xcry1, Xcry2a, Xcry2b) (Anderson
and Green 2000) have been cloned and have been shown to be expressed in retina. Many
of these clock genes are also expressed in the retinas of birds. Transcripts encoding
cCLOCK (Larkin et al. 1999; Chong et al. 2000; Larkin et al. 1999), cBMAL1 and
cMOP4 (Chong et al. 2000) have been isolated from chicken retina and transcripts
encoding qCLOCK, qPER2 and qPER3 have been isolated from quail retina (Yoshimura
et al. 2000).

Taken together, the results of the studies of the expression of clock genes and
melatonin secretion in retina suggest that vertebrate photoreceptor cells contain
functional circadian clocks and output pathways.

**Light Entrainment of Retinal Photoreceptor Clocks**

Most information about the responses of retinal circadian clocks to light comes
from analyses of processes within the retina that are regulated by these clocks. Studies of
melatonin and iodopsin synthesis, both of which are produced by photoreceptor cells,
suggest that retinal photoreceptors contain functional input pathways that allow
entrainment of the oscillators in these cells to light. For example, in their studies of
reduced Xenopus retina cultures, Cahill and Besharse (Cahill and Besharse 1992; 1993)
noted that the phase of the melatonin rhythms in photoreceptor cells could be reset by
light. In chicken retina, the rhythms of iodopsin transcription in cone photoreceptors can be entrained to the external cyclic lighting conditions and the phase of the rhythms can be shifted by 6 hour light pulses delivered during the dark period (Larkin and Semple-Rowland 2001). These observations together with those mentioned above suggest that photoreceptor cells contain a complete circadian system.

**Synchronization of Circadian Clocks**

### Synchronization of Circadian Clocks in SCN

The mammalian SCN contains 20,000 neurons that work in concert to drive the coordinated circadian rhythms of electrical activity (Herzog et al. 1997), gene expression (Panda et al. 2002), glucose metabolism (Schwartz et al. 1983), and behavior (LeSauter and Silver 1998; Herzog et al. 1997). Individual neurons in SCN dissociated cultures exhibit self-sustained rhythms of spontaneous firing activity, suggesting that functional circadian oscillators are present within these single neurons. However, the firing rhythms of individual oscillators exhibit variable periods and different phase relationships with one another under these culture conditions (Welsh et al. 1995). In SCN explant cultures, in which synapses and cellular appositions are largely preserved, the firing rhythms of individual SCN neurons exhibit periods with significantly less variability and are in phase with each other. Furthermore, the range of the periods in explants is almost identical to that observed for behavioral rhythms (Herzog et al. 2001). These observations suggest that intercellular communication is required to generate and maintain coordinated circadian rhythms in SCN.

Synapse- and gap junction-mediated intercellular communications represent two possible mechanisms that could synchronize populations of autonomous oscillator cells. Accumulating indirect evidence suggests that synaptic transmission does not play a
central role in the synchronization of the circadian oscillators within the SCN neurons. For example, circadian glucose metabolism in the SCN is observed before chemical synapses become functional in the SCN (Reppert and Schwartz 1984; Moore and Bernstein 1989). Consistent with this observation are the observations that disruption of synaptic transmission within the SCN using either tetrodotoxin (TTX) (Shibata and Moore 1993) or calcium-free medium (Bouskila and Dudek 1993) do not alter the ability of the SCN neurons to generate synchronized bursts of activity.

Gap junction channels provide another means for intercellular communication. Gap junction channel consists of a hemichannel (a connexon) in the membrane of one cell that is paired with a similar hemichannel in another adjoining cell. A hydrophilic pore at the core of the connexon allows the passage of small ions and low molecular weight metabolites (up to 1 kD) between the cells and functions to connect the cells both electrically and metabolically. Each connexon, in turn, is composed of 6 similar protein subunits known as connexins (Cx). The connexins are members of a multigene family. Connexins 26, 32 and 43 are the first members of this family that are identified and are the most abundant isoforms expressed in the developing CNS (Cook and Becker 1995).

Gap junction communication has been shown to play a role in the coordination and synchronization of the activity of SCN neurons in vivo. Studies of connectivity of SCN neurons show that SCN neurons are coupled by low resistance pathways (Colwell 2000; Shinohara et al. 2000; Jiang et al. 1997), the permeability of which are modulated by cell activity. Studies using dyes capable of traversing gap junctions show that SCN cells are extensively dye coupled during the day when the cells exhibit synchronous neural activity and are minimally dye coupled during the night when the cells are electrically
silent, a coupling rhythm that is also maintained under constant dark conditions (Colwell 2000). Evidence for a role of gap junctions in the synchronization of the SCN cell oscillators comes from a recent study that shows that the gap junction blockers, octanol and halothane, can reversibly block vasopressin and vasoactive intestinal polypeptide rhythms in SCN slice cultures (Shinohara et al. 2000). Interestingly, there is a growing body of evidence that suggests that gama-aminobutyric acid (GABA) may modify the responses of the SCN neuronal circadian clocks to light and other entraining stimuli by altering cell-cell coupling through gap junctions. GABA, acting through type A receptors, has recently been shown to be capable of phase shifting and synchronizing the oscillators within individual SCN clock cells in vitro (Liu and Reppert 2000) and of modulating the permeability of gap junction channels in SCN slice cultures (Shinohara et al. 2000)

**Expression and Function of Gap Junctions in Retina**

The vertebrate retina is a highly laminated assemblage of five major classes of specialized neurons: a vertical pathway connects photoreceptors to bipolar cell to ganglion cells, while horizontal and amacrine cells provide lateral interactions in the outer and inner retina, respectively. Tracer studies have revealed that in addition to these pathways, many types of retinal neurons are interconnected through gap junctions. In addition to the widespread coupling that is observed between identical cell types, heterologous coupling between rods and cones, between amacrine cells and cone bipolar cells, between different types of bipolar cells, between different types of amacrine cells, and between ganglion cells and amacrine cells is also observed (Vaney and Weiler 2000). There is a growing body of evidence that gap junctions are present and functional in developing retinas. Cx43, 26 and 32 are the major connexin isoforms expressed in developing chicken retina. Among them, Cx43 is the first one to be expressed in the early
neuroepithelium of the eyecup, followed by the expression of Cx32 and 26 at E4-4.5 (Becker et al. 1998). As early as E7, fluorescent dye injected into individual retinal ganglion cells spreads into the cells within the ganglion cell layer, the inner nuclear layer, and also to the cells traversing the whole thickness of retina, results that suggest that an extensive network of gap junctions has been formed in chicken retina at this developmental stage (Catsicas et al. 1998). Functional studies have also shown that gap junction communication is involved in the regulation of synchronized spontaneous neural activity that occurs both before (Catsicas et al. 1998) and during (Wong et al. 1998) synaptogenesis in embryonic chicken retina.

Neurotransmitters play a major role in regulation of gap junction permeability in inner retina. For example, dopamine and GABA have both been shown to modulate the permeability of gap junctions that exist between amacrine cells in rabbit retina (Hampson et al. 1992) and between horizontal cells in rabbit and turtle retina (Hampson et al. 1994; Piccolino et al. 1982; Piccolino et al. 1984). Modulation of gap junction coupling either through changes in ambient illumination or through light-induced changes in dopamine release have been postulated to play a role in regulating retinal sensitivity (Li and Dowling 2000; Manglapus et al. 1998; Manglapus et al. 1999).

Vertebrate photoreceptor cells are also coupled by gap junctions (Gold and Dowling 1979; Raviola and Gilula 1973; Tsukamoto et al. 1992). There is evidence that the strength of junction coupling between photoreceptors can be modulated by light (Yang and Wu 1989) and by dopamine (Krizaj et al. 1998); however, the effectiveness of these stimuli to alter coupling may be species dependent (Schneeweis and Schnapf 1999). While potentially important in processing of light signals for vision (Schneeweis and
Schnapf 1999; Lebedev et al. 1998), changes in gap junction communication may also play a role in the synchronization of photoreceptor circadian oscillators in retina. We have observed that iodopsin rhythms in dispersed cultures of chicken retina are not as robust as those observed in retinal explant cultures, the rhythms in dispersed cells becoming negligible after one week in culture (Semple-Rowland, unpublished observation). This observation also indicates that, as in the case of SCN, gap junction communication may play a role in generation and maintaintence of iodopsin transcript rhythms in retinal explant cultures.
CHAPTER 2

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CHARACTERIZATION OF CIRCADIAN OSCILLATOR FUNCTION IN EMBRYONIC RETINA AND RETINAL EXPLANT CULTURES

Introduction

The retinas of several vertebrate species contain light entrainable circadian oscillators that regulate 24-hour cyclic changes in retinal function (Besharse and Iuvone 1983; Cahill and Besharse 1991; Tosini and Menaker 1996; Tosini and Menaker 1998). Currently, there is significant interest in identifying the retinal cells that contain these oscillators and understanding how these oscillators are entrained to light.

Cahill and Besharse obtained the first evidence that vertebrate photoreceptor cells contain light-entrainable circadian oscillators (Cahill and Besharse 1993). Using a reduced *Xenopus* eyecup preparation, these investigators obtained support for the hypothesis that the circadian oscillators controlling melatonin release in *Xenopus* retina are located in the photoreceptors, and that light entrainment of these oscillators does not require input from cells within the inner retina. Direct evidence for the presence of functional oscillators in *Xenopus* photoreceptor cells has recently been obtained in a study of transgenic *Xenopus* tadpoles that express a dominant negative form of Clock. Photoreceptor-specific expression of the mutant Clock protein was found to disrupt rhythmic production of melatonin by these cells (Hayasaka et al. 2002). These data and the observations that transcription of the cone-specific iodopsin gene is regulated in a circadian manner in dispersed, low-density cultures of chicken and quail retina (Pierce et
al. 1993; Pierce 1999) suggest that it is likely that the retinal photoreceptor cells of several vertebrate species contain autonomous circadian oscillators.

The nature of the biochemical cascades that entrain retinal oscillators to light and the extent to which these oscillators influence each other within the context of the intact retina are currently unknown; however, significant progress has recently been made toward understanding light entrainment of oscillators in avian pineal cells. Using immunocytochemical, molecular and biochemical techniques, Fukada and his colleagues have obtained convincing evidence that an opsin-\(G_{11}\alpha\)-mediated signaling pathway is involved in light entrainment of chicken pineal circadian oscillators (Kasahara et al. 2002). They also found that \(G_{11}\) is expressed in chicken retinal photoreceptors and that it associates with rhodopsin, one of the opsins in retina, in a light- and GTP-dependent manner (Kasahara et al. 2002). Together, these data suggest that an opsin-\(G_{11}\) signaling pathway may be involved in mediating the phase shifting effects of light on circadian oscillators in chicken retina.

Organ culture systems, which have been successfully used to study circadian regulation of melatonin in *Xenopus* and hamster retina (Cahill and Besharse 1991; Tosini and Menaker 1996), may also prove valuable in studies of the retinal oscillators that drive iodopsin transcription. In this series of experiments, iodopsin mRNA rhythms in embryonic retinal explants maintained under cyclic light, constant dark, and light reversal conditions were compared to those in retinas from age-matched chicken embryos and in post-hatch (< 2 weeks old) chickens. Our data show that embryonic retinas maintained as explant cultures exhibit robust iodopsin rhythms that are driven by light entrainable circadian oscillators. These observations support the use of explant cultures in studies to
investigate mechanisms responsible for generating and maintaining iodopsin transcript rhythms in retina.

**Materials and Methods**

**Preparation of Retinal Explant Cultures**

All experimental procedures mentioned in this dissertation were approved by the University of Florida IACUC Committee and were in accordance with the National Institutes of Health guidelines. Fertile White Leghorn chicken eggs obtained from the University of Florida Poultry Sciences Unit were incubated on a 12 hour light: 12 hour dark (12L:12D) cycle in the incubators illuminated by 20 Watt cool white fluorescent bulbs (90 lux). The lights were on at 9:00 AM (Zeitgeber time, ZT0) and off at 9:00 PM (ZT12) EST. Retinal explant cultures were prepared from embryonic day 9 (E9) and E10 chickens during the 12-hour light period. Dissection and preparation of the cultures was carried out according to a method previously described for preparation of neonatal mouse retina cultures (Ogilvie et al. 1999). The eyes were dissected from the embryos and placed in a pool of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (130 U/ml penicillin and 130 µg/ml streptomycin). After the sclera, choroid and retinal pigmented epithelial tissues were removed, the remaining structure, consisting of the vitreous body and the retina, was transferred to a 35 mm culture dish that contained a Millicell membrane insert (0.2 µm; Millipore, Bedford, MA) filled with media. The retina was gently peeled away from the vitreous body and several small cuts were made around the periphery of the retina to facilitate flattening of the retina photoreceptor side down onto the membrane. The retinal explants were incubated on a 12L:12D cycle at 37°C in 5% CO₂ and were fed every two
days. The 12L:12D period beginning the day after the cultures were prepared was designated day 1 \textit{in vitro} (1 DIV).

**Explant Morphology**

Retinal cultures designated for histological analyses were fixed for 1-2 days in 4% paraformaldehyde at 4°C. Retinas were left on the Millicell membrane during the fixation step and in some cases remained attached to the membrane throughout the sectioning process. The tissues were infiltrated with 30% sucrose and sectioned (16 μm) using a cryostat. Sections were placed onto glass slides and stored at -20°C until processed for staining or immunohistochemistry.

Cresyl violet stained sections were examined to assess the general morphology of the retinal explants. To determine if there was significant cell loss within the inner nuclear layer (INL) of these cultured retinas, the mean cell density (number of cells per \( \mu m^2 \)) within the INL was determined for three prescribed regions of three different sections from each explant and the average of these values was corrected by multiplying by the mean thickness of the INL layer (μm). The resulting values were compared using Kruskal-Wallis ANOVA on Ranks (SigmaStat, Jandel, CA). The percent pyknotic cells in the INL was estimated in 3, 5 and 12 DIV retinal explants by counting the pyknotic nuclei present in three separate prescribed regions of three different sections from each explant and dividing by the mean cell density.

To determine if dopaminergic amacrine cells could be detected in the explant cultures, cryosections taken from the retinal explants used in the morphological analyses were immunostained using a monoclonal antibody (1/10,000 dilution in PBS containing 1% goat serum and 0.1% Triton X-100) for tyrosine hydroxylase (TH; Chemicon,
Temecula, CA), a marker for dopaminergic cells, and a goat anti-mouse secondary antibody (1/500 dilution in PBS) tagged with the Alexa-488 fluorophore (Molecular Probes, Eugene, OR). Sections were incubated with the anti-TH primary antibody for 2 hours at room temperature, rinsed three times with PBS, and then incubated with the secondary antibody for 1 hour at room temperature followed by three additional rinses. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). The stained tissues were viewed using the appropriate fluorescent filter sets and digital images were acquired using a SPOT 2 Enhanced Digital Camera System mounted on a Zeiss Axioplan 2 fluorescence microscope.

**Iodopsin Gene and Protein Expression in Retinal Explant Cultures**

To identify the earliest time that iodopsin transcripts could be detected in retinal explant cultures, cultures prepared from E9 embryos and maintained under 12L:12D conditions for 1-6 DIV were collected at ZT 8 (8 hours after lights came on). To compare iodopsin expression onset in cultures and *in ovo*, retinas from chicken embryos were also collected at ZT 8 from E10 to E16. The retina samples were quickly frozen in liquid nitrogen and stored at -75°C until processed for northern blot analyses.

Immunohistochemical staining of retinal explants was carried out to examine expression of iodopsin protein in the explants. Tissue sections were blocked using PBS containing 10% goat serum and incubated overnight at 4°C with a polyclonal antibody for chicken iodopsin (CERN874; 1/5000 dilution in PBS containing 1.0% BSA and 0.3% Triton X-100) (Geusz et al. 1997). The primary antibody was visualized by incubating the sections with a goat anti-rabbit secondary antibody (1/1000 dilution in PBS) tagged
with the Alexa-594 fluorophore (Molecular Probes) for 1 hour at room temperature. Sections were counterstained with DAPI and imaged as described above.

**Lighting Paradigms**

**Cyclic light**

Explant cultures maintained under 12L:12D conditions were collected from 3 DIV to 9 DIV at ZT0 and ZT12. For the *in ovo* experiments, the retinas of White Leghorn chicken embryos maintained *in ovo* under 12L:12D conditions were harvested at ZT0 and ZT12 from E17 to E20. In all experiments, sample collection during the dark period was carried out under a low intensity red safe light (15 Watt bulb, Kodak #2 filter).

**Constant dark**

Retinal explants were maintained under 12L:12D conditions for the first 5 days in culture and were placed in constant darkness on days 6-7. Cultures were collected on 4 and 5 DIV at ZT0 and ZT12 and on 6 and 7 DIV just after the lights would have been turned on (circadian time 0 - CT0) and just after the lights would have been turned off (CT12). For the *in ovo* experiments, chicken embryos were incubated under 12L:12D conditions through E17 and were then placed in constant dark conditions from E18 to E20. The retinas of E17 embryos were collected at ZT0 and ZT12. The retinas of E18 to E20 embryos were collected at CT0 and CT12.

**Reversal of the light cycle**

Retinal explants were maintained on a 12L:12D cycle for 5 DIV. On 6 DIV the light cycle was reversed to a 12D:12L cycle and the cultures remained on this reversed light cycle through 8 DIV. Cultures were collected at ZT0 and ZT12 from 4 to 8 DIV. For the *in ovo* experiments, chicken embryos were incubated under 12L:12D conditions through E17. On E18, the light cycle was reversed and the embryos remained on this
reversed cycle through E20. The retinas of E17 through E20 embryos were collected at ZT0 and ZT12.

**RNA Analyses**

Retinal explants and age-matched embryo retinas were placed in screw top tubes, frozen in liquid nitrogen and stored at -75°C until further processing. Total RNA was extracted from the retinas using an RNeasy kit (Qiagen, Valencia, CA). Northern blots were prepared as previously described (Semple-Rowland and van der 1992), each lane containing 8 µg total RNA. RNA slot blots were prepared using a BioSlot apparatus (BioRad). The RNA samples, each containing 2µg of total RNA from a retinal explant, were diluted with RNase-free water to a final volume of 10 µl. The following solutions were then added to each sample; 20 µl of 100% formamide, 7µl of 37% formaldehyde, and 2ml of 20X SSC (3M NaCl, 0.3M sodium citrate, pH 7.0). The samples were then incubated at 68°C for 15 minutes. During the incubation period, the slot blot apparatus was assembled according to the manufacturers’ instructions and each sample well was rinsed twice with 1 ml 20X SSC. Following the incubation, the denatured RNA samples were cooled on ice, diluted by adding 2 volumes of 20X SSC, and loaded into the sample wells. A gentle vacuum was applied to the apparatus to load the RNA onto a Magnacharge nylon membrane (MSI, Westburough, MA) and to complete the wash steps. Following application of the samples, each well was rinsed twice with 1 ml 20X SSC. The vacuum was kept on for an additional 5 min following the last wash to dry the membrane. The RNA samples were then cross-linked to the blot using UV light (UV Stratalinker, Stragene). The effectiveness of the transfer was examined by staining the blot with methylene blue. Finally, the blot was dried at 37°C for 30 minutes and stored at
room temperature until hybridization. The northern and slot blots were prepared in duplicate and were probed consecutively with radiolabeled cDNA probes specific for iodopsin and 18S rRNA that were synthesized as described previously (Larkin et al. 1999). The amount of probe hybridized to the blots was measured using a BioRad Molecular Imager FX system. Iodopsin transcript levels in individual samples were normalized to the amount of 18S rRNA present in that same sample. These values were then expressed relative to the mean iodopsin/18S rRNA value for each blot. Data were analyzed using two-way ANOVA (SigmaStat).

**Results**

**Morphology of Developing Retinal Explant Cultures**

The cellular architecture and structural integrity of the retinal explants were examined as a function of time in culture by comparing cresyl violet-stained sections of explants to those of retinas from embryos.

The outer nuclear (ONL), inner nuclear (INL), and ganglion cell (GCL) layers of E13 retinas were readily discernable and fairly well organized (Fig.2-1, top panel). From E15 to E18 there was a gradual decrease in the overall thickness of the nuclear layers, a change due in part to a decrease in extracellular space volume and to the rapid growth of the eye that occurs during this period of development. Retinas cultured for 3, 5 and 12 days (Fig.2-1, bottom panel) resembled those obtained from embryos (Fig.2-1, top panel); however, there were two major changes in the structure of the cultured retinas. First, the ganglion cells, which were detectable in 3 DIV cultures, rapidly degenerated and were no longer evident at 5 DIV. Concomitant with the disappearance of the GCL was a reduction in the overall thickness and gradual loss of the inner plexiform layer
Second, the cells within the ONL and INL of the explants remained relatively disorganized compared to those present within these layers in retinas in ovo.

Figure 2-1. Morphological comparison of chicken embryonic retina and retinal explant cultures. Images of E13, E15 and E18 retinal cross-sections (top panels) show the cellular organization and development of chicken embryonic retinas in ovo. Images of 3 DIV, 5 DIV and 12 DIV retinal cross-sections (middle panels) show the cellular organization and development of chicken retinal explants harvested from E10 embryos. The bottom panel contains magnified images of the INL from explant cultures that show evidence of cell death within the INL (arrows indicate pyknotic nuclei). DIV – days in vitro; RPE – retinal pigment epithelium; ONL – outer nuclear layer; OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. Scale bars = 25 µm.

To determine if loss of the IPL in cultured retinas was accompanied by a significant decrease in the number of cells within the INL, we measured both the cell density and thickness of the INL layers in retinas cultured for 3, 5 and 12 days. The results of these
analyses showed that there was not a significant change in the number of cells within the INL ($H = 0.924$, $df = 2$, $p = 0.63$) even though there were significant changes in the mean densities ($H = 21.0$, $df = 2$, $p < 0.0001$) and widths of the INL layer ($H = 16.1$, $df = 2$, $p = 0.003$) over time (Fig. 2-2A).

![Bar chart](image)

**Figure 2-2.** Quantification of the morphological integrity of retinal explant cultures. (A) Cell densities (yellow bars) and widths (blue bars) of INL layers in 3, 5 and 12 DIV explant cultures. The mean number of cells per µm in the INL calculated from the density and width values is also shown in red. (B) Percent pyknotic cells within the INL of 3, 5 and 12 DIV explant cultures. Bars represent mean ± SE of measures taken from three separate prescribed regions within three different sections of representative 3, 5 and 12 DIV retinal explants.

However, there was evidence of cell death within the INL throughout the 12-day culture period. Pyknotic nuclei were detected within this cell layer as early as 3 DIV, the number significantly increasing by 5 DIV and then dramatically falling off by 12 DIV.
(Fig. 2-1, bottom panel). This temporal pattern of cell death is similar to what has been reported in analyses of neural cell death in retinas of developing chick (Cook et al. 1998) and quail embryos (Marin-Teva et al. 1999) and suggests that the loss of cells in the INL may reflect normal changes in cell number that accompany development. The majority of the pyknotic nuclei were located in the inner strata of the INL closest to the IPL. The percent of the total number of INL cells that were pyknotic was relatively small, with mean values ranging from 0.09% (12 DIV) to 3.2% (5 DIV) (Fig. 2-2B). Virtually no pyknotic nuclei were detected in the photoreceptor cell layer in any of the explant cultures examined, consistent with previous studies of normal avian retina development (Cook et al. 1998; Marin-Teva et al. 1999).

We were also interested in determining if we could detect dopaminergic amacrine cells in our retinal explants because of their known importance in retinal circadian biology (Besharse and Iuvone 1920). Gardino et al. (Gardino et al. 1993) have previously shown that E13 is the earliest time at which amacrine cells expressing the TH phenotype can be detected immunohistochemically in developing chicken retina.

We did not detect any TH-positive amacrine cells in cross-sections of the 3, 5, 8 and 12 DIV retinal explants stained with a monoclonal antibody for TH. TH-positive cells exhibiting the morphology of amacrine cells were detected in the inner margin of the INL in E18 and post-hatch retinas using this antibody, but not in E13 retina (Fig. 2-3). The morphology and the location of the TH-positive cells that we observed in E18 and post-hatch retina were the same as previously described (Gardino et al. 1993). Our inability to detect TH-positive cells in E13 retinal sections may have been due to a combination of factors including low levels of expression of TH and the unique spatial
distribution of these cells across the retina at this stage of development (Gardino et al. 1993).

Figure 2-3. TH immunostaining of explants and embryonic and post-hatch retinas. No TH-immunoreactive cells were detected in 3, 5, 8 or 12 DIV retinal explant cultures. Scattered TH-immunoreactive cells exhibiting amacrine cell morphology were identified in E18 and 4 days post-hatch (DPH) retinas along the border between the INL and the IPL. ONL – outer nuclear layer; OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. Scale bars = 10 µm.

Comparison of Iodopsin Expression Onset in Retinal Explant Cultures and in ovo

Prior to initiating the studies of clock function in the retinal explant cultures I identified the earliest time at which iodopsin gene expression could be detected in these cultures. Northern blot analyses showed that iodopsin transcripts could be detected in E9 explants that had been cultured for 3 days (Fig.2-4A, left panel), a time point approximately equivalent to E12. On the other hand, iodopsin gene expression was first detected by Northern blots on E15 in embryonic chicken retina (Fig.2-4A, right panel). However, it should be noted that recent studies of normal developing chicken retina place the age of onset earlier at approximately E6-E8 when expression is analyzed using RT-PCR (Adler et al. 2001). The acceleration of the onset of iodopsin gene expression that we observed in the explant cultures is consistent with the results of previous studies of
iodopsin gene expression in cultured chicken retina (Belecky-Adams et al. 1999) and has been proposed to occur as a result of the absence of inhibitory factors that normally delay expression of this gene in vivo (Adler et al. 2001).

Figure 2-4. Onset of iodopsin expression in retinal explant cultures. (A) Northern blot analyses of iodopsin gene expression. Blots were probed consecutively for iodopsin and 18S rRNA. Each lane contained 8 μg total RNA. The iodopsin mRNA signal was first detected in retinal explants cultured from E9 embryos on 3 DIV, a time roughly equivalent to E12 in ovo. In contrast, iodopsin transcript signal could not be detected in retinas from chicken embryo until E15. (B) Immunohistochemical analyses of iodopsin expression in retinal explants cultured from E10 embryos. Iodopsin immunostaining was not above background in 3 DIV explants but was easily detected in the rudimentary OS of 5 DIV explants. DIV – days in vitro; ONL – outer nuclear layer; OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. Scale bars = 25 μm.

Iodopsin protein was not detected immunohistochemically in E9 retinas maintained in culture for 3 DIV and, in contrast, was easily observed in the photoreceptor cells of 5
DIV cultures. Iodopsin immunoreactivity was primarily localized to the rudimentary outer segments of the photoreceptor cells (Fig.2-4B).

**Iodopsin Transcript Rhythms**

Iodopsin transcript levels in dispersed embryonic retinal cultures and in post-hatch chicken retinas exhibit a circadian rhythm with minimum levels of the transcript at ZT0 and maximum levels at ZT12 (Larkin and Semple-Rowland 2001; Pierce et al. 1993). Analyses of iodopsin transcription in our explant cultures in which we examined transcript levels at ZT0, ZT6, ZT12 and ZT18 revealed that the same dynamics of the rhythm were preserved in explanted retina. Based on these observations, we chose to examine iodopsin transcript levels at ZT0 and ZT12 in our analyses of retinal circadian oscillator function *in ovo* and in explant culture.

**Cyclic light**

**Retinal explants.** The levels of iodopsin mRNA from 3DIV to 9DIV in retinal explant cultures maintained under 12L:12D conditions exhibited a robust rhythm. On culture day 3, iodopsin mRNA levels were 2.5 fold higher at ZT12 than at ZT0 (Fig.2-5A). From culture day 4 to 7, the total amount of iodopsin transcript increased dramatically compared to that observed on 3DIV, but the relative increase in iodopsin mRNA levels that occurred between ZT0 and ZT12 remained relatively constant from Day 3 to Day 7. On 8 and 9 DIV, iodopsin transcript levels at ZT0 were similar to that on Day 7, but the relative increase of iodopsin mRNA levels from ZT0 to ZT12 was reduced. The differences observed in levels of iodopsin mRNA between ZT0 and ZT12 were significant over the time period examined (F = 31.03, df = 1, p < 0.0001).
Figure 2-5. Iodopsin transcript rhythms in explant cultures and embryos maintained under 12L:12D and constant dark conditions. (A) Relative iodopsin mRNA levels in explant cultures prepared from E9 embryos and examined at 3, 5, 7 and 9 DIV and (B) in retinas of embryos examined at E17 – E20. For panels A and B, the explants and embryos were maintained under 12L:12D conditions throughout the experiment. Retinas were analyzed at ZT0 (white bars) and at ZT12 (black bars).
bars). (C) Relative iodopsin mRNA levels in explant cultures maintained for 5 DIV under 12L:12D and then transferred into constant dark conditions on days 6 and 7. (D) Relative iodopsin mRNA levels in embryos raised under 12L:12D until E18 and then transferred into constant dark conditions from E18 to E20. (E) Comparison of explant cyclic light data shown in panel A (red symbols) and the constant dark data shown in panel C (black symbols). (F) Comparison of embryonic cyclic light data shown in panel B (red symbols) and the constant dark data shown in panel D (black symbols). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. In panels E and F, plotted data and light cycles are matched by color, red indicating 12-hour dark periods. In panels A – D, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels E and F, only the mean values are plotted for each group.

**Retina in ovo.** Retinal iodopsin transcripts were first detected in ovo on E15. No significant rhythms were detected on E15 or E16 and the mean values for relative iodopsin levels at these stages were 0.185 and 0.258, respectively (data not shown). The first evidence of a rhythm in iodopsin transcript levels was detected on E17; the transcript levels measured at ZT12 were 1.6 fold higher than those measured at ZT0 (Fig.2-5B). The amplitude of the rhythm increased with developmental age. On E18, iodopsin mRNA levels at ZT12 were 1.8 fold higher than those at ZT0. By E19 and E20, two-fold increases in iodopsin expression were observed over the course of the 12-hour light period. The emergence of the iodopsin transcript rhythm during the late stages of development was paralleled by a steady and significant increase in the total amount of iodopsin transcript, the levels of which appeared to reach a plateau by E20. The differences observed in levels of iodopsin mRNA between ZT0 and ZT12 were significant over the time period from E17 to E20 (F = 97.57, df = 1, p < 0.0001).

**Constant dark**

**Retinal explants.** Under 12L:12D conditions, the iodopsin transcript levels in retinal explant cultures at 4 and 5DIV exhibit robust rhythms. In the absence of light, a
significant rhythm persisted in retinal cultures for 48 hours, the iodopsin levels measured at CT0 being significantly lower than those measured at CT12 on 6 and 7 DIV (F = 9.86, df = 1, p = 0.003) (Fig.2-5C). The relative increase of iodopsin transcript levels observed from CT0 to CT12 under constant dark conditions was 70% of that observed under cyclic light conditions and the temporal characteristics of the iodopsin mRNA rhythm were similar to those observed under cyclic light (Fig.2-5E). These data suggest that the iodopsin rhythms observed in explant cultures under 12L:12D conditions are being driven by functioning retinal circadian oscillators.

**Retina in ovo.** Significant iodopsin transcript rhythms were observed in E17 chicken embryos that had been maintained in ovo under cyclic light conditions. On E18 when the lights were turned off, iodopsin transcript levels at CT12 were 1.3 fold higher than those at CT0 (Fig.2-5D). This increase was half that observed at the corresponding time point in embryos maintained under cyclic light conditions (Fig.2-5B). These incremental increases in transcript levels continued through E19 and E20. At CT12 on E20, the amount of iodopsin transcript in the retinas of embryos maintained in constant dark had reached levels similar to those observed in embryos that had been maintained under cyclic light. Direct comparisons of the cyclic light and constant dark data (Fig.2-5F) revealed that there was no detectable iodopsin mRNA rhythm present in embryos placed in constant darkness. These data suggest that light and developmental mechanisms act synergistically to up-regulate iodopsin transcription in developing embryonic chicken retina, and that in the absence of light, increases in iodopsin transcript levels are predominantly, if not completely, regulated by developmental mechanisms.
Reversal of the light:dark cycle

Retinal explants. Reversal of the light cycle on culture day 6 was followed by a 48-hour transition period during which time the iodopsin transcript levels remained at levels intermediate to those observed during this same time period in the explants that had been maintained under cyclic light conditions (Fig. 2-6A, B).

Figure 2-6. Iodopsin transcript rhythms in explant cultures and embryos following reversal of the light:dark cycle. (A) Relative iodopsin mRNA levels in explant cultures prepared from E9 embryos and examined at 4-8 DIV. Explants were maintained under 12L:12D until 6 DIV when they were transferred to a 12D:12L reversed cycle. (B) Comparison of iodopsin rhythms obtained from explants exposed to a reversed 12D:12L cycle (black symbols) and those from explants maintained on a 12L:12D cycle (red symbols; Fig. 5E). (C) Relative iodopsin mRNA levels in retinas of embryos maintained under 12L:12D until E18 when they were transferred to a 12D:12L reversed cycle. (D) Comparison
of iodopsin rhythms obtained from embryos exposed to a reversed 12D:12L cycle (black symbols) and those obtained from embryos maintained on a 12L:12D cycle (red symbols; Fig 5F). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. In panels B and D, plotted data and light cycles are matched by color, red indicating 12-hour dark periods. In panels A and C, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels B and D, only the mean values are plotted for each group.

Over the course of the first 12-hour dark period of the reversed cycle (6 DIV), iodopsin mRNA levels increased 1.4 fold to levels comparable to those observed in cultures maintained under cyclic light conditions at 6 DIV (Fig.2-6A, B). No further increase in iodopsin mRNA levels was observed in the reversed cycle cultures during the 12-hour light period on 6 DIV. Comparisons of the iodopsin transcription levels in cultures exposed to the reversed light cycle to those in cultures maintained on the 12L:12D cycle at 7 and 8 DIV showed that the oscillators that drive the iodopsin rhythms in the explant cultures are capable of entraining to a new light cycle and suggest that entrainment is completed within 36-48 hours following light reversal (Fig.2-6B).

Retina in ovo. During the first 12-hour dark period of the reversed cycle on E18, the increase in iodopsin transcript levels was minimal. By the end of the subsequent 12-hour light period on E18, the levels of iodopsin mRNA had increased 2-fold (Fig.2-6C). Comparisons of the iodopsin rhythms obtained from these embryos and those obtained from embryos maintained on a normal light:dark cycle (Fig.2-6D) show that reversal of the light cycle produces an immediate shift in the iodopsin transcript rhythm. These data, which are consistent with our constant dark in ovo results, support the hypothesis that iodopsin transcript rhythms in ovo are primarily, if not entirely, driven by light.
Discussion

Two major conclusions can be drawn from the results of these experiments. First, circadian oscillators regulate iodopsin transcription in embryonic retinal explant cultures and the rhythms that are observed are similar to those observed in post-hatch chicken retinas (Larkin and Semple-Rowland 2001). Second, iodopsin transcript rhythms in the retinas of chicken embryos *in ovo* are driven by light and not by circadian oscillators.

The iodopsin rhythms in retinal explants share several attributes with those measured in post-hatch chicken retina. In both paradigms the rhythms are robust with the peaks of the rhythms occurring around ZT12 and the troughs at ZT0. Importantly, reversal of the light cycle induced similar shifts in the iodopsin transcript rhythms in both explant cultures and in post-hatch retina. Together, these observations show that the essential components for circadian regulation of iodopsin transcription remain intact and functional in retinas maintained in explant cultures.

It is generally accepted that the circadian oscillators that drive the iodopsin rhythm in chicken retina are located within the photoreceptor cells. This conclusion is based on the observation that transcription of this cone pigment gene remains rhythmic in dispersed retinal cell cultures (Pierce et al. 1993). Although our analyses of retinal explants do not provide unequivocal evidence that proves that circadian oscillators are located in photoreceptor cells, the results are consistent with this point of view. It is clear from our analyses that neither removal of the retinal pigment epithelium (RPE) nor degeneration of the ganglion cells is sufficient to abolish the generation or entrainment of circadian iodopsin transcription in retinal explants. Thus, neither retinal ganglion cells nor the RPE are essential for maintaining the iodopsin rhythm in chicken retina.
Furthermore, the absence of dopamine-secreting amacrine cells in these explant cultures suggests that dopamine signaling does not play a central role in regulating this rhythm.

In addition to questions related to the location of circadian oscillators in retina, it remains to be determined how the oscillators that drive the iodopsin rhythm are entrained to light. If we assume that the oscillators that drive iodopsin transcription are located within the photoreceptor cells, then a logical starting point for the search for the phototransduction cascades that entrain these oscillators to light would focus on biochemical pathways in these cells. We have previously shown that the absence of guanylate cyclase – 1 in retinas of GUCY1*B chickens, an enzyme essential for visual phototransduction, significantly delays but does not prevent light entrainment of iodopsin rhythms to a reversal of the light cycle (Larkin and Semple-Rowland 2001). These data show that the visual phototransduction cascade mediated by the G-protein, transducin, does not directly play a role in entrainment of the oscillators that drive the iodopsin rhythm. Recent analyses of chicken pineal and retina provide provocative new evidence that suggests a possible role for a phototransduction pathway mediated by the pertussis toxin-insensitive G-protein, G₁₁, in light entrainment of circadian oscillators in these tissues (Kasahara et al. 2002). Activation of a G₁₁-mediated pathway would be expected to lead to changes in phosphatidylinositol turnover and calcium mobilization, changes that have been documented to occur in photoreceptors of several vertebrate species in response to light stimulation (Ghalayini and Anderson 1984; Hayashi and Amakawa 1985; Millar et al. 1988). Pharmacological manipulation of this cascade in retinal explant cultures may help to determine if, in fact, this cascade is involved in light entrainment of the oscillators that drive the iodopsin rhythm in chicken retina.
We were surprised, in view of our explant data, to find that light is the predominant regulatory signal that drives iodopsin transcript rhythms in the retinas of chicken embryos in ovo. These data, together with those obtained in our previous studies (Larkin and Semple-Rowland 2001), suggest that the transition from light to circadian regulation of iodopsin transcription in chicken retina occurs at or shortly after hatching. The onset of circadian regulation of the activity of serotonin N-acetyltransferase (NAT) in chicken retina, the product of another photoreceptor-specific gene, is also delayed in ovo (Iuvone 1990). However, unlike iodopsin transcription, significant light driven changes in NAT activity do not appear until late in development (E20, just/one day prior to hatching). Our observations and those of other investigators show that the emergence of circadian regulation of iodopsin transcription and NAT activity in chicken retina is accelerated in vitro {Pierce, Sheshberadaran, et al. 1993 PIERCE1993 /id} {Pierce 1999 PIERCE1999 /id} {Ivanova & Iuvone 2003 IVANOVA2003A /id}. The mechanism responsible for this acceleration is unknown. The culture conditions may accelerate the maturation of retinal circadian oscillators in vitro. It is also possible that preparation of the retinas for culture results in the removal of tissues (e.g. RPE) that normally produce regulatory signals that delay the onset of circadian regulation in the intact, developing retina.

In conclusion, the results of our analyses show that chicken retinal explant cultures can be used as an experimental paradigm for studies of retinal circadian oscillator function. The structure and organization of many of the retinal cell and synaptic layers remains intact in the explants, allowing studies of entrainment and synchronization of circadian oscillators under various lighting regimens.
CHAPTER 3
GAP JUNCTION BLOCKERS ABOLISH CIRCADIAN RHYTHMS OF GENE
EXPRESSION IN RETINAL PHOTORECEPTORS

Introduction

Circadian oscillators in vertebrate retina regulate many aspects of retinal function, including the synthesis and release of melatonin (Cahill et al., 1991; Cahill and Besharse, 1991; Tosini, 2000), photoreceptor disk shedding (Nguyen-Legros and Hicks, 2000), retinomotor movement (Burnside, 2001), and gene expression (Pierce et al., 1993; Green and Besharse, 1994; Green et al., 1996; Larkin et al., 1999; Chong et al., 2000; Bernard et al., 1999). The transcription of the genes that encode iodopsin, a red sensitive photopigment that is expressed specifically in the cone photoreceptors of chicken retina, and arylalkylamine N-acetyltransferase (AANAT) have been shown to be regulated by circadian oscillators in vivo (Larkin et al., 1999; Bernard et al., 1999; Chong et al., 2000; Liu et al., 2004) and in dissociated retinal cultures (Pierce et al., 1993). We are interested in understanding the mechanisms that coordinate and maintain photoreceptor transcript rhythms in vertebrate retina.

Studies of the SCN provide clues about the mechanisms that may serve to coordinate the activity of populations of autonomous oscillator neurons. Within the intact SCN, 20,000 neurons work in concert to generate coordinated circadian rhythms of electrical activity (Herzog et al., 1997), gene expression (Panda et al., 2002), and glucose metabolism (Schwartz et al., 1983). When examined in dissociated culture, individual SCN neurons exhibit self-sustaining electrical activity rhythms, but the periods and
phases of these rhythms are highly variable between neurons (Welsh et al., 1995). In explant culture in which synapses and cellular appositions are largely preserved, the periods and phases of the firing rhythms of individual SCN neurons exhibit significantly less variability between neurons (Herzog et al., 1998). These observations led to the hypothesis that intercellular communication is required to maintain coordinated circadian rhythms in SCN.

Synapse- and gap junction-mediated intercellular communication represent two possible mechanisms that could coordinate the circadian rhythms generated by populations of autonomous oscillator cells. The observations that circadian rhythms of glucose metabolism appear in the SCN before chemical synapses become functional (Reppert and Schwartz, 1984; Moore and Bernstein, 1989) and that blockade of synaptic transmission using either tetrodotoxin (TTX) (Shibata and Moore, 1993) or calcium-free medium (Bouskila and Dudek, 1993) does not disrupt the ability of SCN neurons to generate synchronized bursts of activity suggest that synaptic transmission is not a central player in the coordination of circadian rhythms in the SCN. Gap junction channels, which allow the passage of small ions, signaling molecules and low molecular weight metabolites between cells, could, on the other hand, serve as mediators of intercellular communication. Goodenough, Goliger, et al. 1996 GOODENOUGH1996 /id.

Gap junction channels are comprised of two paired hemichannels, known as connexons. Each connexon consists of six protein subunits called connexins (Cx) (Cook and Becker, 1995). Gap junction channels form when two connexon hemichannels located on the membranes of adjacent cells couple (Cook and Becker, 1995). Several studies suggest that gap junction communication plays an integral role in maintaining
coordinated rhythms in SCN. Studies using dyes capable of traversing gap junctions show that SCN cells are extensively coupled during the day when the cells exhibit synchronous neural activity and are minimally coupled during the night when the cells are electrically silent, a coupling rhythm that is also maintained under constant dark conditions (Colwell, 2000). The importance of this gap junction coupling in maintaining circadian rhythms in SCN is demonstrated by the observation that the gap junction blockers, octanol and halothane, disrupt the circadian rhythm of vasopressin and vasoactive intestinal polypeptide secretion from SCN slice cultures that is restored upon removal of the blockers (Shinohara et al., 2000).

Extensive networks of gap junctions are present in developing chicken retina as early as embryonic day 7 (Becker et al., 1998; Catsicas et al., 1998). Prior to synaptogenesis, these junctions have been shown to play a role in the propagation of transient Ca$^{2+}$ waves that spread across the developing chicken retina (Catsicas et al., 1998). We have recently developed a retinal explant culture system that allows the study of circadian regulation of gene transcription in vitro (Zhang et al., 2003). Using this culture system, we have successfully monitored the coordinated, self-sustained, and light-entrainable iodopsin transcript rhythms normally present in chicken retina (Zhang et al., 2003; Larkin et al., 1999). In the present study, we have conducted a series of experiments to determine if, as in SCN, the gap junction network present in chicken retina plays a role in maintaining coordinated iodopsin and AANAT transcript rhythms in this tissue. To test this hypothesis, we have examined the effects of two reversible gap junction blockers, carbenoxolone (ACO) and 18β-glycyrrhetinic acids (18-βGA), on iodopsin and AANAT transcript rhythms in explant cultures maintained under different lighting conditions.
Both gap junction blockers are the derivatives of glycyrrhetinic acid. ACO has been reported to reversibly block the gap junction channels comprised of Cx26 (Kamermans et al., 2001), Cx32 (Szente et al., 2002), and Cx43 (Goldberg et al., 1996), the three major connexins that are expressed in developing chicken retina (Becker et al., 2002) and hence the three potential targets of ACO in the retinal explant cultures. More importantly, 100µM ACO has been shown to effectively block gap junction channels in outer (Kamermans et al., 2001) and inner retina (Sekaran et al., 2003). Another gap junction blocker 18-βGA has been shown to induce phosphotase-mediated dephosphorylation of Cx43 and subsequent disassembly of gap junction plaques (Guan et al., 1996). Based on these observations, we hypothesized that application of these blockers would block the gap junction channels and desynchronize the circadian oscillators driving the gene expression rhythms, and the transcript levels of both genes would assume the average of the peak and trough values of their respective intrinsic rhythms (Kunz and Achermann, 2003).

Methods and Materials

Chemicals and Reagents

The culture media for the explants consisted of Dulbecco's modified Eagle's media (DMEM, Gibco # 11995-065) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and antibiotics (130U/ml penicillin, 130 g/ml streptomycin) (Gibco). The gap junction blockers, carbenoxolone (ACO), 18β-glycyrrhetic acids (18-βGA), and the chemically- related inactive compound, glycyrrhizic acid (GA), were purchased from Sigma (St. Louis, MO). Stock solutions of carbenoxolone (163 mM) and GA (5 mM) were dissolved in deionized water. The stock solution of 18-βGA (100 mM) was
dissolved in dimethyl sulfoxide (DMSO; Sigma). All blockers were used at a final concentration of 100 µM in these experiments.

**Retinal Explant Cultures**

All experimental procedures were approved by the University of Florida IACUC Committee and were carried out in accordance with the National Institutes of Health guidelines. Fertile White Leghorn chicken eggs (Charles River Laboratories) were incubated on a 12 hour light: 12 hour dark (12L:12D) cycle in incubators illuminated by 20 Watt cool white fluorescent bulbs (90 lux). The lights were turned on at 9:00 AM (Zeitgeber time, ZT0) and shut off at 9:00 PM (ZT12). Retinal explant cultures were prepared from embryonic day 9 (E9) chickens during the 12-hour light period. Dissection and preparation of the cultures were carried out using methods developed in our laboratory (Zhang et al., 2003). During the first 5 days of culture, all explants were incubated on a 12L:12D cycle at 37°C in 5% CO2, and were fed every two days. The 12L:12D period beginning the day after the cultures were prepared was designated day 1 *in vitro* (1 DIV).

**Lighting and Blocker Delivery Paradigms:**

**12L:12D:**

Retinal explant cultures were maintained on a 12L:12D cycle throughout the experiments. In experiments utilizing gap junction blockers, ACO or 18-βGA were added to the media at ZT12 on 4DIV and removed at ZT12 on either 5DIV or 6DIV. Alternatively, ACO was added at ZT0 on 5DIV and removed 24 hrs later. The affects of ACO on AANAT expression were examined by adding ACO at ZT15 on 4DIV and removing it 24 hours later. Control groups included untreated explants or explants treated
with either 0.1% DMSO or 100 µM GA. The final percent of DMSO in the control cultures was equivalent to that in cultures treated with 18β-GA. DMSO was added at ZT12 on 4DIV and remained on the cultures for either 24 or 48 hrs. GA was added to the cultures at ZT12 on 4DIV and was removed 48 hrs later. Prior to treatment, the cultures were fed every two days. During the treatment period, the media bathing both the experimental and control cultures was replaced every 12 hours. The cultured retinas were collected every 12 hours from 4DIV to 7DIV for analyses of iodopsin expression. In the experiments designed to examine AANAT mRNA levels, the cultured retinas were collected at ZT0 and ZT15 from 4DIV to 7DIV. The retinal cultures were snap frozen in liquid nitrogen, and stored at -75°C until further processing. In all experiments, retinas collected during the dark period were processed under a low intensity safe red light (15 Watt bulb, Kodak #2 filter).

12L:12D followed by constant darkness:

Retinal explant cultures were maintained under 12L:12D lighting conditions for 5DIV. ACO was added to the media either at ZT12 on 4DIV or at ZT0 on 5DIV and was removed 24 or 12 hours later, respectively. The cultures were kept in constant dark conditions following treatment and were collected every 12 hours from 4DIV to 7DIV.

RNA Analysis

Total RNA was extracted from the retinal explant cultures using an RNeasy kit (Qiagen, Valencia, CA, USA). The RNA samples, 2 µg total RNA per sample, were loaded onto a Magnacharge nylon membrane (MSI, Westburough, MA) and analyzed using a slot blot format as described previously (Zhang et al., 2003). Blots were prepared in duplicate and were probed consecutively with radio-labeled cDNA probes specific for
iodopsin, and/or guanylate cyclase activating protein 1 (GCAP1) and/or AANAT, and 18S rRNA. The iodopsin, GCAP1, and 18S rRNA cDNA fragments used for probes have been described previously (Semple-Rowland and van der, 1992; Zhang et al., 2003). The AANAT probe was generated using a 1.4 kb BamHI fragment of cDNA clone #9A (kindly provided by David Klein) that contained the entire AANAT open reading frame. The $^{32}$P-labeled cDNA probes were generated using a Stripeasy DNA Kit (Ambion the RNA company, Austin, TX, USA). The amount of probe hybridized to the blots was measured using a BioRad Molecular Imager FX system. Levels of iodopsin, GCAP1, and AANAT transcript present in individual samples were normalized to the amount of 18S rRNA present in that same sample. These values were then expressed relative to the mean normalized value for the corresponding gene on each blot. Data were analyzed using two-way ANOVA (SigmaStat).

Results

Iodopsin and AANAT Transcript Rhythms in Untreated Explant Cultures:

Previous studies have shown that iodopsin transcript levels in dispersed embryonic retinal cultures and in post-hatch chicken retinas exhibit a robust rhythm with minimum levels at ZT0 and maximum levels at ZT12 under cyclic light conditions that is maintained in the absence of light (Pierce et al., 1993; Larkin et al., 1999; Larkin and Semple-Rowland, 2001). To examine the temporal regulation of iodopsin transcript rhythms in the retinal explant cultures, iodopsin mRNA levels were examined every 6 hours on 6DIV. The results revealed that the temporal dynamics of the iodopsin rhythm observed in our explant retina cultures were very similar to those previously observed in dispersed retinal cultures and in post-hatch chicken retinas (Fig 3-1A, B). In the retinal explant cultures maintained under 12L:12D conditions, analyses of iodopsin mRNA
levels in selected cultures at ZT0 and ZT12 over a 7-day culture period revealed well-defined rhythms that persisted under constant darkness for at least 48 hrs (Fig 3-1C, D). These results show that functional circadian oscillators regulate iodopsin mRNA rhythms in our explants and that these rhythms can be monitored by measuring transcript levels at ZT0 and ZT12.

Figure 3-1. Iodopsin mRNA rhythms in retinal explant cultures. (A) Diurnal iodopsin transcript rhythms every 6 hours observed in the retinal explant cultures on 6DIV. (B) Data in panel A are shown in line graph. (C) Iodopsin transcript rhythms observed in the cultures maintained in 12L:12D conditions for 5DIV and were then transferred to constant darkness on 6 DIV. Relative iodopsin mRNA levels were analyzed every 12 hours. (D) Comparison of iodopsin transcript rhythms observed in the cultures maintained under 12L:12D conditions for 7DIV (black lines) to the data shown in panel C (red lines). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. In panel D, plotted data and light cycles are matched by color. In panels A and C, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels B and D, only the mean values are plotted for each group.
Rhythmic changes in the levels of AANAT mRNA in chicken retina have also been shown to be driven by endogenous retinal circadian oscillators (Bernard et al., 1999; Haque et al., 2002).

Figure 3-2. AANAT mRNA rhythms in retinal explant cultures. (A) Diurnal AANAT transcript rhythms every 3 hours observed in the retinal explant cultures on 6DIV. (B) Data in panel A are shown in line graph. (C) AANAT transcript rhythms observed in the cultures maintained in 12L:12D conditions for 5DIV and were then transferred to constant darkness on 6 DIV. Relative AANAT mRNA levels were analyzed every 12 hours. (D) Comparison of AANAT transcript rhythms observed in the cultures maintained under 12L:12D conditions for 7DIV (black lines) to the data shown in panel C (red lines). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. In panel D, plotted data and light cycles are matched by color. In panels A and C, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels B and D, only the mean values are plotted for each group.
Analyses of AANAT transcripts every 3 hours in our explant cultures on 6DIV that had been maintained under 12L:12D conditions showed that AANAT mRNA levels are lowest at ZT0 and reach peak levels at ZT15 (Fig 3-2A, B). In the retinal explant cultures maintained under 12L:12D conditions, analyses of AANAT mRNA levels in selected cultures at ZT0 and ZT15 over a 7-day culture period revealed that diurnal rhythms with an amplitude of 1.8-fold in the levels of AANAT mRNA were first detected on 5DIV (Fig 3-2D) and continued in constant dark conditions (Fig 3-2C, D). These results show that functional circadian oscillators in our explants regulate AANAT mRNA rhythms and that these rhythms can be monitored by measuring transcript levels at ZT0 and ZT15.

Iodopsin Transcript Levels in Explant Cultures Treated With ACO For 48 Or 24 hrs Maintained Under A 12L:12D Cycle

ACO, a derivative of glycyrrhetinic acid that has been shown to reversibly block gap junction communication in vertebrate retina (Pottek et al., 2003; Kamermans et al., 2001), was added to the media of selected cultures at the beginning of the dark period (ZT12) on 4DIV. Analyses of iodopsin mRNA levels at ZT0 on 5DIV, 12 hours after addition of ACO, revealed that the iodopsin mRNA levels in these cultures were similar to those that had been observed in untreated cultures (Fig 3-3A, D). The affects of ACO on iodopsin mRNA levels were first evident in cultures examined at ZT12 on 5DIV. In untreated cultures, iodopsin mRNA levels increase over the course of each 12hr light period, peaking at ZT12 (Fig.3-3D) (Pierce et al., 1993; Larkin et al., 1999). Treatment of cultures with ACO prevented this increase. In treated cultures, the amount of iodopsin mRNA measured at ZT12 on 5DIV was the same as that measured at ZT0 on 5DIV and remained low throughout the 48 hr treatment period. Removal of ACO at ZT12 on 6DIV produced a rapid, two-fold increase in the amount of iodopsin transcript in the cultures.
examined at ZT0 on 7DIV. Surprisingly, this increase occurred over the course of a 12-hour dark period, a time during which iodopsin transcript levels normally fall to their lowest values. The amount of iodopsin mRNA in cultures examined at ZT12 on 7DIV did not increase significantly over the values measured at ZT0 on 7DIV.

Figure 3-3. Iodopsin expression in explant cultures treated with ACO for 48 and 24hrs
All the explant cultures were maintained under 12L:12D conditions in this series of experiments. (A) Cultures were incubated with 100 µM ACO for 48 hrs. ACO was added to the cultures at ZT12 on 4DIV and was removed at ZT12 on 6DIV. (B) Cultures were incubated with 100 µM ACO for 24 hrs. ACO was added to the cultures at ZT12 on 4DIV and was removed at ZT12 on 5DIV. (C) Cultures were incubated with 100µM ACO for 24 hrs, ACO was added to the cultures at ZT0 on 5DIV and was removed at ZT0 on 6DIV. In panels A to C, retinal explant cultures were analyzed at ZT0 (white bars) and at ZT12 (black bars). (D) Comparison of the data shown in panel A (red line) and the iodopsin transcript rhythms observed in the untreated cultures (black line). (E) Comparison of data shown in panel B (red line) and the iodopsin transcript rhythms observed in the untreated cultures (black line). (F) Comparison of data shown in panel C (red line) and the iodopsin transcript rhythms observed in the untreated cultures (black line). The 12-hour light and
dark periods are indicated below each panel using white and black bars, respectively. The dashed lines in each panel indicate the period of blocker treatment. In panels A – C, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels D – F, only the mean values are plotted for each group.

The rapid suppression of iodopsin mRNA levels by ACO was unexpected. To determine if iodopsin mRNA rhythms would reappear following removal of the blocker and to further examine the temporal characteristics of this phenomenon, cultures were treated with ACO for a shorter period of time. ACO was added to cultures at ZT12 on 4DIV or at ZT0 on 5DIV. In both experiments, ACO was removed 24 hours later. In both paradigms, the iodopsin mRNA levels observed 12 hours following addition ACO were comparable to the low levels observed at ZT0 in control cultures. Importantly, within 24 hours of the removal of the blocker, iodopsin transcript rhythms reappeared in the treated cultures that were indistinguishable from those observed in control cultures (Fig. 3-3E, F). Moreover, introduction of ACO at ZT0 on 5DIV completely blocked the increase in iodopsin mRNA that normally occurred over the course of the 12-hour light period in control cultures (Fig 3-3C, F). Together, these data show that the effect of ACO on iodopsin mRNA levels is reversible and occurs within 12 hours of application.

Iodopsin Transcript Levels in Explant Cultures Treated With ACO For 24hr Or 12hr Followed By Constant Darkness

To determine if there was any evidence that ACO altered the function or phase of the circadian oscillators that drive iodopsin transcript rhythms, iodopsin mRNA levels were measured in cultures that were treated with ACO and were then maintained in constant darkness. As expected from our previous experiments, addition of ACO at the ZT12 on 4DIV or at ZT0 on 5DIV reduced iodopsin transcript levels (Fig 3-4A, B). Importantly, following removal of the blocker, iodopsin mRNA rhythms re-emerged in
the cultures in the absence of the 12L:12D cycle that were congruent with those observed in the untreated cultures (Fig 3-4C, D).

Figure 3-4. Iodopsin expression in explant cultures treated with ACO followed by constant darkness (A) ACO (100 µm) was added to the cultures at ZT12 on 4DIV and was removed at ZT12 on 5DIV. (B) ACO (100 µm) was added to the cultures at ZT0 on 5DIV and was removed at ZT12 on 5DIV. In panels A and B, retinal explant cultures were analyzed at ZT0 (white bars) and at ZT12 (black bars). (C) Comparison of the data shown in panel A (red line) and the iodopsin transcript rhythms observed in the untreated cultures (black line). (D) Comparison of data shown in panel B (red line) and the iodopsin transcript rhythms observed in the untreated cultures (black line). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. The dashed lines in each panel indicate the period of blocker treatment. In panels A and B, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels C and D, only the mean values are plotted for each group.
These data suggest that short-term treatment of cultures with ACO does not alter the function or temporal characteristics of the circadian oscillators that drive iodopsin mRNA rhythms in chicken retina.

**Effects of ACO Treatment On AANAT and GCAP1 Transcript Levels**

In this series of experiments, we examined the specificity of the effect of ACO on gene transcript levels by examining the affects of ACO on transcript levels of AANAT and GCAP1, genes also expressed in chicken retinal photoreceptors (Chong et al., 2000; Semple-Rowland et al., 1999).

![Figure 3-5. AANAT expression in explant cultures treated with ACO for 24hrs. The explant cultures were maintained under 12L:12D conditions. (A) ACO (100 µm) was added to the cultures at ZT15 on 4DIV and removed at ZT15 on 5DIV. Relative AANAT mRNA levels at ZT0 (white bars) and ZT15 (black bars) in the ACO treated cultures were shown. (B) The AANAT expression in ACO treated cultures (red line) was compared to that observed in untreated cultures (black lines). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. The dashed lines in each panel indicate the period of blocker treatment. In panel A, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panel B, only the mean values are plotted for each group.](image)

Treatment of the cultures with ACO beginning at ZT15 on 4DIV produced a significant suppression of AANAT mRNA levels in cultures examined at ZT15 on 5DIV, the values of which were similar to those observed at ZT0 in untreated cultures (Fig. 3-
5A, B) Within 24 hours of removal of the blocker, the AANAT rhythms paralleled to those observed in the untreated cultures.
Figure 3-6. GCAP-1 expression in explant cultures treated with ACO for 48 or 24 hrs and maintained under 12L:12D conditions. (A) GCAP1 expression in the untreated cultures from 4 to 7DIV. (B) The data in panel A are shown in line graph. (C) ACO (100µm) was added to the cultures at ZT12 on 4DIV and removed at ZT12 on 6DIV. (D) The data in panel C are shown in line graph. (E) ACO (100 µm) was added to the cultures at ZT12 on 4DIV and removed at ZT12 on 5DIV. (F) The data in panel E are shown in line graph. The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. The dashed lines indicate the period of ACO treatment. In panels A, C and E, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panels B, D and F, only the mean values are plotted for each group.

In explant cultures, GCAP1 mRNA levels gradually increase over the culture period, reaching relatively stable levels by 5DIV (Fig. 3-6A, B). Incubation of cultures with ACO for either 24 (Fig. 3-6E, F) or 48 hours (Fig. 3-6C, D) did not produce any significant changes in GCAP1 mRNA levels in the cultures.

Together, the result of our analyses of AANAT and GCAP1 mRNA levels in explants treated with ACO indicate that ACO does not produce a generalized reduction in mRNA levels in retinal photoreceptors and suggest that the action of ACO on transcript levels may be restricted to genes that are regulated by retinal oscillators.
Iodopsin Transcript Levels in Explant Cultures Treated With 18-βGA For 48 Or 24 hrs Maintained Under A 12L:12D Cycle

A 18β-GA 48hrs

B 18β-GA 24hrs

C 18β-GA 48hrs

D 18β-GA 24hrs

E 0.1% DMSO 48hrs

F 0.1% DMSO 24hrs
Treatment of explant cultures with the glycyrrhetinic acid derivative, 18-βGA, also significantly reduced iodopsin mRNA levels in the explant cultures maintained under cyclic light conditions. Treatment of cultures with 18-βGA for either 48 or 24 hours reduced iodopsin mRNA levels to values significantly below the trough values observed in untreated cultures at ZT0 (Fig. 3-7A, B, C, D). Removal of the blocker produced a rapid 3 to 3.5-fold increase in iodopsin mRNA levels over the course of the first 12 hours following removal of the blocker. In cultures treated with the blocker for 24 hours, iodopsin mRNA rhythms similar to those observed in untreated cultures were re-established within 24 hours of removal of the blocker (Fig. 3-7D). Unlike cultures treated with 18-βGA for 24 hours, the levels of iodopsin mRNA measured in cultures 24 hours following 48-hour treatment with 18-βGA were not rhythmic and highly variable (Fig. 3-6B). This result is reminiscent of the desynchronized rhythms observed in SCN following...
treatment with octanol and halothane (Shinohara et al., 2000) and suggests that longer exposures of cultures to 18-βGA might affect the coordination of the retinal oscillators that drive iodopsin rhythm. Treatment of cultures with 0.1% DMSO, the amount equivalent to that applied to the cultures treated with 100 μM 18-βGA, did not alter iodopsin mRNA rhythms in the retinal cultures (Fig. 3-6C, D).

Figure 3-8. Iodopsin expression in explant cultures treated with GA for 48hrs. The explant cultures were maintained under 12L:12D conditions in this experiment. (A) GA (100 μm) was added to the cultures at ZT12 on 4DIV and removed at ZT12 on 6DIV. Relative iodopsin mRNA levels at ZT0 (white bars) and ZT12 (black bars) in the GA treated cultures were shown. (B) The iodopsin expression in GA treated cultures (red line) was compared to that observed in untreated cultures (black lines). The 12-hour light and dark periods are indicated below each panel using white and black bars, respectively. The dashed lines in each panel indicate the period of GA treatment. In panel A, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panel B, only the mean values are plotted for each group.

In a second control experiment, we examined the specificity of the actions of ACO and 18-βGA on the cultures. Treatment of cultures for 48 hrs with 100 μM GA, a chemically related inactive compound, did not alter iodopsin mRNA rhythms in the cultures (Fig. 3-8A, B). This result suggests that the actions of ACO and 18-βGA on iodopsin transcript levels are specific to these compounds.
Discussion

The results of these experiments show that iodopsin and AANAT mRNA levels exhibit coordinated rhythms in cultures maintained under cyclic light conditions and that these rhythms are maintained under constant darkness. In contrast to iodopsin and AANAT, GCAP1 mRNA levels in cultured retinas gradually increase over the course of the culture period and do not exhibit a discernible rhythm. The presence of the iodopsin and AANAT mRNA rhythms in cultures maintained in constant darkness data confirm that circadian oscillators contribute to the regulation of the transcript levels of these genes in our culture paradigm. Addition of 100 μM ACO to the cultures rapidly reduced the amount of iodopsin and AANAT mRNA in the cultures, an effect that was maintained as long as the blocker was present. The chemically related gap junction blocker, 18-βGA, produced similar effects on iodopsin transcript rhythms in the retinal explant cultures. Within 24 hours of removal of ACO, the iodopsin and AANAT transcript rhythms reappeared in the cultures. ACO did not alter GCAP1 mRNA levels in the cultured retinas, a result that suggests that the actions of this blocker cannot be attributed to general transcription suppression.

The hypothesis that we set out to test was that gap junctions play a role in maintaining coordinated iodopsin and AANAT transcript rhythms in retina. We expected that treatment of retina cultures with gap junction blockers would result in a desynchronization of the retinal oscillators that drive these rhythms and a subsequent loss of the iodopsin and AANAT transcript rhythms. The transcript levels of these two genes would assume the average of the peak and trough values of their rhythms in the presence of the blockers. However, the effects of the blockers were unexpected in light of the
results of similar studies that were conducted in SCN slice cultures. The effects of ACO and 18-βGA on iodopsin transcript rhythms in retina exhibited two major differences from the effects of two other gap junction blockers, octanol and halothane, on rhythmic AVP and VIP secretion in SCN slice cultures. First, the treatment of ACO or 18-βGA suppressed iodopsin transcript levels to the trough or the values significantly lower than the trough of its rhythms observed in the untreated cultures. Hence, unlike the effects of octanol and halothane on AVP and VIP release in SCN cultures (Shinohara et al., 2000), the total amount of iodopsin mRNA generated in retinal photoreceptors over the treatment period is significantly less than that in the untreated cultures (Fig 3-3, 3-4, 3-7C, D). Second, the reduction of iodopsin transcript levels in response to the addition of the blockers occurs within 12 hours (Fig. 3-3C, F). This time period is much shorter than that observed in the study of SCN slice cultures, the exposure of which to octanol or halothane for 42 hours had no observable effects on the circadian rhythms of peptide secretion. The loss of the peptide release circadian rhythms was not observed until the SCN cultures were treated with the blockers for 7 days (Shinohara et al., 2000). Therefore, the effects of the two gap junction blockers on iodopsin transcript rhythms cannot be attributed to desynchronization of the circadian oscillators, which would be expected to take much longer for the subtle phase angle differences among individual oscillators to become significant enough to affect the overt ensemble gene expression rhythm.

The reduction in the levels of iodopsin and AANAT transcripts in the presence of the blockers could result from cytotoxicity of the blockers, disruption of gene transcription or elevation of transcript degradation.
Several of our experimental observations argue strongly against the possibility that the suppressing effects of ACO and 18-βGA on iodopsin and AANAT transcript levels were due to cytotoxicity of the two gap junction blockers. First, transcription of the photoreceptor-specific gene GCAP1 was not suppressed by ACO. Second, the amount of iodopsin and AANAT transcript in the cultures rapidly recovered to the intermediate or peak levels 12 hours following the removal of the blockers. Finally, circadian rhythms of iodopsin transcript re-appeared within 24 hours after the block was removed, suggesting that retinal circadian oscillators are functional and the explant cultures are physiologically healthy during and after the treatment of ACO. Together, these observations do not support the thesis that the reduction in iodopsin and AANAT mRNA levels that we observed is due to massive cell death.

It is possible that treatment of the cultures with the gap junction blockers disrupted gene transcription. This could occur if the gap junction blockers disrupted oscillator function or if they disrupted the coupling mechanism that normally allows the oscillator to drive transcription of these genes. Disruption of circadian oscillator function is unlikely because recovery of iodopsin rhythms following removal of the blockers was rapid (Fig 3-3). If the blockers altered the function of the oscillators driving iodopsin and AANAT transcript rhythms, we would have observed slower recovery of the rhythms following removal of the blockers. More definitively, iodopsin transcript rhythms were maintained in constant darkness after either 24 or 12hr treatment of ACO (Fig 3-4), suggesting that ACO did not disrupt the ability of the circadian oscillators to regulate iodopsin transcription. On the other hand, ACO may uncouple the functional circadian oscillators and iodopsin or AANAT transcription. A recent study of chick dispersed cell
cultures has shown that the Ca\(^{2+}\) influx stimulates the formation of cAMP, which in turn couples the circadian oscillators and the rhythms of AANAT enzyme activity that bear the similar temporal characteristics to AANAT transcript rhythms (Ivanova and Iuvone, 2003). How does ACO affect Ca\(^{2+}\) influx to the photoreceptor cells? It has been shown that blockade of gap junction channels by 100µM ACO, reduced Ca\(^{2+}\) influx to photoreceptor cells from horizontal cells (Kamermans et al., 2001). Moreover, 100µM ACO can directly reduce the voltage-gated Ca\(^{2+}\) channel current by 37% in isolated cones, and inhibit the Ca\(^{2+}\) influx by 57% in retinal slice preparations (Vessey et al., 2004). Therefore, it is possible that the reduction of Ca\(^{2+}\) influx in presence of ACO results in the lack of stimulation of cAMP, which uncouples the circadian oscillators and the transcription of the output genes, such as AANAT and iodopsin. The exact molecular mechanisms, however, through which the reduced levels of Ca\(^{2+}\) and/or cAMP suppress iodopsin and AANAT transcription, need to be further investigated.

Based the current data, the possibility that the gap junction blockers increase the transcript degradation cannot be ruled out. It has been shown that a rapid turnover protein increases the degradation of AANAT transcript (Greve et al., 1999). Thus, it is also possible that the gap junction blockers enhance the activity of this protein, and lead to the increased degradation of AANAT and/or iodopsin transcript.
A Real-Time Monitoring Culture System For Circadian-Regulated Gene Expression

The major approaches used in this dissertation for studying the transcription of the genes that are expressed in retinal photoreceptors and regulated by circadian oscillators involve collecting significant number of retinal samples at each time point, homogenizing separate populations of the retinal samples, and measuring steady-state RNA levels through standard RNA assays. These typical methods are straightforward and have helped me observe interesting phenomena to prove the hypothesis.

However, these methods have limitations for studying the dynamics of clock-regulated gene transcription. First, sample collections every 12hrs were performed in most experiments studying iodopsin and AANAT mRNA rhythms, the time-resolution of the experiments might not be high enough to reveal the complete temporal characteristics of the clock-regulated gene expression. Especially when the explan cultures were subjected to the changes of light cycle or the treatment of gap junction blockers or both, subtle changes in the phase of iodopsin or AANAT rhythms or the immediate transcriptional response of either genes may not be observed in the two-point analyses every light-dark or circadian cycle. Although the problem can be partly overcome by adding more time points during the period of light changes or blocker treatment, this solution makes the typical methods less efficient and more labor-intensive and time-consuming. Moreover, these approaches exclude the possibility to monitor the dynamics of clock-regulated gene expression in individual retinal explant cultures. The iodopsin
transcript level shown at each time point in the end results is the ensemble average of iodopsin mRNA levels from the population of retinal cultures collected at that time point. The average value can reliably reflect iodopsin expression levels in individual cultures only if are the cultures in a certain experiment synchronized, which is the case in the experiments in which the cultures are exposed to light. Nonetheless, in the constant dark experiments, when the lights are turned off, the iodopsin mRNA rhythms in individual cultures begin to desynchronize. Therefore, taking the ensemble average of the desynchronized rhythms leads to the apparent reduction in amplitude across the retinal samples collected during the darkness. This could account for the damped amplitude of iodopsin transcript rhythms observed in the constant dark experiments (Fig2-5C, E).

Finally, since the typical approaches measure the steady-state mRNA levels, it is difficult to discern if the manipulations act on transcriptional level or on posttranscriptional level. Although it has been shown that circadian oscillators regulate iodopsin expression at the transcriptional level in retinal cultures maintained under different lighting conditions (Pierce et al. 1993), the sites of actions of the gap junction blockers need further investigation.

The monitoring of temporal characteristics of iodopsin transcription could be greatly improved and simplified by establishing a retinal culture system that carries a transgene of iodopsin promoter linked to firefly luciferase coding sequence (Iod:luc). The iodopsin promoter can be isolated from chicken genomic DNA library that is available in my current lab. The luciferase reporter has been used successfully for monitoring the transcription of Per1 with high time-resolution in nervous system of both transgenic Drosophila (Brandes et al. 1996; Stanewsky et al. 1997) and transgenic rats (Abe et al.
2002; Yamazaki et al. 2000). The short half-life (about 2 hrs) of luciferase in vertebrate
and the automated quantification system for measuring luciferase activity makes it an
excellent reporter for real-time monitoring circadian-regulated iodopsin transcription in
chicken retina. The modified lentiviral vector can be used as a novel tool to generate
“transgenic” chicken retina. The lentiviral vector has been shown to be able to transduce
both retinal progenitor cells and terminally differentiated cells in chicken embryo with
high efficiency (> 80% cells transduced) (Coleman et al. 2002; Coleman et al. 2003). The
packaged lentivirus carrying the Iod:luc transgene will be injected into the neural tubes of
chicken embryos at stage 10 to 12 (~embryonic day 2; E2). Following injection, the eggs
will be incubated under 12L:12D conditions until E9. The embryonic retinas will be
dissected on E9 and cultured with the media supplemented with luciferin under 12L:12D
conditions. By continuously measuring the bioluminescence emitted from the retinal
explant cultures, changes of iodopsin expression at the transcriptional level in response to
the manipulations, such as light and pharmacological agents, can be monitored with high
resolution from individual samples over one or two circadian cycles.

**Possible Mechanisms of Light Entrainment in Embryonic Chicken Retinas**

The phase of iodopsin transcript rhythms was reversed following the exposure of
the retinal explant cultures to the reversed light cycle (Fig2-6A, B). This observation
suggests that the circadian oscillators driving iodopsin mRNA rhythms can be entrained
to the environmental light changes. Then what are the possible mechanisms underlying
the light entrainment in the retinal explant cultures?

The recent remarkable progress in the mechanisms responsible for light
entrainment of behavioral rhythms in mammals provides clues for this question. A subset
of retinal ganglion cells that express a novel photopigment, melanopsin, has been
discovered to be intrinsically photoresponsive (ipRGCs). The dendrites of ipRGCs form extensive reticular networks to maximally detect light irradiance (Hattar et al. 2002; Provencio et al. 2002). The ipRGCs also arborize in the inner plexiform layer, suggesting that these cells receive synaptic input from the classical rod and cone photoreceptors (Belenky et al. 2003; Provencio et al. 2002). It has been show that either rod and cone image-forming system or ipRGCs system is sufficient to detect and transduce photic information through retinohypothalamic tract to the SCN, but neither system is necessary for the light entrainment process. Therefore, classical photoreceptors and ipRGCs are functionally redundant for light entrainment of the behavioral rhythms in mammals (Van Gelder 2003).

However, it is notable that by the time the reversal of the light cycle is performed on 6DIV (Fig2-6A, B), the ganglion cells in the retinal explant cultures have degenerated, and are not detectable by cresyl violet staining (Fig2-1). The observations indicate that retinal ganglion cells are not necessary for the light entrainment of the circadian oscillators driving iodopsin transcript rhythms in the explant cultures. On the other hand, circadian oscillator functions in the explant cultures prepared from GUCY1*B embryonic chicken retinas were also characterized. GUCY1*B chicken carries a null mutation in Guanylate Cyclase–1 gene, hence the classical phototransduction pathway is disabled in the retinal photoreceptors. The morphology of *B retinal explant cultures is indistinguishable from that of the cultures prepared from White Leghorn chicken retinas. The ganglion cells also degenerate by 5DIV (data not shown). Interestingly, the iodopsin mRNA rhythms in *B retinal explants exhibit similar dynamics of phase reversal to those observed in Leghorn explant cultures (Fig 4-1). These data suggest that both ganglion
cells and classical phototransduction pathway are not required to entrain the circadian oscillators driving iodopsin transcription in the retinal explant cultures.

Another candidate that might be important for entraining circadian oscillators driving iodopsin rhythms in the retinal explant cultures is Cryptochromes (Crys). Crys are flavin-based photopigments that are first identified as members of photolyase family in plants (Sancar 2000). Although lack of photolyase activity in animals, Crys have been shown to serve as circadian photopigments for light entrainment in both Drosophila (Sancar 2000) and zebrafish (Cermakian et al. 2002). More directly, both chicken Cry1 (Haque et al. 2002) and Cry2 (Bailey et al. 2002) highly express in retinal photoreceptor cells. The dual regulation of chicken Cry1 transcription by circadian oscillators and light

Figure 4-1. Iodopsin transcript rhythms in GUCY1*B and White Leghorn chicken retinal explant cultures maintained under 12L:12D followed by reversal of the light cycle. The explant cultures were prepared from E9 *B and leghorn chicken embryos. The cultures were maintained under 12L:12D until 6 DIV when they were transferred to a 12D:12L reversed cycle. The iodopsin mRNA rhythms from *B explant cultures were analyzed every 12 hours. The iodopsin transcript rhythms from *B retinal explant cultures (red line) were compared to those from Leghorn explant cultures (black line). The 12-hour light and dark periods are indicated below using white and black bars, respectively. In panel A, the group means ± SE at each time point are shown and the number of retinas in each group is indicated on the bars. In panel B, only the mean values are plotted for each group.
suggests its involvement in the function of circadian oscillator and/or circadian photoreception in the photoreceptors of chicken retina (Haque et al. 2002).
REFERENCE LIST


Provencio I, Rollag MD, Castrucci AM (2002) Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. Nature 415: 493.


BIOGRAPHICAL SKETCH

Yan Zhang was born in Tianjin, the third largest city in China, on Dec 28th, 1972. After receiving the award for the best student in the primary school in Tianjin city for three consecutive years, he entered Nankai high school, one of the best high schools in China. During three years of his junior high school, he ranked first of 265 students in 8 out of 12 comprehensive exams. Consequently, he was admitted to Nankai senior high school with the exemption of the final exam. After another three years of endeavors in that highly competitive environment, he chose to enter Tianjin Medical University to study clinical medicine with the intention of his parents and with his own hope of a better future, although he loved and was good at mathematics at the time of graduating from high school.

He did not understand the value of clinical medicine until the last two years of medical training when he did probation in the Department of Internal Medicine and an internship in the Department of Surgery. It was during those two years that he truly realized that a good doctor could relieve suffering and save the lives of patients, thus gaining respect from people. However, at the time he graduated from medical school in 1996, molecular biology had just become the hottest area in China. Additionally, he thought that because he was still very young, his education should not end at the age of 23. He then chose the National Key Laboratory of Hormone and Brain Development in China to pursue the MS degree in molecular endocrinology. During three years of work in the laboratory, he participated in the purification of glutamic acid decarboxylase from
human brain and the development of an ELISA system for early detection of Type I diabetes using the purified protein as antigen. His thesis, “Cloning and Expression of Human Somatostatin Gene in *E. coli*,” summarized his work in molecular biology. After receiving his MS degree in August 1999, he came to the University of Florida, joined the Department of Neuroscience in the interdisciplinary program in the College of Medicine, and began his PhD study under the supervision of Dr. Susan Semple-Rowland. His research during the Ph.D. study is reflected in this dissertation.