REVIEW

Retinal Circadian Clocks and Control of Retinal Physiology

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Abstract Retinas of all classes of vertebrates contain endogenous circadian clocks that control many aspects of retinal physiology, including retinal sensitivity to light, neurohormone synthesis, and cellular events such as rod disk shedding, intracellular signaling pathways, and gene expression. The vertebrate retina is an example of a "peripheral" oscillator that is particularly amenable to study because this tissue is well characterized, the relationships between the various cell types are extensively studied, and many local clock-controlled rhythms are known. Although the existence of a photoreceptor clock is well established in several species, emerging data are consistent with multiple or dual oscillators within the retina that interact to control local physiology. A prominent example is the antiphasic regulation of melatonin and dopamine in photoreceptors and inner retina, respectively. This review focuses on the similarities and differences in the molecular mechanisms of the retinal versus the SCN oscillators, as well as on the expression of core components of the circadian clockwork in retina. Finally, the interactions between the retinal clock(s) and the master clock in the SCN are examined.

Key words circadian clocks, retinal physiology, gene expression, SCN, retinal clock

It has been more than 20 years since the original demonstration that *Xenopus* retinas contained an endogenous circadian clock that continued to oscillate in constant conditions in a culture dish (Besharse and Iuvone, 1983). This finding substantiated several other studies on intact animals, suggesting local, ocular control of retinal circadian rhythms (reviewed in Besharse, 1982), and led to the idea that this local clock controls many aspects of retinal physiology. Although it is now well known that vertebrates have many clocks distributed throughout their bodies (e.g., see Balsalobre et al., 1998; Whitmore et al., 1998; Yamazaki et al., 2000; Yagita et al., 2001), the functions of "peripheral" clocks are not well understood. However, in the retina, a wealth of data exists on cellular, molecular, and system-level events that are regulated by local circadian clocks. Many of these daily rhythms are thought to allow the retina to anticipate the more than 6–log unit change in illumination between day and night.

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JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 19 No. 2, April 2004 91-102
DOI: 10.1177/0748730404263002

A MULTIPLICITY OF RETINAL CIRCADIAN RHYTHMS

A survey of the many rhythms that have been described in vertebrate retinas is beyond the scope of this review. Interested readers are referred to references in several earlier reviews to this rich body of physiological data (Besharse, 1982; Besharse et al., 1988; Cahill and Besharse, 1995; Anderson and Green, 2000). Recently, studies of retinal circadian rhythms have emphasized physiology, as revealed by the electroretinogram (ERG) (Manglapus et al., 1998; McGoogan and Cassone, 1999); melatonin and dopamine content (Doyle et al., 2002a; Doyle et al., 2002b); pH (Dmitriev and Mangel, 2001); phototransduction events, including iodopsin expression (Pierce et al., 1993) and cGMP-gated channel sensitivity (Ko et al., 2001, 2003); and gene expression (Pierce et al., 1993; Green and Besharse, 1994; Green and Besharse, 1995a, 1995b, 1996a, 1996b; Zhuang et al., 2000; Bailey et al., 2002; Chong et al., 2003). The principal conclusion to be drawn from the physiological data is that circadian physiology is critical to retinal function and that understanding the underlying mechanisms is of fundamental importance.

MOLECULAR NATURE OF THE RETINAL CLOCK

In rodents, a "master" clock that drives locomotor rhythms resides in the SCN, and the molecular mechanism of this clock has been studied extensively (reviewed in Dunlap, 1999; Reppert and Weaver, 2001, 2002; Takahashi et al., 2001). The mammalian clock comprises 2 interlocking feedback loops, 1 negative and 1 positive. Two bHLH-PAS transcription factors, CLOCK and BMAL1 (also called MOP3), form heterodimers and bind to specific E-box elements in the promoters of *Period* (*Per*) *1* and *2*, *Cryptochrome* (*Cry*) *1* and *2*, and *Rev-erb*α genes, resulting in transcriptional activation. As the PER proteins accumulate, they form complexes with the CRY proteins and with Casein Kinase I ε/δ (CKIε/δ) and are phosphorylated. These complexes translocate into the nucleus and interact with the CLOCK/BMAL1 complex, resulting in repression of their transactivation activity—thereby forming the negative feedback loop. The positive loop is composed of the REV-ERBα protein, which increases following CLOCK/BMAL1-induced transcription and translocates into the nucleus to bind an ROR element in the *Bmal1* promoter. Since REV-ERBα is a repressor, this binding causes *Bmal1* messenger RNA (mRNA) and, subsequently, BMAL1 protein levels to fall. Once the CRY protein complex has repressed CLOCK/BMAL1 transcription, the REV-ERBα levels fall (the repressor is inhibited), the *Bmal1* transcription is activated, and the BMAL1 levels begin to rise again.

In parallel with the recent development of the molecular underpinnings of the core circadian oscillator in the SCN (above), it became apparent that molecular components of the SCN clock were expressed in a rhythmic pattern in multiple tissues throughout the body. Furthermore, it was found that sustained circadian oscillations in vitro—formerly measured only for the SCN (Green and Gillette, 1982; Groos and Hendriks, 1982; Earnest and Sladek, 1986), the pineal gland of nonmammalian vertebrates (Deguchi, 1979; Falcon et al., 1989; Cahill, 1996), and retina (Besharse and Iuvone, 1983; Tosini and Menaker, 1996)—were a feature of multiple tissues throughout the body (Balsalobre et al., 1998; Whitmore et al., 1998; Yamazaki et al., 2000; Yagita et al., 2001) as well as multiple brain regions outside the SCN (Abe et al., 2002). Currently, the SCN is referred to as a master clock, whereas independent clocks outside the SCN are called peripheral oscillators. Peripheral clocks generally exhibit sustained oscillations of clock gene expression that are out of phase with the oscillations of the same gene in the SCN clock, and some in vitro rhythms damp rapidly after isolation.

Initial evidence indicating that retinal clocks operate in much the same manner as the SCN at the molecular level came from studies of the *tau* mutant hamsters. These animals exhibit locomotor activity rhythms with dramatically shortened periods resulting from a point mutation in the *ckI*ε gene that results in an enzyme with altered activity and deficiency in its ability to phosphorylate PER (Lowrey et al., 2000). Examination of melatonin rhythms from cultured *tau* mutant hamster retinas and disk-shedding rhythms from intact *tau* mutant hamsters revealed that these retinal rhythms also exhibited short periods, suggesting that they use the same basic molecular mechanism as the SCN clock (Grace et al., 1996; Tosini and Menaker, 1996, 1998b). Recent studies showing that the known "clock" genes identified in the SCN are also expressed in retinas from all species examined to date have provided further support for this prediction (Gekakis et al., 1998; Zylka et al., 1998; Namihira et al., 1999; Kuhlman et al., 2000; Sakamoto et al., 2000;

Sancar, 2000; Yoshimura et al., 2000; Zhu et al., 2000; Zhuang et al., 2000; Namihira et al., 2001; Zhu and Green, 2001; Bailey et al., 2002; Haque et al., 2002; Chong et al., 2003; Witkovsky et al., 2003). Furthermore, a critical role of the CLOCK protein in the *Xenopus* retinal clock mechanism has been directly tested using a "dominant-negative" form of CLOCK protein (Hayasaka et al., 2002). The dominant negative was predicted to prevent *Per* and *Cry* transcription and, therefore, stop clock function based on the model of the SCN clock. Overexpression of this mutant specifically in the retinal photoreceptor cells resulted in a dose-dependent abolition of melatonin rhythms, without affecting overall levels of melatonin production (Hayasaka et al., 2002). These results suggest that a similar clock mechanism must operate in the retina, at least with regard to CLOCK function. These findings that the retinal clocks have mechanisms that are similar to the SCN clocks are consistent with accumulating data that peripheral clocks in many organisms share the same basic molecular properties. Many different cultured tissues from transgenic animals carrying reporter genes driven by the *Per1* promoter exhibit rhythms similar to those observed in the SCN (Yamazaki et al., 2000). Even cultured mouse embryonic fibroblasts appear to use the same mechanism since cells made from mice lacking one or both of the *Cry* genes exhibit rhythms with the same phenotype (altered period or arrhythmicity) observed in the locomotor activity (Yagita et al., 2001).

Despite the broad similarities in the use of a set of rhythmically expressed clock genes, variations in the molecular details of clock organization are rapidly emerging in different circadian systems. For example, the clock in the mammalian forebrain is very similar to the SCN clock but appears to use NPAS2 in place of CLOCK (Reick et al., 2001). Likewise, several reports indicate that some aspects of the clock in the retina may work differently than the SCN clock. For example, in *Xenopus* retina, *xPer2* mRNA is driven by light and dopamine and, unlike that in mammalian systems, is out of phase with the circadian rhythm of *xPer1* mRNA (Steenhard and Besharse, 2000).

LOCALIZATION OF THE CLOCK(S) WITHIN PHOTORECEPTORS

Because the retina is so well characterized physiologically and the cells are morphologically distinct and organized in clearly stratified layers (Fig. 1), it is possible to examine the cellular localization of circadian properties within this complex tissue and to relate those properties to circadian physiology. Although a definitive understanding of the relative importance of different retinal cell types in circadian control is not yet possible, 2 general conclusions (discussed in detail below) can be reached based on currently available data. First, photoreceptors have all of the properties of endogenous circadian clocks and are responsible for circadian release of melatonin, which could drive circadian rhythms in other aspects of retinal physiology. Second, circadian clock genes are widely expressed in most, if not all, retinal cell types; thus, in principal, retinal circadian rhythmicity could result from the interaction of multiple circadian clocks residing in different cell types.

The initial evidence that photoreceptors are circadian clocks came from tissue reduction and cell culture experiments. In *Xenopus* eye cups, a lesioning procedure was developed that permitted isolation of photoreceptor layers capable of generating sustained circadian oscillations of melatonin for many days in vitro (Cahill and Besharse, 1993). Light and dopamine acting through D2-like receptors caused phase resetting in those cultures, demonstrating that both a circadian clock mechanism and entrainment pathways were present in photoreceptors. Consistent with this was the demonstration that a dominant negative form of CLOCK, expressed specifically in photoreceptors, resulted in ablation of the melatonin outflow rhythm in transgenic *Xenopus* (Hayasaka et al., 2002). An alternative approach involving photoreceptor-enriched cultures from embryonic chick retinas (Pierce et al., 1993) showed sustained oscillations in the expression of iodopsin mRNA, indicating that chicken cone photoreceptors exhibited clock properties. These direct demonstrations of photoreceptor clock properties confirmed suggestions based on kainic acid lesioning experiments (Thomas et al., 1993), and both approaches have subsequently been exploited to define essential features of photoreceptor oscillators such as a role for cAMP in phase setting (Hasegawa and Cahill, 1998, 1999a, 1999b), the regulation of the melatonin synthetic enzyme serotonin N-acetyltransferase (e.g., arylalkylamine N-acetyltransferase, AANAT) (Iuvone et al., 1997; Greve et al., 1999; Haque et al., 2003; Ivanova and Iuvone, 2003a, 2003b), and regulation of a circadian rhythm in sensitivity of the cone cGMP-gated channel (Ko et al., 2001).

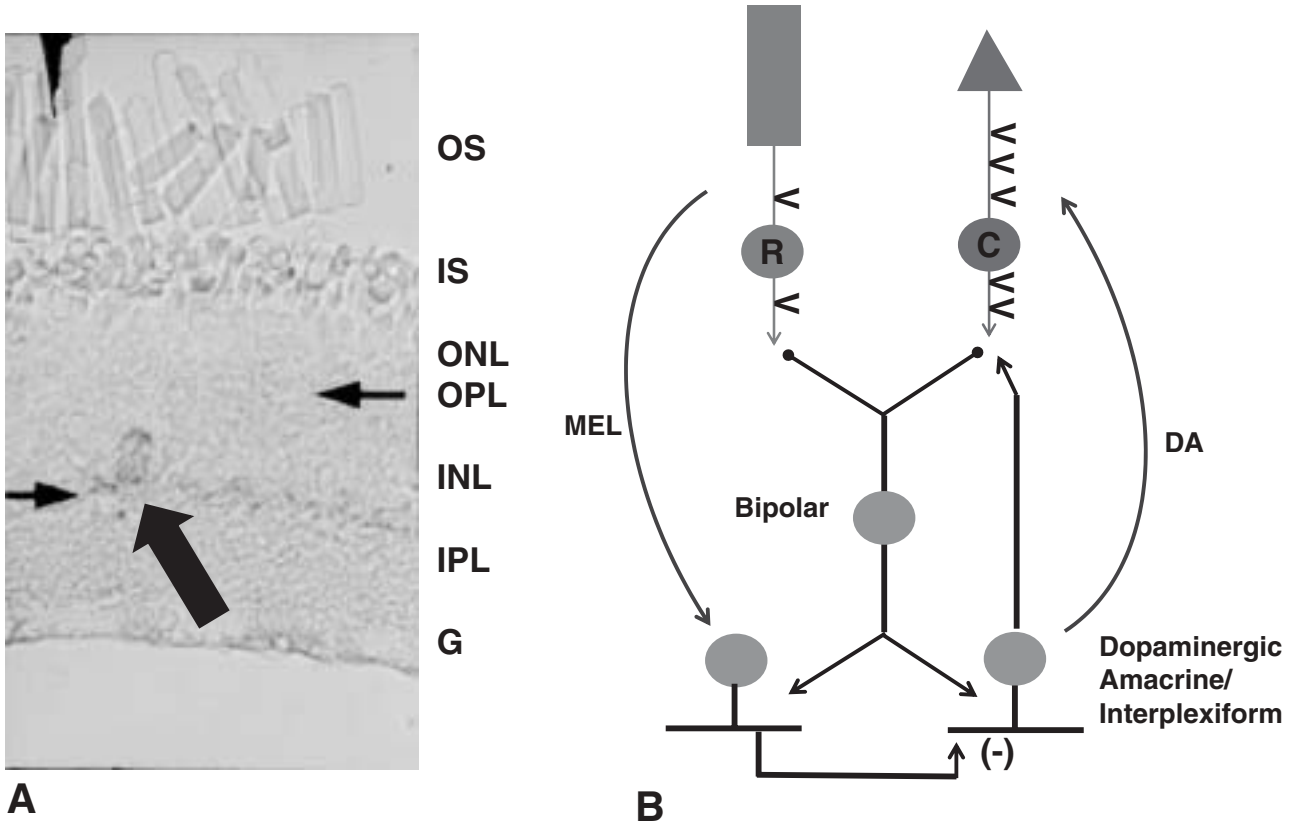

Figure 1. Organization of retinal cell types. (A) Histological section of a *Xenopus laevis* retina stained with an antibody to tyrosine hydroxylase using the peroxidase technique. Large arrow indicates a dopaminergic amacrine cell with processes extending along the junction of the inner plexiform and inner nuclear layers (lower small arrow). OS, photoreceptor outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer (upper small arrow); INL, inner nuclear layer; IPL, inner plexiform layer; G, ganglion cell layer. (B) Diagrammatic representation of photoreceptors (R, rod; C, cone), bipolar neuron, and dopaminergic amacrine/interplexiform cells. Dopamine (DA) from the inner retina regulates photoreceptors by binding D2-like receptors (<), while melatonin (Mel) from photoreceptors regulates dopaminergic cells. B is redrawn and modified from Figure 9 in Besharse and Witkovsky (1992).

Similar studies of isolated photoreceptors have not been reported for mammalian systems. However, several lines of evidence suggest that rodent photoreceptors have circadian properties similar to those in *Xenopus* and chicken. First, hamster (Tosini and Menaker, 1996), rat, and mouse (Tosini and Menaker, 1998a) retinas in vitro have been shown to exhibit circadian release of melatonin in a manner remarkably similar to that originally described in *Xenopus* (Cahill and Besharse, 1991). This rhythm appears to depend on the integrity of the photoreceptor layer because it is lost as rod cells degenerate in mice carrying the *rd* mutation (Tosini and Menaker, 1998a). This, along with data localizing AANAT to rodent photoreceptors (Liu et al., in press), indicates that rhythmic release of melatonin is a feature of mammalian photoreceptors. Nonetheless, direct demonstration that this rhythm is controlled by an endogenous photoreceptor oscillator is lacking. Given the facts that melatonin release is regulated in rodent retina in a manner similar to that described for *Xenopus* (Tosini and Dirden, 2000) and that dopamine metabolism is circadian (Wirz-Justice et al., 1984; Doyle et al., 2002a), it remains possible that rhythmic melatonin release from rodent photoreceptors is controlled by an inner retinal oscillator driving dopamine release (see below).

CLOCKS WITHIN OTHER RETINAL CELL TYPES

Given the widespread expression of clock properties throughout the body, it seems likely that retinal cell types in addition to photoreceptors have endogenous circadian properties as well. The best evidence

for this comes from studies of the mutual antagonism of the retinal dopamine and melatonin systems (Fig. 1). Dopamine is produced and released by retinal amacrine and interplexiform cells (Dowling and Ehinger, 1978) and inhibits melatonin synthesis and release in photoreceptors by binding to D2-like receptors (Iuvone and Besharse, 1986; Cahill and Besharse, 1991). In contrast, melatonin produced in photoreceptors inhibits the release of dopamine (Dubocovich, 1983). These findings, along with studies showing that dopamine and melatonin are potent modulators of rhythmic retinal physiology (e.g., see Pierce and Besharse, 1985; Manglapus et al., 1999), led to the idea that melatonin-dopamine antagonism was at the heart of rhythmic retinal physiology (see Fig. 10 and review in Besharse et al., 1988). A central feature of this model was the idea that dopamine, like melatonin, was synthesized rhythmically (Iuvone et al., 1978; Wirz-Justice et al., 1984). However, as elegantly modeled in a simultaneous analysis of the dopamine and melatonin rhythms in the pigeon retina (Adachi et al., 1998), these two tightly coupled circadian rhythms could be accounted for equally well by either one clock driving a single neuromodulator (i.e., melatonin in photoreceptors) or independent oscillators driving each neuromodulator (Fig. 2). Direct tests to distinguish between these models are difficult and, with the exception of those directly showing that photoreceptors are oscillators, have not yet been fully accomplished. However, available data indicate that dopamine rhythms are not just driven by melatonin rhythms (Adachi et al., 1999) and that loss of photoreceptors in the Royal College of Surgeons rat does not result in a loss of the circadian rhythm of dopamine metabolism (Doyle et al., 2002b). These data, together with the recent finding that the circadian clock gene, *Per1*, is rhythmically expressed in dopaminergic amacrine cells (Witkovsky et al., 2003), are consistent with endogenous circadian control within the inner retina and at least a dual-oscillator mechanism within the retina.

LOCALIZATION OF CLOCK GENE EXPRESSION IN RETINA

Numerous studies have documented that virtually all genes considered part of the core clockwork in the SCN are expressed in the retina (reviewed in Tosini and Fukuhara, 2002) as well as in other tissues containing peripheral oscillators (reviewed in Reppert and Weaver, 2002). Here we focus on studies that provide insight into localization of clock gene expression in different retinal cell types. In *Xenopus*, *Clock* (Zhu et al., 2000) and 3 *Cry* homologues (Zhu and Green, 2001) are expressed predominantly in photoreceptors, and photoreceptor localization of *Cry1* (Haque et al., 2002) and *Cry2* (Bailey et al., 2002) has been reported in chicken as well. In addition, a recent in situ analysis of *Per1* and *Per2* indicates that both are expressed in *Xenopus* photoreceptors (Besharse et al., in preparation). These localization studies are consistent with the known oscillator functions of photoreceptors in *Xenopus* and avian retinas. However, all in situ studies using *Xenopus* show that clock genes are more globally expressed. *Clock*, *Bmal1*, *Cry*, and both *Pers* are expressed widely in the inner nuclear layer and ganglion cells. *Xenopus Per2* is also rhythmically expressed in the retinal pigment epithelium (Zhuang et al., 2000). Likewise, both *Cry1* and *Cry2* are expressed in ganglion cells of chickens (Bailey et al., 2002; Haque et al., 2002). The in situ analysis of *Per1* and *Per2* is particularly interesting in that in *Xenopus*, they are expressed out of phase with one another (Zhuang et al., 2000). In addition, in *Xenopus* and quail, *Per2* is regulated by light in a manner expected if *Per2* were playing a key role in circadian phase regulation (Steenhard and Besharse, 2000; Yoshimura et al., 2000).

Several studies reporting a different pattern of circadian clock gene expression in rodents raise the possibility that retinal circadian organization is different in mammals. In one of the earliest studies of mammalian clock gene expression, *Per1*, *Clock*, and *Bmal1* mRNA were reported to be expressed predominantly in the inner retina of mouse with a lower level of expression in photoreceptors (Gekakis et al., 1998). This result was confirmed, at least for inner retina in the rat for both *Per1* and *Per2* (Namihira et al., 2001). These initial studies suggested widespread clock gene expression in all retinal layers in rodents. However, mouse and human *Cry1* and *Cry2* expression was found to occur exclusively in the ganglion cells and a subset of cells of the inner nuclear layer (Miyamoto and Sancar, 1998; Thompson et al., 2003). Furthermore, a recent detailed analysis of *Per1* has demonstrated rhythmic *Per1* expression in the inner nuclear layer, including dopaminergic amacrine cells (Witkovsky et al., 2003). This study failed to detect *Per1* expression in either photoreceptors or ganglion cells and suggested that retinal circadian organization in the mouse differs from that of chickens or *Xenopus*.

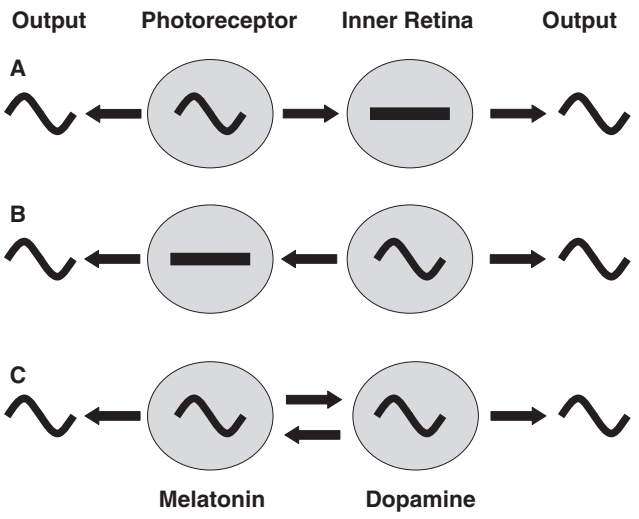

Figure 2. Hypothetical models comparing single- and multi-oscillator models for control of retinal rhythmicity. In (A), a photoreceptor clock controls circadian rhythmicity in the inner retina, while the reverse is shown in (B). (C) illustrates control by oscillators in both inner and outer retina. Sine waves inside circles indicate location of clocks. All 3 systems could generate circadian rhythms in both the inner and outer retina such as those for melatonin and dopamine. Drawing adapted from Figure 6 in Adachi et al. (1998).

Although further analysis of clock gene expression in mammalian retina is clearly needed, current data lead to the conclusion that individual components of the core oscillator, as defined for the SCN, are differentially expressed in different cell types in retina. Thus, the retina is an ideal site for further analysis of the molecular role played by different clock genes.

DIFFERENTIAL EXPRESSION OF *Per* GENES IN THE RETINA

In *Drosophila*, a single *Per* gene plays a central role in the molecular clockwork, while in vertebrates, 3 *Period* homologues have been identified. The existence of 3 rhythmically expressed *Period* homologues suggests that they are likely to serve fundamentally different circadian functions, but overlapping temporal and spatial patterns of expression of these genes have made it difficult to delineate their separate roles. However, in *Xenopus* retina, both the spatial and temporal patterns of expression of *Per1* and *Per2* differ (Steenhard and Besharse, 2000; Zhuang et al., 2000; Besharse, in preparation). Retinal *Per1* mRNA is expressed in a circadian pattern, peaking near the end of the dark period and, in contrast to findings in mammalian systems, is not regulated by light. *Per2*, on the other hand, is not regulated in a circadian pattern in darkness; instead, *Per2* exhibits a light-driven diurnal rhythm peaking out of phase with *Per1* during the light period. Furthermore, *Per2* is induced by both light and dopamine (Steenhard and Besharse, 2000) under conditions known to cause phase shifts of the photoreceptor oscillator (Cahill and Besharse, 1991). Although both *Per1* and *Per2* are expressed in multiple cell types in the retina, light and dopamine induction of *Per2* occurs specifically in photoreceptors (Besharse et al., in preparation). The differential expression of the two *Per* genes in this case has led to the conclusion that *Per2* plays an important role in phase resetting of the photoreceptor clock, while circadian expression of its mRNA is dispensable as far as clock function is concerned.

HOW ARE THE RETINAL CLOCKS ENTRAINED?

One of the major unanswered questions in retinal rhythmicity relates to the mechanism of local entrainment. Vertebrate retinas that have been shown to exhibit circadian rhythmicity in vitro also retain their ability to entrain to light, indicating that the input pathways are present within the retina. The photoreceptors that mediate this entrainment are not known, but this input pathway is present in pure photoreceptor layers in *Xenopus* (Cahill and Besharse, 1993), suggesting that light can entrain these photoreceptor clocks via a photoreceptor present in the rods or cones. In *Xenopus*, dopamine acting at D2-like receptors on rods and cones can also reset the clock in a manner similar to light but uses a distinct pathway that involves changes in cAMP levels (Hasegawa and Cahill, 1999b). As discussed above, recent observations that light and dopamine induce *Per2* specifically in photoreceptors have led to the idea that changes in PER2 protein expression alter the circadian phase through interaction with other components of the molecular clockwork. Although light, like dopamine, has been reported to inhibit adenylate cyclase in photoreceptors, light-mediated resetting uses a cAMP-independent mechanism. The photoreceptor mechanism remains unexplored.

Although mammalian retinas can also be reset by light in a culture dish (Tosini and Menaker, 1996, 1998a), the cell types or signaling pathways involved

are not known. Photoreceptors in the rods, cones, and a light-sensitive class of ganglion cells all contribute to entrainment of the SCN clock (Hattar et al., 2003; Panda et al., 2003), but their roles in entrainment of local retinal clocks have not been investigated.

HOW DO THE RETINAL CLOCKS CONTROL LOCAL PHYSIOLOGY?

Many retinal rhythms are known to be regulated, at least in part, by rhythms in melatonin and/or dopamine (see above discussion), but how the clock controls the rhythms of these neuromodulators, particularly dopamine, is not well defined. The enzymes involved in melatonin synthesis are under circadian control at several levels, including the transcriptional and posttranscriptional levels, although the details vary somewhat between species (Hamm and Menaker, 1980; Thomas and Iuvone, 1991; Green and Besharse, 1994; Roseboom et al., 1996; Bernard et al., 1997; Chong et al., 1998; Ivanova and Iuvone, 2003a). It is currently unclear what mechanisms regulate circadian dopamine metabolism. In mice, the circadian rhythm of dopamine is dependent on the presence of retinal melatonin since mouse strains lacking melatonin also lack dopamine rhythms (Doyle et al., 2002a). This would suggest that a circadian rhythm of melatonin release from photoreceptors drives retinal dopamine rhythms. However, this conclusion is made ambiguous by the finding that in RCS rats, loss of at least rod cells does not alter circadian dopamine metabolism (Doyle et al., 2002b). The recent finding of rhythmic expression of *Per1* in dopaminergic amacrine cells has led to the suggestion that a local clock controls dopamine metabolism (Witkovsky et al., 2003), but the details by which this regulation occurs has not been investigated.

Retinal clocks can control downstream rhythms through transcriptional control using either a clock "E-box system" or a cAMP response element. In chicken photoreceptors, the *aanat* mRNA exhibits circadian rhythms in abundance, which underlies the rhythm in melatonin synthesis (Bernard et al., 1997) The chicken *aanat* gene has an E-box enhancer element in its 5′-flanking region and can be transcriptionally induced by CLOCK/BMAL1 (and MOP4/BMAL1) in transient transfections of heterologous cell cultures (Chong et al., 2000). These data suggest that the same "clock E-box" mechanism used within the core oscillator is also used to control rhythmic transcription of a "clock-controlled gene." Although this has not been confirmed in retinal cells, these data suggest that *aanat* may be regulated directly by components of the circadian clock loop, a mechanism that could potentially be extended to include additional "clock-controlled genes."

AANAT is also regulated at the posttranslational level. The enzymatic activity of AANAT is activated by cAMP, and recent work in chick photoreceptor cultures has demonstrated that cAMP level varies with a circadian rhythm (Ivanova and Iuvone, 2003a) reminiscent of the cAMP rhythms that had been observed previously in chick pineal cells (Nikaido and Takahashi, 1989). Recent studies have also shown that expression of the type 1 adenylyl cyclase and the synthesis of cAMP in rat retinas are under circadian control (Tosini et al., 2003). Although these data suggest that the clock controls melatonin rhythmicity (and possibly other photoreceptor rhythms) through cAMP rhythms, further details about this signaling pathway remain to be defined.

A rhythm of cAMP in photoreceptors provides an alternative mechanism for regulating circadian gene transcription. In *Xenopus* retina, the *nocturnin* gene, which encodes a deadenylase (Baggs and Green, 2003), is expressed in the photoreceptor cells with a high-amplitude rhythm of transcription (Green and Besharse, 1996a, 1996b; Liu and Green, 2001, 2002). This gene contains an enhancer sequence that resembles a cAMP response element (CRE), which binds to CRE binding protein (CREB) in both its phosphorylated and nonphosphorylated forms (Liu and Green, 2002). Phospho-CREB (but not unphosphorylated CREB) activates *nocturnin* gene expression in transfected cells, and within *Xenopus* photoreceptors (but not in other retinal neurons), levels of phosphorylated CREB fluctuate rhythmically with a peak at midnight, which correlates well with the peak of *nocturnin* transcription. Although these data suggest that the photoreceptor circadian clock drives rhythmic *nocturnin* transcription via this mechanism, it is not known whether the clock drives the rhythms in phospho-CREB via changing cAMP levels or through some other mechanism.

Although rhythms of cAMP, *nocturnin*, and *aanat* have clear elements of transcriptional control, there is strong evidence for posttranslational pathways as well. Perhaps best defined is the circadian regulation of cGMP-gated channel ligand affinity observed in chick cone photoreceptors. In this case, the channel regulation occurs at the posttranslational level, and

the rhythmicity is driven by rhythms in 2 protein kinases, Erk and Ca/calmodulin-dependent protein kinase II (CaMKII) (Ko et al., 2001). Dopamine or D2 agonists can alter the sensitivity of these channels, but the effects are different at different times of day, resulting from the differential use of the clock-driven Erk and CaMKII signaling pathways (Ko et al., 2003).

Although specific output pathways are not completely defined in retinas, the demonstration of rhythmic signaling pathways—such as CREB phosphorylation, cAMP levels, and Erk and CaMKII activities discussed above, as well as reported rhythms in protein kinase C immunoreactivity (Gabriel et al., 2001)—suggests that these mechanisms will be used widely within the retina for rhythmic control of many cellular processes. Therefore, further definition of these pathways will likely have widespread impact on the understanding of the rhythmic physiology in the retina.

DO RETINAL CLOCKS INTERACT WITH THE SCN?

Although peripheral clocks, such as those in the retina, can generate rhythms endogenously, they normally operate within the context of the whole organism. Little is known about the signals that couple these oscillators, but it is clear that they influence each other to generate a coordinated circadian "system" (this idea is reviewed in Reppert and Weaver, 2002). The clock in the SCN acts as a "master" clock in mammalian systems by influencing the phase relationships of the various peripheral oscillators. However, it is clear that feedback from peripheral clocks also influences the SCN (Stokkan et al., 2001; Dudley et al., 2003). The relationship between the retina and the SCN is of particular interest because the retina has direct projections to the SCN via the retino-hypothalamic tract and serves as the route by which light signals entrain the SCN pacemaker. It was recently reported in mice that the light-sensitive retinal ganglion cells that project directly to the SCN and contain the putative circadian photoreceptor melanopsin do not express *Per1*, suggesting that these cells do not contain circadian clocks (Witkovsky et al., 2003). It is not known whether the circadian clock within the retina modulates the entraining effects of light on the SCN, perhaps by gating the input signals in some way.

Although the retina's role in light input to the SCN is well known, there is also evidence that the retina affects the SCN in other ways, independent of its role as a photoreceptor. Enucleation of hamsters results in modification of the locomotor activity rhythms, with these animals showing significantly more variable free-running periods than intact animals maintained in constant darkness (Yamazaki et al., 2002). Although these experiments did not directly demonstrate that the effects required a functional clock in the retina, the authors speculated that the precision of the behavioral period may require a coupled oscillator system in which the SCN clocks interact continuously with retinal clocks to determine the free-running period of the system. Changes in several features of circadian locomotor behavior were also observed in *rdta* mice, in which rod photoreceptors are specifically ablated, and were interpreted to support the hypothesis that the SCN and the retina interact to generate the normal circadian phenotype (Lupi et al., 1999).

The eyes have also recently been shown to contribute to normal molecular phenotypes within the SCN. Phosphorylation of MAPK exhibits a robust rhythm in the anatomically distinct "core" region of the hamster SCN, and this rhythm is lost following enucleation (Lee et al., 2003). In contrast, removal of the eyes from rats results in amplification of FOS rhythmicity in the SCN, suggesting that the eyes normally contribute to the "dampening" of this rhythm (Beaule and Amir, 2003).

In most species examined, retinal rhythmicity is not controlled by extraretinal clocks, as shown, for example, in the elegant studies on melatonin rhythms in Japanese quail retinas (Underwood et al., 1988; Underwood et al., 1990). However, in some cases, rhythms in the retina may also be driven or modulated by the SCN or other parts of the brain, reminiscent of what has been observed in invertebrate systems such as *Limulus*, in which a brain oscillator drives retinal rhythms (Chamberlain and Barlow, 1987). In the iguana, circadian rhythms of b-wave amplitude of the ERG and melatonin and dopamine contents have been reported in intact animals, but these rhythms disappear upon optic nerve sectioning, suggesting that they are generated by the brain (Miranda-Anaya et al., 2002). ERG rhythms in rabbits are also under at least partial control by the brain since bilateral sectioning of the cervical sympathetic nerves (but not the optic nerves) abolishes these rhythms (Brandenburg et al., 1981). Influence of the brain has also been reported for rod disk-shedding rhythms in rats, in that this rhythm was not reset by light in animals with severed optic nerves (Teirstein et al., 1980). Similarly, it has been

reported that the retinal rhythm of *Per2* mRNA in rats with SCN lesions is lost while the rhythm of *aanat* is retained (Sakamoto et al., 2000). Although this result suggests that the retinal *Per2* rhythm is driven by the SCN and that a divergent circadian mechanism without cycling *Per2* drives *aanat*, it is also possible that the Northern analysis used in this study lacked the sensitivity to detect the rhythm of *Per2* in the small subset of photoreceptors synthesizing *annat*.

CONCLUSIONS

Although a significant body of data about retinal clock systems has been acquired, many new questions still need to be addressed. Are the clocks in retinas from different species localized differently? Do photoreceptors have clocks in mammals? Do inner retinal neurons have clocks in *Xenopus*, chicken, and fish? Do these individual cellular clocks control individual (different) cellular rhythms? Or do the individual clocks work together to form a rhythmic milieu (melatonin, dopamine, or something else) that drives all the rhythms?

What is clear is that within the retina, there exists an entire circadian "system" composed of input pathways for light and dopamine, multiple clocks that most likely are coupled with each other, and many physiologically relevant outputs. Available data suggest that these retinal clocks have many characteristics in common with clocks in the SCN at the molecular level but also have some very interesting distinctions. These differences may reflect specialized needs of the retinal clocks and, with further study, may also provide insight into the mechanism and organization of other peripheral clocks.

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