Cell Tissue Res (2002) 309:35–45
DOI 10.1007/s00441-002-0571-6

REVIEW

Arjun Natesan · L. Geetha · Martin Zatz

Rhythm and soul in the avian pineal

Received: 3 January 2002 / Accepted: 2 April 2002 / Published online: 25 May 2002
© Springer-Verlag 2002

Abstract The avian pineal gland, like that of mammals, displays a striking circadian rhythm in the synthesis and release of the hormone melatonin. However, the pineal gland plays a more prominent role in avian circadian organization and differs from that in mammals in several ways. One important difference is that the pineal gland in birds is relatively autonomous. In addition to making melatonin, the avian pineal contains photoreceptors and a circadian clock (thus, an entire circadian system) within itself. Furthermore, avian pineals retain their circadian properties in organ or dispersed cell culture, making biochemical components of regulatory pathways accessible. Avian pinealocytes are directly photosensitive, and novel candidates for the unidentified photopigments involved in the regulation of clock function and melatonin production, including melanopsin, pinopsin, iodopsin, and the cryptochromes, are being evaluated. Transduction pathways and second messengers that may be involved in acute and entraining effects, including cyclic nucleotides, calcium fluxes, and protein kinases, have been, and continue to be, examined. Moreover, several clock genes similar to those found in *Drosophila* and mouse are expressed, and their dynamics and interactions are being studied. Finally, the bases for acute and clock regulation of the key enzyme in melatonin synthesis, arylalkylamine N-acetyltransferase (AA-NAT), are described. The ability to study entrainment, the oscillator itself, and a physiological output in the same tissue at the same time makes the avian pineal gland an excellent model to study the bases and regulation of circadian rhythms.

A. Natesan (✉) · L. Geetha · M. Zatz
Section on Biochemical Pharmacology,
Laboratory of Cellular and Molecular Regulation,
National Institute of Mental Health, Bethesda,
MD 20892-4068, USA
e-mail: arjun@codon.nih.gov

A. Natesan
LCMR, NIMH, Bldg 36, Rm 2A-11, Bethesda,
MD 20892-4068, USA

Keywords Pineal · Melatonin · Circadian rhythms · Entrainment · AA-NAT · Phototransduction · Birds

Introduction

‘’...if it is true that people can walk about and do their business without brains, – then certes the soul does not inhabit there.” – Walter Shandy’s assessment of the role of the pineal, a part of the brain, as seat of the soul (Sterne 1760).

It is debatable whether birds have souls or brains as good as ours, but they, and in particular their pineal glands, certainly have endogenous rhythms as good as ours. Although Walter Shandy (a character in Laurence Sterne’s classic “The Life and Opinions of Tristram Shandy”) did not use the scientific method, he was skeptical of Descarte’s assertion that the primary function of the pineal gland is soul storage. Subsequent research has, however, uncovered other characteristics of the pineal gland worthy of contemplation and shown that pineal glands from many species share fascinating features.

The pineal is a glandular structure that is developmentally derived from diencephalic tissue, and contains a number of cell types including pinealocytes, fibroblasts, T-cells, and glia (Korf 1994). The primary function of the gland in all species is to synthesize and release the hormone melatonin. Melatonin synthesis is always rhythmic, and is several-fold higher during the night than during the day in both diurnal and nocturnal species. The brain also regulates the pineal gland, at least in part, as it is innervated by the sympathetic nervous system. These nerves release norepinephrine (NE), but the response to NE differs among species. NE stimulates pineal melatonin synthesis and release in the rat (Axelrod 1974), whereas it suppresses melatonin synthesis and release in the chicken (Binkley 1988).

Although pineal glands in birds and mammals share many features, the avian pineal has three properties that distinguish it from those of mammals and provide it with distinct advantages for the study of circadian regulation.

First, pineal cells from several avian species, including pigeon, chick, and house sparrow, continue to make melatonin spontaneously in vitro in dispersed cell cultures (Deguchi 1979a; Kasal et al. 1979; Murakami et al. 1994). Second, they continue to do so rhythmically under constant conditions. Therefore, the avian pinealocytes contain endogenous oscillators. Third, this rhythm in melatonin release is responsive to the environment. In a light:dark cycle, melatonin release is high in the dark (night) and low in the light (day). Light pulses applied to cultured chick pineal cells maintained in otherwise constant darkness or red light can phase shift the rhythm of melatonin release (Zatz et al. 1988a; Takahashi et al. 1989; Fig. 1). Furthermore, the properties listed above for dispersed cell cultures have been demonstrated in individual chick pinealocytes (Nakahara et al. 1997). Thus, cultured chick pineal cells provide an excellent model for the examination of an entire circadian system at the cellular and molecular level.

Melatonin

Melatonin is an indoleamine hormone rhythmically synthesized and released by pinealocytes. The only other cells so far clearly shown to make melatonin are retinal photoreceptors (Cahill and Hasegawa 1997). The biochemical pathway for the synthesis of melatonin is well known and conserved in all species examined (Axelrod 1974). Melatonin is synthesized in a four-step enzymatic process from dietary tryptophan. Tryptophan is converted in two steps to serotonin, which is then acetylated by the enzyme arylalkylamine *N*-acetyltransferase (AA-NAT) and methylated by hydroxyindole-*O*-methyl transferase (HIOMT) to melatonin. The rhythm of AA-NAT activity correlates strongly with the rhythm of melatonin synthesis. Since melatonin is lipophilic and does not reside in storage vesicles, changes in synthesis lead directly to changes in release. Melatonin is prominently involved in the seasonal timing of reproduction (Goldman 2001; Pévet et al. 2002) and the circadian regulation of many physiological functions, including locomotor activity, heart rate, feeding, and body temperature (Arendt 1995). Although the rhythm in melatonin synthesis reflects the circadian oscillator in pineal cell culture, a number of perturbations can affect melatonin output independently of the oscillator. In practice, perturbations to pineal cells in culture can lead to acute and/or phase-shifting effects on melatonin release. For example, a 3-h light pulse to pineal cells in constant red light, at a certain phase (Fig. 1), causes both an acute decrease in melatonin output and a phase advance in the clock generating the rhythm of melatonin release (Zatz et al. 1988). NE, on the other hand, can only decrease melatonin synthesis acutely and does not affect the phase of the melatonin rhythm in subsequent cycles (Zatz 1996).

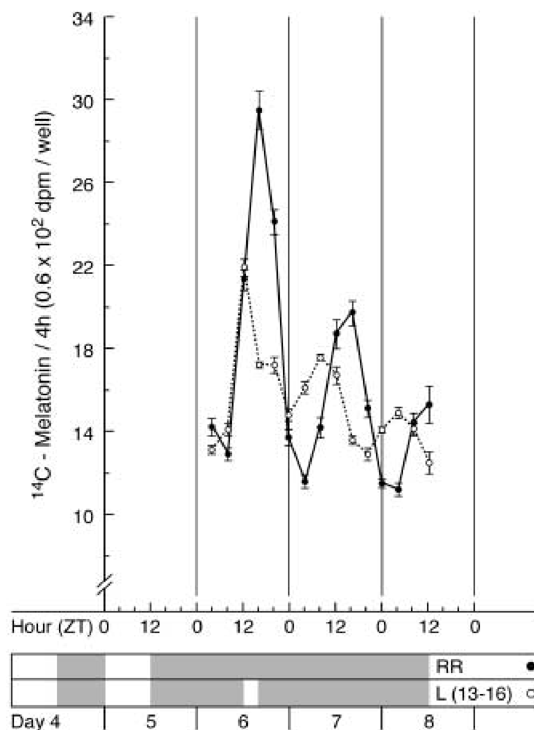

Fig. 1 Effects of a 3-h light pulse on chick pineal melatonin release in vitro. Chick pineal cells were plated on day 1 and maintained for 5 days in a cycle of 12 h white light and 12 h red light (LR 12:12). On day 5, cells were placed in constant red light (*RR*). Experimental cells (*dashed line*) were given a 3-h light pulse beginning at ZT13 on day 6, whereas control cells (*solid line*) were maintained in *RR*. Cells were cultured in medium containing ^{14}C-tryptophan from day 5 onward. Media were collected every 4 h and assayed for ^{14}C-melatonin (Zatz et al. 1988). Note the acute decrease in melatonin output during the light pulse on day 5 and the phase advance evident in subsequent cycles

Circadian organization

In birds, the pineal is one part of a multi-oscillator circadian system that also includes the retina and the suprachiasmatic nucleus (SCN) of the hypothalamus (Fig. 2). The relative contribution of each of these structures to the rhythmicity of the whole organism varies among avian species (Cassone 1990; Underwood et al. 2001). In house sparrows and starlings, pinealectomy alone abolishes or significantly disrupts circadian rhythmicity. In chicks and Japanese quail, however, removal of the pineal has no effect on rhythms of locomotor and feeding activity, whereas enucleation alone in the chick does abolish rhythmicity. In the pigeon, the pineal and retina are more equal partners; both pinealectomy and enucleation are required to abolish rhythmicity.

The contribution of the SCN has not been definitively described in birds because of controversy as to its exact location. Within the hypothalamus, the medial SCN and the visual (or lateral) SCN have both been implicated as possible homologs to the mammalian nucleus, based upon anatomical, biochemical, and molecular evidence (Cassone and Moore 1987; Norgren and Silver 1989).

Lesions of the hypothalamus encompassing both suprachiasmatic nuclei result in arrhythmicity in house sparrows and Japanese quail (Underwood et al. 2001). Yoshimura et al. (2001) applied the measurement of clock gene expression to the question of avian circadian organization. They reasoned that the avian homolog of the mammalian SCN would express clock genes (see next section) more robustly than would other hypothalamic nuclei. They found *per2* mRNA to be strongly expressed and light inducible in the medial SCNs of several bird species (only weak expression was seen for *per3* and *Clock*), whereas no expression was seen in the visual SCN. Although these results support a role for the medial SCN in the avian circadian system, they do not clearly distinguish or allocate circadian functions among hypothalamic nuclei. One possibility remaining, for example, is that the two avian SCN represent the anatomical correlates of the ventrolateral and dorsomedial subdivisions of the mammalian SCN. Another is that functional roles of avian nuclei simply do not correspond to those of mammalian nuclei or subnuclei.

Two models have been constructed to describe the relationship between the circadian oscillators in the avian retina, SCN, and pineal gland. The neuroendocrine loop model advocates mutual inhibition between clock components: melatonin released from the retina and pineal gland is thought to inhibit SCN metabolic activity during the night, whereas the SCN inhibits melatonin synthesis during the day (Cassone and Menaker 1984). The internal resonance model suggests that the three oscillatory components synchronize and amplify each other through resonance to produce a stable high-amplitude circadian output (Gwinner 1989).

Circadian organization in birds differs significantly from that in mammals because of the different properties of the pineal, retina, and SCN in these systems (Fig. 2). In contrast to birds, the rhythm in mammalian pineal melatonin synthesis is entirely driven by the SCN. The pineal has no endogenous oscillator. In mammals, the SCN is the master pacemaker. Endogenous rhythms in mammalian retina have been described (Tosini and Menaker 1996; Tosini and Fukuhara 2002), but the ramifications of these oscillations, beyond local modulation of retinal function, are unclear. Recent evidence of endogenous rhythmicity in peripheral tissues has added a new level of complexity to the hierarchical organization of the mammalian circadian system (Buijs and Kalsbeek 2001). Although lesion and transplantation studies demonstrate unequivocally that the SCN is the mammalian master pacemaker, it may not be driving the rhythmicity of peripheral tissues so much as synchronizing them.

Entrainment in mammals requires the retina (Bellingham and Foster 2002). Photic signals travel exclusively from the retina via the retinohypothalamic tract to the SCN, which in turn drives pineal rhythmicity. In birds, however, the pineal, like the retina, is directly photosensitive. The bird pineal expresses a number of retina-specific proteins, and developmentally, both the retina and pineal derive from diencephalic tissue (Korf et al. 1998).

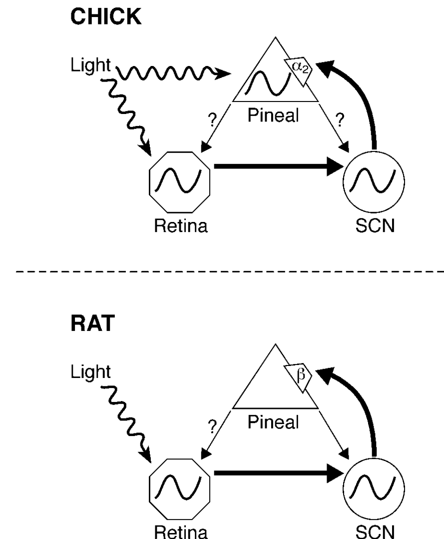

Fig. 2 Comparison of the circadian organization of the chick and the rat. In the chick, the retina, pineal, and suprachiasmatic nucleus (*SCN*) each contains a circadian oscillator. On the other hand, the rat pineal is a slave to the SCN oscillator. In the chick, light input can directly entrain the pineal, whereas in the rat, light acts indirectly. It enters through the retina, which in turn entrains the SCN, and subsequently, the pineal gland. Whether the chick hypothalamic clock is directly light sensitive is uncertain. Adrenergic receptors, which ultimately receive SCN output via NE, differ in the chick and rat. In the chick, they are primarily of the alpha-2 type (α_2) and inhibitory. In the rat, they are primarily of the beta (β)and alpha-1 types and stimulatory. Peak NE release occurs during the day in the chick but at night in the rat. The exact role and strength of feedback from the pineal to retina and SCN is probably variable among both avian and mammalian species and has not been fully determined

Molecular oscillator components

Although there are significant functional differences between specific clock genes across phyla, a general mechanism of interlocked feedback loops generating circadian oscillations is retained (Dunlap 1999; Glossop et al. 1999; Shearman et al. 2000). Increasing numbers of components of circadian oscillators are being isolated and they have revealed remarkable sequence similarity between *Drosophila* and mammals. The regulation and interaction of a number of these genes have been analyzed in detail, and homologous genes have recently been uncovered in chick and quail.

Period and *cryptochrome*

The *period* gene of *Drosophila* was the first component of a circadian oscillator to be found (Konopka and Benzer 1971), and since then, three *per* homologs have been described in mouse (Reppert and Weaver 2001). Abundant evidence indicates that PER acts as a "negative element" in the regulation of its own transcription and that of several other molecular clock components by suppressing the stimulation of the transcription by

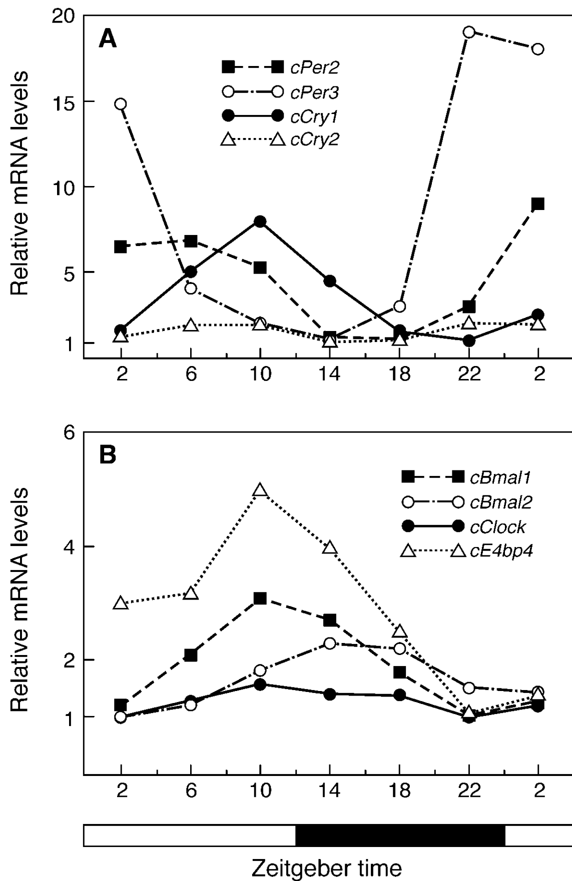

Fig. 3A, B Temporal dynamics of gene expression for clock components in the chick pineal. The patterns for *period* (*per*) and *cryptochrome* (*cry*) mRNAs in a 12:12 LD cycle are shown in **A** and those for *clock*, *bmal*, and *e4bp4* mRNAs are shown in **B**. Note that these are not actual concentrations but values that have been normalized to the level of stable transcripts that differ for the different mRNAs (histone H4 for *cClock*, *cBmal1* and *cBmal2*, gapdh for *cPer2*, *cPer3*, *cCry1*, and *cCry2*, and *cTbp* for *cE4bp4*; from Doi et al. 2001; Okano et al. 2001; Yamamoto et al. 2001). Consequently, only the temporal patterns and relative excursions of the different messages can be compared with each other. Similar results with minor differences have been reported by others for the temporal dynamics of *cBmal1*, *cClock*, and *cCry2* messages (Chong et al. 2000; Bailey et al. 2002)

CLOCK:BMAL heterodimers. However, PER can also promote the transcription of other clock genes, such as *bmal1* in mouse (Shearman et al. 2000) and its homolog in *Drosophila* (Glossop et al. 1999). Homologs of *per2* and *per3* have been isolated and sequenced in chick (Okano et al. 2001) and quail (Yoshimura et al. 2000), but avian *per1* has not yet been reported. Chick *per2* (*cPer2*) mRNA is rhythmic in the pineal in both light/dark (LD) and dark/dark (DD) with a peak in the early morning and a trough in the early night (Fig. 3A). Effects of light pulses on chick *per* expression have not yet been reported. Quail *per2* (*qPer2*) mRNA is also rhythmic in the pineal (and the eye): its levels are higher during the day, but the amplitude is significantly reduced in DD (Yoshimura et al. 2000). Light pulses of 1 h increase *qPer2* gene expression. The rhythms of *per3* expression in chick and quail are similar to those of *per2* (Yoshimura et al. 2000; Yamamoto et al. 2001). However, *qPer3* mRNA is not light inducible.

The temporal pattern of expression of avian *Per* homologs exhibit peaks significantly earlier than that of any of the mouse *per* genes (Okano et al. 2001; Reppert and Weaver 2001), but the expression of chick *per2* mRNA most closely resembles that of mouse *per1*. Such comparisons of the temporal dynamics of clock genes will be important for understanding the similarities and differences between systems in the roles played by various clock genes in generating circadian oscillations. The avian *per* genes also differ from their mouse (and fruit fly) counterparts in their response to light. *per* mRNA in *Drosophila* is not affected directly by light pulses. In contrast, mouse *per1* and *per2* and quail *per2* messages are rapidly induced by light (Yoshimura et al. 2000; Reppert and Weaver 2001). *per* mRNA photoinduction in mouse is gated, restricted to late day or night. However, quail *per2* mRNA induction by light is not temporally gated (Yoshimura et al. 2000). Neither mouse (Reppert and Weaver 2001) nor quail (Yoshimura et al. 2000) *per3* expression is induced by light pulses.

In *Drosophila*, strong genetic evidence indicates that cryptochrome (CRY) serves as the photopigment mediating entrainment (Emery et al. 1998; Stanewsky 2002). In mammals, there are two homolog genes, *cry1* and *cry2*. Although a role for cryptochromes in photoentrainment of mammalian circadian systems has also been proposed (Sancar 2000), most evidence suggests a more prominent role as a "negative element" in the regulation of the transcription of several mammalian clock components (Hall 2000). Fragments of quail *cry1* and *cry2* and full-length chick *cry1* and *cry2* have been uncovered (Yamamoto et al. 2001; Fu et al. 2002; Bailey et al. 2002). Chicken *cry1* and *cry2* mRNA are both rhythmically expressed in the pineal gland, with higher levels during the night, and *cCry2* expression is light inducible. This is in contrast to mouse cryptochromes where only *cry1* is rhythmic, and neither *cry1* nor *cry2* message is induced by light (Miyamoto and Sancar 1999). Although there is no evidence for or against a photoreceptive role for avian cryptochromes, there is evidence suggesting that these genes play a role in clock function similar to that in mammals. Chicken CRY1 and CRY2 inhibit the transactivation of the *cPer2* E-Box by BMAL:CLOCK heterodimers as do their mouse counterparts (Hall 2000; Yamamoto et al. 2001). To date, there is less information about the dynamics, interactions, and roles of molecular clock components in avian pineal than in other systems, but the early characterization of avian *per* and *cry* suggests a functional similarity to the mouse negative elements, *mPer* and *mCry*.

Clock, Bmal, and *E4bp4*

The DNA element thought to be critical for and characteristic of clock transcriptional regulation is called the

E-Box (Kyriacou and Rosato 2000). The "positive elements" of the circadian system are proteins that dimerize and (always) activate transcription at the E-box found in the promoters of *per* and other genes. In mammals, these are called CLOCK and BMAL (Reppert and Weaver 2000, 2001). In chicks, a homolog to the mammalian *clock* gene, *cClock*, and its paralog, *cMop4* (NPAS2), have been sequenced (Larkin et al. 1999; Chong et al. 2000). A *clock* homolog, *qClock*, has also been found in quail (Yoshimura et al. 2000). In the chick pineal, *clock* message levels are only weakly rhythmic, if at all, with a broad peak around late day (Chong et al. 2000; Okano et al. 2001; Fig. 3B). In the quail pineal, however, *clock* mRNA is robustly rhythmic (Yoshimura et al. 2000). Not much is yet known about *cMop4* except for a partial cDNA sequence and that it is weakly expressed in both retina and pineal (Chong et al. 2000).

Genes coding for members of the BMAL family, the dimerization partners to CLOCK, have also been described in chicks. Two chick *bmal* genes have been uncovered, *cBmal1* (MOP3 homolog) and *cBmal2* (MOP9 homolog; Chong et al. 2000; Okano et al. 2001). The abundance of both *cBmal* transcripts is rhythmic in LD and DD in the chick pineal, with a peak in late day (Fig. 3B). The abundance of *cClock* message, however, does not cycle. It appears that, in various species, either *clock* or *bmal* message levels cycle. Assuming that the corresponding proteins also cycle, this provides rhythmic transcriptional activation at the E-Box by CLOCK:BMAL heterodimers. The pattern in mouse is similar to that in the chick: *mBmal1* mRNA is rhythmic, whereas *mClock* message does not cycle (Shearman et al. 2000). In contrast, *Drosophila clock* mRNA cycles, whereas the *Drosophila bmal* homolog does not (Stanewsky 2002).

Chick BMAL proteins can each heterodimerize with cCLOCK and activate *cPer2* transcription via an E-Box (Okano et al. 2001). Furthermore, the addition of cPER2 can inhibit transcriptional activation by either heterodimer. Consistent with this observation, overexpression of cBMAL1 or cBMAL2 in cultured pineal cells eliminates the rhythm of melatonin release (Okano et al. 2001). Importantly, a direct link between clock components and pineal melatonin synthesis has been found. There is an E-Box in the *aa-nat* promoter (Chen and Baler 2000; Chong et al. 2000). cBMAL1:cCLOCK heterodimers can also activate chick *aa-nat* transcription via this E-Box. Our understanding of the role and regulation of the E-Box in transcriptional regulation is still unfolding. E-Box-containing genes are expressed in various ways and with various dynamics (Kyriacou and Rosato 2000). For example, *cAa-nat* and *cPer2* mRNAs are expressed 180° out-of-phase with one another, although the promoters of both genes have E-Boxes sufficient to activate transcription in response to cBMAL1:cCLOCK heterodimers. Clearly, other factors are involved in the differential regulation of these genes.

One of these regulatory factors might be the recently described chicken homolog of *Drosophila vrille* (*vri*), *cE4bp4* (Doi et al. 2001). VRI in *Drosophila* is a negative regulator of *per* expression (Blau and Young 1999). Expression of the *vri* homolog in chicken, *cE4bp4*, is rhythmic in pineal with a peak in the early night in both LD and DD. Interestingly, cE4BP4 represses transcription from the *cPer2* promoter at a defined recognition sequence separate from the E-Box (Doi et al. 2001). This sequence is also found in the mouse *per1* promoter (Yamaguchi et al. 2000). It will be interesting to see whether these regulatory sites are present in the promoter of *cAa-nat*. As stated above, the elucidation of molecular clock mechanisms in avian pineal lags behind that in other systems, but the presence of a clear output pathway via regulation of *cAa-nat* may help clarify both the output pathway from the clock and the features necessary for, or unique to, the generation of circadian rhythmicity.

Phototransduction and entrainment

Abundant evidence (Zatz 1996) indicates that there are two distinct mechanistic pathways mediating the effects of light on melatonin output in the chick pineal (Fig. 4). One of these, mediating the acute suppression of melatonin output, does not act through the clock. The other pathway, mediating phase shifts (entrainment) of the melatonin rhythm, does act through the clock. Indirect experiments have distinguished some aspects of these two pathways. Vitamin-A depletion of chick pineal cells in culture significantly reduces the acute effect of light, and 11-*cis*-retinaldehyde addition restores this response (Zatz 1994). In contrast, phase shifts in response to light pulses are unaffected by vitamin-A depletion or addition. These results suggest, but do not prove, that two different photopigments mediate the acute and phase-shifting effects of light. Corroborating this inference is the effect of

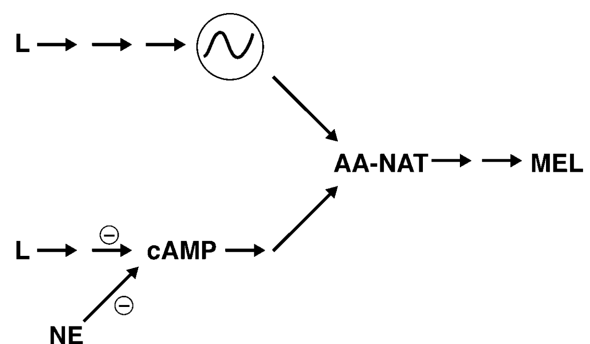

Fig. 4 Separate pathways for acute and phase-shifting effect of light on melatonin output. The acute reduction of melatonin output is mediated by a reduction in cAMP levels and, ultimately, by a reduction of AA-NAT activity. NE also lowers cAMP levels and mimics the acute effect of light (*L*). The pathway for phase shifting (entrainment) by light is poorly understood. Light induces a change in the circadian oscillator that drives the rhythm of AA-NAT activity. cAMP mediates neither the effect of light on the clock, nor the effects of the clock on AA-NAT activity. Evidence suggests that the two pathways are separate from the outset, i.e., they use different photopigments

pertussis toxin on chick pineal cells. Pertussis toxin interferes with a subset of G-proteins with which photoreceptors are often coupled. Pertussis toxin has been shown to block the acute, but not the phase-shifting, effects of light in chick pineal cells (Zatz and Mullen 1988a), again suggesting, but not demonstrating, distinct mechanistic pathways.

Photopigments

Light has two effects on the chick pineal gland, an acute reduction of melatonin synthesis and release and a phase-shifting effect on its rhythm (Fig. 1). The transduction pathways for these effects (one leading "directly" to the regulation of melatonin synthesis, the other to entrainment of the circadian clock generating the rhythm in melatonin synthesis) must each start with a photopigment. The photopigment(s) that mediate these responses have not been identified, but there are several candidates (Bellingham and Foster 2002). An action spectrum for the acute effect has suggested a rhodopsin-like photopigment (Deguchi 1981). One intriguing candidate is pinopsin, an opsin-like protein whose gene is expressed exclusively in chick pineal, but not in retina or brain (Okano et al. 1994; Max et al. 1995). *Pinopsin* mRNA levels increase 6-fold in response to a light pulse, irrespective of the time of day (Takanaka et al. 1998). However, *pinopsin* mRNA levels are not clock-controlled: there is no rhythm in DD (nocturnal low levels are simply maintained), although there is a robust day:night rhythm in LD. Pinopsin protein, on the other hand, is neither responsive to light nor clock-controlled: its level does not cycle in either LD or DD (Takanaka et al. 1998). Pinopsin is localized in structures that are regarded as pinealocyte outer segments in the follicular lumen and in modified photoreceptor cells in the parafollicular layer (Hirunagi et al. 1997). Furthermore, at least half of the pinopsin molecules are colocalized with α-subunits of the G-proteins rod transducin (Gt1) or Gq/11 (Matsushita et al. 2000). In vitro, pinopsin activates bovine retinal Gt1α, whereas in vivo, Gt1α is activated in the chick pineal in a light-dependent manner (Max et al. 1998; Kasahara et al. 2000). These properties strongly suggest that pinopsin can function as a photopigment, but its specific role remains uncertain. The interaction of pinopsin with transducin and the sensitivity of transducin to pertussis toxin may make it more likely that pinopsin mediates the acute effect of light than the phase-shifting effect.

Another candidate pineal photopigment is melanopsin. Chicken *melanopsin* has been cloned out of a chicken pineal cDNA library (I. Provencio, personal communication) and has a high level of homology with *melanopsin* from *Xenopus laevis* (Provencio et al. 1998). Chick *melanopsin* is expressed in the parafollicular cells of the pineal and in nonphotoreceptor cell layers of the retina. *Melanopsin* has also been found in ganglion cells of the mammalian retina (Provencio et al. 2000; Hannibal 2002). The tissue localization of melanopsin raises the possibility that it could mediate the entraining effects of light. The genes for iodopsin, rhodopsin, and three cone visual pigments have also been cloned and sequenced from chick, but none of their proteins are known to be expressed in the pineal gland (Takao et al. 1988; Kuwata et al. 1990; Okano et al. 1992).

If photoentrainment is mediated by a single photopigment, then knocking out the expression of the gene of that photopigment or specifically blocking its function should selectively block the phase-shifting effects of light without interfering with the endogenous rhythm. Increased protein levels or enhanced function might increase sensitivity to light as reflected in the size of phase shifts obtained to a submaximal intensity or duration of a light pulse. Similar considerations apply to the photopigment mediating the acute effect of light. Ongoing work in our laboratory is aimed at testing these predictions. It is also possible, although there is no evidence favoring this less parsimonious hypothesis, that the effects of light are mediated by the combined effects of more than one photopigment.

Cyclic nucleotides

Cyclic nucleotides were the first candidates examined for a role in the transduction of light signals into acute and phase-shifting effects (Deguchi 1979b). The well-known role of cAMP in NE stimulation of rat pineal suggested that a reduction in cAMP levels might mediate the inhibitory effects of NE and light in the chick pineal. Light and NE do rapidly reduce cAMP levels concomitant with a reduction of melatonin synthesis. Furthermore, cAMP analogs, such as 8BrcAMP, and agents that raise cAMP levels, such as forskolin or vasoactive intestinal peptide, acutely increase melatonin synthesis (Zatz and Mullen 1988b). Such evidence implicates cAMP in the acute regulation of melatonin synthesis.

Other experiments have helped to define the relationship between cAMP and the circadian regulation of melatonin output. None of the agents (except light) that raise or lower cAMP levels demonstrably affected the temporal pattern of the melatonin rhythm in subsequent cycles (Zatz and Mullen 1988b); they do not induce phase shifts, and they do not perturb the underlying pacemaker. Thus, cAMP does not act through the clock. These results contrast with those in neuronal systems, such as *Aplysia* eye and SCN, where analogs and perturbations of cyclic nucleotides do induce phase shifts (Eskin and Takahashi 1983; Gilette and Mitchell 2002).

The question then arises as to whether the clock acts through cAMP, regulating melatonin synthesis by raising and lowering cAMP levels. Attempts to demonstrate a free-running rhythm of cAMP levels in intact pineal cells have been unsuccessful. Furthermore, if the clock drives the melatonin rhythm by raising and lowering cAMP levels, then maintaining supramaximal levels of cAMP should abolish the rhythm. Experiments assessing the effects of high concentrations of forskolin or 8BrcAMP

have shown that saturating levels of cAMP do not interfere with the ability of the clock to drive the rhythm in melatonin output (Zatz 1992). Thus, the clock does not act through cAMP (Fig. 4). Rather, cAMP (and its regulation by light and NE) mediates acute effects on melatonin output primarily by acting “directly” at the level of AA-NAT protein (see section on AA-NAT regulation below and Ganguly et al. 2002). NE does, however, contribute to rhythm (amplitude) regulation in the chick pineal (Fig. 2). In vitro, daily exposure to NE (like daily light) helps to prevent damping and sustain a robust rhythm (Zatz 1996). The rhythm also damps rapidly in vivo when there is neither a light cycle nor sympathetic innervation (and thus no rhythm of daytime NE release), but it remains strong in the presence of either of these cues (Cassone and Menaker 1983).

The importance of cGMP in visual phototransduction suggests that changes in cGMP levels might mediate the effects of light in the chick pineal. cGMP activated channels, similar to those in retinal photoreceptor cells, have been shown to be present in chick pineal cells (Dryer and Henderson 1991). However, the addition of cGMP analogs such as 8-BrcGMP (Zatz and Mullen 1988b) or stimulants such as nitroprusside (unpublished observations) have neither acute nor phase-shifting effects on melatonin output. Thus, the role of cGMP in chick pineal phototransduction and melatonin regulation, if any, remains mysterious.

Calcium

The next candidate examined for a role in the transduction of light signals was the flux of calcium ions across the plasma membrane. The presence of voltage-dependent calcium channels, mainly of the L-type, was demonstrated in chick pineal cells (Harrison and Zatz 1989). Calcium flux through these channels affects melatonin output: blockers such as nifedipine or nitrendipine decrease melatonin output, whereas agonists such as Bay K 8644 increase melatonin production (Zatz 1996). None of these agents induce any phase shifts in chick pineal cells. These calcium channels and fluxes appear to modulate, and to be regulated by, cyclic nucleotides. Agents that affect calcium influx such as Bay K 8644 and nitrendipine alter the level of cAMP in chick pineal cells (Zatz 1996). On the other hand, cyclic-nucleotide-gated channels in chick cones and pineal are modulated by Ca^{2+}/calmodulin (Bonigk et al. 1996). The circadian control of certain unusual Ca^{2+} channels has been demonstrated. Calcium channels with long open times (called I_{LOT} channels) have been discovered and identified in chick pineal cells and are synthesized and open during subjective night and degraded and absent during the day (D’Souza and Dryer 1996). Just how (and whether) these channels are involved in the regulation of melatonin output remains to be determined.

In contrast to the lack of effect on the circadian pacemaker of agents acting on the calcium channels of the plasma membrane, agents that affect intracellular calcium fluxes do affect the clock in chick pineal cells. Caffeine, at the high concentrations required to release calcium from intracellular stores, induces phase shifts that resemble those evoked by light pulses (Zatz 1996). At the lower concentrations sufficient to inhibit phosphodiesterase, there is only an acute increase in melatonin output. Cyclopiazonic acid, which blocks replenishment of intracellular Ca^{2+} stores, induces phase shifts that resemble those evoked by dark pulses (unpublished observations). These results suggest a role for intracellular calcium regulation in the entrainment of chick pineal cells. When we examined the response to light of inositol-tris-phosphate (IP_3) levels, a second messenger regulating intracellular calcium fluxes, we found them to go up (unpublished observations). However, there were similar changes in response to agents that had no effect on melatonin output or its rhythm. Unfortunately, the regulation of (and by) intracellular calcium is multifaceted and complex and does not readily lead to specific candidates for second messengers and transduction pathways.

MAP kinases

Another candidate for second messengers in the transduction of light signals is the family of kinases called mitogen-activated-protein-kinases (MAPK) or environmentally regulated-kinases (ERK). These are ubiquitous kinases involved in many responses and functions (Robinson and Cobb 1997; Chang and Karin 2001; Pearson et al. 2001). Activation of these kinases by phosphorylation is part of a cascade that leads to the activation of several transcription factors. One feature that makes them interesting candidates is that they have been shown, in various systems, to be activated by perturbations that “stress” cells, some of which have also been shown to induce phase shifts in the chick pineal melatonin rhythm (Zatz 1996).

Recently, several reports implicating these kinases in photoentrainment and/or clock output pathways have appeared. In mouse SCN, immunoreactivity attributed to the activated form of the kinase, phospho-MAPK (pMAPK), has been reported to cycle with a peak during mid-day and to be increased by light pulses in a phase-dependent manner (Obrietan et al. 1998). In contrast, in chick pineal glands, levels of pMAPK (assayed by immunoprecipitation followed by Western blot) have been demonstrated to cycle with a peak during mid-night and to be rapidly and transiently decreased by light pulses (Sanada et al. 2000). The same laboratory has also reported rhythms of upstream kinases in the MAPK cascade with temporal patterns corresponding to that of pMAPK (Hayashi et al. 2001). A role in photoentrainment is suggested. Another possibility (given the rapidity and transience of pMAPK changes to light relative to the duration-dependent effects of phase shifts) is a role for pMAPK in the acute effects of light. Yet other evidence suggests that changes in MAPK activation are a

driven output of the clock rather than a part of the entrainment pathway. In clock-containing chick retinal cones, the affinity of the cGMP-gated channel for cGMP is cyclically reduced by phosphorylated forms of MAPK and CamKII (Ko et al. 2001).

We have undertaken to determine whether changes in activated MAPK are necessary or sufficient for acute and phase-shifting effects on the melatonin rhythm in chick pineal cells. Our results indicate that changes in pMAPK levels are neither necessary nor sufficient for acute or entraining effects in chick pineal cells (L. Geetha, A. Natesan, M. Zatz, unpublished). One agent that specifically inhibits the activation of MAPK induces phase-dependent phase shifts, whereas another does not. Neither induces marked acute changes in melatonin output. Some agents that increase pMAPK induce phase shifts, whereas others do not. Some agents that induce phase shifts and acute changes in melatonin output change pMAPK levels, whereas others do not. Thus, the role, if any, of these kinases in entrainment and regulation of melatonin output remains problematic.

AA-NAT regulation

Enzyme activity

It has been known for decades that changes in melatonin synthesis and secretion essentially follow changes in the enzymatic activity of AA-NAT (Klein 1985). AA-NAT enzyme activity is dynamic and highly regulated. It is rhythmic and responds to all the factors that regulate melatonin output, including light and NE. Acute changes in melatonin output and phase shifts in the melatonin rhythm are reflected in acute changes in AA-NAT activity and phase shifts in the rhythm of AA-NAT enzyme activity. There is another enzyme, arylamine *N*-acetyltransferase (A-NAT), that can acetylate serotonin (although not as efficiently as AA-NAT). It is present in the chick pineal and probably contributes to daytime serotonin acetylation but does not contribute to the rhythm of melatonin production, since its activity is stable and does not cycle. Activity, protein levels, and mRNA levels for tryptophan hydroxylase do cycle (Green et al. 1996), with a lower amplitude than AA-NAT, and may contribute to daily changes in melatonin synthesis. The activity and mRNA levels of HIOMT, the enzyme that completes the synthesis of melatonin, also change under some conditions (Bernard et al. 1993), but whether this enzyme contributes much to the dynamic regulation of melatonin output is uncertain.

Because of its dominant role, a good deal of effort (mostly in the rat pineal) has been devoted to elucidating the mechanisms regulating AA-NAT. It has long been known that the nocturnal increase in AA-NAT activity requires RNA and protein synthesis (Klein 1985). Enzyme activity is labile and can be rapidly lost after a light pulse. Elevated cAMP levels can prevent such a loss and lowered levels can mimic it. This implies a role

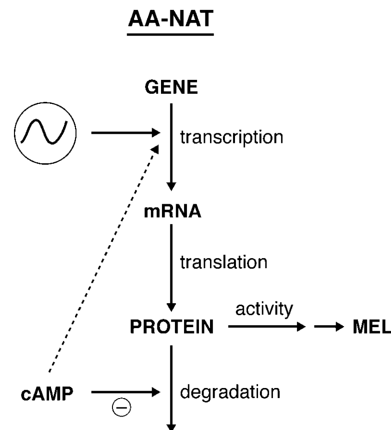

Fig. 5 Different primary sites of action of cAMP and the circadian clock in the regulation of AA-NAT activity. The circadian clock acts at the level of transcription. cAMP acts mainly to protect AANAT from proteasomal proteolysis, with small effects upon *cAa-nat* transcription

for phosphorylation in stabilizing the enzyme. Together, these results make the regulation of AA-NAT at the transcriptional, translational, and posttranslational levels seem likely. However, progress on specific mechanisms was hampered because, at that time, only enzyme activity could be assayed. A breakthrough toward understanding melatonin regulation and rhythmicity occurred in 1995: the *aa-nat* gene was identified, cloned, and sequenced (Borjigin et al. 1995; Coon et al. 1995). The *aa-nat* gene has now been cloned and sequenced from a number of species in several vertebrate classes (Ganguly et al. 2002), including chicken and quail, and specific antibodies to its protein have been generated (Bernard et al. 1997b; Gastel et al. 1998; Kato et al. 1999).

mRNA

We knew that acute and clock effects in the chick pineal converged on the regulation of AA-NAT activity (Fig. 4). The ability to measure *aa-nat* mRNA levels, AA-NAT protein levels, and AA-NAT enzyme activity in parallel has allowed us to determine their sites of action (Fig. 5). Results indicate that the chick pineal clock generates a rhythm in the abundance of *aa-nat* mRNA that can account for the free-running rhythm in AA-NAT activity (Bernard et al. 1997a, 1997b). Remarkably, clock-controlled changes in *aa-nat* expression are apparently mediated by the E-Boxes that have been found in the promoter region of the *aa-nat* gene, as discussed above (Chong et al. 2000). It has also been found that the clock-dependent nocturnal increase in *aa-nat* mRNA requires gene expression but not de novo protein synthesis (Bernard et al. 1997a). In contrast to the effects of the clock, elevating cAMP by forskolin treatment, which acutely increases AA-NAT enzyme activity, does not induce corresponding increases in *aa-nat* mRNA levels. The lowering of cAMP, by NE treatment, does not de-

crease *aa-nat* mRNA levels much either. Together, these results suggest that cAMP-controlled (acute) changes occur primarily through changes at the protein level.

Different patterns of regulation have emerged in different species (Ganguly et al. 2002). In the rat, there is a large increase in nocturnal *aa-nat* mRNA levels, attributed to cAMP (acting, ultimately, via a cyclic AMP response element in the *aa-nat* gene promoter), but in the sheep, there is little diurnal change in message levels. In the chick, the nocturnal increase in *aa-nat* mRNA levels is intermediate but cannot be attributed to cAMP. One important mechanism that has been elucidated in the rat pineal helps explain the fall of AA-NAT activity in the second half of the night. In addition to inducing *aa-nat* expression, the nocturnal elevation of cAMP induces the transcription factor, inducible cAMP early repressor (ICER), which feeds back to turn off further *aa-nat* expression (Stehle et al. 1993; Maronde et al. 1999). The presence and role of ICER has not yet been studied in the chick pineal; doing so might further clarify the relationship between clock and cAMP regulation of AA-NAT.

Protein and posttranslational modification

These inferences concerning chick pineal AA-NAT regulation have been confirmed by direct measurement of AA-NAT protein levels (Zatz et al. 2000). Protein levels and AA-NAT enzyme activity in cultured chick pineal cells correlate well under all conditions. They cycle in parallel and are both raised by agents that increase cAMP, such as forskolin, and lowered by agents that decrease cAMP, such as light and NE (subsequent unpublished work in our laboratory, with a more specific antibody and a simpler assay, have confirmed these results without the quandaries, e.g., the doublet bands for AA-NAT encountered previously). Thus, cAMP controls AA-NAT activity primarily by altering the total amount of AA-NAT protein.

As mentioned, it has also long been known that elevating cAMP levels can reduce the lability of AA-NAT enzyme activity. No evidence for active and inactive forms of the enzyme itself (e.g., differences in activity based on its phosphorylation state) has been obtained. However, effects of proteasomal proteolysis inhibitors suggested, first in the rat pineal (Gastel et al. 1998) and then in the chick pineal (Zatz et al. 2000) and bovine pineal (Schomerus et al. 2000), that rapid changes in AA-NAT activity and protein levels reflect changes in the rate at which the protein is destroyed by proteasomal proteolysis. These inhibitors are as effective as forskolin in elevating and protecting AA-NAT protein and enzyme activity. Thus, one of the effects of cAMP, which is apparently widespread among species (Ganguly et al. 2002), is to protect AA-NAT against proteasomal degradation (Fig. 5). In sheep, a shift to less degradation relative to synthesis may be the major mechanism for the nocturnal rise in activity. In chick, changes in cAMP levels increase or decrease degradation rates of AA-NAT protein and mediate acute changes in AA-NAT activity. In rat, both transcriptional activation and protection against degradation are provided by cAMP. In contrast, transcriptional regulation of the *aa-nat* gene in the chick pineal is mediated primarily by the clock, whereas protection against degradation is provided, as in the rat pineal, by cAMP. Thus, acute and clock-mediated regulation of melatonin synthesis and output occur at different levels in the chick pineal's regulation of AA-NAT.

It is reasonable to suspect that the phosphorylation of some factor protects the AA-NAT protein from degradation. Recent studies suggest that such phosphorylation may affect the binding of rat AA-NAT to a protein called 14-3-3 or the stability of the complex (Ganguly et al. 2002). This interaction has not yet been investigated in chick pineal.

Final comments

In conclusion, we have learned a good deal about the regulation of melatonin synthesis and output in the avian pineal, but major components and mechanisms remain to be determined and the site of the soul of the pineal rhythm remains elusive.

References

Arendt J (1995) Melatonin and the mammalian pineal gland. Chapman and Hall, London

Axelrod J (1974) The pineal gland: a neurochemical transducer. Science 184:1341–1348

Bailey MJ, Chang NW, Xiong J, Cassone VM (2002) Chicken's Cry2: molecular analysis of an avian cryptochrome in retinal and pineal photoreceptors. FEBS Lett 513:169

Bellingham J, Foster RG (2002) Opsin and mammalian photoentrainment. Cell Tissue Res DOI 10.1007/s00441-002-0573-4

Bernard M, Guerlotte J, Cogne M, Greve P, Collin JP, Voisin P (1993) Transcriptional regulation of hydroxyindole O-methyltransferase in the chicken pineal gland: day/night changes and long-term effects of light and darkness. Biochem J 290:661–664

Bernard M, Klein DC, Zatz M (1997a) Chick pineal clock regulates serotonin N-acetyltransferase mRNA rhythm in culture. Proc Natl Acad Sci USA 94:304–309

Bernard M, Iuvone PM, Cassone VM, Roseboom PH, Coon SL, Klein DC (1997b) Avian melatonin synthesis: photic and circadian regulation of serotonin N-acetyltransferase mRNA in the chicken pineal gland and retina. J Neurochem 68:213–224

Binkley S (1988) The pineal. Prentice-Hall, Englewood Cliffs, N.J.

Blau J, Young MW (1999) Cycling vrille expression is required for a functional *Drosophila* clock. Cell 99:661–671

Bonigk W, Muller F, Middendorff R, Weyand I, Kaupp UB (1996) Two alternatively spliced forms of the cGMP-gated channel alpha-subunit from cone photoreceptor are expressed in the chick pineal organ. J Neurosci 16:7458–7468

Borjigin J, Wang MM, Snyder SH (1995) Diurnal variation in mRNA encoding serotonin N-acetyltransferase in pineal gland. Nature 378:783–785

Buijs RM, Kalsbeek A (2001) Hypothalamic integration of central and peripheral clocks. Nat Rev Neurosci 2:521–526

Cahill GM, Hasegawa M (1997) Circadian oscillators in vertebrate retinal photoreceptor cells. Biol Signals 6:191–200

Cassone VM (1990) Effects of melatonin on vertebrate circadian systems. Trends Neurosci 13:457–464
Cassone VM, Menaker M (1983) Sympathetic regulation of chicken pineal rhythms. Brain Res 272:311–317
Cassone VM, Menaker M (1984) Is the avian circadian system a neuroendocrine loop? J Exp Zool 232:539–549
Cassone VM, Moore RY (1987) Retinohypothalamic projection and suprachiasmatic nucleus of the house sparrow, *Passer domesticus*. J Comp Neurol 266:171–182
Chang L, Karin M (2001) Mammalian MAP kinase signaling cascades. Nature 410:37–40
Chen W, Baler R (2000) The rat arylalkylamine N-acetyltransferase E-box: differential use in a master vs a slave oscillator. Mol Brain Res 81:43–50
Chong NW, Bernard M, Klein DC (2000) Characterization of the chicken serotonin N-acetyltransferase gene. Activation via clock gene heterodimer/E box interaction. J Biol Chem 275: 32991–32998
Coon SL, Roseboom PH, Baler R, Weller JL, Namboodiri MA, Koonin EV, Klein DC (1995) Pineal serotonin N-acetyltransferase: expression cloning and molecular analysis. Science 270:1681–1683
D'Souza T, Dryer SE (1996) A cationic channel regulated by a vertebrate intrinsic circadian oscillator. Nature 382:165–167
Deguchi T (1979a) A circadian oscillator in cultured cells of chicken pineal gland. Nature 282:94–96
Deguchi T (1979b) Role of adenosine 3',5'-monophosphate in the regulation of circadian oscillation of serotonin N-acetyltransferase activity in cultured chicken pineal gland. J Neurochem 33:45–51
Deguchi T (1981) Rhodopsin-like photosensitivity of isolated chicken pineal gland. Nature 290:706–707
Doi M, Nakajima Y, Okano T, Fukada Y (2001) Light-induced phase-delay of the chicken pineal circadian clock is associated with the induction of cE4bp4, a potential transcriptional repressor of cPer2 gene. Proc Natl Acad Sci USA 98:8089–8094
Dryer SE, Henderson D (1991) A cyclic GMP-activated channel in dissociated cells of the chick pineal gland. Nature 353: 756–758
Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290
Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679
Eskin A, Takahashi JS (1983) Adenylate cyclase activation shifts the phase of a circadian pacemaker. Science 220:82–84
Fu Z, Inaba M, Noguchi T, Kato H (2002) Molecular cloning and circadian regulation of cryptochrome genes in Japanese quail (*Coturnix coturnix japonica*). J Biol Rhythms (in press)
Ganguly S, Coon SL, Klein DC (2002) Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. Cell Tissue Res DOI 10.1007/s00441-002-0579-y
Gastel JA, Roseboom PH, Rinaldi PA, Weller JL, Klein DC (1998) Melatonin production: proteasomal proteolysis in serotonin N-acetyltransferase regulation. Science 279:1358–1360
Gillette MU, Mitchell JW (2002) Signaling in the SCN: selectively responsive and integrative. Cell Tissue Res DOI 10.1007/s00441-002-0576-1
Glossop NR, Lyons LC, Hardin PE (1999) Interlocked feedback loops within the *Drosophila* circadian oscillator. Science 286:766–768
Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283–301
Green CB, Besharse JC, Zatz M (1996) Tryptophan hydroxylase mRNA levels are regulated by the circadian clock, temperature, and cAMP in chick pineal cells. Brain Res 738:1–7
Gwinner E (1989) Melatonin in the circadian system of birds: model of internal resonance. In: Hiroshige T, Honma K (ed) Circadian clocks and ecology, Hokaido Press, Sapporo, pp 127–145
Hall JC (2000) Cryptochromes: sensory reception, transduction, and clock functions subserving circadian systems. Curr Opin Neurobiol 10:456–466
Hannibal J (2002) Neurotransmitters of the retino-hypothalamic tract. Cell Tissue Res DOI 10.1007/s00441-002-0574-3
Harrison NL, Zatz M (1989) Voltage-dependent calcium channels regulate melatonin output from cultured chick pineal cells. J Neurosci 9:2462–2467
Hayashi Y, Sanada K, Fukada Y (2001) Circadian and photic regulation of MAP kinase by Ras- and protein phosphatase-dependent pathways in the chick pineal gland. FEBS Lett 491:71–75
Hirunagi K, Ebihara S, Okano T, Takanaka Y, Fukada Y (1997) Immunoelectron-microscopic investigation of the subcellular localization of pinopsin in the pineal organ of the chicken. Cell Tissue Res 289:235–241
Kasahara T, Okano T, Yoshikawa T, Yamazaki K, Fukada Y (2000) Rod-type transducin alpha-subunit mediates a phototransduction pathway in the chicken pineal gland. J Neurochem 75:217–224
Kasal CA, Menaker M, Perez-Polo JR (1979) Circadian clock in culture: N-acetyltransferase activity of chick pineal glands oscillates in vitro. Science 203:656–658
Kato H, Fu Z, Kotera N, Sugahara K, Kubo T (1999) Regulation of the expression of serotonin N-acetyltransferase gene in Japanese quail (*Coturnix japonica*). I. Rhythmic pattern and effect of light. J Pineal Res 27:24–33
Klein DC (1985) Photoneural regulation of the mammalian pineal gland. Ciba Found Symp 117:38–56
Ko GY, Ko ML, Dryer SE (2001) Circadian regulation of cGMP-gated cationic channels of chick retinal cones. Erk MAP kinase and Ca2+/calmodulin-dependent protein kinase II. Neuron 29:255–266
Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116
Korf HW (1994) The pineal organ as a component of the biological clock. Phylogenetic and ontogenetic considerations. Ann N Y Acad Sci 719:13–42
Korf HW, Schomerus C, Stehle JH (1998) The pineal organ, its hormone melatonin, and the photoneuroendocrine system. Springer, Berlin Heidelberg New York
Kuwata O, Imamoto Y, Okano T, Kokame K, Kojima D, Matsumoto H, Morodome A, Fukada Y, Shichida Y, Yasuda K, et al (1990) The primary structure of iodopsin, a chicken red-sensitive cone pigment. FEBS Lett 272:128–132
Kyriacou CP, Rosato E (2000) Squaring up the E-box. J Biol Rhythms 15:483–490
Larkin P, Baehr W, Semple-Rowland SL (1999) Circadian regulation of iodopsin and clock is altered in the retinal degeneration chicken retina. Brain Res Mol Brain Res 70:253–263
Maronde E, Pfeffer M, Olcese J, Molina CA, Schlotter F, Dehghani F, Korf HW, Stehle JH (1999) Transcription factors in neuroendocrine regulation: rhythmic changes in pCREB and ICER levels frame melatonin synthesis. J Neurosci 19:3326–3336
Matsushita A, Yoshikawa T, Okano T, Kasahara T, Fukada Y (2000) Colocalization of pinopsin with two types of G-protein alpha-subunits in the chicken pineal gland. Cell Tissue Res 299:245–251
Max M, McKinnon PJ, Seidenman KJ, Barrett RK, Applebury ML, Takahashi JS, Margolskee RF (1995) Pineal opsin: a non-visual opsin expressed in chick pineal. Science 267:1502–1506
Max M, Surya A, Takahashi JS, Margolskee RF, Knox BE (1998) Light-dependent activation of rod transducin by pineal opsin. J Biol Chem 273:26820–26826
Miyamoto Y, Sancar A (1999) Circadian regulation of cryptochrome genes in the mouse. Brain Res Mol Brain Res 71:238–243
Murakami N, Nakamura H, Nishi R, Marumoto N, Nasu T (1994) Comparison of circadian oscillation of melatonin release in pineal cells of house sparrow, pigeon and Japanese quail, using cell perfusion systems. Brain Res 651:209–214

Nakahara K, Murakami N, Nasu T, Kuroda H, Murakami T (1997) Individual pineal cells in chick possess photoreceptive, circadian clock and melatonin-synthesizing capacities in vitro. Brain Res 774:242–245

Norgren RB Jr, Silver R (1989) Retinohypothalamic projections and the suprachiasmatic nucleus in birds. Brain Behav Evol 34:73–83

Obrietan K, Impey S, Storm DR (1998) Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat Neurosci 1:693–700

Okano T, Kojima D, Fukada Y, Shichida Y, Yoshizawa T (1992) Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proc Natl Acad Sci USA 89:5932–5936

Okano T, Yoshizawa T, Fukada Y (1994) Pinopsin is a chicken pineal photoreceptive molecule. Nature 372:94–97

Okano T, Yamamoto K, Okano K, Hirota T, Kasahara T, Sasaki M, Takanaka Y, Fukada Y (2001) Chicken pineal clock genes: implication of BMAL2 as a bidirectional regulator in circadian clock oscillation. Genes Cells 6:825–836

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22:153–183

Pévet P, Bothorel B, Slotten H, Saboureau M (2002) The chronobiotic properties of melatonin. Cell Tissue Res DOI 10.1007/s00441-002-0584-1

Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD (1998) Melanopsin: an opsin in melanophores, brain, and eye. Proc Natl Acad Sci USA 95:340–345

Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD (2000) A novel human opsin in the inner retina. J Neurosci 20:600–605

Reppert SM, Weaver DR (2000) Comparing clockworks: mouse versus fly. J Biol Rhythms 15:357–364

Reppert S, Weaver D (2001) Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63:647–676

Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9:180–186

Sanada K, Hayashi Y, Harada Y, Okano T, Fukada Y (2000) Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. J Neurosci 20:986–991

Sancar A (2000) Cryptochrome: the second photoactive pigment in the eye and its role in circadian photoreception. Annu Rev Biochem 69:31–67

Schomerus C, Korf HW, Laedtke E, Weller JL, Klein DC (2000) Selective adrenergic/cyclic AMP-dependent switch-off of proteasomal proteolysis alone switches on neural signal transduction: an example from the pineal gland. J Neurochem 75:2123–2132

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, Horst GT van der, Hastings MH, Reppert SM (2000) Interacting molecular loops in the mammalian circadian clock. Science 288:1013–1019

Stanewski R (2002) Clock mechanisms in *Drosophila*. Cell Tissue Res DOI 10.1007/s00441-002-0569-0

Stehle JH, Foulkes NS, Molina CA, Simonneaux V, Pevet P, Sassone-Corsi P (1993) Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. Nature 365:314–320

Sterne L (1760) The life and opinions of Tristram Shandy, 3rd edn. Penguin Books, London

Takahashi JS, Murakami N, Nikaido SS, Pratt BL, Robertson LM (1989) The avian pineal, a vertebrate model system of the circadian oscillator: cellular regulation of circadian rhythms by light, second messengers, and macromolecular synthesis. Recent Prog Horm Res 45:279–352

Takanaka Y, Okano T, Iigo M, Fukada Y (1998) Light-dependent expression of pinopsin gene in chicken pineal gland. J Neurochem 70:908–913

Takao M, Yasui A, Tokunaga F (1988) Isolation and sequence determination of the chicken rhodopsin gene. Vision Res 28:471–480

Tosini G, Fukuhara C (2002) The mammalian retina as a clock. Cell Tissue Res DOI 10.1007/s00441-002-0578-z

Tosini G, Menaker M (1996) Circadian rhythms in cultured mammalian retina. Science 272:419–421

Underwood H, Steele CT, Zivkovic B (2001) Circadian organization and the role of the pineal in birds. Microsc Res Tech 53:48–62

Yamaguchi S, Mitsui S, Yan L, Yagita K, Miyake S, Okamura H (2000) Role of DBP in the circadian oscillatory mechanism. Mol Cell Biol 20:4773–4781

Yamamoto K, Okano T, Fukada Y (2001) Chicken pineal *Cry* genes: light-dependent up-regulation of cCry1 and cCry2 transcripts. Neurosci Lett 313:13–16

Yoshimura T, Suzuki Y, Makino E, Suzuki T, Kuroiwa A, Matsuda Y, Namikawa T, Ebihara S (2000) Molecular analysis of avian circadian clock genes. Brain Res Mol Brain Res 78:207–215

Yoshimura T, Yasuo S, Suzuki Y, Makino E, Yokota Y, Ebihara S (2001) Identification of the suprachiasmatic nucleus in birds. Am J Physiol Regul Integr Comp Physiol 280:R1185–1189

Zatz M (1992) Does the circadian pacemaker act through cyclic AMP to drive the melatonin rhythm in chick pineal cells? J Biol Rhythms 7:301–311

Zatz M (1994) Photoendocrine transduction in cultured chick pineal cells. IV. What do vitamin A depletion and retinaldehyde addition do to the effects of light on the melatonin rhythm? J Neurochem 62:2001–2011

Zatz M (1996) Melatonin rhythms: trekking toward the heart of darkness in the chick pineal. Cell Dev Biol 7:811–820

Zatz M, Mullen DA (1988a) Two mechanisms of photoendocrine transduction in cultured chick pineal cells: pertussis toxin blocks the acute but not the phase-shifting effects of light on the melatonin rhythm. Brain Res 453:63–71

Zatz M, Mullen DA (1988b) Photoendocrine transduction in cultured chick pineal cells. II. Effects of forskolin, 8-bromocyclic AMP, and 8-bromocyclic GMP on the melatonin rhythm. Brain Res 453:51–62

Zatz M, Mullen DA, Moskal JR (1988) Photoendocrine transduction in cultured chick pineal cells: effects of light, dark, and potassium on the melatonin rhythm. Brain Res 438:199–215

Zatz M, Gastel JA, Heath JR IIIrd, Klein DC (2000) Chick pineal melatonin synthesis: light and cyclic AMP control abundance of serotonin N-acetyltransferase protein. J Neurochem 74: 2315–2321