Hypothalamic Circadian Organization in Birds. II.
Clock Gene Expression

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ABSTRACT

While the site of the major circadian pacemaker in mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus, is very well characterized, little is known about hypothalamic circadian organization in birds. This paper reviews recent findings on clock gene expression in the hypothalamus of several bird species focusing on circadian pPer2 expression in the house sparrow. In contrast to mammals, rhythmic Per2 gene expression in the house sparrow hypothalamus is not restricted to a single cell group but occurs in two distinct hypothalamic nuclei, the SCN and the lateral hypothalamic nucleus (LHN). The complex temporal and spatial distribution of pPer2 expression suggests a longitudinal compartmentalization of the SCN with period gene expression being initiated in the most rostral portion before lights on. In the lateral hypothalamus, phasing of pPer2-rhythmcity appeared delayed. In pinealectomized house sparrows, the overall circadian pPer2 expression pattern is maintained indicating that rhythmic pPer2 transcription in the SCN and LHN of the house sparrow are not driven by the pineal gland. Rather, they reflect the activity of autonomous hypothalamic circadian oscillators. Certain changes in peak expression levels and the expression phase, however, suggest that the pineal melatonin rhythm affects both the phase and the amplitude of rhythmic hypothalamic pPer2 expression.

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The discovery of genes as essential components of endogenous circadian clocks initiated tremendous progress in understanding the regulatory mechanisms of daily rhythms in a variety of animals. Initially, in *Drosophila* a mutation with an abnormally long period of the activity rhythm was discovered (Konopka and Benzer 1971). The responsible gene was called *Period* (*Per*) and its deletion resulted in the abolishment of the circadian activity rhythm under constant conditions (Bargiello et al., 1984; Reddy et al., 1984). The first mammalian gene associated with circadian function was discovered in mice by means of mutagenesis and subsequent screening for circadian clock mutations. It was called *Clock* (Vitaterna et al., 1994). Like *Per* in *Drosophila*, a mutation of *Clock* lengthened the circadian period and abolished circadian rhythms of mice kept in constant conditions. Since then, a number of other clock genes have been identified in several mammalian species including three different homologues (*Per1*, *Per2*, and *Per3*) of the *Drosophila period* gene (Albrecht et al., 1997; Nagase et al., 1997; Sun et al., 1997; Takumi et al., 1998a; Takumi et al., 1998b; Tei et al., 1997; Zylka et al., 1998).

In the major circadian pacemaker of mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1991), a molecular “clockwork” that involves interacting positive and negative transcriptional and post-transcriptional feedback loops driving recurrent rhythms in mRNA and protein levels of clock genes provides the basis for circadian rhythm generation (Okamura et al., 2002; Reppert and Weaver, 2002). Clock genes are rhythmically expressed in several other brain areas as well, including the pineal gland and the retina (Abe et al., 2002; Dunlap, 1999). Although the circadian expression patterns differ between genes and tissues, they display the same phase relationship to each other in every cell, indicating that all clock genes are apparently linked and controlled by interacting autoregulatory transcriptional–translational feedback loops. Loss or mutation of specific clock genes can lead to altered period length and gradual or immediate loss of circadian behavior under constant conditions (Bae et al., 2001; Cermakian et al., 2001; Shearman et al., 2000; Zheng et al., 1999; Zheng et al., 2001). For example, *Per1*/*Per2* double mutant mice (Bae et al., 2001; Zheng et al., 2001) lose circadian rhythmicity immediately under constant conditions, demonstrating the importance of these genes in the clock mechanism. However, *Per3* seems to be of less importance because deletion of *Per3* has only a weak effect on circadian behavior (Shearman et al., 2000). Additionally, both *mPer1* and *mPer2* seem to be involved in the phase shifting and resetting properties of light (Albrecht et al., 1997; Albrecht et al., 2001; Shearman et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998). While *Per1* is rhythmically expressed in several other brain areas, including the pituitary gland, some hypothalamic and thalamic nuclei, olfactory bulb, cerebellum, and retina (Reppert et al., 2002; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997), rhythmic *Per2* expression is predominantly found in the mammalian SCN (Takumi et al., 1998).

The discovery of the core autoregulatory feedback loop and the subsequent search for clock genes in tissues outside the central nervous system revealed that molecular oscillations are almost ubiquitous. They have been demonstrated in a variety of tissues including liver, skeletal muscle, kidney, lung, heart, testis, and even in cultured rat fibroblasts following a serum shock (Balsalobre et al., 1998; Yamazaki et al., 2000;
Zylka et al., 1998). Although recent studies indicate that circadian function in peripheral tissues has more tissue-specific characteristics than expected (Panda et al., 2002; Storch et al., 2002), the molecular core mechanism seems to be similar in both central and peripheral clocks, with the phase of circadian clock gene expression in peripheral tissues (including the pineal gland and retina) being delayed by approximately 4 hours as compared to the SCN (Abe et al., 2002; Yamazaki et al., 2000). In contrast to the SCN, oscillations in peripheral tissues (with exception of the retina) damp in constant conditions in vitro (Balsalobre et al., 1998; Stokkan et al., 2001; Yamazaki et al., 2000) and are lost or desynchronized after SCN lesions in vivo (Sakamoto et al., 1998).

In birds, a certain number of clock genes with high-sequence identities to mammalian clock genes have recently been cloned and localized in retina, pineal gland, brain, and several peripheral tissues in different species, including chicken, Japanese quail, and house sparrow. The identified genes include Per2, Per3, Clock, Bmal1, Cry1, and Cry2 (Bailey et al., 2002; Brandstätter et al., 2000; Chong et al., 2000; Yoshimura et al., 2000; Doi et al., 2001; Fu et al., 2002; Okano et al., 2001; Yamamoto et al., 2001). However, little is known about the molecular mechanisms orchestrating circadian function in birds and various differences to mammals suggest that the molecular clockwork in birds is indeed distinct from that in mammals as, for example, indicated by the lack of an avian homologue of Per1 and differences in phasing of some clock genes (Abraham et al., 2002; Brandstätter et al., 2001; Yoshimura et al., 2000).

In general, the circadian pacemaking system of birds is built up by circadian oscillators located in the pineal gland, the retina, and the hypothalamus [Fig. 1(A)]. The functional significance of these individual oscillators for regulating rhythmicity of the organism varies considerably between those species that have been investigated until now. However, melatonin released from the pineal gland alone or from the pineal gland and the retina plays a major role in most avian species with the obvious exception of some galliform birds, such as the Japanese quail (for a recent review see Brandstätter, 2002). Additionally, lesions targeted on the suprachiasmatic hypothalamus resulted in severe disruptions of circadian activity rhythms in house sparrows, Java sparrows, pigeons, and Japanese quail (Ebihara and Kawamura, 1981; Ebihara et al., 1987; Simpson et al., 1981; Takahashi and Menaker, 1982). Until recently, no coherent information was available on whether the avian hypothalamus indeed contains circadian oscillators and where they might be localized. With regard to the question of a hypothalamic circadian oscillator in birds, the present paper reviews recent findings on Per2 expression in the hypothalamus of house sparrows as compared to other bird species as well as mammals.

We cloned a 688 bp fragment of the house sparrow Per2 [pPer2; sequence identity of 77% to mouse Per2 and 96% to quail Per2 (Brandstätter et al., 2001)]. Using in situ hybridization pPer2 expression was detected in the retina, the pineal gland, as well as various hypothalamic cell groups, including the preoptic nucleus, the SCN and the lateral hypothalamic nucleus (LHN) [Fig. 1(B), (C), (D)]. Quantitative investigation of pPer2 expression patterns in the house sparrow hypothalamus revealed a rhythm of transcription in LD as well as in DD conditions in the SCN and LHN (Abraham et al., 2002) (Figs. 2 and 3). In LD, pPer2 expression was detected in the most rostral portion of the SCN before “light on” at Zeitgeber time (ZT) 24, followed by an extension of the signal throughout the whole nucleus at ZT6 (middle of the light phase). The pPer2 signal declined to low levels at ZT12 (shortly before “lights off”) and background levels at ZT18 (middle of the dark phase) (Figs. 2 and 3). A similar temporal and spatial expression pattern was observed in...
DD, however, peak expression at circadian time (CT) 6 was reduced as compared to LD and pPer2 transcription at CT12 had already reached background levels (Figs. 2 and 3). This complex spatio-temporal pattern of clock gene expression suggests a certain compartmentalization of the SCN, with transcription being initiated in the most rostral portion of the SCN and subsequently expanding to the more caudal parts of the nucleus and to the LHN.

The LHN displayed a different phasing of pPer2 expression than the SCN: while no in situ hybridization signal was found at ZT24, pPer2 expression was high at ZT6 and ZT12 and declined to background levels at ZT18 (Figs. 2 and 3). This expression pattern was retained in DD. In contrast to the situation in the SCN, peak expression levels at CT6 and CT12 were not reduced as compared to LD, but appeared even slightly elevated (Figs. 2 and 3).

pPer2 expression in the rostral SCN appears in phase with the pineal gland, a well known circadian pacemaker in the house sparrow, whereas transcription in the medial SCN

Figure 1. pPer2 expression in circadian oscillators of the house sparrow. (A) Diagram of the major components of the avian circadian pacemaking system. Oscillatory components are indicated by clock symbols, photoreceptive structures are indicated by yellow arrows. Hormonal signal pathways are drawn in red; neural pathways are drawn in blue; EP, encephalic photoreceptors; the diagram is not indicative for a certain species but summarizes information that has been obtained in chicken, house sparrow, pigeon, and Japanese quail. For details see (Brandstätter, 2003). B, C, D: Cross-sections through the retina (B), pineal gland (C), and suprachiasmatic hypothalamus (D) showing pPer2 expression (blue = Hoechst-stained cell nuclei; red = in situ hybridization signal, probe: anti sense pPer2; red). Inserts in B and C show control sections (probe: sense pPer2). In the hypothalamus (D), pPer2 is expressed in the preoptic nucleus (yellow arrows), in the SCN (light blue arrow), and in the LHN (green arrows). Scale bars = 200 µm. (See color insert at end of issue.)
and in the LHN appear phase-delayed to the pineal gland and the rostral SCN (Fig. 2). In regard of the situation in mammals, where oscillations in various brain regions and in peripheral organs have been found to be phase-delayed to those in the SCN we may assume that the temporal sequence of clock gene expression is a certain indication for the hierarchical organization of the system as a whole. Oscillators that are driven or synchronized by others are delayed to those that act as the major pacemakers. Thus, the pineal gland and the most rostral portion of the SCN, where *pPer2* expression is in phase with each other, might represent the sites of autonomous circadian oscillations in the house sparrow. To test for this hypothesis, hypothalamic *pPer2* expression was investigated in pinealectomized house sparrows. Initiation of the *pPer2* signal at CT24 in the most rostral portion of the SCN was indeed unaffected by pinealectomy whereas other features of the *pPer2* rhythm such as peak expression levels at CT6 and the decline at CT12 were different from intact animals. In the LHN, *pPer2* expression showed identical phasing but a reduced amplitude when comparing pinealectomized to intact house sparrows (Fig. 3). These data suggest that the region of the SCN where the *pPer2* signal starts before “light on” in light/dark conditions as well as before subjective “light on” in constant conditions is less dependent on the pineal oscillator as compared to the medial SCN and the LHN. The damped but elongated *pPer2* expression in the medial SCN of pinealectomized birds goes along with a gradual extension of the activity phase into arrhythmicity following pinealectomy (Brandstätter, 2002; Ebihara and Kawamura, 1981; Ebihara et al., 1987).

Figure 2. Temporal and spatial variations of *pPer2* expression in the pineal gland, SCN and LHN of the house sparrow. Cross-sections through the pineal gland (A), the rostral SCN (B), the medial SCN (C), and the LHN (D) at four different Zeitgeber times in LD and at four different circadian times in DD. White = in situ hybridization signal. *Per2* expression in the pineal gland is in phase with the rostral SCN, whereas it is delayed in the medial SCN and in the LHN. For details see text.
Figure 3. *pPer2* expression patterns in the SCN and LHN of the house sparrow in LD, DD, and DD following pinealectomy. Average areas (%) covered by in situ hybridization product in the rostral (●) and medial (○) SCN (upper graphs) as well as in the LHN (▼; lower graphs) at four different time points in intact birds in LD and DD and in pinealectomized birds in DD (PinX). For details see text and Abraham et al. (2002).
Considering that two anatomically distinct cell groups, the SCN and the LHN (Brandstätter and Abraham, 2003), exhibit circadian rhythms in clock gene expression, we may assume the presence of at least two circadian oscillators in the hypothalamus of the house sparrow. However, to which degree these cell groups are involved in regulating circadian rhythmicity of the animal is still elusive but a certain role in circadian pacemaking is strongly indicated by SCN lesions disturbing circadian activity rhythms in all species investigated until now (Ebihara and Kawamura, 1981; Ebihara et al., 1987; Simpson et al., 1981; Takahashi and Menaker, 1982). Although no obvious sign of a diurnal or circadian rhythmicity was found until now in other parts of the hypothalamus, it cannot be excluded that structures like the preoptic nucleus are also linked to circadian function. Additionally, further parts of the brain that express putative clock genes in birds, such as the cerebellum and the optic tectum (Yoshimura et al., 2000), have to be investigated in future studies. Nevertheless, in connection with the ongoing discussion about the localization of the circadian oscillator in the avian hypothalamus, our studies expand current knowledge by demonstrating that, unlike in mammals, rhythmic Per gene expression in the SCN is not confined to a single cell group in birds. Rather, our present results indicate a complex organization of the circadian oscillator in the hypothalamus of the house sparrow, such that molecular circadian oscillations can be found in two cell groups, the SCN and the LHN.

Our data on clock gene expression in the SCN of the house sparrow are comparable to circadian rhythms of Per2 transcription in the SCN of the Japanese quail (Yasuo et al., 2002; Yoshimura et al., 2001) with the phasing of quail Per2 (qPer2) expression being similar to that in house sparrows. Additionally, circadian rhythms of qPer3 transcription have been found in the SCN of the Japanese quail with peak expression levels at ZT/CT24, whereas qClock did not exhibit a pronounced rhythmicity. However, Per2 expression could not be detected in the lateral hypothalamus of the Japanese quail, the Java sparrow (Padda oryzivora), the chicken (Gallus gallus domesticus) and the pigeon (Columba livia) (Yasuo et al., 2002; Yoshimura et al., 2001). In contrast, cloning and localization of another clock gene in the chicken that is part of the circadian autoregulatory feedback loop in mammals, Cry2, support the findings in the LHN of the house sparrow since Cry2 expression was found in the chicken SCN but also in the retinorecipient part of the LHN (Bailey et al., 2002; Yamamoto et al., 2001). However, Cry2 expression is widespread throughout structures associated with the visual system in the chicken brain, and whether avian Cry2 is a component of the core clock mechanism as in mammals, or whether it is predominantly associated with photic input to the circadian system as inferred from findings in other non-mammalian vertebrates is still unknown (Bailey et al., 2002).

As compared to mammals, the phasing of pPer2 transcription in birds appears more similar to mammalian Per1 than to mammalian Per2, or at least “intermediate”: Mammalian Per1 starts to increase before CT24, peaks at CT4, and declines to lower levels by CT12 (Yan and Okamura, 2002; Yan et al., 1999), whereas the rhythm of mammalian Per2 expression is phase-delayed by approximately 4 hours (Albrecht et al., 1997; Takumi et al., 1998; Yan and Okamura, 2002; Zheng et al., 1999). Thus, we may assume that Per2 in birds is the functional correlate of Per1 in mammals and that only two Per genes (Per2, Per3) are present in birds, suggesting that the molecular autoregulatory feedback loop is indeed different from mammals.

When summarizing available data on clock gene expression in the avian hypothalamus it becomes evident that birds possess an autonomous hypothalamic circadian
oscillator. Since hypothalamic lesions had effects on behavioral rhythmicities in all species investigated, we may assume that hypothalamic circadian oscillations indeed play an important role in regulating circadian rhythmicity of behavior. This role may vary between species as it has been shown that the circadian pacemaking system is highly diverse in birds and the functional importance of individual components is distinct between species. For example, the importance of the pineal gland in the circadian system of house sparrows becomes evident in pinealectomized birds whose circadian rhythms of hopping, feeding, and body temperature gradually become arrhythmic after release into constant conditions (Binkley et al., 1971; Gaston and Menaker, 1968). Arrhythmicity in pinealectomized house sparrows in DD is due to the lack of a melatonin rhythm, consistent with the fact that the pineal gland is the only source of rhythmic plasma melatonin (Janik et al., 1992). Periodic administration of melatonin synchronized the activity of pinealectomized house sparrows in constant conditions (Heigl and Gwinner, 1994; Lu and Cassone, 1993b) but not that of intact birds (Murakami et al., 2001). Moreover, the abolishment of the endogenous melatonin rhythm by continuous melatonin administration clearly destabilized the circadian activity rhythm (Binkley and Mosher, 1985; Hendel and Turek, 1978; Turek et al., 1976). Thus, at least in the house sparrow, a rhythmic melatonin signal is a prerequisite for circadian rhythmicity in constant conditions; nevertheless, animals with an intact pineal gland bearing hypothalamic lesions are not able to maintain circadian activity (Gaston and Menaker, 1968; Takahashi and Menaker, 1982). Hence, circadian behavior is either driven by two spatially separated oscillators in the pineal gland and in the hypothalamus with the necessity of dual input signals at target sites that regulate circadian rhythmicity of the organism, or the hypothalamic oscillator functions as a gate for the pineal output rhythm and mediates the melatonin signal to target sites. Melatonin receptors and melatonin binding sites are widespread throughout the avian brain, with great similarity among species. 2[125I]iodomelatonin binding occurs in most areas associated with tectofugal, thalamofugal, accessory, and hypothalamic visual pathways in a variety of avian species, including the house sparrow. Most notably, melatonin binding was found in the retinorecipient part of the LHN but not in the SCN (Aste et al., 2001; Cassone and Brooks, 1991; Cassone et al., 1995). The damped rhythm of Per gene expression in the LHN of pinealectomized house sparrows corresponds well with the presence of melatonin binding sites as well as previous studies reporting a gradual damping of the rhythm of 2-deoxyglucose uptake and of iodomelatonin binding in the retinorecipient part of the LHN of pinealectomized house sparrows and chicken (Brooks and Cassone, 2002; Cassone, 1988; Lu and Cassone, 1993a). However, it remains to be investigated whether hypothalamic Per2 expression is damping all over the SCN and LHN concomitantly with the loss of activity rhythms of pinealectomized birds or whether it continues to oscillate despite arrhythmic behavior after a longer time in constant conditions. Nevertheless, these data indicate a certain role for melatonin in the modulation of molecular and physiological circadian oscillations in the house sparrow hypothalamus. Altered Per gene rhythms in the LHN as well as in the medial portion of the SCN after pinealectomy suggest that not only the LHN but also the SCN may contain functional melatonin receptors or, if this is not the case, that the LHN transmits information about the presence of melatonin to the SCN. Recently, rhythmic melatonin receptor mRNA expression was found in the SCN of the Japanese quail but exogenous melatonin had no effect on clock gene expression (Yasuo et al., 2002). In contrast to house sparrows, disrupted locomotor rhythms of Japanese quail kept in constant dim light did not entrain to daily melatonin application (Murakami et al., 2001).
2001) and neuronal signals from the eyes have been shown to play a major role in maintaining circadian rhythmicity leaving only a minor role for melatonin in this species (Underwood et al., 1990). Additionally, the demonstration of melatonin receptor mRNA does not necessarily imply the presence of a functional melatonin receptor (Reppert et al., 1995). The presence of consistently high melatonin caused by implants (Yasuo et al., 2002) might anyhow be without effect when receptor up- and down-regulation is controlled by a cell-autonomous circadian mechanism and, thus, melatonin responsiveness rather than the presence of melatonin is critical for mediating a possible effect of melatonin.

Taken together, effects of melatonin could be mediated by melatonin responsive components of damped circadian oscillators in the hypothalamus that require a rhythmic melatonin signal to maintain a high-amplitude rhythm of activity. Without any doubt, hypothalamic pPer2 expression is not directly driven by the pineal gland since pPer2 rhythmicity is present in birds lacking a pineal gland. Moreover, reduced peak levels in the SCN and LHN and extensions of the phase of elevated expression of pPer2 in the medial portion of the SCN are, at least to a certain degree, compatible with the hypothesis that the abolition of the endogenous melatonin rhythm following pinealectomy may affect self-sustainment of the hypothalamic oscillator and, thus, of the circadian pacemaking system as a whole (Gwinner, 1989).

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