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Modulation of metabolic and clock gene mRNA rhythms by pineal and retinal circadian oscillators

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Abstract

Avian circadian organization involves interactions between three neural pacemakers: the suprachiasmatic nuclei (SCN), pineal, and retina. Each of these structures is linked within a neuroendocrine loop to influence downstream processes and peripheral oscillations. However, the contribution of each structure to drive or synchronize peripheral oscillators or circadian outputs in avian species is largely unknown. To explore these interactions in the chick, we measured 2-deoxy [¹⁴C]-glucose (2DG) uptake and mRNA expression of the chick clock genes *bmal1*, *cry1*, and *per3* in three brain areas and in two peripheral organs in chicks that underwent pinealectomy, enucleation, or sham surgery. We found that 2DG uptake rhythms damp under constant darkness in intact animals, while clock gene mRNA levels continue to cycle, demonstrating that metabolic rhythms are not directly driven by clock gene transcription. Moreover, 2DG rhythms are not phase-locked to rhythms of clock gene mRNA. However, pinealectomy and enucleation had similar disruptive effects on both metabolic and clock gene rhythms, suggesting that both of these oscillators act similarly to reinforce molecular and physiological rhythms in the chicken. Finally, we show that the relative phasing of at least one clock gene, *cry1*, varies between central and peripheral oscillators in a tissue specific manner. These data point to a complex, differential orchestration of central and peripheral oscillators in the chick, and, importantly, indicate a disconnect between canonical clock gene regulation and circadian control of metabolism.

Keywords

Circadian; Avian; Chicken; Pineal; Eyes; Retina; Metabolism; 2DG uptake; Clock gene; Peripheral; Brain; Heart; Liver

1. Introduction

Avian circadian organization is complex, consisting of an interplay between at least three separate oscillators located in the pineal, the eyes, and the avian suprachiasmatic nucleus (SCN) (Gwinner and Brandstätter, 2001; Underwood et al., 2001). It has been proposed that these

three structures contain damped oscillators which interact within a neuroendocrine loop to sustain rhythmicity over multiple cycles (Cassone and Menaker, 1984). Specifically, this model hypothesizes that the avian pineal and retina inhibit SCN activity during the night by secretion of melatonin and/or via neurotransmission, while the SCN inhibits melatonin production in the pineal during the day.

This is only a generalized model for avian species, however, as the specific interactions between circadian oscillators are hierarchical and species dependent. For example, pinealectomy has a greater effect on overt rhythms in passerine birds such as the house sparrow than on galliform species such as chicken and quail (Underwood et al., 2001; Bell-Pedersen et al., 2005). In the latter two species, the eyes have been demonstrated to play a greater role, as ocular enucleation, but not pinealectomy, abolishes activity rhythms in these animals (Nyce and Binkley, 1977; Underwood, 1994). This regulatory role for avian eyes does not correlate with their contribution to circulating melatonin, however. For instance, the retinæ of quail secrete up to 50% of the plasma levels of melatonin, the pineal being responsible for the remaining half (Underwood, 1994). In contrast, the eyes of chickens release very little, if any, detectable amounts of melatonin in the bloodstream (Reppert and Sagar, 1983; Cogburn et al., 1987). Overall, these studies highlight a major organizational difference between birds and mammals, where an intact SCN is necessary and sufficient to sustain rhythmicity under constant conditions. Still, in birds, pinealectomy and/or enucleation often abolishes rhythms gradually, suggesting a damping of rhythmic SCN activity occurs (Underwood et al., 2001).

Radioligand binding studies using 2-[¹²⁵I]iodomelatonin (IMEL) indicate that melatonin binding is widespread in the avian nervous system, most prominently within visual structures, including the retina and the visual suprachiasmatic nuclei (vSCN) (Dubocovich and Takahashi, 1987; Rivkees et al., 1989; Cassone et al., 1995). Birds express three melatonin receptor subtypes, Mel_{1A}, Mel_{1B}, and Mel_{1C}, which also have widespread but differential spatial distributions in the brain (Reppert et al., 1995, 1996; Natesan and Cassone, 2002). Furthermore, melatonin receptor mRNA levels, as well as IMEL binding, are rhythmic in some neuronal structures, including the SCN (Lu and Cassone, 1993b; Natesan and Cassone, 2002).

As an endocrine output of the circadian system, melatonin plays an important role in synchronizing internal rhythms. For instance, melatonin entrains behavioral and metabolic rhythms (as measured by uptake of the metabolic marker 2-deoxy[¹⁴C]-glucose, or 2DG) in birds (Lu and Cassone, 1993a,b; Adachi et al., 2002; Cantwell and Cassone, 2002) as well as activity rhythms in mammals and neuronal firing rhythms of mammalian SCN tissue *in vitro* (Redman et al., 1983; Starkey et al., 1995). In house sparrow, both metabolic and IMEL binding rhythms in the SCN are abolished by pinealectomy and restored by rhythmic melatonin administration (Lu and Cassone, 1993a,b). These studies provide good evidence that the SCN pacemaker is entrained by melatonin.

Less is known about how melatonin influences peripheral tissues, although studies characterizing autoradiographic binding and molecular receptor distribution in birds and mammals suggest melatonin may act on heart, lung, kidney, gut, gonads, and circulatory vasculature, although the density and distribution of receptors in these sites are highly species dependent (Pang et al., 1993, 1996; Lee et al., 1995; Wan and Pang, 1995; Drew et al., 2001; Poon et al., 2001; Naji et al., 2004). In rodents, melatonin is known to regulate the expression of multiple clock genes within the pars tuberalis, a site with a high density of melatonin receptors (Pévet et al., 2006).

In addition to regulating glucose metabolism, circadian clocks are linked to numerous other metabolic processes, including lipo-genesis, xenobiotic metabolism, and cellular redox state (Rutter et al., 2001; Duffield, 2003; Wijnen and Young, 2006; Duez and Staels, 2007; Kohsaka

and Bass, 2007). Also, circadian mutant mice exhibit a range of metabolic defects, including hyperphagia, obesity, and impaired carbohydrate metabolism (Rudic et al., 2004; Turek et al., 2005; Oishi et al., 2006; Kohsaka and Bass, 2007). These data highlight an intimate linkage between circadian clocks and metabolism. Many of these processes are likely controlled via tissue specific circadian regulatory pathways.

In peripheral tissues, the core transcriptional feedback loop based on the positive and negative regulatory limbs (composed of *bmal/clock* and *per/cry* genes, respectively) is preserved (Yagita et al., 2001; Stratmann and Schibler, 2006; Hastings et al., 2007). As with pacemaker tissues, these gene products oscillate autonomously, though they damp over time as a result of desynchronization between individual cellular oscillators (Balsalobre et al., 1998; Yamazaki et al., 2000; Yoo et al., 2004; Guo et al., 2005, 2006). Presumably, it is the oscillations of these canonical clock gene products which drive rhythms in local physiological processes within peripheral tissues.

In both birds and mammals, the SCN coordinates the circadian physiology of multiple organ systems by synchronizing peripheral clocks via both neural and humoral mechanisms (Bell-Pedersen et al., 2005; Stratmann and Schibler, 2006; Kalsbeek et al., 2007). However, it is not known how multiple pacemakers interact to coordinate peripheral oscillators in complex avian systems. In this study, we profile mRNA rhythms of both positive and negative clock genes in multiple central and peripheral tissues in chick, and monitor 2DG uptake as an important circadian output in these tissues. Although numerous peripheral tissues contain self-sustaining circadian oscillators (Yoo et al., 2004; Stratmann and Schibler, 2006), we have decided to focus on liver and heart, since these tissues are commonly studied in mammalian systems, where they have been shown to be regulated autonomously from SCN (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). We also investigate the roles of the eyes and the pineal in synchronizing peripheral rhythms, and explore the relationship between metabolic rhythms and transcription of avian clock genes.

2. Materials and methods

2.1. Animals and surgeries

All animals were treated in accordance with ILAR guidelines; these procedures have been approved by the Texas A&M University Laboratory Animal Care Committee (AUP No. 2005–110). Male White Leghorn chicks (W36) were obtained from Hyline International (Bryan, TX) on the first day post-hatch and maintained under a 12:12 LD cycle in heated brooders with continuously available food and water. Surgeries (pinealectomy, enucleation, or sham surgery) were performed 7–8 days post-hatch. Prior to each surgery, chicks were deeply anaesthetized with an intramuscular injection of a ketamine/xylazine (9:1) cocktail (100 mg/kg).

Pinealectomies ($n = 72$) were performed as follows: anaesthetized chicks were secured in an avian stereotaxic apparatus, a small mid-sagittal incision was made in the skin above the cranium, and then a small portion of skull was removed using a dental drill to expose the pineal gland. Meninges were cut away using microsurgical Vannas scissors, the pineal was gently removed with forceps, and the opening was packed with gel foam to reduce bleeding. The skull cap was placed back on the birds and the wound was then closed with surgical sutures and treated with a topical antibiotic ointment. For enucleation surgeries, animals ($n = 72$) were anaesthetized and then bilaterally enucleated using curved iridectomy scissors. To maintain hemostasis, the orbits were packed with gel foam while pressure was applied with surgical gauze, and animals were placed at an angle to allow wound drainage during recovery from the anesthetic. Sham surgeries ($n = 72$) were performed exactly the same way as pinealectomies, except that the pineal was left intact. There was no specific sham control for enucleation

surgery. All animals were allowed to recover for one week in LD with food and water provided *ad libitum*.

2.2. 2DG injections and tissue sampling

After recovery, chickens were kept in LD or DD three days prior to tissue collection ($n = 108$ total, each treatment). Tissues were harvested every 4 h at six timepoints (ZT or CT 2, 6, 10, 14, 18, or 22), beginning 2 h after lights on (LD birds) or 2 h after the onset of subjective day (DD birds). Because the internal circadian clock of the chick runs at approximately 24 h, we assumed subjective day began at the time the lights had previously come on for those birds being sampled in DD.

One hour prior to tissue collection, birds were given an intraperitoneal injection of 2-deoxy [^{14}C]glucose (100 $\mu\text{Ci/kg}$; American Radiolabeled Chemicals, St. Louis, MO). Exactly 1 h after being injected, animals were killed by CO_2 asphyxiation and the following tissues were excised and frozen on dry ice: telencephalon, diencephalon, optic tectum, liver, and heart. Serum was isolated from trunk blood and frozen with the tissue samples. All tissues were transferred to an ultrafreezer for long-term storage at -80°C . All injections and euthanizations done during the dark phase were performed using infrared optical viewers. Because of the possibility of photoreceptors present in brain, a dim red light source was used when harvesting brain tissue during dark phases. Heart and liver were harvested under light.

2.3. Tissue processing and 2DG uptake

Frozen tissue samples (100 mg) were placed into Trizol reagent (Invitrogen, Carlsbad, CA) and disrupted using a roto-stator homogenizer. An aliquot of tissue homogenate or serum was placed into scintillant and beta emission was measured using a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). 2DG uptake levels were determined based on the specific activity of the isotope (300 mCi/mmol), and then normalized to tissue weight and serum 2DG levels.

2.4. Real-time PCR analysis

Total RNA was extracted from lysis homogenates using a Qiagen RNeasy kit (Qiagen, Valencia, CA). RNA was DNase treated, then primed with oligo dT primers using a Superscript II First Strand Synthesis RT-PCR kit (Invitrogen). Quantitative real-time PCR (qPCR) amplification and detection was performed on an ABI 7500 Fast instrument (Applied Biosystems, Foster City, CA) using SYBR green master mix. Target gene mRNA levels were determined by the standard curve method of relative quantification, using *cyclophilin* as an endogenous control gene. Each PCR plate included control samples lacking template and samples which lacked reverse transcriptase during the cDNA synthesis reaction. Primer sequences are listed in Supplementary Table 1.

2.5. Statistical analysis

All 2DG and qPCR time course data were subjected to ANOVA (Sigma Stat software), as well as cosinor analysis utilizing a linear harmonic regression algorithm (CircWave software). F-testing probability values (α) were set to ≤ 0.05 , and expression was considered to be rhythmic when a cosine fit was statistically significant ($p \leq 0.05$). Multivariate analysis was performed in parallel using 2-way ANOVA (Sigma Stat) to assess effects of time and surgery on 2DG uptake and gene expression.

3. Results

3.1. 2DG uptake in brain

2DG uptake rhythms were observed in both telencephalon and diencephalon of intact birds under LD, with greatest uptake occurring during midday, at approximately ZT 8, based on the best fit cosinor wave (Fig. 1A and B and Supplementary Table 2). This observation corroborates and extends previous reports from our laboratory (Cantwell and Cassone, 2002). Surprisingly, 2DG uptake rhythms were not detected in optic tectum using our criteria for cosinor analyses (Fig. 1C and Supplementary Table 2). However, 2DG uptake in optic tectum did vary significantly over time, as determined by ANOVA. Metabolic rhythms did not persist in any brain tissue after three days in DD, with 2DG uptake being highly variable in the day and during the late subjective night. This may indicate that damping of the metabolic rhythms occurred in individual birds or, alternatively, birds may have drifted out of phase from each other but maintained coherence within individual brain tissues.

Using 2-way ANOVA, no effect of surgery was indicated for telencephalon or diencephalon, although a significant interaction between time and surgery was found for diencephalon under LD (Supplementary Table 3). However, cosinor analysis reveals both surgeries had disruptive effects on one or more tissues, though the effects of pinealectomy were more subtle. In telencephalon, pinealectomy had a moderately disruptive effect on the metabolic rhythm, resulting in a 50% decrease in the rhythm amplitude, but with no effect on acrophase. In contrast, pinealectomy had little effect on 2DG uptake rhythms in diencephalon, although the acrophase was delayed considerably (Supplementary Table 2). Enucleation had a large effect on metabolic rhythms in both rhythmic brain structures, as it abolished 2DG rhythms even under LD conditions (Fig. 1A and B and Supplementary Table 2). Optic tectum was an aberration in this data set, as rhythmic 2DG uptake was only detected under constant conditions in pinealectomized animals (Fig. 1C). This may indicate that weak 2DG rhythms occur in the tissue in other treatment groups, but that we were unable to detect them despite having relatively strong statistical power.

3.2. 2DG uptake in peripheral tissues

A daily rhythm of 2DG uptake was measured in both liver and heart of intact chickens under LD, but with a considerable delay in phase for both tissues compared to neural structures (Fig. 2). 2DG uptake in liver peaked 9–10 h after brain metabolic rhythms, while the rhythm was delayed approximately 7 h in heart (Supplementary Table 2). The relative amplitude of this rhythm was greater in heart (~4-fold) compared to liver (<2-fold). As with brain, 2DG uptake was highly variable in DD, and metabolic rhythms did not persist after three days in constant darkness for any treatment group.

A significant effect of enucleation on 2DG uptake was observed in liver under LD, compared to sham animals, when data were subjected to 2-way ANOVA (Supplementary Tables 3 and 4). Surprisingly, closer scrutiny of the data using cosinor analysis reveals that surgical removal of the pineal or eyes had opposing effects on metabolic rhythms in these two peripheral tissues. In heart, pinealectomy and enucleation reduced the overall rhythm amplitude, with enucleation having a greater effect (Fig. 2B and Supplementary Table 2). In contrast, both surgeries actually increased the rhythm amplitude in liver, with enucleation again producing a greater effect (Fig. 2C and Supplementary Table 2).

3.3. Clock gene expression in brain

3.3.1. Telencephalon—We measured the temporal expression of mRNA levels of the clock genes *cry1*, *per3*, and *bmal1* in the same three subdivisions of the chicken brain. In telencephalon, all three clock genes exhibited circadian rhythms in mRNA levels. Contrary to

the metabolic rhythms, clock gene mRNA rhythms in intact animals persisted for three days in DD, with a similar phase and amplitude as rhythms observed in LD (Fig. 3 and Supplementary Table 5). *Cry1* mRNA levels peaked between mid and late day/subjective day, at around ZT/CT 8–9 (Fig. 3A). *Per3* mRNA levels peaked about 10 h earlier, during late night, at ZT/CT 22–23 (Fig. 3B). *Bmal1* mRNA peaked just before the day/night transition, approximately anti-phase to the *per3* rhythm (Fig. 3C). The phases of these rhythms match clock gene mRNA profiles reported for neural tissues of chick and other avian species (Yoshimura et al., 2000; Okano et al., 2001; Yamamoto et al., 2001; Fukada and Okano, 2002; Bailey et al., 2003; Chong et al., 2003; Helfer et al., 2006).

Surgical interventions affected the clock gene rhythms differentially in telencephalon. Overall, pinealectomy had little effect on telencephalic clock gene expression. In LD, pinealectomy actually increased the amplitude of *per3*, whereas in DD the surgery resulted in minor reductions in mRNA rhythm amplitudes for *cry1* and *per3* (Fig. 3A and B). Additionally, while pinealectomy had no apparent effect on the *bmal1* rhythm in LD, the surgery resulted in a broadened daytime peak for *bmal1* in DD (Fig. 3C). Enucleation had a more potent effect, abolishing free-running rhythms of *cry1* and *bmal1* mRNA and reducing rhythm amplitudes of *cry1* and *per3* mRNA in LD as well as *per3* under constant darkness (Fig. 3). 2-way ANOVA also revealed a statistically significant difference between *bmal1* rhythms in enucleated animals and sham or pinealectomized animals under DD, but was unable to detect other differences between surgical treatments. However, a significant interaction was found between time of day and surgical treatments for *per3* rhythms under LD (Supplementary Tables 6 and 7).

3.3.2. Diencephalon—Clock gene expression in diencephalon was very similar to what was observed in telencephalon, although *bmal1* peaked earlier in sham animals, and *cry1* peaked later in DD (Fig. 4 and Supplementary Table 5). No statistically significant differences were found for rhythms between different surgical groups when analyzed using 2-way ANOVA, though significant interactions between time and surgery were observed for *cry1* and *per3* under LD (Supplementary Tables 6 and 7). However, cosinor analyses indicate that *bmal1* mRNA rhythms were abolished by both enucleation and pinealectomy (Fig. 4C), though *cry1* rhythmicity persisted undisturbed under DD (Fig. 4A). The peak of the *cry1* rhythm, however, occurred earlier under DD for animals in both surgery groups compared to sham birds (Supplementary Table 5). Generally, where clock gene rhythms were not abolished, either surgical procedure reduced the amplitude of the oscillations.

3.3.3. Optic tectum—While metabolic rhythms were not detected in the optic tectum of sham birds, all three clock genes exhibited rhythmic mRNA expression in this tissue, with rhythm amplitudes comparable to those measured in telencephalon and diencephalon (Fig. 5 and Supplementary Table 5). The *per3* rhythm amplitude was, however, reduced in DD compared to other tissues. Overall, as in other brain tissues, pinealectomy and enucleation reduced the amplitude of clock gene rhythms in optic tectum, both in LD and DD. The exception to this was the *per3* rhythm in DD, perhaps because the rhythm amplitude was relatively low in sham animals compared to other brain structures. Enucleation did not abolish any of the clock gene rhythms in optic tectum although *bmal1* expression was disrupted considerably (Fig. 5C). 2-way analysis detected a significant interaction between time and surgery for *per3* expression, but no other effects of surgery were found for this tissue (Supplementary Tables 6 and 7).

3.4. Clock gene expression in peripheral tissues

3.4.1. Liver—*Cry1*, *per3*, and *bmal1* mRNA levels exhibited daily rhythms in liver under LD, though only the *bmal1* rhythm persisted in DD (Fig. 6). The phases of the clock gene rhythms were delayed relative to brain. *Bmal1* and *per3* mRNA levels in liver peaked

approximately 4–5 h after their peak levels in brain, at ~ZT 15 and ~ZT 3, respectively. *Cry1* mRNA peaked 2–4 h later, at ~ZT 11 (Supplementary Table 2).

All three hepatic clock gene expression profiles were similar in pinealectomized animals in LD, although the *bmal1* mRNA rhythm amplitude was reduced by about half in DD (Fig. 6C and Supplementary Table 5). In enucleated animals, *cry1* and *per3* rhythms were attenuated, and *bmal1* expression appeared to be disrupted (Fig. 6 and Supplementary Table 5). Temporal expression of *per3* mRNA levels fit a cosinor function, although these data did not show significant variation over time when subjected to ANOVA. 2-way ANOVA detected a significant effect of enucleation on *per3* rhythms in LD, but did not detect any other differences between rhythms (Supplementary Tables 6 and 7).

3.4.2. Heart—Unlike in liver, all three clock genes exhibited circadian rhythms in heart, and these persisted in constant darkness (Fig. 7). These rhythms had a different phase relationship to each other and to rhythms in the brain and liver. For instance, the phase of the *per3* mRNA rhythm in LD was delayed only 1.5–2 h with respect to brain (peaking at around ZT 0), less than half the delay observed in liver. In DD, *per3* was approximately in phase with brain rhythms (Fig. 7B and Supplementary Table 5). Likewise, *bmal1* mRNA rhythms in heart exhibited a similar phase to *bmal1* rhythms in brain structures, both in LD and DD (Fig. 7C and Supplementary Table 5). Cardiac rhythms of *cry1*, however, were advanced by 2–3 h in LD and 6–7 h in DD, with peaks at ZT 5–6 and CT 2–3, respectively (Fig. 7A and Supplementary Table 5).

Overall, both types of surgery had a greater effect on clock gene rhythms in heart than in liver. While pinealectomized birds had normal clock gene rhythms in LD, cosinor analysis could not detect rhythms for either *cry1* or *bmal1* in free-running conditions. Also, the cardiac *per3* mRNA rhythm was diminished in pinealectomized birds under DD, though it was slightly increased for birds in LD (Fig. 7 and Supplementary Table 5). Enucleation had a more potent effect on cardiac tissue, abolishing mRNA rhythms of all three clock genes in DD, and reducing rhythm amplitudes by 40–50% in LD (Fig. 7 and Supplementary Table 5). A significant effect of surgery (*cry1*) or interaction of surgery with time (*bmal1*, *cry1*, *per3*) was indicated for clock genes under LD, based on 2-way ANOVA (Supplementary Tables 6 and 7).

3.5. Circadian phase analysis

To facilitate comparison of circadian phases of metabolic and clock gene rhythms between different tissues, acrophases determined for statistically significant rhythms were plotted onto polar graphs. These graphs reveal that all three brain structures possess a similar pattern of clock gene expression, in which the phases of individual rhythms, along with their respective phase angles, are conserved (Fig. 8). Phases were most tightly clustered in LD, with rhythms damping or drifting out of phase in DD. Overall, while removal of the pineal or eyes was shown to abolish or reduce the amplitudes of some genes, they did not consistently affect the phasing of clock genes. The only metabolic rhythm detected in brain tissue after three days in DD was in the optic tectum of pinealectomized animals, which was approximately antiphase to the 2DG uptake rhythms detected in other tissues (Fig. 8). Interestingly, the phases of the clock gene rhythms in these animals were not significantly different from those in sham animals.

In peripheral tissues, the phase angles between different clock gene rhythms and 2DG uptake rhythms were not conserved, and were distinct in liver and heart (Fig. 9). In liver, the phases of *bmal1* and *per3* were both delayed by 4–5 h in LD, and thus maintained the same phase angle in liver and brain. *Cry1* rhythms, however, exhibited a different phase relationship to other genes in liver and brain. Also, *bmal1* and *per3* were delayed by ~2 h in enucleated animals, but this was not correlated with a similar shift in 2DG rhythms in these animals (Fig. 9A). In

constant darkness, most clock gene mRNAs were not rhythmic, although the few rhythms that were detected maintained a similar phase.

In heart, *bmal1* and *per3* mRNA expression was similarly correlated, having circadian phases coincident with their expression in brain tissues. In contrast, the rhythm in *cry1* was at a different phase angle to the *bmal1* and *per3* rhythms, and to the 2DG uptake rhythm (Fig. 9B). Moreover, although all clock genes were rhythmic in heart, the phases of these rhythms became desynchronized in DD.

4. Discussion

Metabolism is controlled by the circadian clock in animals, allowing them to synchronize bioenergetic processes with anticipated daily activities. For instance, plasma levels of glucose and glucose uptake are controlled by the clock in many species, with levels being higher during the day or night in diurnal or nocturnal animals, respectively (la Fleur, 2003). One exception is the SCN, where glucose utilization is higher during the day in both diurnal and nocturnal species (Refinetti, 2005). In this study, we demonstrate that overt daily rhythms of 2DG uptake occur in at least two subdivisions of the chicken brain and two different peripheral organs. In brain, uptake was high during the day (with a peak at ~ZT 8), as has been reported for the SCN in house sparrows and mammals (Schwartz et al., 1980; Cassone, 1988; Schwartz, 1990), and several brain structures in chickens (Cantwell and Cassone, 2002). Moreover, we show that both heart and liver exhibit rhythms in 2DG uptake, which are phase delayed by up to 10 h compared to brain. These nocturnal rhythms did not persist in constant darkness, however, and are likely driven by light stimulation. Similar findings have been made in chick brain, where multiple anatomical brain regions, including six different visual structures, exhibited daily rhythms but only two of these rhythms persisted in constant darkness (Cantwell and Cassone, 2002).

One structure which has been reported to have circadian rhythms of 2DG uptake but was not found to be rhythmic in our study was optic tectum, the only brain structure in which metabolic rhythms were not detected even in LD. However, examination of the data suggests 2DG may be rhythmic, but that greater statistical power is needed to resolve these rhythms from background variation. Secondly, the previous study utilized autoradiographic techniques to measure rhythms in specific areas of these structures, whereas our methods measure the average 2DG uptake in whole tissue samples. Therefore non-uniformly distributed 2DG uptake rhythms in tissue may have resulted in a diminished signal: noise ratio with our technique. Also, we measured 2DG uptake after three days in DD, not one day as in the previous study. It is therefore likely that these weak metabolic rhythms had damped out by the third day. Indeed, autoradiographic data reveal considerable damping of rhythms occurs after one day in DD (Cantwell and Cassone, 2002). Another possibility is that 2DG uptake was still rhythmic in these tissues by the third day in DD, but that individual chickens had drifted out of phase, resulting in the appearance of arrhythmicity in the population. However, this seems unlikely, given that the mRNA rhythms of most clock genes were easily detectable and had similar phases as rhythms measured under LD. Still, we must be cautious in our interpretation, as 2DG rhythms could be less tightly coupled with the molecular clock under DD, resulting in them drifting out of phase from other clock components. In any case, it is interesting that 2DG uptake rhythms were delayed by 7–10 h in peripheral tissues compared with brain, such that uptake peaked at night when chickens are less active. Unlike some species, plasma glucose levels in chicks do not vary throughout the day (Raheja, 1973), suggesting that overall glucose utilization is reduced in these peripheral organs during the period of activity. It is unclear what adaptive benefit this may have for a diurnal species such as chicken.

Glucose uptake rhythms in heart and liver were not phase-locked to clock gene mRNA rhythms or to metabolic rhythms in brain. Similar findings were reported for NIH3T3 cells entrained by co-culture with SCN cells. In that study, 2DG uptake rhythms in fibroblasts lagged SCN uptake rhythms by 4 h, while *per2* mRNA levels were delayed by 12 h (Allen et al., 2001). While we observe a different lag time in peripheral clock gene and metabolic rhythms, both studies demonstrate that the phase of molecular clock gene oscillations do not predict the phase of the presumed output of this molecular clock.

Regulation of metabolic and clock gene rhythms in peripheral tissues may occur as a result of endocrine signals ultimately regulated by the SCN, or via autonomic efferents originating in the SCN (Bartness et al., 2001; la Fleur, 2003; Hastings et al., 2007). The pineal likely regulates peripheral clocks via the nightly synthesis and secretion of melatonin. Pineal regulation may occur solely through an indirect influence of melatonin on the SCN (Lu and Cassone, 1993a,b; Starkey et al., 1995), or may result from the direct action of melatonin on peripheral tissues. Functional melatonin receptors have been detected in heart (Pang et al., 1998, 2002) but not liver of chicken, and Western analyses from our laboratory confirm these data (unpublished data). In contrast, since the retinae do not contribute significantly to circulating melatonin levels in chicks (Reppert and Sagar, 1983; Cogburn et al., 1987), regulation by the eyes most likely involves only neural connections, either indirectly via the retinohypothalamic tract (RHT), or through their effects on other neural structures.

Chickens are able to entrain to a 12:12 light:dark cycle using only their eyes or pineal (MacBride, 1973), as animals in all three surgical groups exhibited daily rhythms in clock gene expression in all structures examined. However, our data reveal differential effects of surgical removal of the pineal or eyes on brain and peripheral rhythms, suggesting the roles of these pacemakers are not entirely redundant, and may involve regulation independent of the SCN. Overall, enucleated animals exhibited greater disruptions in both clock gene and metabolic rhythms compared with pinealectomized animals, with some rhythms being abolished (see Fig. 1A and B, Fig 3A and C). Enucleation abolished rhythms of 2DG uptake in brain even under an LD cycle, while clock gene mRNA levels were still rhythmic, though some were only reduced in amplitude in LD. This disconnect suggests that either: (1) parallel pathways exist by which the eyes reinforce circadian oscillators and their output; or (2) a minimum amplitude in clock gene transcriptional rhythms is necessary to drive rhythms in clock controlled metabolic output. Moreover, enucleation actually increased the amplitude of 2DG and *per3* rhythms in liver (Fig. 2A and Fig 6B). This may indicate that central pacemakers can actually inhibit endogenous rhythms in this organ, perhaps by maintaining tighter coupling between liver and brain rhythms.

Pinealectomy had a disruptive effect on rhythms in brain and peripheral tissues, consistent with reported behavioral effects (Nyce and Binkley, 1977; McGoogan and Cassone, 1999), but the effects were more subtle than with enucleation. Multiple rhythms disrupted by enucleation were similarly affected by pinealectomy (Fig. 1A, Fig 2A and B, Fig 3B, Fig 4B and C, Fig 7A–C), suggesting the eyes and the pineal have similar reinforcing or coupling effects. It is therefore tempting to speculate that the eyes and pineal affect overt rhythms solely through their partially redundant roles as coupling elements within the neuroendocrine loop, with the pineal acting as a weaker element. However, SCN independent regulation by the pineal cannot be ruled out, given that melatonin receptor distribution is widespread in the brain and many peripheral tissues (Rivkees et al., 1989; Pang et al., 1993). Additionally, injections of melatonin into chick brain have been reported to cause acute suppression of 2DG uptake in some structures when administered during the day (Cassone and Brooks, 1991; Cantwell and Cassone, 2002). However, our data do not support a similar endogenous role for melatonin in the long term, as 2DG uptake levels were not elevated in any tissues in pinealectomized chickens.

A final point of consideration is that this current study provides the most in depth characterization of avian clock gene expression to date, which has allowed us to make two important observations. First, a comparative analysis of metabolic rhythms and clock gene expression demonstrates that the transcriptional clockwork (along with a presumed circadian output) is organized in a similar fashion throughout multiple brain tissues. Thus, it will be interesting to see if functional anatomical differences in molecular clockworks will be found in the brains of birds, as has been documented for the mammalian forebrain (Reick et al., 2001). Secondly, our study reveals that in liver and heart, this canonical transcriptional ensemble is structured differently, not only between these two organs, but also between brain and periphery. Particularly, *bmal1* and *per3* mRNA rhythms appear to be phase-locked, whereas *cry1* expression is not. The antiphasic relationship between *bmal1* and *per3* is consistent with their putative roles as positive and negative elements of the avian feedback loop. *Cry1*, however, is transcriptionally connected to the loop in a tissue specific manner. Whether this differential regulation is a product of control by specific neuroendocrine pathways or is intrinsic to peripheral tissues is unclear.

Taken together, these data provide new insights into the connectivity of the avian circadian system. To illustrate the expanding complexity of avian clocks, we have incorporated these observations into an illustrative model (Fig. 10). According to this model, three pacemakers differentially feedback into one another to influence brain and peripheral rhythms via the SCN or via direct entrainment by melatonin. Although there is no evidence that avian eyes can directly entrain slave oscillators, they appear to have a greater effect on the circadian system as a whole, presumably by stronger connectivity to the SCN compared to the pineal. Finally, at least two important peripheral tissues have different and uniquely patterned transcriptional clockworks compared to brain, which may be weakly coupled to metabolic rhythms. This model presents a new and updated view of the circadian clock in chicken.

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Appendix

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2008.12.015.

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Fig. 1. 2DG uptake in brain. 2DG uptake is shown for telencephalon (A), diencephalon (B), and optic tectum (C). Data are plotted as a function of zeitgeber time, or hours after lights on, for birds in LD 12:12 (top row in each panel). For birds in DD (bottom row in each panel), the abscissa displays hours after the onset of the third subjective day. In each panel, the left column indicates sham operated (Sham) animals (circles), the middle column indicates pinealectomized (PINX) animals (squares), and the right column indicates enucleated (EX) animals (triangles). Data points are plotted with SEM at each timepoint. For time series that significantly fit a cosine function ($\alpha, p \leq 0.05$), a fitted curve is plotted along with data points in the graph.

Fig. 2. 2DG uptake in peripheral tissues. 2DG uptake is shown for liver (A) and heart (B). For other details see Fig. 1.

Fig. 3. Clock gene expression in telencephalon. mRNA levels of chick *cry1* (A), *per3* (B), and *bmal1* (C) are shown for telencephalon as measured by qPCR. For other details see Fig. 1.

Fig. 4. Clock gene expression in diencephalon. mRNA levels of chick *cry1* (A), *per3* (B), and *bmal1* (C) are shown for diencephalon as measured by qPCR. For other details see Fig. 1.

Fig. 5. Clock gene expression in optic tectum. mRNA levels of chick *cry1* (A), *per3* (B), and *bmal1* (C) are shown for optic tectum as measured by qPCR. For other details see Fig. 1.

Fig. 6. Clock gene expression in liver. mRNA levels of chick *cry1* (A), *per3* (B), and *bmal1* (C) are shown for liver as measured by qPCR. For other details see Fig. 1.

Fig. 7. Clock gene expression in heart. mRNA levels of chick *cry1* (A), *per3* (B), and *bmal1* (C) are shown for heart as measured by qPCR. For other details see Fig. 1.

Fig. 8.

Circadian phase plots for brain. Polar plots show circadian acrophases for statistically significant rhythms as determined by cosinor analysis. Acrophases of 2DG uptake and clock gene mRNA rhythms are plotted for telencephalon (A), diencephalon (B), and optic tectum (C). Acrophases measured under LD cycles are plotted on the left column of each panel, and acrophases measured under DD are plotted on the right column. The angular axis (θ) displays hours after lights on (LD), or hours after the beginning of subjective day (DD). Radial values are shared by data points within common surgical treatment groups. Circadian phases of metabolic and clock gene rhythms are distinguished by shape, whereas surgical treatment groups are distinguished by color (see key).

Fig. 9. Circadian phase plots for peripheral tissues. Acrophases of 2DG uptake and clock gene mRNA rhythms are plotted for liver (A) and heart (B). For other details see Fig. 8

Fig. 10.

Model of chicken circadian system. Pacemakers and slave oscillators are denoted by large and small wave symbols, respectively. Proposed information flow between circadian pacemakers and target tissues is indicated by block arrows, while yellow bolts indicate photoreceptive inputs. White arrows indicate putative autonomic or endocrine pathways originating from the SCN, gray arrows indicate endocrine melatonin secretion by the pineal, and blue arrows indicate neural pathways from the eyes to the SCN, including the retinohypothalamic tract (RHT). The wider arrow originating from the eyes indicates a stronger influence on the SCN compared to the pineal pacemaker. Faded and dotted gray arrows indicate possible direct signaling to oscillator tissues via pineal melatonin. Arrows in brain, liver, and heart correspond

to phases of rhythms in 2DG uptake and clock gene expression as shown in the key. Arrow direction corresponds to phases plotted on a 24 h circular plot as follows: (12 o'clock = time 0/24; 6 o'clock = time 12). Dotted arrows for 2DG uptake rhythms emphasize that they are light driven or uncouple from clock gene rhythms under DD conditions.