General and Comparative Endocrinology 161 (2009) 179–192

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

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

Stephen P. Karaganis [1,*], Paul A. Bartell [2], Vikram R. Shende, Ashli F. Moore [3], Vincent M. Cassone [4,*]

Center for Biological Clock Research, Department of Biology, Texas A&M University, College Station, TX 77843-3258, USA

ARTICLE INFO

Article history:
Received 19 June 2008
Revised 25 October 2008
Accepted 11 December 2008
Available online 24 December 2008

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

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[^{14}C]-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.

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 retinae 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-[^{125}I]iodomelatonin (IMEL) indicate that melatonin binding is widespread in the avian nervous

* Corresponding authors. Fax: +1 214 902 2448 (S.P. Karaganis).
E-mail addresses: skaraganis@hotmail.com (S.P. Karaganis), vcass2@uky.edu (V.M. Cassone).
[1] Present address: 4065 N Beltline Rd. #2066, Irving, TX, 75038, USA.
[2] Present address: Department of Poultry Science, Pennsylvania State University, University Park, PA 16802-3501, USA.
[3] Present address: Department of Biology, University of Virginia, Charlottesville, VA 22904-4328, USA.
[4] Present address: Department of Biology, University of Kentucky, Lexington, KY, USA.

0016-6480/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2008.12.015

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[^{14}C]-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 lipogenesis, 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 μCi/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.