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Extra-hypothalamic brain clocks in songbirds: Photoperiodic state dependent clock gene oscillations in night-migratory blackheaded buntings, *Emberiza melanocephala*

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ABSTRACT

The avian circadian pacemaker system is comprised of independent clocks in the retina, pineal and hypothalamus, as shown by daily and circadian oscillations of core clock genes (*Per2*, *Cry1*, *Bmal1* and *Clock*) in several birds including migratory blackheaded buntings (*Emberiza melanocephala*). This study investigated the extra-hypothalamic brain circadian clocks in blackheaded buntings, and measured *Per2*, *Cry1*, *Cry2*, *Bmal1* and Clock mRNA expressions at 4 h intervals over 24 h beginning 1 h after light-on in the left and right telencephalon, optic tectum and cerebellum, the brain regions involved in several physiological and cognitive functions. Because of seasonal alterations in the circadian clock dependent brain functions, we measured daily clock gene oscillations in buntings photoperiod-induced with the non-migratory state under short days (SDnM), and the pre-migratory (LDpM), migratory (LDM) and post-migratory (refractory, LDR) states under long days. Daily *Per2* oscillations were not altered with changes in the photoperiodic states, except for about 2–3 h phase difference in the optic tectum between the SDnM and LDpM states. However, there were about 3–5 h differences in the phase and 2 to 4 fold change in the amplitude of daily *Bmal1* and *Cry1* mRNA oscillations between the photoperiod-induced states. Further, Cry2 and Clock genes lacked a significant oscillation, except in Cb (*Cry2*) and TeO and Rt (*Clock*) under LDR state. Overall, these results show the presence of circadian clocks in extra-hypothalamic brain regions of blackheaded buntings, and suggest tissue-dependent alterations in the waveforms of mRNA oscillations with transitions in the photoperiod-induced seasonal states in a long-day species.

1. Introduction

Birds like other vertebrates show adaptations to the day-night environment, which are overtly expressed in daily cycles in physiology and behaviour governed by circadian clocks [1]. Nearly five-and-half decades ago, the principal circadian clock was discovered in the suprachiasmatic nuclei of anterior hypothalamus in rodents [2]. Later, the principal circadian clock in birds was found to be a multi-oscillatory system with interacting independent circadian clocks located in the eyes, pineal and hypothalamus [3,4,5]. At the mechanistic level, a circadian clock is comprised of a set of core clock genes, *Period*, *Per*; *Cryptochrome*, *Cry*; *Brain and muscle arnt like protein 1*, *Bmal1*, and *circadian locomotor output cycles kaput*, *Clock* [6]. These clock genes interact in a transcriptional-translational feedback loop and produce a near 24 h (circadian; *circa* = about, *dian* = day) time [6,7]. Using transcriptional oscillations of the core clock genes, particularly *Per2*, *Cry1*, *Bmal1* and *Clock* genes, a number of investigations have found circadian clocks in both the central clock tissues (retina, pineal and hypothalamus) and peripheral organs like liver and muscle in birds [8,9]. Recently, we have reported differences in the phase and amplitude of circadian oscillations of *Per2*, *Cry1* and *Bmal1*, not *Clock*, in the central (retina, pineal, hypothalamus) and peripheral (liver, muscle) clock tissues between the photoperiod-induced seasonal life history states (LHSs) in a migratory songbird, the blackheaded bunting (*Emberiza melanocephala*) [9]. Most interestingly and importantly, the altered waveforms of clock gene expressions in all tissues paralleled with the migration associated shifts in daily activity behaviour (from day activity to predominantly night activity with the onset of the migratory phase) in blackheaded buntings [9].

The extra-hypothalamic brain areas also play significant role in controlling different physiological and behavioural functions. For example, the olfactory and visual sensory systems play significant roles in avian migration, particularly in the orientation and navigation. The olfactory perception deprived swifts (*Apus apus*) and starlings (*Sturnus vulgaris*) do not show the

Abbreviations: Bmal, brain and muscle arnt like protein; Cry, cryptochrome; Cb, cerebellum; CPl, piriform cortex; CPP, peripiriform cortex; EH, extra-hypothalamic; GLd, dorsal lateral geniculate nucleus; LDpM, long day premigratory; LDM, long day migratory; LDR, long day refractory; LC, lateral olfactory cortex; LOT, lateral olfactory tract; Lt, left telencephalon; OA, anterior olfactory nucleus; Per, period; Rt, right telencephalon; ROT, nucleus rotundus; SCN, suprachiasmatic nuclei; SDnM, short day non-migratory; TeO, optic tectum.

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homeward navigation [10,11]. Also, there is loss of migratory orientation ability in anosmic (individuals with smell sense loss) catbirds, *Dumetella carolinensis* [12]. Similarly, night-migratory songbirds use a vision-mediated pathway to find their direction [13]. Both olfactory and visual sensory systems are comprised of several forebrain regions [14,15]. The brain region involved in the perception of olfactory cues includes the olfactory bulb (OB), anterior olfactory nucleus (OA), prepiriform cortex (CPP), lateral olfactory tract (LOT) and olfactory cortex (piriform cortex, CPl; lateral cortex, LC). Similarly, the visual sensory system includes both the thalamofugal (eye > dorsal geniculate complex, GLd > telencephalic wulst, tW) and tectofugal (eye > optic tectum, OT, nucleus rotundus, ROT > entopallium) pathways [16]. The cluster N (cN) of tW has been suggested playing a key role in the magnetic compass orientation in migratory European robins, *Erithacus rubecula* [17], although the study on migratory blackheaded buntings suggests the possible involvement of entire thalamofugal visual pathway including cN in the nocturnal migration [18].

In parallel with shifts in the daily activity behaviour from predominantly day activity to profoundly night activity with the onset of the photoperiod-induced migratory state, the phase inversion in the neural activity of the olfactory and visual sensory circuits, as shown by Fos-immunohistochemistry, has also been reported in migratory blackheaded buntings [18]. Notably, the neural activity of the medial (mSCN), the hypothalamic site of the circadian clock in songbirds was not phase inverted [18]. This may suggest that the olfactory and visual sensory systems have their own independent circadian clocks, and perhaps linked upstream to a common regulatory pathway governed by the principal circadian clock system. This has not been investigated however. Also, it is unknown whether the left and right telencephalons, which are not connected with each other in birds, unlike by carpus callosum in the mammalian brain [19], would show independent circadian clocks. This is not unlikely since each cerebral hemisphere independently control rhythmic sleep-wakefulness in birds [20]. Further, avian cerebellum has been found associated with the motor and cognitive functions [21], which may have significant roles during migration in songbirds.

Very little is known about extra-hypothalamic (EH) brain clocks in birds, except for the demonstration of circadian gene oscillations in the optic tectum of Japanese quails, *Coturnix c. japonica* [22,23]. However, circadian gene oscillations have been shown in the olfactory bulb, lateral hypothalamus, paraventricular nuclei, epithalamus, cerebellum in the rodent brain [24,25,26,27]. Here, therefore, we investigated the EH circadian clocks in the left and right telencephalons, optic tectum and cerebellum of blackheaded buntings. In captive buntings under long days, gonadal growth-regression cycle runs in parallel with transitions between nonmigratory to migratory states [9]. Buntings show non-migratory state under short days (SDnM), and sequentially undergo through pre-migratory (LDpM), migratory (LDM) and post migratory (refractory, LDR) states under long days [9]. The SDnM and LDR birds with reproductively inactive small testes and predominantly day activity represent the photosensitive and photorefractory states, respectively. Similarly, LDpM birds with day activity and LDM birds with predominantly night activity represent the early and late photostimulated states of testis maturation, respectively [9]. In this study, we measured daily cycle in mRNA expression of *Per2*, *Cry1*, *Cry2*, *Bmal1* and *Clock* genes in central and peripheral tissues in blackheaded buntings [9]. We expected, in particular, the photoperiodic state dependence of the alterations in daily *Per2*, *Cry1* and *Bmal1* oscillations in EH brain regions, since these genes have been reported to have a significant daily rhythm in the pineal and hypothalamus in an earlier study on blackheaded buntings [9].

2. Material and Methods

2.1. Experiment and Tissue Samples

This study was carried out in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) and used brain samples collected from adult male blackheaded buntings during different photoperiod-induced seasonal states, as described in detail in an earlier publication [9]. Briefly, we used 3 groups of buntings (groups 1–3; $n = 24$ each) that were maintained under short days (8 h light: 16 h darkness, 8L:16D) and were photosensitive, and one group of buntings (group; $n = 24$) that were maintained under long days (16L:8D) and following growth-regression cycle had become photorefractory. Groups 1 and 4 birds were maintained under 8L and 16L photoperiods, as before, and represented the short day non-migratory (SDnM) and long day refractory (LDR) seasonal states. Thus, birds in these two different seasonal states had small reproductively inactive small testes. Of remaining photosensitive birds, group 2 was exposed to 16L:8D for 7 days and they remained still day active. Group 3 birds were exposed to 16L:8D until the time (18–25 days) when each individual had shown 7 nights of nighttime migratory restlessness, *Zugunruhe* (characterized by intense night activity and wing whirring) [28]. Thus, groups 2 and 3 represented the long day pre-migratory (LDpM) and migratory (LDM) states and had partial and fully-grown testes, respectively. We continuously monitored activity-rest pattern as a behavioural assay of non-migratory and migratory states in individually housed buntings in activity cages (dimension: 60 × 45 × 35 cm) using The Chronobiology Kit software program (Stanford Software Systems, Santa Cruz, CA, USA) [9]. The electronic timers controlled the times of light onset and offset, and the temperature was maintained at 22 ± 2 °C. Food and water were present all the time, and replenished only during the light hours.

Fig. 1 provides a summary of the experimental protocol. In each photoperiod-induced state, birds ($n = 3$–4) were decapitated at 4 h intervals during the day, beginning at 1 h after the light onset (zeitgeber time 1, ZT1). Brains were quickly removed and stored in RNA later and then frozen at −80°C until further processed. The left and right telencephalon (Lt, Rt), optic tectum (TeO) and cerebellum (Cb) were excised out and processed for gene expression study. Thus, in total we had six daily samples collected at ZTs 1, 5, 9, 13, 17 and 21.

2.2. RNA Isolation, cDNA Synthesis and Measurement of Gene Expressions

The total RNA was extracted using Tri reagent (Ambion AM9738), as per the manufacturer's protocol. A 1 μg RNA aliquot treated with RQ1 DNase (Promega M6101) to remove genomic DNA contamination, if any, was used for cDNA synthesis by the first strand cDNA synthesis kit (Thermo scientific, K1622). Using gene specific primers, we measured *Per2*, *Cry1*, *Cry2*, *Bmal1* and *Clock* with *β-actin* as the endogenous control, mRNA expressions by qPCR run on the Applied Biosystems ViiA7 thermal cycler [9]. We used a total reaction volume of 10 μl for each gene comprising 1 μl each of cDNA (10 ng/μl) and primers (conc. 500 nM – *β-actin*, *Per2*, *Cry1*, *Bmal1*), 5 μl Power Syber Green PCR mastermix (ABI 4387669) and 2 μl of nuclease free water. The fold change in the gene (relative mRNA) expression levels was calculated using the formula $\mathbf{2}^{-(\Delta\Delta Ct)}$ [8,29].

2.3. Statistics

Unless specified otherwise, all statistics were done using GraphPad prism version 5.0. One-way analysis of variance (1-way ANOVA) tested the significance of variation in mRNA levels across the day. When appropriate, we used Newman-Keuls post hoc test for comparison between the time points. Also, mRNA levels from different states were compared by 2-way ANOVA with Bonferroni correction to test for the effects of photoperiod-induced state, time of day, and their interaction. Further, to test the rhythmicity in daily expression patterns, mRNA profiles were subjected to the unimodal cosinor regression analysis ($y = A + [B. \cos(2\pi (x\text{-}C) / 24)]$), where A, B and C denoted the mean (mesor), amplitude and acrophase of the rhythm, respectively [30], as has been described in our earlier studies [8]. This analysis applies harmonics to basic sinusoidal function on 24 h assumption. The significance of nonlinear cosinor regression analysis was calculated using the