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Bird eyes distinguish summer from winter: Retinal response to acute photoperiod change in the night-migratory redheaded bunting

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ABSTRACT

Eyes are the part of the circadian timekeeping system but not involved in the photoperiod regulated seasonal physiology in songbirds. Here, two experiments tested whether eyes detect and respond to seasonal change in the photoperiod environment, by examining gene and protein expressions in the retinas of redheaded buntings exposed to a single long day (LD, 16L:8D), with controls on short days (SD, 8L:16D). In the first experiment, mRNA expression of genes implicated in the light perception (opsins, rhodopsin, neuropsin, melanopsin, peropsin) and photoperiod induction (*eya3, tsh-β, dio2, dio3*) was measured at hours 15 and 19 (hour 0 = light on) on the first long day. There was a significant increase in the *eya3, tsh-β* and *dio2* mRNA expression, albeit with a temporal difference, and decrease in the neuropsin mRNA expression in buntings on the first long day. There was no change in the *dio3*, rhodopsin, melanopsin and peropsin mRNA expressions on exposure to long days. The second experiment immunohistochemically examined the *eya3*, *tsh-β* and rhodopsin peptide expressions. *eya3* was expressed in both light conditions, but with a significant higher levels in the retinal photoreceptor layer (PRL) under LD, as compared to SD. Similarly, *tsh-β* was expressed in the PRL of LD retinas only. Rhodopsin levels were not significantly different between SD and LD conditions, however. These results for the first time show photoperiod-dependent molecular switches in the bunting retina, similar to the well documented thyroid hormone response genes based molecular cascades in the avian hypothalamus.

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1. Introduction

Eyes are part of the photoperiodic response system that regulates daily and seasonal functions in mammals. Blind mammals do not respond to the external photoperiod environment. They fail to synchronize circadian rhythms with the light–dark (LD) cycles and recrudesce gonads under stimulatory photoperiods (Arendt, 1995; Goldman, 2001). Both circadian and photoperiod effects are mediated by the retina, the third and inner coat of the eye which is a light-sensitive layer of tissue. The retina is considered part of the central nervous system, and converts light input to the eyes into a neural signal that the brain can understand. Structurally, the retina consists of five cell types (photoreceptor, horizontal, bipolar, amacrine and retinal ganglion cells) and organized in seven layers. Inside to outside, these layers are the retinal pigment epithelium (RPE), photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL).

Unlike in mammals, the absence of eyes fails to block the circadian and photoperiodic responses in birds (see review Kumar et al., 2004). Blind sparrows synchronize circadian activity rhythms with external LD cycles and respond to the stimulatory effects of long photoperiods, as do the sighted birds (Menaker, 1971; McMillan et al., 1975; Wilson, 1991; Valdez et al., 2013). Thus, the extra-retinal photoreceptors in the pineal and hypothalamus are more directly involved in the avian circadian and photoperiodic responses (Menaker, 1971; Kumar et al., 2004; Surbhi and Kumar, 2014). The functional redundancy of avian retina in the photoperiod-regulated physiology, therefore, raises an important question, as to whether the retina which is a part of the circadian timekeeping in birds (Kumar et al., 2004), responds to

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change in the photoperiod environment. Recent findings on molecular cascades of gene activation underlying photoperiod induction in mediobasal hypothalamus (MBH) have made it possible to answer this. As early as hour 14 on the first long day (hour 0 = light on), there is an activation of *eya3* and *tsh-β* genes, and this is followed at hour 18 by an change in the transcription of *dio2* and *dio3* genes (Nakao et al., 2008). Reciprocal switching of *dio2* and *dio3* gene activations in tanycytes surrounding the third ventricle affects GnRH release from the median eminence (ME; Yamamura et al., 2004) and in turn the pituitary release of gonadotropins (Cassone and Yoshimura, 2015). This molecular pathway appears an evolutionary conserved mechanism for the photoperiod regulated seasonal gonadal development in birds and mammals (Nakane and Yoshimura, 2014; Cassone and Yoshimura, 2015).

Interestingly, there are suggested overlaps in the functional roles of some of the molecules, described above. For example, *eya3* possibly acts as a photoreceptive molecule, as evidenced by recent studies on redheaded buntings, *Emberiza bruniceps* (Majumdar et al., 2014, 2015). There was a positive correlation of hypothalamic rhodopsin on *eya3* mRNA expression (Majumdar et al., 2015), and the *eya3* peptide was found expressed in the septal lateralis (SL; Majumdar et al., 2014) in buntings exposed to a single long days. The SL has also been shown expressing the rhodopsin in ring doves, *Streptopelia risoria* (Silver et al., 1988) and house sparrows, *Passer domesticus* (Wang and Wingfield, 2011). Similarly, the light sensitive neuropsin (Nakane et al., 2010) can be involved in sensing the photoperiod change; the neuropsin mRNA expression was decreased after a single long light exposure in the border canaries, *Serinus canaria* (Stevenson and Ball, 2012) and redheaded buntings (Majumdar et al., 2015).

This study investigated whether the retina in photoperiodic songbirds responds to the photoperiod change involving thyroid hormone responsive genes. Particularly, we measured mRNA and peptide of known candidate molecules linked with the photoperiod perception and induction in the avian hypothalamus (see above) in the retinal of photosensitive redheaded buntings exposed to a single long light hour.

2. Material and methods

Two experiments were performed on redheaded buntings (*Emberiza bruniceps*) that were maintained under short days (SD, 8 h light: 16 h darkness, 8L:16D, $L = 250 \pm 5.0$ lux; $D = \sim 0.3$ lux). Under SD, buntings remain photosensitive and unstimulated i.e. they do not show fattening and significant gain in body mass (body mass = 22–24 g), and maintain the small reproductively inactive testes (testis volume = 0.52–0.78 mm^3) (Rani et al., 2005).

2.1. Experiment 1: mRNA expression of genes involved in photoperiod perception and induction

Photosensitive buntings ($n = 16$) were singly housed in cages (size = $45 \times 32 \times 35$ cm^3) individually placed inside photoperiodic boxes (size = $70 \times 50 \times 70$ cm^3) and exposed to 8L:16D, as before. On day 6, to half of them light was extended until they were sacrificed at hour 15 ($n = 4$) or until hour 16 and sacrificed at hour 19 ($n = 4$); these birds thus received a single 15 or 16 h long light exposure (long day, LD). Also, of the other eight buntings maintained on SD, 4 birds each were sacrificed at hours 15 and 19; these served as controls. Thus, except 4 birds that were sampled at immediately after 15 h light exposure (hour 15, LD group), all birds were sampled during the darkness under a background illumination as in the photoperiodic boxes. The retina was immediately dissected out from quickly removed eyes and stored in the RNA later (Ambion Inc., Cat No. AM7020) until further processed. Total mRNA was extracted from the homogenized retina in Tri reagent (Ambion Inc., Cat No. AM7020) by the chloroform extraction method. 1 μg of DNAse (DNA free; Ambion Inc.,Cat No. AM1906) treated RNA was used to prepare cDNA using MAXIMA single strand cDNA prep kit (Fermentas, Cat no. K1641).

The mRNA expression of genes implicated in the light perception (opsins; rhodopsin, neuropsin, melanopsin, peropsin) and photoperiod induction in the MBH *(eya3, tsh-β, dio2, dio3)* was measured by the real-time PCR (qPCR, Applied Biosystems Step One Plus real-time PCR system). This used standardized primers designed from the partially cloned genes from redheaded bunting's cDNA, and SYBR green chemistry as described in Majumdar et al. (2015). Relative mRNA expression was calculated by subtracting individual ct values for each sample from the ct value of β-actin (internal control), and the ΔC_t value was further subtracted from ΔC_t value of a sample comprising the sample mix of a group. $\Delta\Delta C_t$ value thus obtained was plotted as 2^{-DDC_t} (Livak and Schmittgen, 2001; Majumdar et al., 2014, 2015).

2.2. Experiment 2: eya3, tsh-β and rhodopsin peptide expressions

This experiment differed from the experiment 1 in the sense that birds were sampled after they experienced one full long day (16L:8D). Briefly, to a group ($n = 4$) of buntings on SD, the light period on day 6 was extended until hour 16; thus these birds received day 6 as 16L:8D. Then during the first hour on day 7 when light did not come on, these birds as well as a group ($n = 4$) of birds on SD were transcardially perfused with 4% paraformaldehyde under the background illumination as was during the dark period in preceding LD cycles, as described previously in Majumdar et al. (2014). Eyes were dissected out, post fixed over night in the same paraformaldehyde solution and cryoprotected by passing through ascending gradients (10%, 20% and 30%) of sucrose solution.

Cryoprotected eyes were sectioned on a Leica 1850 cryostat and 20 μm thick coronal sections procured on polylysine-coated slides were processed for the immunohistochemistry (IHC) of *eya3*, *tsh-β* and rhodopsin peptides, using the standard avidin-biotin protocol (Majumdar et al., 2014). A separate set of H–E (haematoxylin and eosin) stained retinal sections were used for histology of the bunting retina. The polyclonal antibodies used for the IHC of *eya3* (Cat No. ab22835, 1:500) and *tsh-β* (Cat. No. ab80784, 1:500; Abcam Inc. USA) have been validated and previously used in another study on the bunting hypothalamus (Majumdar et al., 2014). However, for rhodopsin IHC, the polyclonal antibody used was specifically raised against the bunting antigen, as follows. Bunting's full-length rhodospin mRNA was cloned using the degenerate primers (for primer details see supplementary Table 1). A specific 1056 bp PCR amplified product was further cloned in the pGEMT vector (Promegap GEM-T Easy Vector System 1; Cat No. A1360) and commercially sequenced by MWG Bangalore, India. The rhodopsin sequence, found identical in both the retina and hypothalamus tissues, was translated to the corresponding peptide sequence using AnnHyb 4.942 (http://bioinformatics.org/annhyb) and analyzed for trans-membrane/cytoplasmic domain search by Phobius prediction (http://phobius.sbc.su.se/) and Tm prediction software (http://www.ch.embnet.org/software/TMPRED_ form.html). The cytoplasmic domains were located and a 15-mer peptide sequence with an overhanging cysteine was commercially synthesized and used for raising polyclonal rhodopsin antibody in the rabbit by Imgenex India (http://www.imgenexindia.com/). Further details are outlined in the supplementary Fig. 1. ELISA results from the validation tests by Imgenex, India using the 15-mer peptide antigen (200 ng) at 1: 5000 dilution at 450 nm of the enzyme substrate reaction against the antibody, pre immune, first and third bleeds, supported the specificity of the rhodopsin antibody.

In spite of the same batch of *eya3* and *tsh-β* antibodies that were used in our previous study on bunting hypothalamus (Majumdar