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Genome-wide Analysis of Seasonal Reproduction in Birds

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Many seasonal breeding animals can estimate the day length (photoperiod) and prepare for breeding in the optimal season. The Japanese quail (*Coturnix japonica*) is an excellent model for studying photoperiodism because of its rapid and dramatic response to the photoperiod. Recent molecular analysis using the quail has revealed that local thyroid hormone activation by thyroid hormone deiodinases (*DIO2* and *DIO3*) in the mediobasal hypothalamus (MBH) plays a critical role in the regulation of seasonal reproduction in birds. However, the molecular dynamics of gene expression that regulates photoperiodic thyroid hormone activation in the MBH during the photoinduction process remains unclear. The chicken genome project has enabled the analysis of the conservation of the genetic interaction networks for studying photoperiodism. This review focuses on genome-wide transcriptional studies of avian photoperiodism.

Key words: deiodinase, mediobasal hypothalamus, pars tuberalis, photoperiodism, thyrotropin

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Introduction

The photoperiod affects the seasonal changes in endocrine and metabolic physiology. This phenomenon is termed as "photoperiodism". The photoperiodic characteristics in birds include seasonal reproduction, passage, characteristic plumage, and changes in body mass. We focus on the photoperiodic regulation of seasonal reproduction in our consideration of avian photoperiodism. Most seasonally reproductive animals use changes in the photoperiod to time their breeding seasons. It has been thought that photoperiod is measured in birds (as well as in mammals) with the use of a circadian clock (Follett and Sharp, 1969). In long-day breeders, increasing photoperiod in the spring stimulates secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus and hence of gonadotropins (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) from the pituitary gland, and gonadal growth (hypothalamic-pituitary-gonadal axis). Among birds, the Japanese quail exhibits a rapid response to the photoperiod. As little as a single long day has been shown to stimulate the release of both LH and FSH in the Japanese quail (Nicholls *et al.,* 1983). This increase in plasma LH lasts for approximately 10 days after the animals are transferred back to short-day conditions (Follett *et al.,* 1998). Further, when quail kept under short-day conditions are transferred to long-day conditions, the testicular weight increases more than 100-fold within 2 weeks. For these reasons, the Japanese quail is an excellent model for studying photoperiodism. Many studies using the Japanese quail have shown that the mediobasal hypothalamus (MBH), including the infundibular nucleus (IN), the ependymal cells (EC) lining the ventrolateral walls of the third ventricle, and the median eminence (ME), is an important centre for controlling photoperiodism (summarized by Yoshimura, 2004) (Fig. 1).

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Local Activation of Thyroid Hormone is the Key for the Photoperiodic Response

Although it is known that thyroid hormones are also deeply involved in seasonality, almost nothing is known about the molecular timekeeper of the photoperiod (Dawson *et al.,* 2001). Recent molecular analysis has revealed that local thyroid hormone activation by 2 thyroid hormone deiodinases (a thyroid hormone-activating enzyme, type 2 deiodinase, [*DIO2*] and an inactivating enzyme, type 3 deiodinase [*DIO3*]) in the MBH play a critical role in the regulation of seasonal reproduction in birds (Yoshimura *et al.,* 2003; Yasuo *et al.,* 2005; Watanabe *et al.,* 2007). The upregulation of *DIO2* in the MBH under long-day conditions is associated with an increase in the concentration of triiodothyronine (T_3) and the consequent induction of testicular growth, whereas *DIO3* expression is maintained at a low level. The increased T_3 concentration within the MBH by *DIO2* has been proved to induce morphological changes in GnRH neuron terminals and glial processes, which result in LH secretion and

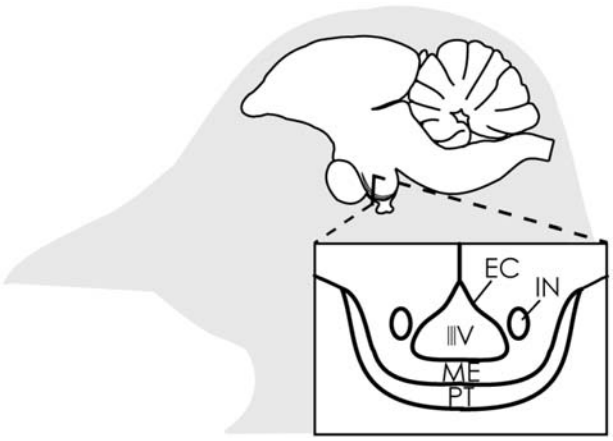

Fig. 1. **The mediobasal hypothalamus and pars tuberalis.** Sagittal view of the quail head and brain section. A boxed area (frontal section) indicates the location of the mediobasal hypothalamus and pars tuberalis. EC, ependymal cell layer lining the ventrolateral walls of third ventricle; ME, median eminence; IN, infundibular nucleus; IIIV, third ventricle and PT, pars tuberalis of the pituitary gland.

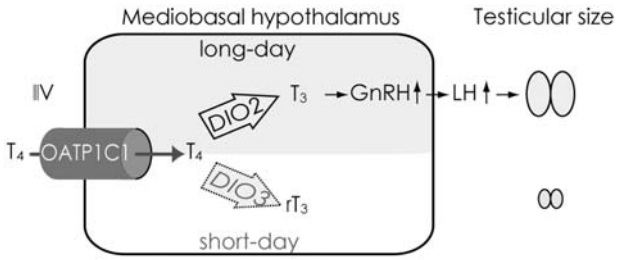

Fig. 2. **Fine-tune the concentration of thyroid hormones in the MBH.** Thyroxine (T_4) is transported to the ependymal cells lining the ventrolateral wall of the third ventricle under both short-day and long-day conditions by OATP1C1. T_4 is metabolized to rT_3 by DIO3 under short-day conditions, while DIO2 converts T_4 to the bioactive form, T_3, under long-day conditions. IIIV, third ventricle. Modified from Nakao *et al.* (2006).

testicular growth in the quail (Yoshimura *et al.,* 2003, 2004, 2006)(Fig. 2). Further, the expression profiles of *DIO2* and *DIO3* are altered in opposition to one another by a single long-day stimulation preceding the first increase in the plasma concentration of LH (Yasuo *et al.,* 2005). Thus, the local activation of the thyroid hormone by DIO2 and DIO3 is the key for both the initiation and the maintenance of photoperiodically induced reproductive neuroendocrine function. Furthermore, the cellular uptake of thyroxine (T_4) from the cerebrospinal fluid (CSF) to EC under both short-day and long-day conditions is transported by organic anion transporting polypeptide 1c1 (OATP1C1) (Nakao *et al.,* 2006).

Thyroid Hormone-independent Pathway in the Photoperiodism

Although there is no doubt about the involvement of thyroid hormone in photoperiodism, T_3 infusion did not maximize testicular size and iopanic acid (DIO2 inhibitor) did not block testicular growth completely in the quail (Yoshimura *et al.,* 2003). Thyroidectomized European starling and house sparrow became photoperiodically blind (Dawson, 1993, 1998). However, thyroidectomized quail could still respond to photostimulation (Follett and Nicholls, 1985). These suggests the existence of other regulatory mechanisms involving photoperiodism. Differential subtractive hybridization analysis using MBH of the quail kept under short-day and long-day conditions identified that transforming growth factor alpha (*TGFα*) was induced in the ME and EC lining the ventro-lateral walls of third ventricle by photostimulation (Takagi *et al.,* 2007). Further, timing of *TGFα* mRNA expression was similar to *DIO2* expression. Namely, *TGFα* was also induced in both stimulation of the long-days and a single long-day. Further, the long-day-induced activation of the TGFα signaling pathway was mediated by a thyroid hormone-independent pathway because T_3 administration did not affect *TGFα* expression in the ME and TGFα infusion did not affect *DIO2* expression. Furthermore, intracerebroventricular administration of TGFα for ten days induced LH secretion and testicular growth under short-day conditions. Importantly, the combined administration of TGFα and T_3 to the MBH induced testicular growth to a larger extent than that induced by the infusion of TGFα or T_3 alone (Takagi *et al.,* 2007).

Genome-wide Analysis of Genes Involved in Photoinduction

The method for the measurement of photoperiodic time is unclear for most animals. In the quail, a single long photoperiod results in the induction of *DIO2* expression and an increase in LH secretion, which are first detected at approximately 18 h and 22 h after dawn, respectively (Follett *et al.,* 1977; Yasuo *et al.,* 2005; Nakao *et al.,* 2008) (Fig. 3a). However, testicular growth and gonadotrophin secretion begin when the day length reaches approximately 12 h (critical day length) (Follett and Maung, 1978; Follett *et al.,* 1998); thus, it is assumed that there are genes involved in the association of photoperiodic response and the early expression of *DIO2.* In order to analyze the molecular dynamics of gene expression that regulates photoperiodic thyroid hormone activation in the quail MBH during the photoinduction process, a search for the candidate gene (s) for the photoinduction process was performed using high-density oligonucleotide microarray for chicken (Affymetrix chicken genome array). The quail is a galliform bird closely related to the chicken and the nucleotide sequences between the 2 species are highly conserved; thus, we can use the chicken genomic information for the molecular genetic analysis of the quail. Further, over 83% of the probes of the Affymetrix chicken

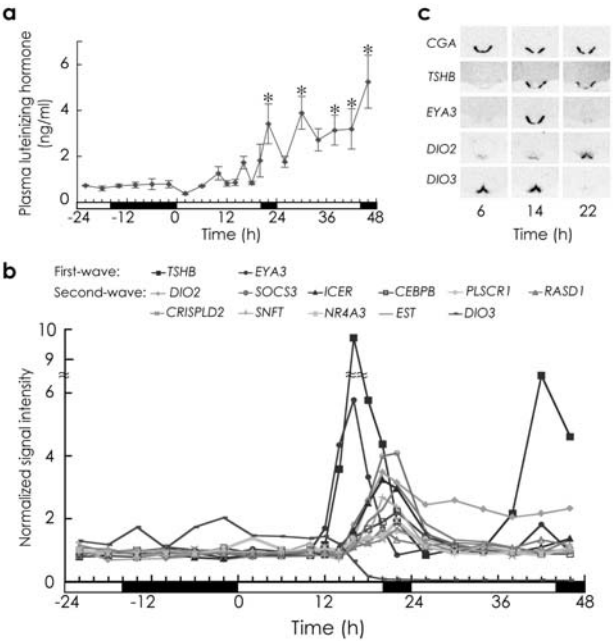

Fig. 3. **Plasma luteinizing hormone and the spatiotemporal profile of photoinduction genes.** a, Long-day induced plasma luteinizing hormone. b, Identification of waves of gene expression during the photoinduction process. Data were normalized such that the median signal strength for each gene over all time points was 1.0. The average signal strength at each point was then displayed as a ratio relative to the median signal strength of that gene. c, Spatiotemporal expression pattern of *TSHB, EYA3, DIO2, DIO3* and *CGA*. Expression of *TSHB, EYA3* and *CGA* genes were observed in the pars tuberalis, whereas *DIO2* and *DIO3* genes were observed in the ependymal cell layer lining the ventrolateral walls of third ventricle and the infundibular nucleus. Time 0h is dawn of the first long day. Modified from Nakao *et al.* (2008).

genome array are useful for the analysis of the quail genomic DNA (Nakao *et al.,* 2008).

We compared the genome-wide gene expression profiles for the photoinduction process in the MBH using the Affymetrix chicken genome array when animals were transferred from short-day (6 h light: 18 h dark) to long-day (20 h light: 4 h dark) conditions. This experimental schedule enables the observation of the initial molecular events in the photoinduction process. Two waves of gene were identified before the increase in the plasma LH secretion during the first long day by using the Robust Multichip Average (RMA) algorithm and one-way analysis of variance (ANOVA) for the quantification of gene expression analysis (Fig. 3b). The initial waves of gene consist of thyroid-stimulating hormone, beta chain (*TSHB*) and eyes absent 3 (*EYA3*) (designated as the first-wave), which are induced at approximately 14 h of the first long day. At approximately 4 h after the first wave gene expression, the expression of 11 genes, including *DIO2* and *DIO3,* is induced (designated as the second-wave) (Fig. 3 b). EYA3 is a transcriptional coactivator involved in the development of the eye, which seems to regulate the second-wave genes. However, a spatiotemporal expression analysis of these genes revealed that the 2 wave genes are expressed in different locations in the MBH. The first wave genes are expressed in the pars tuberalis (PT), which covers most of the outer walls of the basal hypothalamus above the posterior median eminence (Wingstrand, 1951). The second wave genes are expressed in the EC lining the ventrolateral walls of the third ventricle and in the adjacent IN (Fig. 3c). Thus, EYA3 cannot gain access to the EC or IN. A functional TSH consists of 2 noncovalently linked subunits: CGA (common alpha subunit) and TSHB. The *CGA* gene is rhythmically expressed in the PT unlike the *TSHB* gene (Fig. 3c). The expression of *CGA* before *TSHB* prevents the intracellular degradation of TSHB since free TSHB is degraded intracellularly (Matzuk *et al.,* 1988). Therefore, studies focused on the functional significance of TSH action (Nakao *et al.,* 2008). Additionally, a single long-day stimulus leads to the expression of *TSHB* in the PT of the red jungle fowl (*Gallus gallus*), a predecessor of the domestic chicken. This result suggests that the red jungle fowl may be an ideal model animal for the genome-wide transcription analysis of photoperiodism in the future because draft sequences and the initial analysis of the genome have been reported (Ono *et al.,* 2009).

In seasonally reproductive mammals, the circadian system regulates the rhythmic secretion of the pineal hormone, melatonin, which is a potentially crucial component of the mammalian photoperiodic mechanism, acts in the hypothalamus to mediate the control of the seasonal changes in gonadotropin secretion and gonadal activity (Goldman, 2001). However, in birds, melatonin has no distinct effects on the regulation of photoperiodic time measurement (Kumar *et al.,* 1993). The circadian clock is involved in the photoperiodic time measurement (Follett and Sharp, 1969, Pittendrigh, 1972). Furthermore, clock genes in the quail MBH exhibited stable rhythmic expression patterns under various light conditions (Ball and Balthazart, 2003; Yasuo *et al.,* 2003), which are assumed to play a role in the photoperiodic time measurement. Therefore, a set of rhythmically expressed candidate genes encoding the putative components of photoperiodic time measurement in the photoinduction process were analyzed. By using statistical cosine filters in the microarray data sets, 77 rhythmically expressed genes, including clock genes, were identified (Nakao *et al.,* 2008). Further studies are needed to evaluate the interaction between the 77 genes and the transcriptional mechanism of the *TSHB* gene.

Target Site of Photoinduced TSH

It is widely accepted that actions of TSH are mediated by G-protein-coupled thyroid stimulating hormone receptor (TSHR), which is the most important factor for the regulation of the production of thyroid hormones in the thyroid gland. However, the TSH-binding sites and TSHR

mRNA expression site are present in nonthyroid tissues such as the adipose tissue, lymphocytic tissue, ependymal cell layer, and subependymal zone in mammals (Crisanti *et al.,* 2001; Davies *et al.,* 2002). Additionally, photoperiodic stimuli influence the secretory activity of TSH-like immunoreactive cells in the PT of the Djungarian hamster (Wittkowski *et al.,* 1988). These observations suggest the presence of a local function of TSH in the central nervous system. In the quail, *TSHR* expression was also observed in the EC and PT when they are kept at a transition of short-day and long-day conditions. This expression of *TSHR* in the EC coincides with the expression sites of the second wave genes. Furthermore, ^{125}I-labeled TSH-binding assay revealed that TSH expression was associated with the EC, which is consistent with the localization of the *TSHR* mRNA (Nakao *et al.,* 2008). Median eminence is one of the circumventricular organs (CVOs) that permit hypothalamic polypeptide hormones to leave the brain without disrupting the blood-brain barrier (BBB) and permit substances that do not cross the BBB to trigger changes in the brain function (Ganong, 2000). Hence, these observations lead to the speculation that photoinduced upregulation of TSH in PT acts through TSHR on the time-dependent second-wave gene regulation in the MBH.

TSH can Trigger a Photoinduced Neuroendocrine Response

In order to assess whether the photoinduced second-wave gene expression was driven by TSH, we investigated the effect of intracerebroventricular infusion of bovine TSH on the second wave gene expression. Several doses of TSH were infused into the third ventricle at 16 h after dawn under short-day conditions (i.e., at the time of *TSHB* expression at the first long-day stimulation); the results revealed that the expression levels of *DIO2, ICER, CEBPB,* and *NR4A3* mRNAs were increased in a TSH dose-dependent manner, whereas the immunoneutralization of TSH by the administration of anti-TSHβ IgG impaired the expression that had been induced by the first long-day conditions (Nakao *et al.,* 2008).

It has been reported that the expression of *DIO2* in the human thyroid gland is regulated through a TSHR-Gsα-cAMP regulatory cascade (Murakami *et al.,* 2001). The 5′ upstream regions of *DIO2, ICER, CEBPB* and *NR4A3* genes contain putative cAMP responsive elements (CRE). In addition, the transcriptional activity of the *DIO2* promoter is mediated by the TSH-TSHR-CRE signaling cascade when the 293 cells contransfected with the reporter gene constructs and quail *TSHR* expression constructs are treated with TSH. These findings suggest that the photoperiodic regulation of second wave gene expressions in MBH by TSH involves a cAMP signaling pathway through TSHR (Nakao *et al.,* 2008).

Photoperiodically Regulated Output Genes

For the better understanding of avian photoperiodism, we next examined the expression profiles of genes involved in the chronic effects of photostimulation in the MBH under short-day and long-day conditions using microarray experiments. A total of 183 genes, including *DIO2* and *DIO3,* were differentially expressed as determined by two-way ANOVA when quail were exposed to 2 weeks of photostimulations. These genes, including *CCK, CRH, FSTL4, RLN3, STC2, PNOC, OPRL1, POMC, MC4R, GHR* and *PRLR,* are assumed to be involved in several photoperiodic responses such as the reproductive status, changes in body weight, and plumage. Importantly, *TSHB* and *CGA* were also highly expressed in the PT under long-day conditions, indicating that the continuous presence of TSH affects the photoperiodism. In fact, the intracerebroventricular administration of bovine TSH into the third ventricle for 2 weeks under short-day conditions induces the expression of *DIO2* in the MBH (Nakao *et al.,* 2008). *TGFα* has been known to be a long-day-induced gene that is involved in a thyroid hormone-independent pathway for the photoperiodic regulation of reproduction (Takagi *et al.,* 2007), even though the microarray experiments failed to detect the *TGFα* gene. The administration of TSH also induced *TGFα* gene expression and testicular growth to the same extent as that under long-day conditions. These findings indicate that photoinduced TSH in the PT is not only the initial step in the photoinduction process but also a regulator of the photoperiodic response of gonad maturation in birds.

Conclusion

Many studies on the photoperiodic response have shown that MBH is important for controlling the photoperiodic response and photoperiodic signal. The recent functional genomic analysis of avian photoperiodism has demonstrated that TSH in the PT is used to transmit the signal for seasonal timing to MBH (Fig. 4). Recently, it is reported that Soay sheep exposed to summer day length causes melatonin-responsive cell in the PT to increase production of TSH. TSH then induces *DIO2* expression in the ME (Hanon *et al.,* 2008). Furthermore, melatonin-dependent regulation of *DIO2* and *DIO3* expression in the mice MBH involves TSH signaling (Ono *et al.,* 2008). Thus, TSH in the PT appear to be the key to an understanding of photoperiodism in vertebrate although the photic signaling pathway involved in seasonal photoperiodic responses is different between mammalian and birds. It is known that the transcriptional activity of *TSHB* gene is regulated by thyrotropin-releasing hormone (TRH) or pituitary specific transcription factor 1 (Pit-1/GHF-1) (Shupnik *et al.,* 1986; Steinfelder *et al.,* 1991). However, *in situ* hybridization analysis has revealed that quail *TRH* and *TRHR* mRNAs are not present in the PT (Ono and Yoshimura, unpublished observation). Thyrotrophs in the rat PT lack the Pit-1 (Sakai *et al.,* 1999). Further, the primary objective of immediate future research should be to identify the photoperiodic signal pathway that activates TSH in the avian PT.

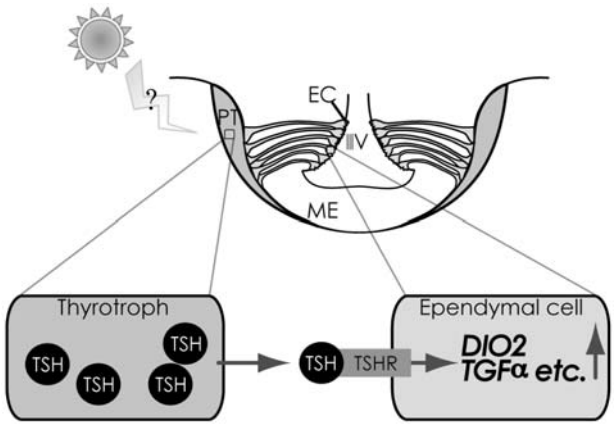

Fig. 4. **Model of the mechanisms regulating photoperiodic time measurement in birds.** Light information received by the deep brain photoreceptors induces expression of *TSHB* in the pars tuberalis (PT) of the pituitary gland. The light input pathway to the PT remains unknown. Long day-induced *TSHB* and cycling *CGA* (common pituitary glycoprotein alpha subunit) form TSH in the PT and act on TSH receptor (TSHR) localized in EC. Expression of second-wave gene include *DIO2* is induced by TSH through TSHR signaling pathway.

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