ARTICLES

Thyrotrophin in the pars tuberalis triggers photoperiodic response

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Molecular mechanisms regulating animal seasonal breeding in response to changing photoperiod are not well understood. Rapid induction of gene expression of thyroid-hormone-activating enzyme (type 2 deiodinase, *DIO2*) in the mediobasal hypothalamus (MBH) of the Japanese quail (*Coturnix japonica*) is the earliest event yet recorded in the photoperiodic signal transduction pathway. Here we show cascades of gene expression in the quail MBH associated with the initiation of photoinduced secretion of luteinizing hormone. We identified two waves of gene expression. The first was initiated about 14 h after dawn of the first long day and included increased thyrotrophin (TSH) β-subunit expression in the pars tuberalis; the second occurred approximately 4 h later and included increased expression of *DIO2*. Intracerebroventricular (ICV) administration of TSH to short-day quail stimulated gonadal growth and expression of *DIO2* which was shown to be mediated through a TSH receptor–cyclic AMP (cAMP) signalling pathway. Increased TSH in the pars tuberalis therefore seems to trigger long-day photoinduced seasonal breeding.

Animals living outside the tropics use changes in photoperiod to adapt to seasonal changes in environment, but the molecular mechanisms underlying photoperiodic time measurement are not fully understood[1]. The Japanese quail is a robust model for the study of these mechanisms because of its rapid and dramatic response to changes in photoperiod. When quail are transferred from short to long days, plasma luteinizing hormone increases at the end of the first long day: this photoperiodic response is the core feature of the avian 'first day release model' of reproductive photoperiodism[2,3]. In birds, the components required for photoperiodic signal transduction are located in the mediobasal hypothalamus (MBH) and include a deep brain photoreceptor[4], a clock to measure daylength[5], and output pathways to regulate the secretion of gonadotrophin-releasing hormone (GnRH)[6,7]. Recently, we have reported that long-day-induced local activation of thyroid hormone metabolism in the quail MBH is an early event in photoperiodic signal transduction[8,9]. Under short-day conditions, expression of type 2 deiodinase (*DIO2*), which converts the prohormone thyroxine (T_4) to bioactive triiodothyronine (T_3), is maintained at a low level, whereas expression of type 3 deiodinase (*DIO3*), which metabolizes T_4 and T_3 to reverse (r)T_3 and T_2, respectively, is maintained at a high level. When quail are transferred from short to long days, rapid reciprocal switches in *DIO2* and *DIO3* expression occur at the end of the first long day, resulting in a local increase in T_3 concentration. This increase in MBH T_3 concentration precedes the first rise in the concentration of photoinduced plasma luteinizing hormone and is causally related. Administration of T_3 to short-day quail stimulates secretion of luteinizing hormone and testicular growth, whereas conversely, administration of a DIO2 inhibitor inhibits photoinduced testicular growth[8,10]. The question now is the identity of the photoperiodic transduction pathway regulating *DIO2* expression in the MBH.

To address this, we have dissected the molecular dynamics of gene expression regulating photoinduced thyroid hormone metabolism in the quail MBH during the first day of photoinduced luteinizing hormone secretion by using a chicken high-density oligonucleotide microarray. Quail and chicken are both galliforms with predicted high interspecific DNA sequence conservation. To test this prediction, we applied biotinylated chicken and quail genomic DNA to the array. Signals for 82.2% of the probes were statistically indistinguishable between the two species (Welch's *t*-test, Benjamini and Hochberg false discovery rate (FDR) multiple test, $P > 0.05$, $n = 3$) (Supplementary Fig. 1).

Genome-wide expression analysis

To study changes in gene expression during the first long day, eight-week-old male quail kept under short days (6/18 h light/dark cycle) for four weeks were transferred to long days (20/4 h light/dark cycle). Plasma samples and brains were collected from six birds every 4 h for three days during this transition. In addition, samples were collected every 2 h between 10 and 22 h after dawn of the first long day to cover the period during the initiation of the photoperiodic response when the most rapid changes in gene expression were predicted to occur[2,3]. The first increase in plasma luteinizing hormone was observed at 22 h after dawn of the first long day as previously reported[3,11] (Fig. 1a)

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(one-way analysis of variance (ANOVA), $F_{20,105} = 5.860$, $P < 0.0001$, Fisher's least significant difference (LSD) post-hoc test, $P < 0.05$, $n = 6$).

For each time point, biotinylated antisense RNAs (cRNAs) prepared from pooled MBH were hybridized to duplicate sets of arrays to minimize experimental error. Using the RMA algorithm and statistical cosine filters[12], we identified 77 cycling genes which were disqualified from consideration as long-day-induced genes (Fig. 1b and Supplementary Table 1). These genes included eight circadian clock genes (Supplementary Fig. 2). We next focused on genes showing 1.5-fold or more changes in expression during the first long day, and found two waves of expression initiated at around 14 h (peak time 16.46 h, Fig. 1a) and the other initiated at around 18 h after dawn (peak time 21.15 h, Fig. 1a) (Supplementary Table 2 and Supplementary Fig. 3) (Welch's one-way ANOVA, FDR $P < 0.01$).

The first wave comprised two genes encoding thyrotrophin-β (TSH-β) and eyes absent 3 (EYA3); the second wave comprised 11 genes including *DIO2* and *DIO3* which showed inversely related changes in expression (Supplementary Fig. 4). Using *in situ* hybridization, the expression of the two first-wave genes (*TSHB* and *EYA3*) was observed in the pars tuberalis of the pituitary gland, whereas expression of six of the second-wave genes including *DIO2* and *DIO3* was observed in the ependymal cells lining the ventro-lateral walls of third ventricle and in the adjacent infundibular nucleus (Fig. 1c).

We also noted rhythmic expression of the gene encoding common pituitary glycoprotein alpha subunit (CGA) in the pars tuberalis (Fig. 1c and Supplementary Fig. 2). The expression of this gene with that encoding the TSH-β suggests that the proteins encoded by these genes associate in the pars tuberalis to form TSH. This view is supported by the observation that TSH-β protein occurs in pars tuberalis cells (Fig. 2a). We therefore deduced that increased TSH in the pars tuberalis may be functionally significant for photoperiodic signal transduction. To test this hypothesis, we first determined whether TSH receptor (*TSHR*) gene expression occurs in the MBH.

TSH receptor in the MBH

Strong expression of *TSHR* was observed in the ependymal cells and pars tuberalis; weak expression was observed in the infundibular nucleus (Fig. 2b). *TSHR* expression in the pars tuberalis was detected at 6 and 22 h after dawn of the first long day but not at 14 h, whereas it occurred in the ependymal cells all the times examined ($n = 2$). To verify these results, we further performed a ^{125}I-labelled TSH binding assay. We first demonstrated specific binding of ^{125}I-labelled TSH in the thyroid gland of quail as a positive control (Fig. 2c). Specificity of the binding assay was also confirmed by radioreceptor assay (Supplementary Fig. 5). We then observed specific binding of ^{125}I-labelled TSH in the ependymal cells, infundibular nucleus and the pars tuberalis. This observation is consistent with expression sites of *TSHR* mRNA at these loci. Although TSH binding in the ependymal cells was observed at all the times examined, that in the pars tuberalis was undetectable at time 22 h (Fig. 2d). Because the median eminence is one of the circumventricular organs and is outside the blood–brain barrier[13], long-day-induced TSH in the pars tuberalis has the potential to enter the brain to interact with TSHR in the ependymal cells and infundibular nucleus. We therefore predicted that the

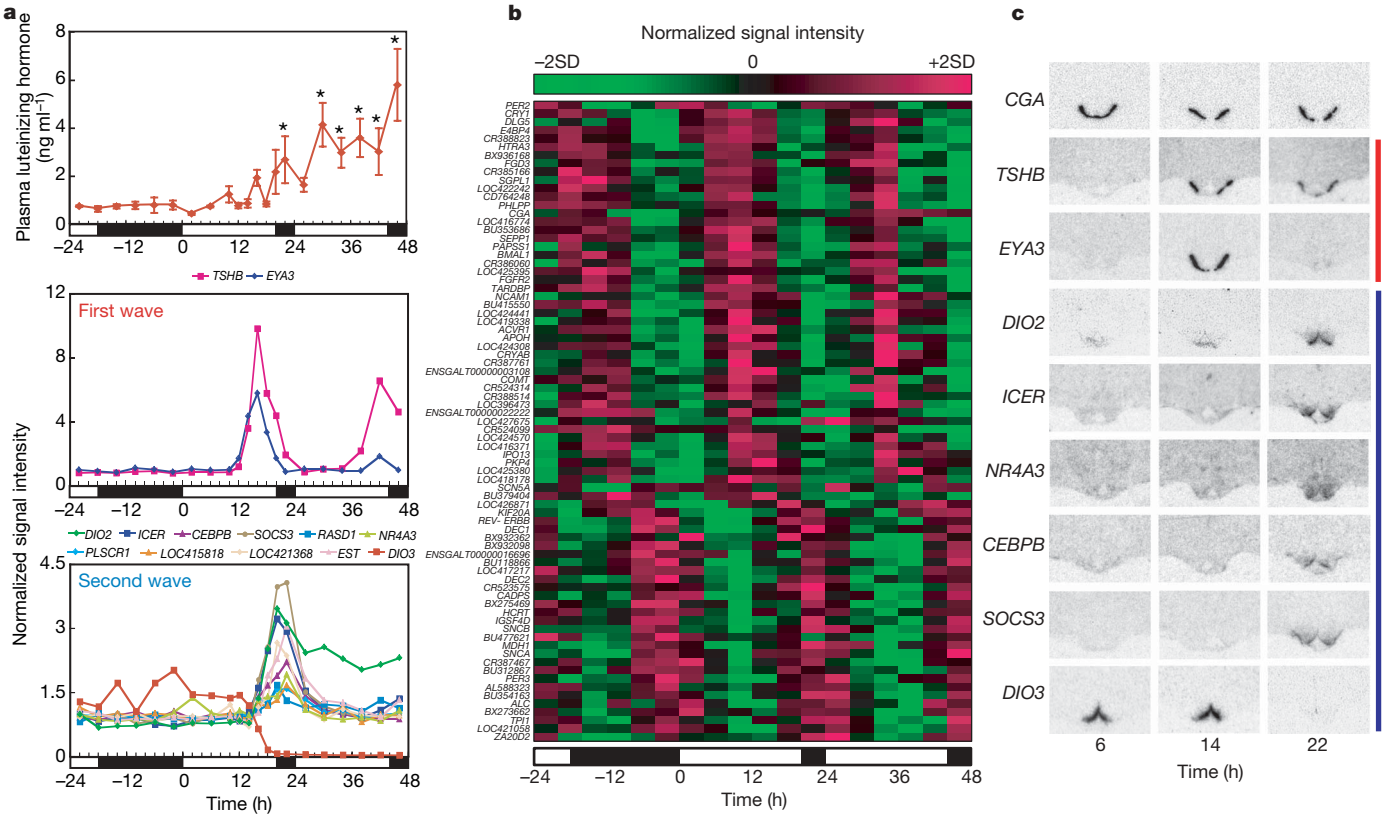

Figure 1 | Plasma luteinizing hormone and genome-wide analysis of genes expressed in the quail MBH during the first day of photostimulation (time 0 h is dawn of the first long day). **a**, Changes in plasma luteinizing hormone (mean ± s.e.m., $n = 6$, $^*P < 0.05$ versus the value at time −22 h); and timing of first- and second-wave gene expression. Data are 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. **b**, Organization of 77 genes showing 24 h rhythmic changes in expression. Data are normalized such that the mean and s.d. of log expression values over all time points for each gene are 0 and 1, respectively. Italic script, gene identities and accession numbers. **c**, Spatio-temporal expression of common glycoprotein hormone subunit (*CGA*), first-wave (red bar) and second-wave genes (blue bar). Expression of *CGA* and first-wave genes was observed in the pars tuberalis, whereas that of second-wave genes was observed in the ependymal cells and the infundibular nucleus.

photoinduced increase in pars tuberalis TSH may function to stimulate the expression of *DIO2* and possibly other second-wave genes.

TSH regulation of *DIO2* gene

To test this prediction, a range of doses (0.01, 0.1, 1.0 mIU) of bovine TSH[14] dissolved in 10 µl saline was administered 16 h after dawn to short-day quail to correspond with the time that *TSHB* expression is at its highest in the pars tuberalis after dawn of a first long day. Brains were collected 4 h after the injection when the induction of second-wave genes was predicted to be maximal. As shown in Fig. 3a, b, intracerebroventricular (ICV) TSH injection induced the expression of the *DIO2* and three other second-wave genes in a dose-dependent manner (one-way ANOVA, Fisher's LSD post-hoc test, $P < 0.05$, $n = 3$–6). Induction of these genes was observed in the dorsal and ventrolateral ependymal cells and in the infundibular nucleus (Figs 3a and 5a) and was more prominent in the ependymal cells than in photostimulated birds (Fig. 1c and Supplementary Fig. 6). This is likely to be a consequence of the periventricular ependymal cells being more assessable to ICV TSH than to TSH originating from the pars tuberalis. We further confirmed this physiological effect of TSH by ICV injection of TSH-β antibody to long-day quail. Anti-chicken/quail TSH-β IgG[15] or pre-immune serum IgG (1 µg per 10 µl) was administered every 2 h (ref. 16) from 12 h to 18 h after dawn of the first long day, and brains were collected 2 h after the last injection. As shown in Fig. 3c, d, anti-TSH-β IgG injections suppressed the expression of the four second-wave genes, including *DIO2*, shown to be induced by ICV TSH.

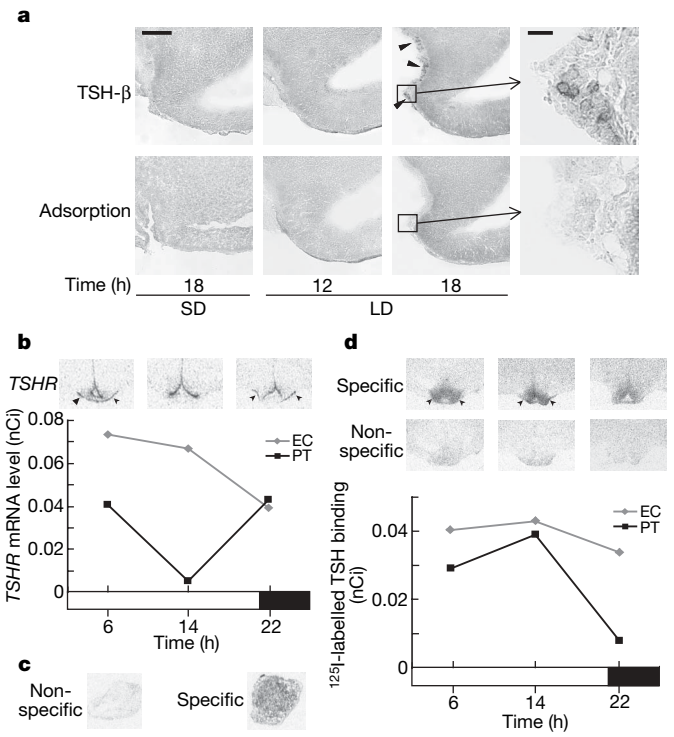

Figure 2 | Localization of TSH-β and TSHR in the pars tuberalis and MBH. **a**, Positive immunolabelling for TSH-β in the pars tuberalis (arrowhead) induced by the long-day stimulus was eliminated by pre-adsorption of the anti-TSH-β antibody with the synthetic TSH-β peptide sequence used to produce the antibody. Scale bars: left, 100 µm; right, 10 µm. SD, short day; LD, long day. **b**, Expression of *TSHR* mRNA in the ependymal cells was observed at all the times examined, whereas that in the pars tuberalis (arrowhead) was not observed at time 14 h ($n = 2$). **c**, **d**, Binding of ^{125}I-labelled TSH to quail thyroid gland (**c**) and the MBH (**d**). Although TSH binding in the ependymal cells and the infundibular nucleus was observed at all the times examined, that in the pars tuberalis was not observed at time 22 h ($n = 2$).

Involvement of cyclic AMP signalling pathway

To further address the mechanism through which TSH might regulate the expression of *DIO2* and three other second-wave genes we first determined the transcriptional start sites using the oligo-capping method[17] and mapped them to quail and chicken genome sequences (Fig. 4a, Supplementary Fig. 7a). It is reported that the expression of *DIO2* in human thyroid gland is regulated through a TSHR-Gsα-cAMP regulatory cascade[18]. We found several putative cAMP responsive elements (CREs) in the 1.5 kilobase (kb) 5′ upstream regions of *DIO2*, in quail and chicken (Fig. 4a) and in the three other second-wave genes (Supplementary Fig. 7a). Conservation of CREs between the two species suggests the functional significance of this element (Fig. 4b and Supplementary Fig. 7b). To validate whether these CREs are involved in the regulation of *DIO2* gene by TSH, we analysed the promoter activity of the *DIO2* gene transfected into the 293 cell line. TSH administration induced expression of *DIO2* reporter activity in a dose-dependent manner only when *TSHR* was co-transfected (Supplementary Fig. 8). However, when CREs were mutated, induction by TSH was not observed (Fig. 4c). These results demonstrate that induction of the *DIO2* gene by TSH involves a cAMP signalling pathway through TSHR.

Photoperiodically regulated output genes

We next performed a microarray analysis on quail kept under short- and long-day conditions for two weeks to assess the chronic effects of photostimulation on MBH gene expression. MBH samples were collected from six birds every 4 h during a 24 h lighting cycle. This analysis identified 183 differentially expressed genes (Welch's two way ANOVA, FDR $P < 0.05$) (Supplementary Fig. 9a, b and Supplementary Table 3). Among these genes, 124 were upregulated and 59 were downregulated under long-day conditions (Supplementary

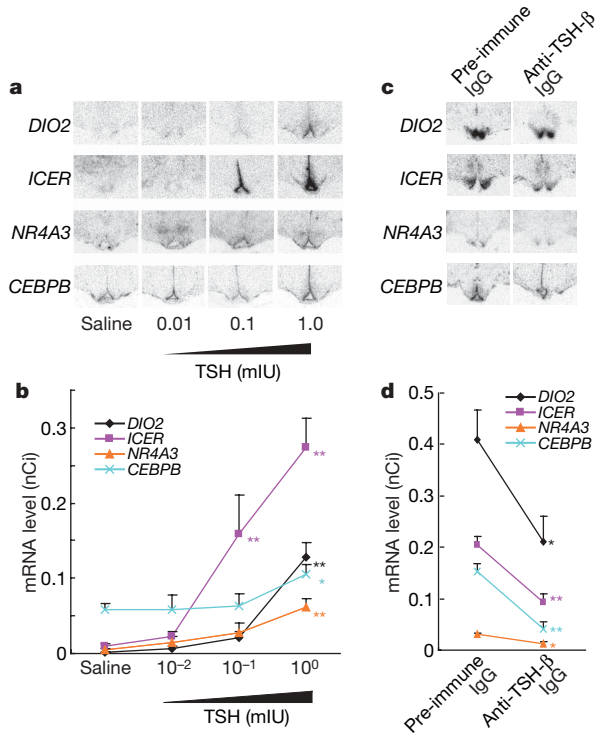

Figure 3 | Induction of the expression of *DIO2* and three other second-wave genes by ICV injection of TSH and inhibition by ICV injection of anti-TSH-β IgG. ICV injection of TSH (**a**, **b**) and anti-TSH-β IgG (**c**, **d**). Representative autoradiograms (**a**, **c**) and densitometric quantification (**b**, **d**) showing the effect of ICV injections of TSH/anti-TSH-β (**b**, $*P < 0.05$, $**P < 0.01$, ANOVA, Fisher's LSD post-hoc test, $n = 3$–6; **d**, $*P < 0.05$, $**P < 0.01$, *t*-test, mean + s.e.m., $n = 3$).

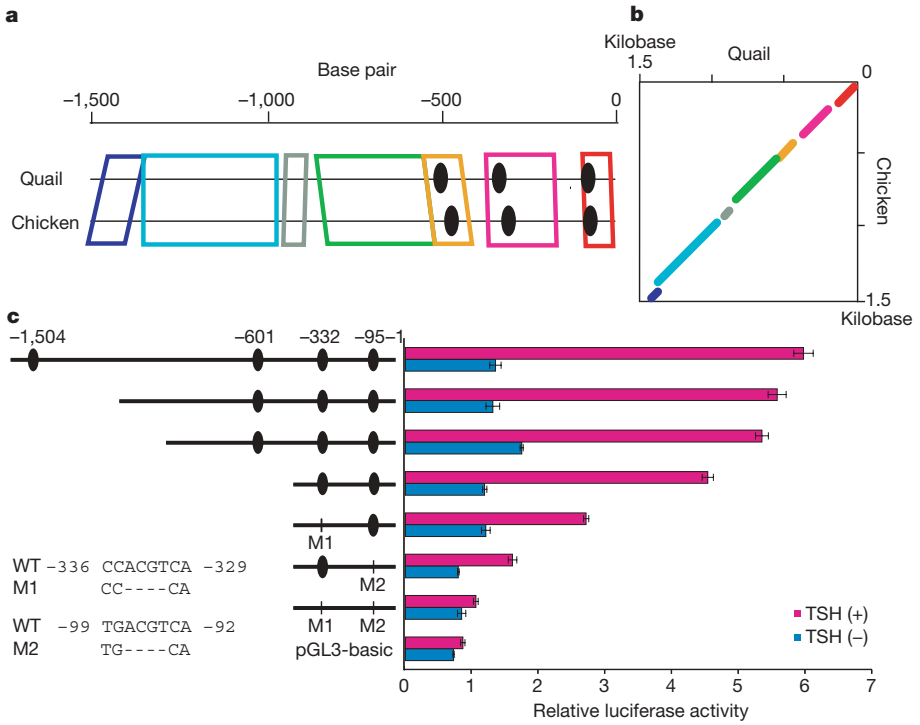

Figure 4 | Involvement of a cAMP signalling pathway in TSH induction of *DIO2* gene expression. **a**, Comparison of quail and chicken *DIO2* gene 5′ upstream sequences. Conserved segments are boxed. Filled ovals, putative CRE sites. **b**, Dot plot analysis of 5′ upstream regions using BLASTN, with greater than 85% identity. **c**, Promoter activity of quail *DIO2*. Wild-type and deletion/mutant reporters fused to the luciferase gene were assayed for their activities in response to TSH. Each value represents the mean ± s.e.m. of three replicates for a single assay.

Fig. 9a, b). We found long-day-induced expression of *DIO2* and reduced expression of *DIO3*, as previously reported[8,9] (Supplementary Fig. 10). *In situ* analysis of genes with known functions and showing differences in expression between long and short days, in the MBH, other than *DIO2* and *DIO3*, confirmed the microarray analysis (Supplementary Fig. 9c). In addition, we found a set of genes encoding various hormones and hormone receptors, which included *TSHB* and *CGA* (Supplementary Fig. 10 and Supplementary Table 3). Because high expression of *TSHB* and *CGA* was observed under chronic long-day conditions, we deduced that increased pars tuberalis TSH may not only play a role in initiating photoinduced secretion of luteinizing hormone, but may also be necessary to maintain the expression of other genes required to support a full reproductive response. We therefore investigated this possibility by prolonged ICV infusion of TSH (1.2 mIU per day) in short-day quail between 8 and 10 weeks of age. This treatment simulated MBH *DIO2* expression and gonadal development (Fig. 5).

Discussion

We have used the 'first day release model' of photoinduced luteinizing hormone release in quail to dissect the temporal pattern of changes in gene expression in the MBH associated with the initiation of photoinduced reproductive function. We found 77 genes that displayed a temporal pattern of expression under short and long days that would be expected of clock genes or clock-driven genes. Because most cycling genes are tissue specific[12,19], future analyses of the relations between the functions of these genes are likely to reveal further details of the molecular basis of the photoperiodic response. Our most important observation was the photoinduction of a first wave of gene expression initiated about 14 h after dawn of the first long day, comprising *TSHB* and *EYA3* in the pars tuberalis. These changes in gene expression are the earliest yet reported, to our knowledge, for the photoperiodic signal transduction pathway. This was followed approximately 4 h later by a second wave of gene expression in the ependymal cells and infundibular nucleus and included an increase in *DIO2*, a key element in the photoperiodic signal transduction pathway[8]. EYA3 is a transcriptional co-activator involved in the development of the eye and forms a nuclear complex with SIX (sine oculis) DNA-binding homeodomain factor and DACH (dachshund) nuclear cofactors[20]; we considered the possibility that the photoinduction of *EYA3* may induce expression of second-wave genes. However, this is unlikely because if *EYA3* is involved in second-wave gene expression it would need to be co-localized with these genes to exert its function. In the present study, *EYA3* was expressed in the pars tuberalis whereas second-wave genes were expressed in the ependymal cells and infundibular nucleus. Several *SIX* genes were observed in the pars tuberalis (Supplementary Fig. 11), suggesting that these may interact with long-day-induced *EYA3* to regulate the expression of genes, the identity of which remains to be established. We therefore focused on the possibility that *TSHB* in the pars tuberalis might be involved in the initiation of *DIO2* and the expression of other second-wave genes. Among the various cycling genes, we found rhythmic expression of *CGA*, and the peak of *CGA* preceded that of

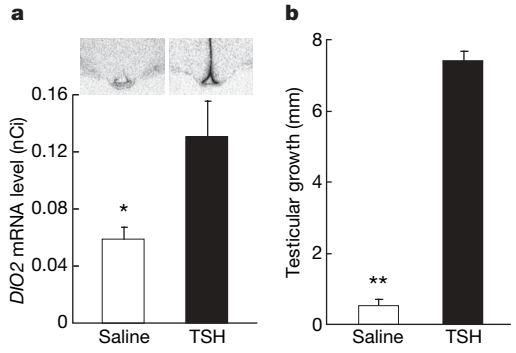

Figure 5 | Effect of chronic ICV infusion of TSH on MBH *DIO2* expression and testicular growth under short-day conditions. **a**, MBH *DIO2* expression. **b**, Testicular growth. (*$P < 0.05$, **$P < 0.01$, *t*-test, mean + s.e.m. $n = 5$).

long-day-induced *TSHB*. The biological activity of TSH requires a non-covalent association of CGA and TSH-β[21]. Thus it appears that the cycling CGA is available for dimerization with long-day-induced TSH-β to form bioactive TSH. It is also of note that translation of TSH occurs at least within 20 min[21]. In addition, unlike luteinizing hormone, dimerization of TSH-β and CGA, and secretion of TSH is very rapid and efficient, with a value of $t_{1/2}$ for the intracellular disappearance (that is secretion) of about 1 h (ref. 21). The expression of *CGA* and *TSHB* in the pars tuberalis therefore indicates that this is a source of biologically active TSH which may be transported into the third ventricle, possibly through tanycytes which abut the pars tuberalis[22]. The target site for pars tuberalis TSH was suggested by the presence of *TSHR* gene expression in the ependymal cells, infundibular nucleus and the pars tuberalis. This observation was supported by a ^{125}I-labelled TSH binding assay which showed specific TSH binding in these loci. Observations on the effects of ICV injection of TSH and anti-TSH-β IgG on *DIO2* and three other second-wave genes demonstrated that TSH triggers the expression of these genes in the ependymal cells. Furthermore, promoter analysis indicated that the induction of *DIO2* is likely to be mediated by the cAMP-signalling pathway. This observation is consistent with the action of TSH on human thyroid gland and rat brown adipose tissue, where *DIO2* expression is regulated by a TSHR–cAMP mediated mechanism[18,23]. Our study revealed a similar TSHR–cAMP mediated mechanism in the quail MBH. In addition to the acute effect of ICV TSH, chronic administration of TSH maintained increased *DIO2* expression and induced testicular growth under short-day conditions. This suggests that elevated TSH in the pars tuberalis may be required to maintain photoinduced reproductive function.

Although it is known that several species become photoperiodically blind after thyroidectomy, quail can respond to photoperiod even after thyroidectomy[1]. Recently, we have reported the involvement of TGF-α in the photoperiodism, and that the TGF-α signalling pathway is not dependent on thyroid hormone activity[24]. Interestingly, the similarity in expression profile between *DIO2* and *TGF-α* suggested that these two genes share the same transcriptional regulation. Although we failed to detect *TGF-α* gene expression in the present microarray analysis, we found TSH induced expression of *TGF-α* (Supplementary Fig. 12a, b). It is, therefore, possible that pars tuberalis TSH may signal photoperiodic information through both DIO2 and TGF-α. The magnitude of testicular growth induced by ICV TSH administration in short-day quail was indistinguishable from that of intact birds kept under long-day conditions (Supplementary Fig. 12c). This suggests that TSH is important not only for triggering photoperiodic responses, but also for the maintaining photoperiodically induced reproductive neuroendocrine function.

Since the discovery of dense melatonin receptors in the pars tuberalis in most mammalian species, but not birds, the pars tuberalis in mammals is considered to be involved in the transmission of photoperiodic stimuli to endocrine outputs through melatonin[25,26]. Further, the thyrotrophe cell type in the mammalian pars tuberalis expresses a high density of melatonin receptors and may regulate seasonal prolactin secretion[27]. However, unlike mammals, there is no evidence that circulating melatonin plays a role in photoperiodic transduction in birds[28]. Consequently, the mechanism transducing photoperiodic information to the avian pars tuberalis remains to be discovered. However, pars tuberalis TSH may be an evolutionarily conserved element of a photoperiodic signal transduction pathway in birds and mammals. This view is consistent with an earlier finding in sheep that expression of TSH-β in the pars tuberalis is not regulated by classical thyrotrophe receptors and their intracellular pathways, but through a novel, photoperiod-dependent mechanism[29].

Our view that photoinduced pars tuberalis TSH in the quail enters the cerebrospinal fluid to induce a photoperiodic response is consistent with the finding in the hamster that photoperiod-dependent changes in TSH-like immunoreactivity occur in the pars tuberalis[30] whereas TSH is found in the cerebrospinal fluid and central nervous system (CNS) of mammals[31,32]. The expression of *TSHR* has been reported in the mammalian brain[33,34], but no function has been proposed in relation to the control of photoperiodic responsiveness. In the present study, we show that long-day-induced TSH in the pars tuberalis triggers the expression of *DIO2* in the ependymal cells (Supplementary Fig. 13). To our knowledge, this is the first demonstration of the likely functional significance of pars-tuberalis-derived TSH in the CNS. Recently, it has been proposed that the pars tuberalis in sheep may be the circannual pacemaker for seasonal prolactin secretion[35]. Thus, the pars tuberalis appears to be the locus for the control of seasonality both in birds and other vertebrates.

In conclusion, one of the most important questions in photoperiodism is the identity of the molecular basis of the mechanism underlying the photoinducible phase that in the quail occurs 12–16 h after dawn[2]. Because photoinduction of *TSHB* expression was observed from about 12 h after dawn of the first long day, increased *TSHB* expression in the pars tuberalis may be the key molecular event defining the onset of the photoinducible phase. Our study presents the first comprehensive analysis of changes in hypothalamic gene expression likely to be involved in the regulation of the long-day reproductive photoperiodic response, and identifies pars tuberalis TSH as a key factor controlling photoperiodic signal transduction. The identification of a key role for *TSHB* expression in the pars tuberalis in reproductive photoperiodic time measurement marks a major advance in our knowledge of molecular mechanisms controlling seasonal breeding.

METHODS SUMMARY

Animals. We used Japanese quail (*C. japonica*) obtained from a local dealer and chicken (*Gallus domesticus*) (WL-G) kept in our colony. Because female birds have ZW chromosomes, they were used for genomic DNA analysis. In all other experiments, male quail were used. The present study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Microarray experiments. We used Affymetrix Chicken Genome Array. This array contains over 38,000 probe sets representing 32,773 transcripts. Genomic DNA individually extracted from liver using DNeasy tissue kit (QIAGEN) was labelled by BioPrime DNA labelling system (Invitrogen). The MBH was punched out (2.5 mm diameter) from 3 mm quail brain slices generated using a mouse brain matrix. Total RNA was prepared from two pools of three MBH at each time point to duplicate our observations on two arrays, using Trizol reagent (Invitrogen); cDNA synthesis and cRNA labelling reactions were performed with One-Cycle Target Labelling and Control Reagents Kit (Affymetrix). Hybridization, wash and stain protocols and scanning were performed using standard Affymetrix protocols. Data were analysed by using GeneSpring GX7.3 software (Agilent Technologies).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions T.Yo. conceived and directed the work. N.N., H.O., T.Ya., T.A., T.T., K.H., S.Y., Y.K., S.K., Y.U. and T.Yo. performed the microarray analysis and *in situ* hybridization. N.N., T.K., H.R.U. and T.Yo. analysed the microarray data. N.N. performed the quantitative PCR and promoter assay. M.I. and P.J.S. determined the luteinizing hormone assay. H.O. and M.I. performed the ^{125}I-labelled TSH binding assay. T.Ya. and A.I. performed the immunocytochemistry. T.Ya., T.A. and A.I. examined the ICV injection and infusion. N.N., H.O., Y.S. and S.S. determined transcriptional start sites and genomic DNA sequences. T.Ni. cloned EYA, SIX and DACH family. M.M., T.Na. and S.E. provided laboratory facilities and new materials. All authors discussed the results and commented on the manuscript. T.Yo. and P.J.S. wrote the paper.

Author Information The microarray data and DNA sequence information have been deposited in NCBI Gene Expression Omnibus (GEO) (GSE8016–GSE8018) and DDBJ/EMBL/GenBank (AB307676–AB307681), respectively. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to T.Yo. (takashiy@agr.nagoya-u.ac.jp).

METHODS

Luteinizing hormone radioimmunoassay. Plasma luteinizing hormone concentrations of quail were determined by radioimmunoassay (RIA) as previously described[24,36].

Discrimination of long-day waves of gene expression. Pearson's correlation analysis was used to calculate the timing of the first and second waves of photoinduced gene expression. Correlation values were statistically tested and the peak time of the expression of each wave was determined by fitting a quadratic function to expression values of ±6 h around the time points with the maximum expression values.

***In situ* hybridization.** *In situ* hybridization was performed by using antisense and sense 45-nucleotide probes (Supplementary Table 4) as previously described[37]. No hybridization signal was observed in sense controls (data not shown).

Quantitative PCR. Reverse transcription was performed on total RNA (0.5 μg) using ReverTra Ace (Toyobo) and oligo-dT primers. Samples contained 1× SYBR Premix Ex Taq (Takara), 0.3 mM gene-specific primers (Supplementary Table 5) and 1/20 synthesized cDNA in a 25 μl volume. Quantitative PCR was performed in duplicates by using ABI Prism 7000 (Applied Biosystems) as follows: 95 °C for 10 s, then 40 cycles of 95 °C for 5 s, 60 °C for 30 s. We used *GAPDH* as an internal control.

Immunocytochemistry. Coronal frozen sections (20 μm) were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 10 min at room temperature. Immunocytochemistry for the anti-chicken/quail TSH-β (1:5,000) was performed using Vectastain Elite ABC rabbit IgG kit (Vector Laboratories) with a standard protocol.

TSH binding assay. Bovine TSH (AFP8755, NIDDK) was labelled with ^{125}I by use of [^{125}I]Bolton-Hunter reagent (NEX120H, PerkinElmer) and purified by gel filtration using PD-10 column (GE Healthcare). Frozen sections were air-dried for 15 min, preincubated in the binding buffer (50 mM Tris-HCl buffer (pH 7.4) containing 0.1% BSA) at 37 °C for 1 h, and then incubated with ^{125}I-labelled TSH (78,000 c.p.m. per millilitre) in the binding buffer with (non-specific binding) or without (specific binding) cold bovine TSH (550 μg ml^{-1}, Sigma) at 37 °C for 1 h. Slides were rinsed in the ice-cold buffer without BSA (twice, 5 min each) followed by a rapid rinse in ice-cold distilled water to remove buffer salts. Labelled sections were apposed to BioMax MR (Kodak).

ICV TSH and TSH-β antibody administration. One week after the cannula implantation into the third ventricle of seven-week-old quail, we injected bovine TSH (T8931, Sigma) or TSH-β antibody through a guide cannula (24-gauge, 6 mm) and measured resulting changes in gene expression. We used bovine TSH, because avian TSH is unavailable and bovine TSH is known to activate avian TSHR[14]. An infrared viewer (NVR 2015, NEC) was used to facilitate TSH injection in darkness. An Alzet 2002 osmotic minipump was used for the prolonged ICV infusion of TSH, as previously reported[8].

Constructs. The 5′-flanking region of quail *DIO2* was subcloned into pGL3-basic vector (Promega) using 5′-ttgctgcctctcttctgccggatgaattca-3′ and 5′-tgaaagctctctcaatgcctcaaggtctg-3′. Deletion constructs and a mutated CRE site were created by PCR-based site-directed mutagenesis[38]. Mutation in the CRE site was generated by deletion of the central four nucleotides (−99: TGACGTCA → TGCA; −336: CCACGTCA → CCCA), as previously reported[39]. Quail *TSHR* cDNA was subcloned into pcDNA 3.1 vector (Invitrogen) using 5′-catgctgtggctgcctgtcgcct-3′, 5′-tcacagctcagtttgcctgc-3′. Constructs were verified by sequencing.

Transfection and luciferase assay. The 293 cells (RIKEN BRC Cell Bank) were plated in 24-well plates at a density of about 2×10^5 cells per well in 1 ml MEM supplemented with 10% fetal calf serum and 0.1 mM NEAA. *DIO2* promoter–luciferase construct (400 ng) was co-transfected with *TSHR* expression vector (400 ng) and the *Renilla* luciferase (phRL-TK, 2 ng, Promega) (for an internal control for transfection efficiency) using LipofectAMINE 2000 reagent (Invitrogen). After 24 h, cells were washed by PBS and treated with or without 1 mIU ml^{-1} bovine TSH (Sigma) in MEM medium. Five hours after this treatment, these media were washed with ice-cold PBS and transcriptional activity was determined using the dual-luciferase assay system (Promega) with Lumat LB950 (Berthold) according to the manufacturer's protocols. Firefly relative luciferase unit (RLU) measurements were normalized to *Renilla* RLU.

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