0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(6):2551–2554

doi: 10.1210/en.2005-0057

The Reciprocal Switching of Two Thyroid Hormone-Activating and -Inactivating Enzyme Genes Is Involved in the Photoperiodic Gonadal Response of Japanese Quail

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The molecular mechanisms underlying photoperiodic time measurement are not well understood in any organism. Relatively recently, however, it has become clear that thyroid hormones play an important role in photoperiodism, and in a previous study we reported that long daylengths in Japanese quail increase hypothalamic levels of T_3 and of the thyroid hormone-activating enzyme, type 2 iodothyronine deiodinase. The present study extends these observations to measure gene levels of the thyroid hormone-inactivating enzyme, type 3 deiodinase. Levels decreased after exposure to long days, but increased under short days. Changes in the two genes were then analyzed during the precisely timed photoinduction that occurs in quail exposed to a single long day. The two gene switches are the earliest events yet recorded in the photoinduction process, and overall, these reciprocal changes offer the potential to regulate active brain thyroid hormone concentrations rather precisely at the site in the brain where photoinduction is triggered. (*Endocrinology* 146: 2551–2554, 2005)

THE PHOTOPERIODIC CONTROL of seasonal reproduction ensures that offspring are born only when abundant food is available. Species vary in how they use the annual information about length of day to regulate breeding, but for most (birds and small mammals) it is the lengthening days of spring that stimulate gonadotropin secretion. For a few (*e.g.* sheep and deer) it is the decreasing photoperiods after the summer solstice that are stimulatory. Birds have evolved especially sophisticated photoperiodic mechanisms, and among these the Japanese quail (*Coturnix coturnix japonica*) has proved an excellent model for studying the phenomena (1). The chain of events, all of which lie within the brain, involves a photoreceptor, a clock (calendar) to measure daylength, and neural circuitry to trigger the increased secretion of GnRH and hence of LH and FSH from the pituitary gland. Many of these functions involve areas within the medial basal hypothalamus (MBH), including the dorsal hypothalamus (nucleus hypothalamus posterior medialis) and the basal tuberal hypothalamus (BTH; infundibular nucleus plus median eminence). The cell bodies of the GnRH neurons lie within the anterior hypothalamus (reviewed in Ref. 2).

Most intriguingly, thyroid hormones are also deeply involved in seasonality (3–5). Removal of the thyroid glands in birds and mammals profoundly changes photoperiodic responses, and these are restored with replacement therapy. Using Japanese quail as a model species, a molecular substrate for these effects has recently been uncovered. Within the quail's basal hypothalamus, long daylengths induce the gene for type 2 iodothyronine deiodinase (*Dio2*) (4). By outer ring deiodination, this enzyme converts the prohormone T_4 into its bioactive form, T_3. It was then shown that under long-day conditions, the hypothalamic content of T_3 is about 10-fold higher than under short-day conditions, whereas the intracerebroventricular infusion of T_3 induced testicular growth in quail held under nonstimulatory short days. The overall control of thyroid hormones at their site of action involves three iodothyronine deiodinases (6). Types 2 and 1 deiodinase (*Dio2* and *Dio1*) generate active T_3 from T_4 by outer ring deiodination, whereas catabolism of both T_3 and T_4 to inactive metabolites by inner ring deiodination employs a type 3 deiodinase (*Dio3*). Synchronizing the activity of these enzymes could play an important role in regulating appropriate active hormone concentrations locally and be varied according to specific needs. We hypothesized, therefore, that photoperiods might alter the activity of *Dio2* and *Dio3* in opposite directions and investigated this in Japanese quail. We also took advantage of the particular characteristics of the photoperiodic response in quail to reinforce the correlation between gene activity and photoinduction.

Materials and Methods

Animals

Male Japanese quail were obtained from a local dealer at 4 wk of age and kept under short-day conditions [8 h of light, 16 h of darkness

First Published Online March 3, 2005

Abbreviations: BTH, Basal tuberal hypothalamus; Dio1, type 1 iodothyronine deiodinase; Dio2, type 2 iodothyronine deiodinase; Dio3, type 3 deiodinase; 8L:16D; 8 h of light, 16 h of darkness; LD, long day; LSD, least significant difference; MBH, medial basal hypothalamus.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

(8L:16D)] in light-tight boxes held within a room at 24 ± 1 C. Light was supplied by fluorescent lamps delivering an intensity of 200 lux at the level of the bird's head. All birds were maintained under these short-day conditions for 1 month before any exposure to long days of 16L:8D. Food and water were available *ad libitum*, and the quail were treated in accordance with the guidelines of Nagoya University.

Differential subtractive hybridization analysis

The differential analysis compared quail from short days (8L:16D) with birds exposed to long days for 2 wk (16L:8D, beginning at 8 wk of age). Brain slices (3 mm thick) from quail were generated using a mouse brain matrix (ASI, Warren, MI), and the MBH punched out (3-mm diameter). This hypothalamic region contained both the dorsal hypothalamus (nucleus hypothalamus posterior medialis) and the more tuberal region (BTH) containing the infundibular nucleus and the median eminence. Total RNA was prepared from 20 pooled MBHs using a TRIzol reagent (Invitrogen Life Technologies, Inc., Gaithersburg, MD) and the polyadenylated RNA purified using Oligotex-dt30 Super (Takara, Otsu, Japan). Differential subtractive hybridization analysis was performed according to the manufacturer's instructions (PCR-Select cDNA Subtraction Kit, BD Clontech, Palo Alto, CA). Final PCR products were inserted into a TA cloning vector (Invitrogen Life Technologies, Inc., Carlsbad, CA) and sequenced by an ABI PRISM 373 using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA).

In situ hybridization

Animals were killed by decapitation, and the brains removed immediately to avoid acute changes in gene expression. *In situ* hybridization was carried out as previously described (7). Antisense and sense 45-mer oligonucleotide probes were labeled with [^{33}P]deoxy-ATP (NEN Life Science Products, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen Life Technologies, Inc.): *Dio2*, 5′-gatggttcagcctcaatgaatatcaagacggaaatacattctgta-3′; and *Dio3*, 5′-tctcctcctggatgacgtagagccgctcgaagtaggcgccgtagg-3′. Hybridization was carried out overnight at 42 C. After the glass slides were washed, they were air-dried and apposed to Biomax-MR film (Eastman Kodak Co., Rochester, NY) for 2 wk with ^{14}C-labeled standards (American Radiolabeled Chemicals, St. Louis, MO). Relative ODs were measured using a computed image-analyzing system (MCID Imaging Research, St. Catharines, Canada) and were converted into the relative radioactive value (nanocuries) by ^{14}C-labeled standards. Specific hybridization signals were obtained by subtracting background values obtained from adjacent brain areas that did not exhibit a hybridization signal.

Effect of serial long-day and one long-day stimulus on Dio2 and Dio3 expression

In the serial long-day (LD) group, birds were transferred from 8L:16D to continuous long days (16L:8D) at 8 wk of age. In the one long-day (1LD) group, birds were transferred from 8L:16D to 1 d of 16L:8D (dawn at same time) at 8 wk of age and then returned to 8L:16D. In practice, both experiments were carried out at the same time using a single batch of birds (108 quail).

Results

Among the candidate genes obtained by the differential analysis, we focused on the *Dio3* gene. Expression of the *Dio3* gene was found in the BTH of quail taken from short daylengths, but was undetectable in quail under long daylengths (Fig. 1; by Mann-Whitney *U* test, $P < 0.01$). These results with *Dio3* are entirely opposite those reported for the expression of *Dio2* (4). In the case of *Dio2*, there was an 8-fold increase in expression (Fig. 1H in Ref. 4) when quail were on long days, while levels are undetectable under short days.

Figure 2 summarizes the changes in the gene expression of both *Dio3* and *Dio2* when a group of quail was transferred from short to long days and kept under these photoperiods

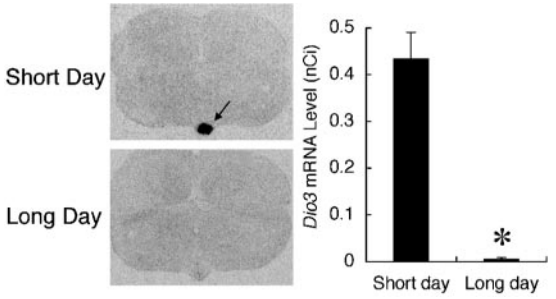

FIG. 1. Long day exposure suppresses *Dio3* expression in the quail's hypothalamus. *Left*, Representative autoradiograms of *Dio3* expression in transverse sections at the level of the BTH (indicated by *arrow*). *Right*, Quantitative results from the autoradiograms (mean ± SEM; n = 3) of *Dio3* expression of quail under long and short days. The high level under short days is significantly greater than that under long days (*, $P < 0.01$, by Mann-Whitney *U* test).

for the next 10 d. A total of 60 quail were shifted from short to long days and killed in batches of four birds on 15 occasions starting at time zero and ending 10 d later. Reciprocal changes occur in both genes, and these are highly significant: *Dio3* expression was reduced upon transfer to long days and remained low [by one-way ANOVA, $F(14,43) = 46.226$; $P < 0.01$; by Fisher's least significant difference (LSD) *post hoc* test, $P < 0.01$], whereas *Dio2* expression was increased upon transfer to long days and remained high [by one-way ANOVA, $F(14,44) = 3.201$; $P < 0.01$; by Fisher's LSD *post hoc* test, $P < 0.01$].

A particular feature of the Japanese quail is its capacity to respond rapidly and precisely to long photoperiods, with measurable effects being detectable within the first long day. Two features of this so-called first day response were analyzed with respect to *Dio2* and *Dio3* expression: the timing of the earliest detectable changes in gene expression during the first long day and the pattern of expression changes after returning the quail to short days. Figure 3A summarizes an

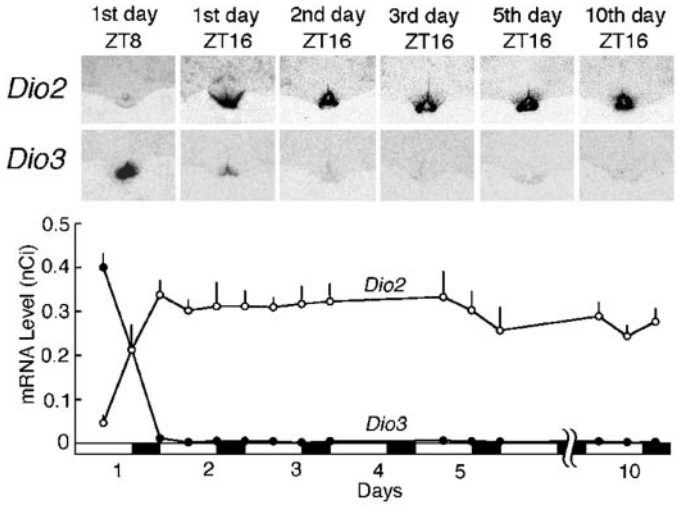

FIG. 2. The photoperiodic responses within the BTH of the genes responsible for thyroid hormone activation (*Dio2*) and inactivation (*Dio3*) in a group of quail transferred from short to long days. Representative autoradiograms are shown in the *upper* panel; the *lower* graph shows the quantitative responses (mean ± SEM; n = 3–4).

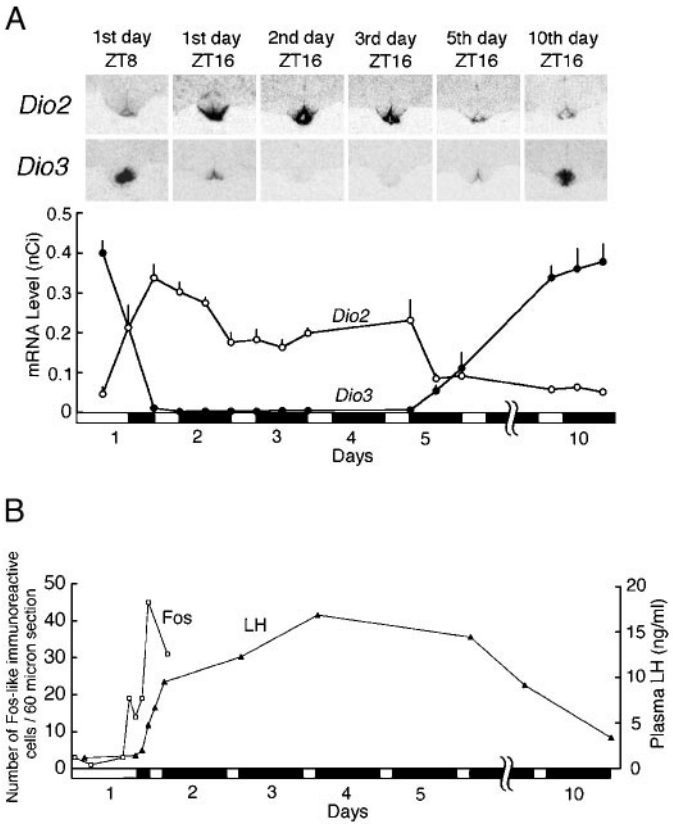

FIG. 3. The first day response in quail: the reciprocal photoperiodic induction of thyroid hormone-metabolizing enzyme gene expression mirrors that of LH secretion. A, A batch of birds was exposed to a single long day and returned to short days. *Dio2* and *Dio3* gene expression are shown at 15 time points. Representative autoradiograms are shown in the *upper* panel, and quantitative responses are shown in the graph (mean ± SEM; n = 3–4). B, Results for the first day response of LH secretion and c-Fos expression in the BTH are redrawn from Ref. 1. Note that the daylength used was slightly different from that in the present study. ZT, Zeitgeber time.

experiment in which a group of intact male quail (n = 48) was exposed to 1 long day of 16L:8D and then returned to 8L:16D. Batches of four quail were killed at 12 time points over the 9 d of the experiment (the data during the first long day are replotted Fig. 2). We have added Fig. 3B to provide a temporal comparison with earlier results on changes during photoinduction in quail. It shows the pattern of LH secretion and also the timing of induction of c-Fos within the BTH as reported by the Bristol group (taken from Ref. 1).

The first detectable change in gene expression occurred by h 16 of the long day and had taken place completely by h 24 (Fig. 3, d 1). The overall experiment showed a wave of *Dio2* expression occurring for 5 d [by one-way ANOVA, $F(14,41) = 16.693$; $P < 0.01$; by Fisher's LSD *post hoc* test and then decreasing to low levels. In contrast, *Dio3* was switched off within the first long day and only began to recover after 4 short days [by one-way ANOVA, $F(14,43) = 40.640$; $P < 0.01$; by Fisher's LSD *post hoc* test]. Although a precise overlap between LH secretion and the gene expression changes clearly requires direct experimental testing, the broad story is very similar, with gene expression mirroring photoinduction.

Discussion

In the present study we report within the tuberal hypothalamus high expression of *Dio3* and low expression of *Dio2* under short-day conditions and low expression of *Dio3* and high expression of *Dio2* under long-day conditions. Although the relationship between *Dio2* and *Dio3* during the development has been discussed in several species (8, 9), to our knowledge, this is the first demonstration of the reciprocal expression of *Dio2* and *Dio3* in adult tissue. Long-day-induced *Dio2* should maximize the local production of T_3, whereas long-day-reduced *Dio3* expression should minimize T_3 metabolism. Such conclusions accord with our previous finding of a 10-fold increase in T_3 content within the quail's MBH when birds are photostimulated. Independently, we examined the expression of all known thyroid hormone transporters within the quail's hypothalamus to clarify the basis for the increase in T_4 content under long-day conditions, but the levels of expression did not appear different between short and long days (Takagi, T., N. Nakao, and T. Yoshimura, unpublished observations). Because *Dio3* transforms not only T_3, but also T_4, to inactive metabolites by inner ring deiodination, high expression of *Dio3* under short-day conditions should actively eliminate substrate (T_4) when it is not necessary.

The exact timing of the changes in gene expression during the very first long day is of particular interest. By h 16 there had been a significant increase in *Dio2* ($P < 0.01$) and a significant decrease in *Dio3* ($P < 0.01$). These gene changes appear to precede the first rise in LH secretion (Fig. 3B) that occurs at about h 22 (1). The only event known to precede LH secretion is immediate early gene activation (c-*fos*) in the MBH, but even this is not detectable before h 18 (Fig. 3B) (10).

At this stage we conclude that thyroid hormone gene switching is the earliest event yet detected in the photoperiodic cascade and that it must occur at or before h 16. This fits with earlier experiments indicating that a single day of 16 h is just long enough to induce LH secretion (1). Our hypothesis remains that long days stimulate local T_3 production within the MBH while simultaneously inhibiting its catabolism. Acting in concert, these opposite effects on gene activation amplify the localized action of thyroid hormones and lead to neuroendocrine changes that cause GnRH secretion a few hours later. The basis for the delay is unknown, but may involve changes in the interaction between GnRH nerve terminals and glial end-feet on the median eminence (11). Finally, we need to discover how photoperiod can switch two genes in opposite directions and by what means this is achieved.

Acknowledgments

We thank Nagoya University Radioisotope Center for the use of facilities.

Received January 14, 2005. Accepted February 22, 2005.

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This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (to T.Y.).

References

1. **Follett BK, King VM, Meddle SL** 1998 Rhythms and photoperiodism in birds. In: Lumsden PJ, Miller AJ, eds. Biological rhythms and photoperiodism in plants. Oxford: BIOS Scientific; 231–242
2. **Juss TS** 1993 Neuroendocrine and neural changes associated with the photoperiodic control of reproduction. In: Sharp PJ, ed. Avian endocrinology. Bristol: Society for Endocrinology; 47–60
3. **Nicholls TJ, Follett BK, Goldsmith AR, Pearson H** 1988 Possible homologies between photorefractoriness in sheep and birds: the effect of thyroidectomy on the length of the ewe's breeding season. Reprod Nutr Dev 28:375–385
4. **Yoshimura T, Yasuo S, Watanabe M, Iigo M, Yamamura T, Hirunagi K, Ebihara S** 2003 Light-induced hormone conversion of T_4 to T_3 regulates photoperiodic response of gonads in birds. Nature 426:178–181
5. **Dawson A, Thapliyal JP** 2001 The thyroid and photoperiodism. In: Dawson A, Chatruvedi CM, eds. Avian endocrinology. New Delhi: Narosa; 141–151
6. **Leonard JL, Visser TJ** 1986 Biochemistry of Deiodination. In: Hennemann G, ed. Thyroid hormone metabolism. New York: Marcel Dekker; 189–229
7. **Yoshimura T, Suzuki Y, Makino E, Suzuki T, Kuroiwa A, Matsuda Y, Namikawa T, Ebihara S** 2000 Molecular analysis of avian circadian clock genes. Brain Res Mol Brain Res 78:207–215
8. **Becker KB, Stephens KC, Davey JC, Schneider MJ, Galton VA** 1997 The type 2 and type 3 iodothyronine deiodinase play important roles in coordinating development in *Rana catesbeiana* tadpoles. Endocrinology 138: 2989–2997
9. **Bates JM, St Germain DL, Galton VA** 1999 Expression profiles of the three iodothyronine deiodinases, D1, D2, and D3, in the developing rat. Endocrinology 140:844–851
10. **Meddle SL, Follett BK** 1997 Photoperiodically driven changes in Fos expression within the basal tuberal hypothalamus and median eminence of Japanese quail. J Neurosci 17:8909–8918
11. **Yamamura T, Hirunagi K, Ebihara S, Yoshimura T** 2004 Seasonal morphological changes in the neuro-glial interaction between gonadotropin-releasing hormone nerve terminals and glial endfeet in Japanese quail. Endocrinology 145:4264–4267

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