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Hypothalamic expression of thyroid hormone-activating and -inactivating enzyme genes in relation to photorefractoriness in birds and mammals

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Watanabe T, Yamamura T, Watanabe M, Yasuo S, Nakao N, Dawson A, Ebihara S, Yoshimura T. Hypothalamic expression of thyroid hormone-activating and -inactivating enzyme genes in relation to photorefractoriness in birds and mammals. *Am J Physiol Regul Integr Comp Physiol* 292: R568–R572, 2007; doi:10.1152/ajpregu.00521.2006.—Photorefractoriness is the insensitivity of gonadal development to the stimulatory effects of long photoperiods in birds and to the inhibitory effects of short photoperiods in small mammals. Its molecular mechanism remains unknown. Recently, it has been shown that reciprocal expression of thyroid hormone-activating enzyme [type 2 deiodinase (Dio2)] and -inactivating enzyme [type 3 deiodinase (Dio3)] genes in the mediobasal hypothalamus is critical for photoperiodically induced gonadal growth. Since thyroid hormones are required not only for photoinduction, but also for the induction of photorefractoriness, we examined the expression of these genes in relation to photorefractoriness in birds and mammals. Transfer of birds to long photoperiods induced strong expression of *Dio2*. This was maintained in tree sparrow when they later became photorefractory, but decreased somewhat in quail. In hamsters, transfer to long photoperiods also induced strong expression of *Dio2*. High values were not maintained under long photoperiods, and, indeed, expression decreased at the same rate as in animals transferred to short photoperiods. There was no renewed expression of *Dio2* associated with testicular growth as animals became refractory to short photoperiods. Expression of *Dio3* was high under short photoperiods and low under long photoperiods in all the animals examined, except for the short photoperiod-refractory hamsters. Our present study revealed complex regulation of deiodinase genes in the photoinduction and photorefractory processes in birds and mammals. These gene changes may be involved in the regulation of photorefractoriness, as well as photoinduction.

Eurasian tree sparrow; Japanese quail; Djungarian hamster

THE MAJORITY OF BIRD AND MAMMAL species living outside the tropics uses a changing photoperiod to time their breeding seasons, but the photoreceptive and neuroendocrine mechanisms involved differ markedly between them (10, 15). Also, the degree of gonadal regression outside the breeding season is greater in birds, which may be an adaptation to flight, and the duration of breeding seasons of birds tend to be more restricted and asymmetrical than those of mammals. Although the times of gonadal maturation and regression are controlled by photoperiod in both birds and mammals, the period of gonadal maturation, the breeding season, is rarely symmetrical with the annual change in photoperiod. The asymmetry is caused by photorefractoriness.

Photorefractoriness is the switch from an active to an inactive reproductive state, or vice versa, that occurs apparently spontaneously at some stage during prolonged exposure to a particular photoperiod (19, 20). In the case of birds, transfer from a short to a long photoperiod initially induces gonadal maturation, but some time later gonadal regression occurs as birds become refractory to the long photoperiod (8, 10, 28). Some species, e.g., Japanese quail (*Coturnix japonica*), do not show spontaneous gonadal regression but become predisposed to undergo regression when the photoperiod is reduced somewhat, but to a photoperiod still longer than earlier required to induce maturation. This is relative, as opposed to absolute, photorefractoriness (25). In the case of small mammals, such as hamsters, transfer from a short to a long photoperiod also induces immediate gonadal maturation, and subsequent transfer to a short photoperiod induces immediate regression. Photorefractoriness in mammals is the spontaneous renewed gonadal maturation that occurs later during prolonged exposure to a short photoperiod (18–20). Although the same term, photorefractoriness, is used for both phenomena, the process in mammals may be opposite to that in birds. Refractoriness to short photoperiods in mammals may be equivalent to the termination of photorefractoriness to long photoperiods in birds. Both occur during exposure to a short photoperiod and involve reactivation of the reproductive system. In birds this reactivation is characterized by renewed photosensitivity, but, unlike mammals, is not normally associated with spontaneous rapid gonadal maturation.

Recently, it has been shown that local activation of thyroid hormone in the mediobasal hypothalamus (MBH) is critical for long photoperiod-induced testicular growth in Japanese quail (32, 35). Long photoperiods induce expression of type 2 deiodinase (*Dio2*) gene and reduce expression of type 3 deiodinase (*Dio3*) gene. Dio2 is the thyroid hormone-activating enzyme; it converts the prohormone thyroxine (T_4) into the bioactive form T_3 by outer-ring deiodination. The inactivating enzyme (Dio3) converts both T_4 and T_3 into inactive metabolites reverse T_3 and T_2, respectively, by inner-ring deiodination. It is well established that thyroid hormones are involved not only in photoinduction, but also in photorefractoriness (5, 10). Removal of the thyroid gland blocks photorefractoriness

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in the European starling, American tree sparrow, European house sparrow, and Japanese quail (6, 11, 16, 23, 27). In the present study, therefore, we examined expression of *Dio2* and *Dio3* in relation to photorefractoriness in Eurasian tree sparrows (*Passer montanus*), which show absolute photorefractoriness, and Japanese quail, which show relative photorefractoriness.

As is the case in birds, thyroid hormones are involved in the regulation of seasonal reproduction in mammals. Thyroidectomy blocks transition of seasonal reproduction in sheep (19, 20), and photoperiodic regulation of *Dio2* is observed in the Djungarian hamster (*Phodopus sungorus*) and goat (*Capra hircus*) (26, 31). Moreover, in hamsters, *Dio2* expression is suppressed by melatonin administration (26), and exogenous thyroid hormone administration elicits a long photoperiod response under short photoperiods (13). Although cDNA microarray analysis identified a class of genes encoding thyroxine-binding proteins whose expression is associated with refractoriness (21), a molecular mechanism for mammalian refractoriness remains elusive. Therefore, we examined expression of *Dio2* and *Dio3* in the hypothalamus of photostimulated and photorefractory hamsters.

MATERIALS AND METHODS

Animals and housing. Male tree sparrows were caught from the wild in Nagoya during August 2003. They were kept under 8 h light: 16 h dark (8L16D) condition for 3 mo at 24 ± 1°C in light-tight boxes (55 × 210 × 62 cm). Male 4-wk-old Japanese quail were obtained from a local dealer and kept in the same conditions until 8 wk old. Djungarian hamsters were kept in our colony under 14L10D conditions until weaning at 3 wk old and then transferred into light-tight boxes. In the light-tight boxes, light was supplied by fluorescent lamps with a light intensity of 200 lux measured at the level of the animal's head. Food and water were provided ad libitum for all animals, and sunflower seeds were given once per week to hamsters. Animals were treated in accordance with the guidelines of Nagoya University.

Light schedules. Sparrows and quail were transferred from short photoperiods (8L16D) to long photoperiods (18L6D). Brains were collected before transfer (0 wk; photosensitive), 6 wk after transfer (photostimulated), and 20 wk after transfer (absolutely photorefractory in sparrows, relative photorefractory in quail). In each case, this was done at the midpoint of the light phase.

To examine the effects of long photoperiods, hamsters were moved to 8L16D after weaning to induce testicular regression as previously described (26). One group of animals was continuously kept under 8L16D, and another group was transferred to 14L10D at 7 wk of age. Brains were collected at 9 wk of age. To examine gene expression in refractory animals, hamsters were kept under 14L10D after weaning. When 7 wk old, animals for the refractory group were transferred to 10L14D, and control animals were kept under 14L10D. Brains were collected (at the midpoint of the light phase) before transfer to 10L14D (0 wk; photostimulated), 6 wk after transfer (gonadal regressed), and 27 wk after transfer (refractory).

In situ hybridization. In situ hybridization was carried out according to previous work (34). Antisense 45-oligomer oligonucleotide probes were labeled with [^{33}P]deoxy-ATP (NEN Life Sciences, Boston, MA) using terminal deoxyribonucleotidyl transferase (Invitrogen Life Technologies): sparrow and quail *Dio2*, 5′-gatggttcagcctcaatgaatatcaagacggaaatacattctgta-3′; sparrow *Dio3*, 5′-ggatgatgtagagcctctcgaagtaggcaccgtaggcggcgctgg-3′; quail *Dio3*, 5′-tctcctcctggatgacgtagagccgctcgaagtaggcgccgtagg-3′; hamster *Dio2*, 5′-tgcttgagtagaatgaccgagtcatagagcgccaggaagaggcag-3′; and hamster *Dio3*, 5′-ctggtaaccgtcggggccacggcctccctggtacatgatggtgcc-3′. Coronal sections (20 μm thick) were prepared using a Cryostat (model CM3050S; Leica, Nussloch, Germany). Hybridization was carried out overnight at 42°C. After the glass slides were washed, they were air-dried and apposed to Biomax-XR film (Eastman Kodak, Rochester, NY) for 2 wk with ^{14}C-labeled standards (American Radiolabeled Chemicals, St. Louis, MO). Relative optical densities were measured using a computerized image analysis system (MCID Imaging Research, St. Catharines, Canada) and were converted into relative radioactive values (nanocuries) using ^{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.

RESULTS

Expression of Dio2 and Dio3 in photorefractory birds. In sparrows, there was significant testicular growth 6 wk after transfer to long photoperiods, followed by complete regression after 20 wk, as birds became absolutely photorefractory [one-way ANOVA, $F(2,9) = 44.6$, $P < 0.0001$, Fisher's least significant difference (LSD) post hoc test, $P < 0.0001$, $n = 4$](Fig. 1*A*). In contrast, in relative photorefractory quail, the increase in testis size after 6 wk of photostimulation was maintained at 20 wk, as expected [one-way ANOVA, $F(2,11)=138.3$, $P < 0.0001$; Fisher's LSD post hoc test, $P < 0.0001$, $n = 4$–5](Fig. 1*B*).

Expression of *Dio2* and *Dio3* was observed in the basal tuberal hypothalamus, consisting of the infundibular nucleus and the median eminence in both sparrow and quail (Fig. 2, *A* and *B*). This is consistent with previous reports. In sparrows, expression of *Dio2* was significantly increased 6 wk after transfer to long photoperiods, and this high expression was maintained at 20 wk [one-way ANOVA, $F(2,10) = 20.6$, $P < 0.0005$; by Fisher's LSD post hoc test, $P < 0.001$, $n = 4$ or 5] (Fig. 2*A*). In quail, expression of *Dio2* was also significantly increased 6 wk after transfer to long photoperiods, but expression was somewhat attenuated at 20 wk [one-way ANOVA, $F(2,11) = 63.5$, $P < 0.0001$; Fisher's LSD post hoc test, $P < 0.005$, $n = 4$–5] (Fig. 2*B*). These experiments were repeated using different series of animals with consistent results (data not shown). In contrast to *Dio2*, expression of *Dio3* was high under short photoperiods (0 wk) but almost undetectable 6 wk and 20 wk after transfer to long photoperiods in both sparrow and quail [sparrow: one-way ANOVA, $F(2,10) = 41.8$, $P < 0.0001$, Fisher's LSD post hoc test, $P < 0.0001$, $n = 4$–5;

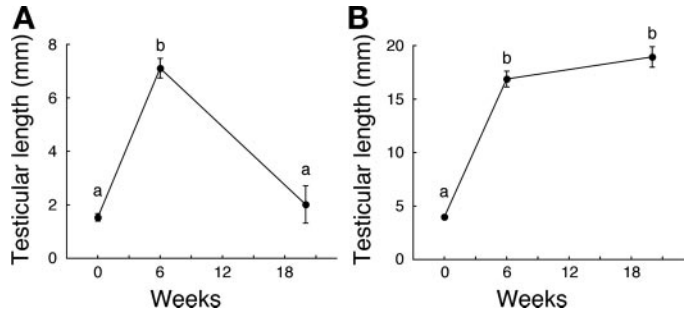

Fig. 1. Effect of long photoperiods on testicular length in absolute photorefractory Eurasian tree sparrow (*A*) and relative photorefractory Japanese quail (*B*). Testicular length was measured before transferring to long photoperiods (0 wk; photosensitive state), 6 wk after transfer to long photoperiods (photostimulatory state), and 20 wk after transferred to long photoperiods (photorefractory state in sparrow). Values are means ± SE ($n = 4$–5). Different characters indicate significant differences [one-way ANOVA and Fisher's least significant difference (LSD) post hoc test].

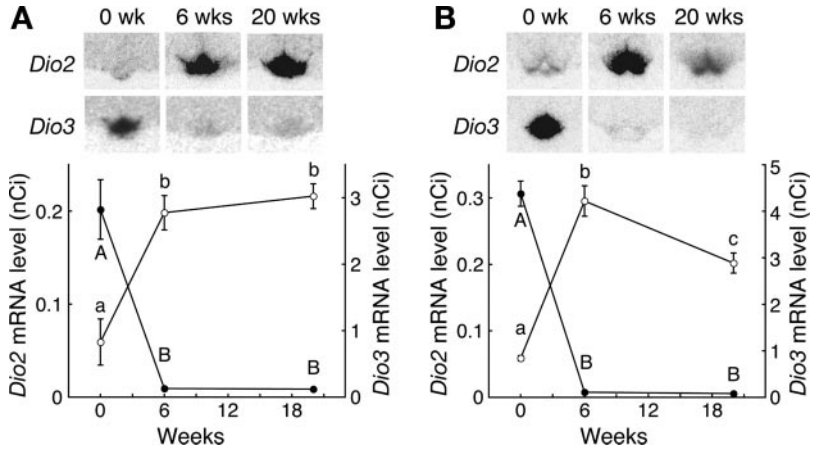

Fig. 2. Expression of thyroid hormone-activating (*Dio2*) and -inactivating (*Dio3*) enzyme genes in sparrows (*A*) and quail (*B*). *Top*: representative autoradiograms for *Dio2* and *Dio3* expressions in the basal tuberal hypothalamus. *Bottom*: quantitative results of *Dio2* (○) and *Dio3* (●). Values are means ± SE (n = 3–5). Different letters (lower case for *Dio2* and upper case for *Dio3*) indicate significant differences (one-way ANOVA and Fisher's LSD post hoc test).

quail: $F(2,8) = 209.2$, $P < 0.0001$, Fisher's LSD post hoc test, $P < 0.0001$, n = 3–4](Fig. 2, *A* and *B*).

Expression of Dio2 and Dio3 in long-day-stimulated and short-day-refractory hamsters. When hamsters were transferred from short to long photoperiods, testicular mass increased (Mann-Whitney U-test, $P < 0.01$, $n = 5$) (Fig. 3). When hamsters were transferred from long to short photoperiods, testicular mass was decreased at 6 wk and then returned to long photoperiod values at 27 wk as hamsters became refractory to short photoperiods. High testicular mass was maintained in animals that had been kept on long photoperiods [two-way ANOVA, $F(2,26) = 55.3$, $P < 0.0001$; Mann-Whitney U-test, $P < 0.01$, n = 5–7] (Fig. 3).

Dio2 expression was found in the ependymal cell layer lining the infralateral walls of the third ventricle and the cell-clear zone overlying the tuberoinfundibular sulcus, as previously described (Fig. 4*A*). *Dio3* expression was observed only in the ependymal cell layer lining the infralateral walls of the third ventricle (Fig. 4*B*). When animals were transferred from short to long photoperiods, a significant increase in *Dio2* expression and decrease in *Dio3* expression were observed (Mann-Whitney U-test, $P < 0.01$, $n = 5$) (Fig. 4). Interestingly, when hamsters were maintained under long photoperiods continuously, expression of *Dio2* decreased [one-way ANOVA, $F(3,16) = 15.9$, $P < 0.0001$, Fisher's LSD post hoc test, $P < 0.05$, n = 5–7] (Fig. 4*A*). When hamsters were transferred from long to short photoperiods, expression of *Dio2* also decreased; there was no significant difference between long and short photoperiod animals [two-way ANOVA, $F(3,18) = 0.712$, $P > 0.5$, n = 5–7] (Fig. 4*A*). Expression of *Dio3* was undetectable under long photoperiods. Strong expression of *Dio3* was observed in hamsters shortly after transfer to short photoperiods, but this was not maintained; there was no expression of *Dio3* in hamsters as they became refractory to short photoperiods [(two-way ANOVA for the comparison between LD→SD and LD→LD), $F(2,26) = 156.0$, $P < 0.0001$; Mann-Whitney U-test, 6 wk: $P < 0.01$ (asterisk); 27 wk: $P > 0.8$; one-way ANOVA for the comparison among the SD group, $F(2,14) = 63.9$, $P < 0.0001$; Fisher's LSD post hoc test, $P < 0.05$, n = 5–7] (Fig. 4*B*).

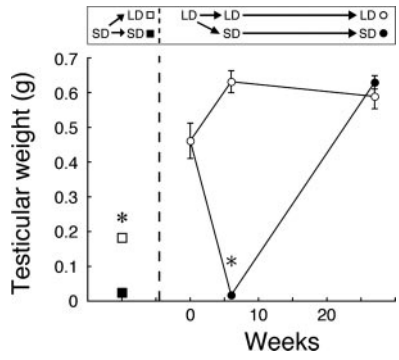

Fig. 3. Effect of different photoperiods on testicular weight in hamster. *Left*: animals were transferred from short (SD) to long (LD) photoperiods (□) or continuously kept under short photoperiods (■) for 2 wk. In the right side of the graph, animals were transferred from long to short photoperiods (●) or continuously kept under long photoperiods (○). Values are means ± SE (n = 5–7). *Significant difference between the short photoperiod and long photoperiod groups (Mann-Whitney U-test).

DISCUSSION

In the present study, we examined expression of *Dio2* and *Dio3* in absolutely photorefractory Eurasian tree sparrows and in relative photorefractory Japanese quail. In these species, expression of *Dio2* and *Dio3* was directly related to photoperiod (i.e., high expression of *Dio3* and low expression of *Dio2* under short photoperiods and high expression of *Dio2* and low expression of *Dio3* under long photoperiods), but did not relate to gonadal status. Differences in reproductive state depend on the amplitude and frequency of pulsatile secretion of gonadotropin-releasing hormone (GnRH). Unlike mammals, there is a profound physiological switch-off of the GnRH system in seasonally breeding birds (10), including sparrows (6). In absolutely photorefractory birds, a dramatic decline in hypothalamic GnRH content is observed by radioimmunoassay and immunocytochemistry, suggesting that the GnRH system is regulated at the level of synthesis, as well as secretion (3, 10, 22). In contrast to species that become absolute photorefractory, relative photorefractory quail show no decline in hypothalamic GnRH (12). This suggests a fundamental difference between the mechanisms underlying the two forms of photorefractoriness (10).

Thyroid hormones are involved in both photoinduction and photorefractoriness. In the previous study, we observed seasonal morphological changes in the neuro-glial interaction between GnRH nerve terminals and glial endfeet in the median eminence of Japanese quail (29). Since these morphological changes were also caused by T_3 administration, long-photope-

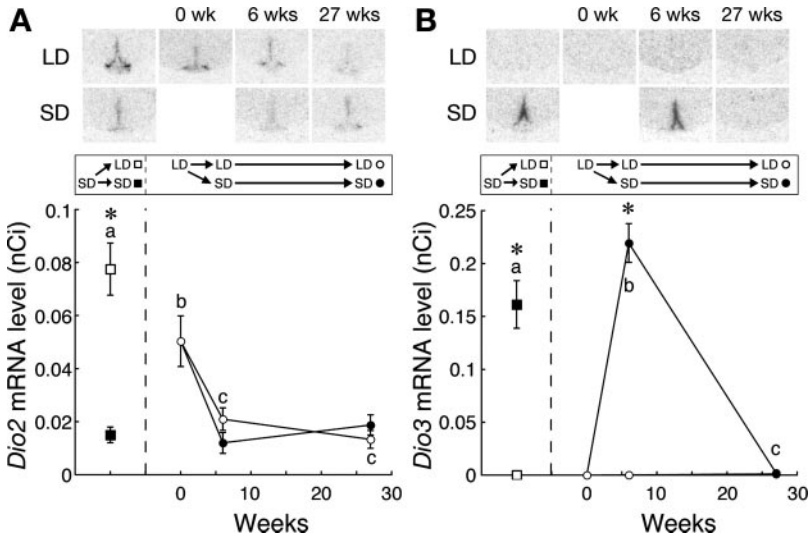

Fig. 4. Effect of different photoperiods on *Dio2* (*A*) and *Dio3* (*B*) expression in hamsters. Representative autoradiograms are shown *top*. Quantitative results are shown *bottom*. *Left*: animals were transferred from short to long photoperiods (□) or continuously kept under short photoperiods (■) for 2 wk. *Right*: animals were transferred from long to short photoperiods (●) or continuously kept under long photoperiods (○). Values are means ± SE (n = 5–7). Different characters indicate significant differences within the long photoperiod group (*A*) and short photoperiod group (*B*), respectively (one-way ANOVA and Fisher's LSD post hoc test). *Significant difference between the short and long photoperiod groups (Mann-Whitney *U*-test).

riod-induced T_4 to T_3 conversion by Dio2 may regulate GnRH secretion in quail during photoinduction (30). Starlings and sparrows become photoperiodically blind following thyroidectomy (5, 6). In these species, prevention of photorefractoriness by thyroidectomy is associated with maintenance of high hypothalamic GnRH levels typical of photosensitive birds (6, 7, 22) and thyroidectomy of photorefractory birds results in an increase in GnRH (9). These reports suggest that thyroid hormones are required to reduce GnRH synthesis during the photorefractory state, rather than a decrease in secretion. The maintenance of high expression of *Dio2* and low expression of *Dio3* as tree sparrows became photorefractory in the present study supports the idea that photorefractoriness is not due to an inhibition of GnRH secretion, i.e., a reversal of what happens during photostimulation. Rather, T_3 may be involved in the long-photoperiod process leading to decreased GnRH synthesis. However, quail also maintained high expression of *Dio2*, although somewhat less than during photostimulation, as they became relatively photorefractory. Yet this is not associated with a decrease in hypothalamic stores of GnRH.

In short-day breeders, such as sheep and goats, thyroid hormones are required for the transition from estrus to anestrus in the spring. In the previous study, we found high expression of *Dio2* in the hypothalamus of goats during this transition stage (31). As possible homologies between photorefractoriness in long-day birds and short-day mammals have been pointed out (19, 20), the present results also appear to suggest that the mechanism regulating short-day breeders and long-day breeders may not be so radically different as previously thought.

In the present study, we have also examined expression of *Dio2* and *Dio3* in Djungarian hamsters. Consistent with our previous report (26), we observed significant induction of *Dio2* expression when transferred from short to long photoperiods. However, expression of *Dio2* decreased when hamsters were continuously kept under long photoperiods. Barrett et al. (1) have reported that they failed to detect photoperiodic change of *Dio2* expression in their Siberian hamster. In the present study, we did not find statistically significant difference in *Dio2* expression between short and long photoperiod hamsters when we transferred animals from long to short photoperiods. This may explain the discrepancy between the results of our previous study and that of Barrett et al., because they examined expression of *Dio2* in animals transferred from long to short photoperiods. Recently, Revel et al. (24) reported photoperiodic regulation of *Dio2* in Syrian hamster (*Mesocricetus auratus*). In contrast to Djungarian hamsters, *Dio2* expression remained elevated in the long photoperiod for at least 28 wk in the Syrian hamsters. Although these two hamsters are known to be good photoperiodic models, differences in photoperiodic responses are reported (4, 14, 17, 33). Although both species exhibit gonadal regression when exposed to short photoperiods, they show opposite body weight changes (i.e., Djungarian hamsters lose weight, but Syrian hamsters gain weight following short photoperiod exposure) (2). In addition, puberty is apparently unaffected by the photoperiod in Syrian hamsters (4, 14), while it is highly affected by photoperiod in Djungarian hamsters (17, 33). Differences in *Dio2* expression profiles may contribute to the different photoperiodic responses between the two hamsters.

In contrast to *Dio2*, marked increase in *Dio3* expression was observed in short photoperiod hamsters. Since Dio3 metabolizes both prohormone T_4 and bioactive T_3, Dio3 may contribute to testicular regression when hamsters are transferred from long to short photoperiods. In short-day refractory hamsters, expression of *Dio2* and *Dio3* was undetectable. Consistent with the present results, a low level of *Dio2* expression is reported in the short-day refractory Syrian hamster (24). It is of note that the expressions of a class of genes encoding thyroxine-binding proteins (TBPs; transthyretin, T_4-binding globulin, and albumin) are downregulated and that T_4 uptake was diminished in the hypothalamus of refractory Siberian hamsters (21). Although the molecular mechanism regulating refractoriness to short photoperiods in hamsters remains unknown, lack of expression of thyroid hormone-activating and -inactivating enzyme genes and TBPs may suggest that refractoriness to short photoperiods in hamsters is not thyroid dependent.

It has been known for several decades that thyroid hormones are involved in regulation of photorefractoriness (6, 11, 16, 23, 27). Our present study revealed complex regulation of deiodinase genes in the photoinduction and photorefractory processes in birds and mammals. This is a first step toward understanding

the molecular mechanism regulating photorefractoriness but many questions remain.

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