Brief Communications

Melatonin Transmits Photoperiodic Signals through the MT1 Melatonin Receptor

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Melatonin transmits photoperiodic signals that regulate reproduction. Two melatonin receptors (MT1 and MT2) have been cloned in mammals and additional melatonin binding sites suggested, but the receptor that mediates the effects of melatonin on the photoperiodic gonadal response has not yet been identified. We therefore investigated in mice whether and how targeted disruption of MT1, MT2, or both receptor types affects the expression level of two key genes for the photoperiodic gonadal regulation, type 2 and 3 deiodinase (*Dio2* and *Dio3*, respectively). These are expressed in the ependymal cell layer lining the infundibular recess of the third ventricle and regulated by thyrotropin produced in the pars tuberalis. In wild-type C3H mice, *Dio2* expression was constantly low, and no photoperiodic changes were observed, whereas *Dio3* expression was upregulated under short-day conditions. In C3H with targeted disruption of MT1 and MT1/MT2, *Dio2* expression was constitutively upregulated, *Dio3* expression was constitutively downregulated, and the photoperiodic effect on *Dio3* expression was abolished. Under short-day conditions, C3H with targeted disruption of MT2 displayed similar expression levels of *Dio2* and *Dio3* as wild-type animals, but they responded to long-day condition with a stronger suppression of *Dio3* than wild-type mice. Melatonin injections into wild-type C57BL mice suppressed *Dio2* expression and induced *Dio3* expression under long-day conditions. These effects were abolished in C57BL mice with targeted disruption of MT1. All data suggest that the melatonin signal that transmits photoperiodic information to the hypothalamo–hypophysial axis acts on the MT1 receptor.

Introduction

In many mammalian species living in temperate zones, changes in photoperiod regulate various functions including reproduction. Transmission of photoperiodic information involves melatonin secreted from the pineal gland during darkness. The duration of the melatonin signal encodes the length of the night (Bartness et al., 1993). Two melatonin receptor subtypes, MT1 and MT2, are identified in mammals (for review, see von Gall et al., 2002b). MT1 plays an important role in rodent brain, and pituitary and [^{125}I]iodomelatonin binding disappears in MT1-deficient mice (Liu et al., 1997). MT2 is also expressed in the brain including the suprachiasmatic nucleus, where it mediates the phase shifting responses to melatonin (Liu et al., 1997; Dubocovich et al., 1998). Moreover, melatonin may act on other binding sites (MT3) (Boutin et al., 2005). The receptor type that mediates the action of melatonin as photoperiodic signal remains unclear. In Djungarian hamsters, which show a strong photoperiodic re-

sponse of gonads, the *MT2* receptor gene cannot encode a functional receptor because of two nonsense mutations within the coding region (Weaver et al., 1996), suggesting that MT2 is not necessary for the photoperiodic response in this species. However, direct evidence for distinct functions of MT1 and MT2 receptor types in photoperiodic regulation is missing.

Experiments with Japanese quail have provided novel data about the molecular mechanisms of photoperiodic gonadal regulation. Long-day stimulus induces the expression of thyrotropin (TSH) β subunit (*TSHB*) in the hypophysial pars tuberalis (PT), and TSH induces *Dio2* expression in the ependymal cell layer (EC) lining the infundibular recess of the third ventricle via TSH receptors (Nakao et al., 2008). DIO2 converts thyroxine into bioactive triiodothyronine, which stimulates the gonadal axis (Yoshimura et al., 2003). Notably, the expression of *Dio3*, which encodes for thyroid hormone-inactivating enzyme, is suppressed by long-day stimulus. Thus, the changing ratio between expression of *Dio2* and *Dio3* would serve as "gene switch" for photoperiodic gonadal regulation (Yasuo et al., 2005). This molecular machinery is conserved in mammals. The expression of *Dio2* and *Dio3* is regulated by the photoperiod and melatonin in Syrian and Djungarian hamsters (Watanabe et al., 2004; Revel et al., 2006; Watanabe et al., 2007b; Yasuo et al., 2007), and *TSHB* is involved in the photoperiodic regulation of *Dio2* in sheep (Hanon et al., 2008). Most interestingly, the expression of *Dio2*, *Dio3*, and *TSHB* is also under photoperiodic control in mice whose gonadal size is not affected by the photoperiod (Ono et al., 2008). This study has established that mice are suitable models to investigate

molecular mechanisms of photoperiodic regulation at the hypothalamo–hypophysial level and has opened the way to use genetically manipulated mice to further analyze the molecular mechanisms underlying photoperiodic regulation. Here, we examined the effects of targeted disruption of melatonin receptors (MT1, MT2, and both subtypes) on the expression of *Dio2* and *Dio3* under various photoperiodic conditions in mice to identify the receptor type through which melatonin exerts its action as a photoperiodic messenger.

Materials and Methods

Animals. All animal experiments reported here were conducted under a protocol in accordance with the Policy on the Use of Animals in Neuroscience Research, and the Policy on Ethics, as approved by the Society for Neuroscience, and were consistent with Federal guidelines of Germany and the European Communities Council Directive. Mice with targeted disruption of MT1, MT2, or both subtypes ($MT1^{-/-}$, $MT2^{-/-}$, and $MT1^{-/-}MT2^{-/-}$) on a melatonin-proficient C3H/HeN genetic background have been described previously (von Gall et al., 2002a, 2005; Jilg et al., 2005). Genotypes were determined by PCR amplification of genomic DNA extracted from the tail as described previously (Liu et al., 1997; Jin et al., 2003). Male mice (4–5 weeks old) of each genotype were housed under short-day condition [8 h of light, 16 h of darkness (8L:16D)] for at least 3 weeks. When mice were 7–8 weeks old, they were separated into two groups: one group was transferred to and kept under long-day condition (16L:8D) for 2 weeks; the other group remained under short-day condition (8L:16D) for an additional 2 weeks. For the investigation of the temporal expression of *Dio2* and *Dio3* in wild-type C3H mice, the animals were killed at Zeitgeber time (ZT: ZT0 corresponds to the light onset) 2, 8, 14, 20 ($n = 3$–4/time point/lighting condition) by decapitation. To investigate the effects of targeted disruption of melatonin receptors on *Dio2* and *Dio3* expression, transgenic C3H mice were killed in the middle of the light phase (ZT4 for 8L:16D, ZT8 for 16L:8D, $n = 5$–6/lighting condition). These time points were selected because the expression of *Dio2*, *Dio3*, and *TSHB* showed significant differences in CBA/N mice at this time (Ono et al., 2008). Animal facilities were maintained at a temperature of 22 ± 1°C. Standard diet pellet for rodents and water were available *ad libitum* and were replenished at least twice a week.

Melatonin injections. In our preliminary experiments, *Dio2* and *Dio3* expression of wild-type C3H mice did not respond to melatonin injections given at ZT14 for 3 weeks (data not shown), whereas *Dio2* and *Dio3* expression was clearly affected by melatonin injections in wild-type C57BL mice (Ono et al., 2008). Therefore, mice with targeted disruption of MT1 on C57BL genetic background (Liu et al., 1997) were used for the melatonin injection experiments. Male mice (4–5 weeks old) of wild-type and $MT1^{-/-}$ genotype were housed under long-day condition (16L:8D) for at least 1 week before the experiments. Thereafter, either melatonin (6 µg melatonin dissolved in 0.1 ml 5% ethanol and 0.9% NaCl) or vehicle (0.1 ml 5% ethanol and 0.9% NaCl) were subcutaneously injected at ZT14 for 1 week. This period was chosen because our preliminary experiments showed that 1 week of injections was sufficient to suppress *Dio2* and to induce *Dio3* expression in wild-type C57BL mice.

In situ hybridization. In situ hybridization was performed according to Yoshimura et al. (2000). Antisense and sense 45-mer oligonucleotide probes for mouse *Dio2* (sense: 5′-ctgcctcttcctggcgctctatgactcggtcatt-ctgctcaagca-3′) and mouse *Dio3* (sense: 5′-ggcaccatcatgtaccagggaggccgt-ggccccgacggttaccag-3′) were labeled with [^{33}P]dATP (NEN Life Science
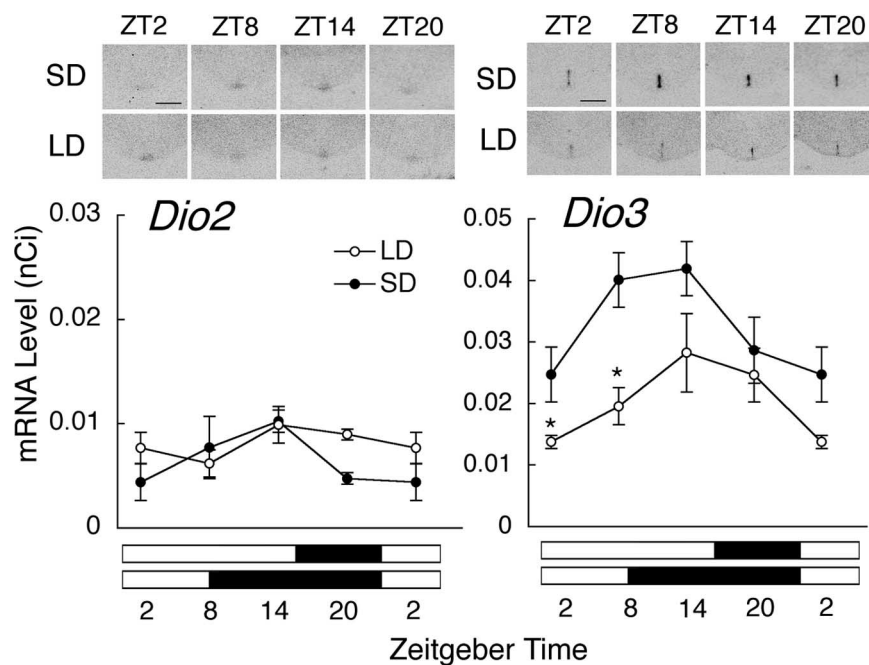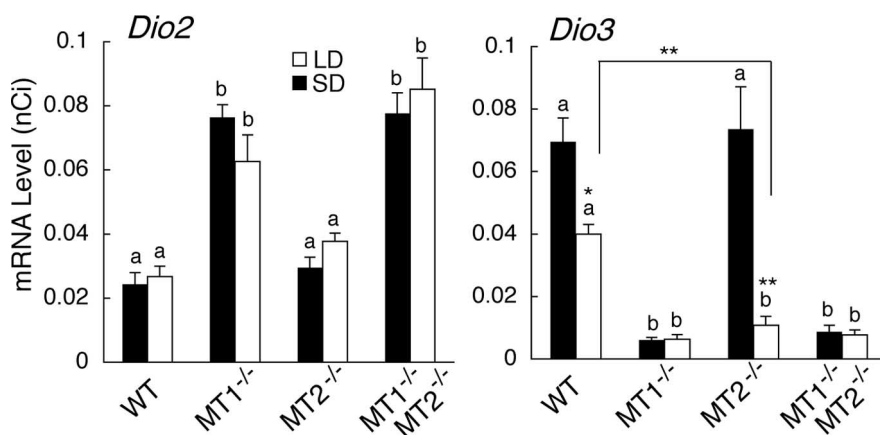

Figure 1. Effects of photoperiod on the expression of *Dio2* and *Dio3* in the ependymal cell layer lining the infundibular recess of the third ventricle. C3H mice of wild type were kept either under short-day (SD, black circles) or under long-day (LD, white circles) conditions for 2 weeks. Representative autoradiograms are shown above the graphs (scale bars, 1 mm). Data at ZT2 are double plotted. The black and white bars below the graphs indicate the light/dark period. Asterisks indicate significant differences between mice kept under SD and LD at each time point (*t* test, *$p < 0.05$; $n = 3$–4). Error bars indicate SEM.

Products) using terminal deoxynucleotidyl transferase (Invitrogen). Coronal frozen sections (20 µm thick) of the mediobasal hypothalamus were prepared using a cryostat. Hybridization was performed overnight at 42°C. After washing, sections were air dried and apposed to Biomax-MR film (Kodak) for 3 weeks. In each experiment, ^{14}C microscales (GE Healthcare Bio-Sciences) were included in the cassette; the relative optical density was measured by use of National Institutes of Health Image software and converted into the radioactivity value (nCi) using the ^{14}C standard measurements. Data were normalized by subtracting the value determined in the lateral hypothalamic area, which is located in the same section and does not exhibit a hybridization signal (Watanabe et al., 2004).

Results

Temporal expression of *Dio2* and *Dio3* in wild-type C3H mice

To examine whether C3H mice in our breeding colony respond to long or short photoperiods with changes in the expression of key photoperiodic genes, we investigated the expression of *Dio2* and *Dio3* in the EC of wild-type C3H mice kept under long- and short-day conditions at four different Zeitgeber time points. *Dio2* was expressed at low levels throughout a 24-h cycle, and its expression showed no diurnal variation in mice kept under long- or short-day conditions (one-way ANOVA, $p > 0.05$) (Fig. 1). Two-way ANOVA did not reveal a significant difference in relation to the photoperiodic condition ($p > 0.05$) (Fig. 1). *Dio3* expression tended to show diurnal variation with high levels during the first half of the day (one-way ANOVA, $p = 0.0791$ for long days; $p = 0.1403$ for short days) (Fig. 1). The expression of *Dio3* differed significantly between mice kept under long- and short-day conditions (two-way ANOVA, $p < 0.01$) (Fig. 1). When each time point was compared, significant differences were found at ZT2 and 8 (*t* test, $p < 0.05$) (Fig. 1). Sense probes for *Dio2* and *Dio3* did not detect any signal in the EC (data not shown).

Figure 2. Effects of targeted disruption of melatonin receptor types on the expression of *Dio2* and *Dio3* in the ependymal cell layer lining the infundibular recess of the third ventricle. C3H wild-type (WT) and C3H mice with targeted disruption of MT1 (MT1 $^{-/-}$), MT2 (MT2 $^{-/-}$), and both receptor types (MT1 $^{-/-}$ MT2 $^{-/-}$) were kept either under short-day (SD, black bars) or under long-day (LD, white bars) conditions for 2 weeks. Different characters (a, b) indicate the significance in each lighting condition (one-way ANOVA followed by Fisher's least significant difference *post hoc* test, $p < 0.05$; $n = 5$–6). Asterisks indicate significant differences between mice kept under SD and LD in each genotype or between mice of different genotypes (*t* test, *$p < 0.05$, **$p < 0.01$; $n = 5$–6). Error bars indicate SEM.

Effects of targeted disruption of melatonin receptors on *Dio2* and *Dio3* expression

We examined photoperiodic changes in *Dio2* and *Dio3* expression in mice with targeted disruption of melatonin receptors ($MT1^{-/-}$, $MT2^{-/-}$, and $MT1^{-/-}MT2^{-/-}$) at one time point in each lighting condition [middle of the light phase: ZT4 for short days (8L:16D), ZT8 for long days (16L:8D)]. These time points were selected because the expression of *Dio2*, *Dio3*, and *TSHB* showed significant differences in CBA/N mice at this time (Ono et al., 2008). *Dio2* expression was constitutively upregulated in $MT1^{-/-}$ and $MT1^{-/-}MT2^{-/-}$ mice regardless of the photoperiodic condition (one-way ANOVA, $p < 0.0001$) (Fig. 2). However, *Dio3* expression was constitutively downregulated in these genotypes (one-way ANOVA, $p < 0.0001$) (Fig. 2). Photoperiodic changes in *Dio3* expression were detected in wild-type C3H and $MT2^{-/-}$ (*t* test, $p < 0.05$ for wild type; $p < 0.01$ for $MT2^{-/-}$), but the changes were abolished in $MT1^{-/-}$ and $MT1^{-/-}, MT2^{-/-}$ mice (Fig. 2). The suppression of *Dio3* expression under long-day condition was stronger in $MT2^{-/-}$ mice than in wild-type mice (*t* test, $p < 0.01$) (Fig. 2).

Effects of melatonin injections on *Dio2* and *Dio3* in MT1-deficient C57BL mice

As also shown by Ono et al. (2008), *Dio2* was suppressed and *Dio3* was upregulated in wild-type C57BL mice injected with melatonin at ZT14 under long-day condition (*t* test, $p < 0.05$) (Fig. 3). These effects were abolished by targeted disruption of MT1 (Fig. 3).

Discussion

Previous work has shown that the mouse circadian system encodes seasonal light cycles at the neural network level (Inagaki et al., 2007; VanderLeest et al., 2007; Watanabe et al., 2007a) although mice are not strongly photoperiodic in their reproductive behavior or physiology. In accordance with a recent report by Ono et al. (2008), our study demonstrates that mice are indeed

suitable models to study molecular mechanisms of photoperiodic regulation at the hypothalamo–hypophysial level. Thus, the expression of genes shown to control the photoperiodic gonadal response at the hypothalamo–hypophysial level in quail and sheep (e.g., *TSHB* in the PT, *Dio2* and *Dio3* in the EC) (Hanon et al., 2008; Nakao et al., 2008) is regulated by photoperiod in melatonin-proficient CBA/N mice and by melatonin in melatonin-deficient C57BL mice (Ono et al., 2008). In this study, we showed that *Dio3* expression in melatonin-proficient wild-type C3H mice was lower under long-day condition than under short-day condition. This was also found in CBA/N mice (Ono et al., 2008), Djungarian hamsters (Barrett et al., 2007; Watanabe et al., 2007b), and Japanese quail (Yasuo et al., 2005). *Dio2* expression did not respond to photoperiodic changes in wild-type C3H, whereas it was strongly induced by long-day condition in CBA/N mice (Ono et al., 2008), Syrian hamsters (Revel et al., 2006; Yasuo et al., 2007), and Japanese quail (Yoshimura et al., 2003; Yasuo et al., 2005). In Djungarian hamsters, *Dio2* expression was induced when animals were transferred from short to long days, but it did not change when animals were transferred from long to short days (Watanabe et al., 2004, 2007b; Barrett et al., 2007). Thus, the effects of photoperiod on gene expression in the EC appear to be specific for species, strain, and experimental schedule, and C3H mice in our breeding colony can be used for research on photoperiodic regulation of *Dio3* expression.

The studies on wild-type mice have opened the way to use genetically manipulated mice to further analyze the molecular mechanisms underlying photoperiodic regulation. Using mice with targeted disruption of melatonin receptor types (MT1, MT2, and both types) on C3H genetic background, we were for the first time able to analyze distinct functions of each receptor type for the photoperiodic response of *Dio3* expression in the EC. In $MT1^{-/-}$ and $MT1^{-/-}MT2^{-/-}$ mice, photoperiodic changes in *Dio3* expression were completely abolished, whereas $MT2^{-/-}$ mice retained the response. Moreover, melatonin injections suppressed *Dio2* and induced *Dio3* expression in wild-type C57BL mice as has also been shown by Ono et al. (2008). These effects were abolished by targeted disruption of MT1. The data indicate that MT1 is crucial for the photoperiodic response of gene expression in the EC and, as a consequence, for the photoperiodic gonadal regulation. Further evidence for the involvement of MT1 in the control of *Dio2* and *Dio3* expression is provided by the observation that overall levels of *Dio2* mRNA were upregulated and those of *Dio3* mRNA were suppressed in C3H mice with targeted disruption of MT1 and MT1/MT2. Notably, these changes were not seen in melatonin-deficient C57BL with targeted disruption of MT1. Together, the data suggest that melatonin affects the expression of *Dio2* and *Dio3* by acting on MT1.

With regard to the location of this receptor, it should be noted that the EC itself does not express melatonin receptors (Schuster et al., 2000; Song and Bartness, 2001). A synopsis of several previous studies and our present results indicates that the expression of *Dio2* and *Dio3* in the EC is regulated via MT1 located in the PT: (1) The PT is the target site for seasonal melatonin signaling
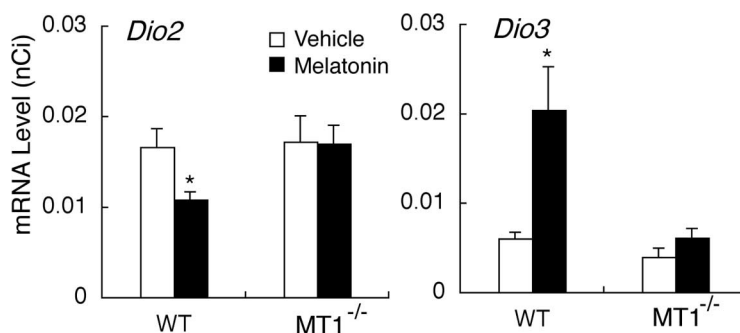
(Reppert et al., 1994; Gauer et al., 1998; von Gall et al., 2002b); (2) it contains high levels of *MT1* mRNA but lacks *MT2* mRNA (Reppert et al., 1995); (3) *MT1* mRNA and TSHB protein are colocalized in the PT of rat (Klosen et al., 2002), and immunosignals of TSHB in the PT of MT1-deficient C3H mice are constitutively upregulated (C. von Gall, unpublished observation); (4) TSH produced in the PT was shown to regulate *Dio2* and *Dio3* expression in the EC in Japanese quail (Nakao et al., 2008), sheep (Hanon et al., 2008), and mice (Ono et al., 2008).

Notably, $MT2^{-/-}$ mice showed a stronger photoperiodic responsiveness of *Dio3* expression than wild-type mice. This effect was not seen in $MT1^{-/-}MT2^{-/-}$ mice, suggesting that MT2 may weaken the photoperiodic responses by interference with MT1 function. This speculation may explain the high responsiveness to photoperiod in hamsters which lack a functional MT2 receptor (Weaver et al., 1996) and imply the adaptation of hamsters to environment by natural mutation of MT2 protein that is disadvantageous for the photoperiodic gonadal regulation.

In summary, we could for the first time provide clear evidence for distinct functions of MT1 and MT2 in photoperiodic regulation at the hypothalamo–hypophysial level: MT1 is crucial for the photoperiodic response of genes in the mediobasal hypothalamus which regulate the photoperiodic gonadal responses. MT2 may weaken the responsiveness of these genes to photoperiod in the presence of MT1. These data provide new insight into the molecular mechanisms of photoperiodic gonadal regulation, and the interaction of MT1 and MT2 would be an interesting research topic in the future.

Figure 3. Effects of melatonin injections on the expression of *Dio2* and *Dio3* in the ependymal cell layer lining the infundibular recess of the third ventricle of wild-type C57BL mice (WT) and C57BL mice with targeted disruption of MT1 ($MT1^{-/-}$). Vehicle (white bars) or melatonin (black bars) were injected 2 h before the light offset under long-day conditions (16 h of light: 8 h of darkness) for 1 week. Asterisks indicate significant differences in each genotype (t test, *$p < 0.05$; $n = 5$–6). Error bars indicate SEM.

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