Genetic variation of melatonin productivity in laboratory mice under domestication

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Melatonin is a pineal hormone produced at night; however, many strains of laboratory mice are deficient in melatonin. Strangely enough, the gene encoding HIOMT enzyme (also known as ASMT) that catalyzes the last step of melatonin synthesis is still unidentified in the house mouse (Mus musculus) despite the completion of the genome sequence. Here we report the identification of the mouse Hiomt gene, which was mapped to the pseudoautosomal region (PAR) of sex chromosomes. The gene was highly polymorphic, and nonsynonymous SNPs were found in melatonin-deficient strains. In C57BL/6 strain, there are two mutations, both of which markedly reduce protein expression. Mutability of the Hiomt likely due to a high recombination rate in the PAR could be the genomic basis for the high prevalence of melatonin deficiency. To understand the physiologic basis, we examined a wild-derived strain, MSM/Ms, which produced melatonin more under a short-condition than a long-day condition, accompanied by increased Hiomt expression. We generated F2 intercrosses between MSM/Ms and C57BL/6 strains and N2 backcrosses to investigate the role of melatonin productivity on the physiology of mice. Although there was no apparent effect of melatonin productivity on the circadian behaviors, testis development was significantly promoted in melatonin-deficient mice. Exogenous melatonin also had the antimodal action in mice of a melatonin-deficient strain. These findings suggest a favorable impact of melatonin deficiency due to Hiomt mutations on domestic mice in breeding colonies.

Hiomt | Asmt | pseudoautosomal | photoperiodism | circadian

M elatonin is a hormone produced primarily in the pineal gland (1). In most animals, melatonin is synthesized and secreted predominantly at night, and it has been implicated in the regulation of the circadian rhythm and seasonal reproduction (2, 3). Melatonin is synthesized by two enzymatic steps from serotonin. First, serotonin is converted to N-acetylserotonin (NAS) by arylalkylamine N-acetyltransferase (AANAT; EC 2.1.3.87). NAS is subsequently methylated by hydroxyindole-O-methyltransferase [HIOMT; EC 2.1.1.4; also known as N-acetylserotonin O-methyltransferase (ASMT)] to form melatonin. The daily rhythm of melatonin synthesis is thought to be due to a marked nocturnal increase of AANAT activity (4). HIOMT activity is, in contrast, almost constant throughout day and night. Although the mechanisms of expression and posttranslational regulation of AANAT have been well studied after the cloning of AANAT or HIOMT (or both) are compromised in mice of the melatonin-deficient inbred strains, such as BALB/c and C57BL/6J (B6J) (Table S1). In the history of domestication of mice, the genetic defects must have been introduced and fixed. Only one defect has so far been found, a point mutation in the Aanat gene, which causes an aberrant splicing and a frameshift in B6J strain (10).

In this study, we identified the mouse Hiomt gene, which is located in the pseudoautosomal region (PAR), a short region of homology between the X and Y chromosomes. PAR mediates proper sex chromosome pairing and segregation, and meiotic recombination between the sex chromosomes occurs in the PAR, which is therefore termed pseudoautosomal (11, 12). Because the PAR of mouse is highly variable due to a high rate of recombination (13), the mouse Hiomt gene may have undergone rapid divergence. We present evidence that melatonin deficiency has a favorable impact on testis development of mice, suggesting some selection for defective alleles of the Hiomt in breeding colonies of fancy/laboratory mice.

Results

Identification of Mouse Hiomt cDNA. TBLASTN search, comparing rat HIOMT protein sequence against the mouse genome database, detected two statistically significant hits (SI Materials and Methods). Both contigs were obtained by Celera’s whole-genome shotgun project. Several PCR primers were designed based on the mouse sequences and rat Hiomt, RT-PCR and rapid amplification of cDNA ends (RACE) techniques were used to clone the mouse Hiomt cDNA (Fig. S14). We have succeeded in obtaining the full-length coding sequence (CDS) of the mouse Hiomt CDS from pineal RNA of C3H/HeJ (C3H), one of the melatonin-proficient strains of classical laboratory mice (Table S1). Although the deduced amino acid sequence of mouse HIOMT (Fig. S1B) is highly divergent from HIOMT of other species (69% and 52% identical to rat and human HIOMT proteins, respectively), the phylogenetic tree constructed on the basis of the amino acid sequences of HIOMT is almost consistent with the expected vertebrate phylogeny (Fig. L4). This analysis reveals the rapid evolution of the Hiomt gene in the rodent species; notably, the theoretical isoelectric point of rodent HIOMT is quite different from (higher than) that of the other species (Fig. L4). The other characteristic aspect of mouse Hiomt is a high GC content, and, in particular, the GC content at the third codon position (GC3) is extremely high (95%) (Fig. L4).

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (DDBJ/EMBL/GenBank accession nos. AB512670–AB512674).

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The mouse Hiomt cDNA that we cloned was expressed in HEK293 cells, and the HIOMT enzymatic activity was assayed. Although the mouse HIOMT has weak sequence homology to those of other species, robust HIOMT activity was detected in the soluble fraction of the transfected cells (Fig. 1B). The activity was inhibited by S-adenosyl-homocysteine, a common inhibitor of methyl transfer reactions involving S-adenosyl-methionine (SAM).

We examined the B6J strain, which reportedly had no HIOMT activity (7). RT-PCR amplified the Hiomt from B6J pineal RNA, but HIOMT protein was not detected in the pineal gland by immunoblot analysis using anti-mouse HIOMT antisemur (Fig. 1C). There are seven SNPs in the Hiomt CDS between C3H and B6J. Two were nonsynonymous SNPs, resulting in amino acid substitutions (R78G and R242C) (Fig. S1A). We overexpressed mouse HIOMT variants, C3H type, R78G, R242C, and B6J type (R78G+R242C), in HEK293 cells. Immunoblot analysis showed that each of the mutations markedly reduced the expression level of HIOMT protein (Fig. 1D).

In addition to C3H and B6J strains, we examined MSM/Ms inbred strain (MSM), which was recently established from wild mice, Mus musculus molossinus, and retains many traits of wild mice (14). MSM mice produced melatonin during nighttime with a peak just before the light came on (Fig. S2A). HIOMT protein in the pineal gland was detected (Fig. 1C) and the two mutations, R78G and R242C, were absent in the Hiomt cDNA sequence of MSM (Fig. S1B). We investigated the daily changes of Aanat and Hiomt expression levels by quantitative RT-PCR analysis of the pineal glands of MSM mice maintained in a 12-h light/12-h dark cycle (LD12:12). The Aanat transcript showed the robust daily fluctuation in abundance with a peak at mid-night [zeitgeber time (ZT) 20] and a trough at early evening (Fig. S2C). In sharp contrast, subtle change was observed in the Hiomt mRNA level, in agreement with reports in other species (4). Tissue distribution study showed that the mouse Hiomt was predominantly expressed in the pineal gland and very slightly in the retina (Fig. S3).

The previous studies showed that two loci responsible for AANAT and HIOMT activities are autosomally located and segregate independently in mice (7, 15, 16). To confirm the genetics of melatonin production using the gene sequence information of Aanat and Hiomt, we analyzed the melatonin productivity trait of (B6J × C3H) F2 intercross mice. The genotypes of the Aanat and Hiomt loci were determined by PCR amplification and direct sequencing (Fig. S4). Both the plasma and the pineal gland were collected from each animal at ZT 22–23, and melatonin levels were assayed. Mice homozygous for the B6J-derived Aanat allele or homozygous for the B6J-derived Hiomt allele produced very little melatonin (Fig. 1E). The result indicated that the gene we cloned is the only gene encoding authentic HIOMT enzyme. Melatonin productivity of mice can be determined by the combination of Aanat and Hiomt genotypes.

**Mouse Hiomt Is a Pseudautosomal Gene.** The mouse Hiomt gene, from the start codon to the stop codon, was amplified by long PCR from the B6J and C3H genomic DNAs. The exon/intron structure of the mouse Hiomt gene, which comprises eight exons and seven introns, corresponds exactly to those of the rat and human genes. The gene size of the mouse Hiomt (B6J, 5.4 kb; C3H, 5.8 kb) differs greatly from the human HIOMT (27.8 kb) but is similar to the rat Hiomt gene (supposedly ~5–6 kb). In contrast to the CDS characterized by a high GC content (Fig. 1A), the intron sequences of the mouse Hiomt gene exhibits 40–60% GC content (Fig. S5A). The intron sequences, however, extensively consist of repeat sequences, which are distinctive of each intron (Fig. S5B).
To obtain further information on the mouse Hiomt gene, we screened a bacterial artificial chromosome (BAC) library of genomic DNA of MSM mice (17). We screened the library (equivalent to ≈60,000 clones) by PCR and isolated two positive clones. One of them (MSMg01-318O12) was used in further studies. Both ends of the BAC clone insert were sequenced and analyzed using BLAST search. One end sequence showed high homology to a number of mouse genomic contigs, almost all of which were mapped to most proximal (subtelomeric) regions of various chromosomes. The other end sequence showed homology to only one genomic contig (NW_001035897). The contig contains a part of the Mid1 gene (also known as Fxy), which spans the pseudoautosomal boundary on X chromosome (18).

The result of the BAC end sequencing suggested that the BAC clone contained a part of the mouse PAR. This is reasonably accounted for by chromosomal synteny between mouse and human; that is, the human HIOMT/ASMT gene is located in the PAR1 that is homologous to mouse PAR.

Using the BAC clone, we performed FISH on metaphase chromosome preparations from B6J mice. We obtained strong hybridization signals of apparently identical intensity on most proximal segments of the X and Y chromosomes corresponding to PAR (Fig. 2A). A weak signal was detected on the most proximal segment of chromosome 9. Mouse PAR and the subtelomeric region of chromosome 9 reportedly share a homologous sequence, which is highly repetitive (19, 20).

Sts (steroid sulfatase; U37545) is the only gene that has been known to be located in the mouse PAR (21, 22), whereas it has not mapped on the reference genome sequence (Build 37). We screened the library which were mapped to most proximal (subtelomeric) regions of homology to a number of mouse genomic contigs, almost all of them. One of them (MSMg01-318O12) was used in further studies. Both ends of the BAC clone insert were sequenced and clones. One of them (MSMg01-318O12) was used in further studies.

Interstrain Variation of Mouse Hiomt. Ebihara et al. (7–9) reported the genetic defects of HIOMT enzyme in many mouse strains as well as B6J (Table S1). To investigate genetic diversity of Hiomt in the divergent lineages, we sequenced the entire CDS of Hiomt gene in 24 inbred strains: nine laboratory strains derived from Lathrop’s stock (23); two strains derived from Japanese fancy mice; four strains derived from wild mice of different Mus musculus subspecies (domesticus, musculus, castaneus, and molossinus); and seven strains of other or unknown origin. We defined the nucleotide sequence of C3H as a reference and sorted out the SNPs and deletions identified. On average, 7.2 SNPs/deletion were found in Hiomt CDS of each strain, and 2.3 of these caused amino acid changes (Fig. 3 and Table S2). The B6J-type allele was shared not only in many strains from Lathrop’s stock but also in several strains of other origins. This cannot exclude the possibility of genetic contamination of mouse strains. Genealogy and mutation analysis of the Hiomt gene in different strains showed that (i) R78G and R242C were found in various strains, whereas synonymous mutations of the B6J-type allele were scarcely present in the other strains having R78G and/or R242C mutations; (ii) M. m. castaneus, M. m. molossinus (a natural hybrid between musculus and castaneus), and JF1 were grouped into a cluster, supporting the validity of the lineage; (iii) the C3H-type allele, shared only by its related strains (CBA/J, DBA/J, and SM/J), was likely to be originated from M. m. musculus; and (iv) the origin of B6-type allele was unclear.

Melatonin Productivity and Circadian Behaviors. Melatonin receptors are highly expressed in the hypothalamic suprachiasmatic nucleus (SCN), where the self-sustained circadian oscillator located, and exogenous melatonin influences the circadian rhythm of mice (24, 25). However, the role of endogenous melatonin on regulation of the circadian oscillator has been unclear. They characterized wheel-running behavior of (B6J × C3H) F2 intercross mice to evaluate the effect of endogenous melatonin on the circadian rhythm and its related behaviors. The male F2 intercrosses were genotyped and classified into two groups, melatonin-proficient and melatonin-deficient mice (each n = 15), according to the result mentioned above (Fig. 1E). Mice were maintained in LD12:12, and all of the mice tested displayed higher activity at night (Fig. S6). Next, mice were transferred to an inverted LD12:12, and all of the mice were re-entrained within 1 week. Both melatonin-proficient and melatonin-deficient mice were entrained also to a short-day photoperiod (LD10:11:11:12), which may be important for some relevant. After a 2-week LD12:12, mice were placed in constant darkness. There was no significant difference in free-running period of circadian locomotor activity (melatonin-proficient, 23.74 ± 0.20 h; melatonin-deficient, 23.77 ± 0.17 h). Mice were exposed to a 1-h light pulse during the early subjective night. Acute suppression of locomotor during the light pulse (light masking effect) and light-induced phase shift were equally observed in melatonin-proficient and melatonin-deficient mice.

Melatonin Levels Under Long- and Short-Day Photoperiods. Next, we focused on a link between photoperiodism, a physiologic response to the length of day or night, and melatonin and Hiomt, because melatonin plays an essential role in conveying temporal information for reproductive function in seasonal mammals (2, 3, 26). In a wild field, mice (Mus musculus) species generally showed seasonal breeding with high reproductive activity in summer; however, the seasonality was considered to be the result of annual fluctuations in environmental conditions such as food and refuge availability (refs. 27–29 and references therein).

We used MSM mice to investigate the photoperiodic change in the melatonin secretion profile. MSM mice were maintained in LD12:12 and divided into two groups: a short-day photoperiod (SP) group transferred in LD8:16, and a long-day photoperiod (LP) group in LD16:8. Four weeks later, we examined nocturnal melatonin levels in the plasma and mRNA expression levels of Aanat and Hiomt in the pineal glands. The amount of plasma

**Fig. 2.** Chromosomal localization of mouse Hiomt gene. (A) FISH using the BAC DNA probe containing Hiomt gene. The strong signals were observed at the distal region of the sex chromosomes with equal signal intensity. Weak signals were found at the distal region of chromosome 9. (B) Schematic map of the mouse PAR. Because the directions of Sts and Hiomt genes are unknown, these genes are shown without arrowheads.
melatonin during nighttime under SP was >3 times higher than that under LP (Fig. 4A) and ≥1.5 times higher than that in LD12:12 (Fig. S2A). Strangely, Aanat mRNA level under SP peaked at dawn, lagging behind the peak of melatonin production (Fig. 4B). Whereas the peak value of Aanat mRNA under SP was similar to that under LP, Hiomt mRNA level under SP was significantly increased compared with that under LP (P = 0.015, general linear model statistics) (Fig. 4C). The increased Hiomt expression may contribute to the elevated melatonin production under SP (Fig. 4D).

**Melatonin and Gonadal Development in Mice.** We examined the effect of melatonin productivity trait and light condition (LP or SP) on mouse gonadal development. Male (B6J × MSM) F1 mice and female B6J were mated to produce melatonin-proficient and melatonin-deficient N2 offspring. The dams were transferred to SP during gestation and maintained in the condition until weaning. The weaned mice were housed individually, and half the mice were transferred to LP. These mice were weighed at 7 weeks of age (Fig. 5A). There was no difference in body weight in males with regard to the melatonin productivity or the light conditions. In females, however, body weights of mice under LP were significantly greater than those under SP [F(1,45) = 9.03, P < 0.01, ANOVA], although the melatonin productivity did not influence the body weight.

Testicular weight was measured at 8 weeks of age (Fig. 5B). The melatonin productivity trait had an impact on the testis development, and testicular weights of melatonin-proficient and melatonin-deficient N2 offspring. The dams were transferred to SP during gestation and maintained in the condition until weaning. The weaned mice were housed individually, and half the mice were transferred to LP. These mice were weighed at 7 weeks of age (Fig. 5A). There was no difference in body weight in males with regard to the melatonin productivity or the light conditions. In females, however, body weights of mice under LP were significantly greater than those under SP [F(1,45) = 9.03, P < 0.01, ANOVA], although the melatonin productivity did not influence the body weight.

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into ZT. For example, 4 AM in a short LD8:16 photoperiod is ZT21, and the same factors, photoperiod and ZT, following to conversion of local time (time of day) which was performed by using R, we used a general linear model with two and 10:00, also shown by ecological studies in the wild (27). Photoperiodic control of reproduction is unlikely in mice, which is a family. Day length (short or long photoperiod), however, did not suggest that mice are potentially a member of long-day breeder although the results are not consistent. As far as we know, however, be stressed that genetically melatonin-deficient mice has not been examined. Ono et al. recently conscious selection. We found that the B6J-type allele of Hiomt is shared in several inbred strains unrelated to B6J (Fig. 3). The expansive prevalence of the B6J-type allele is likely to support the selection pressure rather than genetic drift.

Some researchers claim that melatonin has an antioxidant effect (reviewed in ref. 38). Because laboratory mice were used principally for cancer research in the early years, oncogenic traits due to melatonin deficiency, if any, might also contribute to the artificial selection, although cancer risk caused by melatonin deficiency has not been elucidated. In addition, artificial selection based on other traits could not be ruled out. Melatonin receptors are expressed not only in the pituitary pars tuberalis and SCN, which are crucial sites for photoperiodism and circadian rhythm, but also slightly in several hypothalamic nuclei, hippocampus, cerebral cortex, amygdala, and so on (25). Identification of the mouse Hiomt gene will provide “melatonin knockout” or “melatonin knockin” mice (e.g., melatonin-deficient MSM mice or melatonin-proficient B6J mice), which would tell us the uncharacterized functions involving melatonin such as neural development (39) and mood (40).

Materials and Methods

Mouse Strains. MSM/Ms mice were kindly supplied from T. Shiroishi (National Institute of Genetics, Mishima, Japan) and maintained in the laboratory animal facility in RIKEN Brain Science Institute (BSI). 129+ /SvJcl, BALB/cAJcl, C3H/HeJJcl, and C57BL6/Jcl mice were purchased from CLEA Japan; CBA/JNcrj, DBA/JNcrj, and NCGNgaTndGJj mice were from Charles River Laboratories Japan; and

experiments of melatonin injection have been conducted in mice, although the results are not consistent. As far as we know, however, the effect of melatonin treatment on the developing gonads of melatonin-deficient mice has not been examined. Ono et al. recently reported that a long-day breeder-like photoperiodic signal pathway was retained in laboratory mice (36). The report and our results suggest that mice are potentially a member of long-day breeder family. Day length (short or long photoperiod), however, did not affect testis development of mice, whereas melatonin was secreted more under short photoperiod (ref. 37 and this study). That means photoperiodic control of reproduction is unlikely in mice, which is also shown by ecological studies in the wild (27–29). Again, it should be stressed that genetically melatonin-deficient mice showed accelerated testis growth irrespective of day length (Fig. 5B). Artificial selection of melatonin-deficient mice based on rapid development of testis would be reasonable, whether it is an intentional or unconscious selection. We found that the B6J-type allele of Hiomt is shared in several inbred strains unrelated to B6J (Fig. 3). The expansive prevalence of the B6J-type allele is likely to support the selection pressure rather than genetic drift.

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Cloning of Mouse Hiomt cDNA. A detailed procedure is provided in *SI Materials and Methods*, and the progress of the cloning and the locations of the primers used are shown in Fig. S1. The database accession numbers of mouse *Hiomt* sequences of *B6*, *C3H*, and *MSM* are AB152670, AB152671, and AB152672, respectively.

Cloning of *Hiomt* Gene. The mouse *Hiomt* gene was amplified by PCR from genomic DNA in three overlapping fragments and subjected to direct sequencing. Detailed procedures are provided in *SI Materials and Methods*. The database accession numbers of mouse *Hiomt* sequences of *B6* and *C3H* are AB152673 and AB152674, respectively.

Genotyping of Mice. Genomic DNA was extracted from mouse tail biopsy samples by proteinase K–SDS digestion. Genotyping of the *Hiomt*, *Aanat*, *Mid1*, and *Sts* alleles was performed by PCR and direct sequencing of the PCR products. The primers used are shown in Fig. S4.

Transient Expression of Mouse *Hiomt*, *Hiomt* Enzyme Assay, and Generation of Mouse *Hiomt* Antisense. *Hiomt* activity was determined by a method based on Bernard et al. (42). Detailed procedures are provided in *SI Materials and Methods*.

Melatonin Assay. Mice were deeply anesthetized with diethyl ether in a very dim red light (at night), and blood was collected from the inferior vena cava followed by sampling of the pineal gland in dim red light (at night). A detailed description of the ELISA for melatonin is given in *SI Materials and Methods*.

Quantitative RT-PCR. Details of quantitative RT-PCR are provided in *SI Materials and Methods*.

Recording of Circadian Behaviors. Wheel-running activity was measured as previously described (40), and a detailed procedure is provided in *SI Materials and Methods*.

Administration of Melatonin. Melatonin in drinking water (10 mg/L) was given to dams from the third trimester of pregnancy until weaning (day 21 post-partum). The weaned offspring were given melatonin in drinking water (10 mg/L) for 1 week and thereafter by daily i.p. injection (50 μg) at ZT11–12. The concentration of vehicle (ethanol) was 0.005% (drinking water) and 0.1% (ip injection).

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Cloning of Mouse HIOMT cDNA. The progress of the cloning and the locations of the primers used are shown in Fig. S1. Based on the Celera assembly sequences (NW_0010361817.1 and NW_001030776.1) and rat HIOMT cDNA (LT78306), we designed several PCR primers to obtain a partial sequence of mouse HIOMT from the C3H pineal RNA, which was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo(dt) primer. A primer pair (F1, 5′-CGG GGA AGG GCG GAR CCA GTA-3′ and R1, 5′-AGY RAC AGS AGC GWC CGC-3′) successfully amplified a fragment of mouse HIOMT cDNA. We used several thermostable DNA polymerases in the PCR, and LA Tag (Takara Bio) gave the best result in the PCR and was used in all of the subsequent experiments. Next, 5′ RACE was performed according to cRACE method (1) with mouse HIOMT-specific primers (F2, 5′-TTG AGC GTG ATC TGC GAC CTC GG-3′ and R2, 5′-CAG AGG CTC CAG GTG TCTC TGG-3′) using R3 (5′-AAG AAG TCG CCT GCC AGG AAG-3′)-primed C3H pineal RNA as a template. Because the cRACE product did not contain the start codon of HIOMT, we again performed cRACE with new primers (F2, 5′-TTG AGC GTG ATC TGC GAC CTC GG-3′ and R4, 5′-CTG GGA GGC CAT GAA GCC GTG G-3′), which were designed from the sequence determined by the first cRACE. We obtained a fragment containing a sequence upstream of the start codon that was deduced from the rat HIOMT. Finally, the full-length coding sequence of mouse HIOMT was isolated by RT-PCR using the following primer pair: F3, 5′-CTA GGC TGA GCA GCT CTC GTC-3′ and R5, 5′-CTC ACA GTG TGC GAC CCT ACT GC-3′.

Cloning of Mouse HIOMT Gene. Mouse HIOMT gene was amplified by PCR from genomic DNA in three overlapping fragments and subjected to direct sequencing. Fragment A, from exon 1 to exon 4, was amplified using the following primer pair: AF, 5′-AGG CTS AGT AKG TCG CCT CCC ACC AGT-3′ and AR, 5′-ACC TGT AGA TGG CGG TGA AGG-3′; and sequenced using S1, AGG CTG AGC AGC TCG CTG CCT CCC ACC AGT CAT CAC; S2 (= R4), S3, 5′-CGC CCT CCG CGT GGT GTA CGA-3′; S4, 5′-CAG TGG TGC TTC TCC SCA GCA G3-3′; S5, 5′-CCA ACT CCC CCC TGG CGT CCA-3′; and S6, 5′-CAG TGG TGC GGG ACC AGG TGC CCC CAG C-3′. Fragment B, from exon 3 to exon 6, was amplified using the following primer pair: BF (= S5) and BR, 5′-GTC TGG AAC AGC GTG TTC ACC TGG-3′ and sequenced using S7, 5′-CCC GTG ACC TTG GAC CTT CCT C-3′; S8, 5′-GTT CGG AGG CCG AGC GCC TGC-3′; and S9, 5′-CCA CCG AGG TCG CAG ATG ACC-3′. Fragment C, from intron 5 to exon 8, was amplified using the following primer pair: CF, 5′-CAA GCC CTC AGG GTT GAG CA-3′ and CR, 5′-AGC CCC ACC AGC CCC CRC TTT CTG CCT CT-3′ and sequenced using S10, 5′-GCA AAG TGA AGC ACC ATG TGG-3′; S11, 5′-CCC CAG GCC ACT TCT TC-3′; S12, 5′-GCC CGC AGC AGC TCC ACG CAG-3′; S13, 5′-GTT GTG CTG CTG GTG AGC AGC-3′; and S14, 5′-GGC CAT CAT CAG GTT GTA GAG-3′.

Direct Sequencing of HIOMT cDNA and Gene. A PCR product, which was amplified by LA Tag with GC buffer I (Takara Bio), was reacted with ExoSAP-II (Affymetrix). After heat inactivation, the sample was used in the fluorescence dideoxy terminator cycle sequencing reaction (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit v3.1; Applied Biosystems) and analyzed by ABI 3730xl DNA sequencer. Because of the high GC content of mouse HIOMT, we routinely added DMSO (final concentration, 7.5%) and a higher concentration of a primer (5 μM) in the sequencing reaction, and we used a high denaturing temperature (98 °C for 10 s) of each cycle. If a clear sequencing result was still not obtained, we used dGTP BigDye Terminator v3.0 Kit (Applied Biosystems) or a 1:1 mixture of the two reagents.

Transient Expression of Mouse HIOMT. Silent mutations were introduced by site-directed mutagenesis into the C3H-type mouse HIOMT cDNA for easier handling (ggg ctg ctc cgg aga ggg ggc cgt ctc cgc cgc ggc ggc gtc gtc ggc cct cgT ggg TCT cca GCT corresponding to the region of 75–89 of the encoded protein) before insertion of the cDNA into a mammalian expression vector pcDNA3.1 (Invitrogen). R78G and R242C mutations were subsequently introduced. These constructs were transfected into HEK293 cells using Lipofectamine LTX and PLUS reagent (Invitrogen). HEK293 cells were cultured in DMEM/F12 medium (Sigma-Aldrich) containing 10% FBS and penicillin/streptomycin. Cells were harvested 48 h after transfection.

HIOMT Enzyme Assay. HEK293 cells plated on a 10-cm dish were transfected with the C3H-type mouse HIOMT cDNA as described above. Cells were harvested 48 h after transfection and homogenized with a glass–Teflon homogenizer in 1,300 μL of ice-cold 50 mM sodium phosphate (pH 7.9). After centrifugation at 18,000 × g for 20 min at 4 °C, the supernatant (50 μL) was mixed with NAS (final concentration, 0.5 mM), SAM (0.1 mM), and [3H]SAM (0.5 μCi) in 50 mM sodium phosphate (pH 7.9). SAH (final concentration, 0.1 mM) was added as a potential inhibitor of mouse HIOMT. After incubation at 37 °C for 30 min, the reaction (200 μL) was stopped by addition of 200 μL of 50 mM sodium borate (pH 10.0). Melatonin was extracted with water-saturated chloroform (1 mL), and radioactivity was counted after washing with the borate buffer. NAS-independent, background radioactivity was determined by the reactions without NAS. Protein content was determined by the Bradford method using BSA as a standard.

Generation of Mouse HIOMT Antiserum. A heptadecapeptide (CTS-QtGSGTGTGGAGQD) corresponding to the C-terminal part of mouse HIOMT was synthesized, purified using HPLC, and conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunized with the KLH-conjugated peptide emulsified in complete Freund’s adjuvant. Antiserum was obtained 1 week after the fourth boosting with the same antigen. For immunoblot analysis, the antiserum was diluted with 4% skim milk in PBS at 1:400 and incubated with a blot overnight at 4 °C.

Melatonin Assay. Mice were deeply anesthetized with diethyl ether in a very dim red light (at night), and blood was collected from the inferior vena cava followed by sampling of the pineal gland in dim red light (at night). Plasma was separated from the blood treated with EDTA-2Na by centrifugation. Melatonin was extracted from the plasma by using a C18 column (Sep-Pak Vac 1cc C18 cartridge; Waters). The plasma sample (250 μL) was applied to the column equilibrated with distilled water and passed through the column by centrifugation at 200 × g for 1 min. After washing the column with 10% methanol (1 mL) twice, melatonin was extracted with methanol (1 mL). The extracted melatonin was reconstituted with PBS (250 μL), after methanol was evaporated under N2 gas. Pineal gland was homogenized in PBS (1 mL) with a glass–Teflon homogenizer, and the homogenate was directly assayed. Both plasma and pineal melatonin contents were as-
sayed by ELISA (Direct Saliva Melatonin ELISA kit; Bühlmann Laboratories) according to manufacturer’s instructions.

**Quantitative RT-PCR.** Pineal glands were collected in TRIzol reagent (Invitrogen). Total RNA was extracted according to the manufacturer’s instructions, when two or more glands were used. When RNA was extracted from one pineal gland, we used the PureLink RNA Micro kit (Invitrogen). In every case, RNA was treated with DNase I following to RT using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) primer. Mouse *Aanat*, *Hiomt*, and *Actb* mRNA levels were assayed by real-time PCR technology with Power SYBR Green PCR Master Mix (Applied Biosystems). The relative level of each mRNA was calculated by $2^{-\Delta\Delta Ct}$ (Ct indicating the cycle number at which the signal reached the threshold). Each Ct value used for these calculations was the mean of quadruplicate of the same reaction. The following primers were used: *Aanat*, 5′-GTC ACT GGG CTG GTT TGA GG-3′ and 5′-CTC CGG GCC TGT GTA GTG TC-3′; *Hiomt*, 5′-GCA GCC TCC TGC TCT ACC TG-3′ and 5′-ACC TGT AGA TGG CGG TGA AGG-3′; and *Actb*, 5′-CGY GCC TAC AGC TTC ACC AC-3′ and 5′-AGC TCR TAG CTC TTC TCC AG-3′. An external control standard curve was determined by PCR with serial dilutions of a template plasmid containing mouse (MSM) *Aanat*, *Hiomt*, or *Actb* cDNA (ranging from 3 to $10^6$ molecules in a single well of a 384-well plate).

**Recording of Circadian Behaviors.** Wheel-running activity was measured as previously described (2). Mice were individually housed in cages (24 cm × 11 cm × 14 cm high) equipped with a steel wheel (5 cm wide × 14 cm in diameter). Wheel-running activity (three counts per rotation) was monitored by an online PC computer system (O’Hara & Co.). Light/dark cycles were controlled by an automatic timer, and light was provided by a white fluorescent tube and the light intensity was 30–50 lux at the level of the mouse’s eyes in the cage. Food and water were available ad libitum at all time.


Fig. S1. Cloning of mouse cDNA. (A) Strategy. We cloned the mouse cDNA from C3H pineal RNA by using a PCR-based strategy. (Row 1) Degenerate primers (open triangles) designed based on the rat Hiomt sequence (L78306) and Celera’s data (NW. 001038117 and NW. 001030776) were used to amplify a part of mouse Hiomt cDNA from oligo(dT)-primed RNA. (Rows 2 and 3) cRACE was performed using mouse Hiomt-specific primers (closed triangles) to amplify unknown 5' regions of Hiomt cDNA. (Row 4) The entire CDS of mouse Hiomt was obtained using a mouse Hiomt-specific primer and a degenerate one. (B) Alignment of HiOMT sequences of rat and three mouse strains, B6J, C3H, and MSM. Gaps (-) are introduced in the sequences to optimize alignment. Amino acid residues conserved among more than three boxed in red, and unconserved residues of B6J are indicated in yellow. The domain conserved among S-adenosylmethionine (SAM)–dependent methyltransferases and the amino acid sequence highly conserved among vertebrate HIOMT proteins are indicated by blue and cyan lines, respectively. R242C is located in the functional domain of SAM-dependent methyltransferases, and R78G is at the region with a relatively weak structural organization.
**Fig. S2.** Diurnal variation in plasma melatonin and pineal Aanat and Hiomt mRNA levels of MSM mice. (A) Plasma samples were collected from MSM mice maintained under LD12:12, and melatonin levels were assayed by ELISA. Light and dark periods are indicated by white and gray backgrounds, respectively. (B) A standard curve showing the correlation between PCR cycle threshold (Ct) values and the absolute amounts of plasmid containing mouse Actb, Aanat, or Hiomt cDNA. (C) Diurnal Aanat and Hiomt mRNA profiles. Pineal glands were collected from MSM mice maintained under LD12:12. RNA was extracted from a pool of three glands, and Aanat, Hiomt, and Actb mRNAs were assayed by RT-PCR. Aanat and Hiomt mRNA levels determined by the standard curves (B) and shown as values relative to Actb mRNA level (left axes) and relative to the lowest values (right axes; Aanat, ZT12 and Hiomt, ZT0).

**Fig. S3.** Tissue distribution of mouse Hiomt mRNA. Various tissues were collected from MSM mice at ZT18–19. Total RNA was extracted and reverse transcribed, and Hiomt as well as Aanat and Actb were assayed by PCR. In this assay, almost half length of coding region (CDs) of Hiomt, full length CDs of Aanat, and Actb were amplified using the following primer pairs: Hiomt, 5'-TGA GGG AAG GGC GGC AGC ACT A-3' and 5'-GCC ACA GCA GCA GCG TCC GC-3'; Aanat, 5'-GGG AGG GKT CAG TGG CCA GA-3' and 5'-CAC CCT GCC CAT GCC CAA G-3'; and Actb, 5'-CAT GTT TGA GAC CTT CAA CAC C-3' and 5'-GCC ATA GAG GTC TTT ACG GAT G-3'. Two splicing variants of mouse Aanat were detected: a shorter one encoding authentic AANAT enzyme, and a longer one containing a pseudoexon insertion causing a frameshift and premature termination. The pseudoexon insertion was reported in B6J strain, and the mutant variant was exclusively expressed in the pineal gland of B6J mice (1).

Nucleotide sequences used for genotyping. Primer sequences for PCR amplification of Mid1, Ssts, and Aanat were performed with the forward primers. Nucleotide sequences in lowercase are respectively. Direct sequencing of the PCR products of Mid1, Ssts, and Aanat were performed with the forward primers. Nucleotide sequences in lowercase are not determined, but the corresponding primers were hybridized to the sequences.

**Mid1 (exon 10)**

**Sts (exon 1)**

**Hiomt (intron 5 – exon 6 – intron 6)**

**Aanat (intron 3)**

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Fig. S4. Nucleotide sequences used for genotyping. Primer sequences for PCR amplification and for sequencing are indicated by cyan and green backgrounds, respectively. Direct sequencing of the PCR products of Mid1, Ssts, and Aanat were performed with the forward primers. Nucleotide sequences in lowercase are not determined, but the corresponding primers were hybridized to the sequences.
Fig. S5. Characterization of mouse Hiomt gene. (A) GC content. All exons markedly show higher GC content. (B) Self-similarity dot plot. Introns 1, 2, 4, 5, and 7 have remarkable self-similarity with repetitive sequences.
Fig. S6. Representative records of wheel-running activity of F2 intercrosses (C3H × B6J). Actogram is shown as double plot, and each horizontal sequence of colored bars represents 48 consecutive hours. Melatonin productivity is indicated above the actogram. Mice were maintained under LD12:12 for 3 weeks, then an inverted LD cycle for 2 weeks, a skeleton light condition (L1:D10:L1:D12) for 2 weeks, an LD cycle for 1 week, and finally in a constant darkness condition. Light and dark periods are indicated by white and gray backgrounds, respectively.
**Table S1.** Interstrain variations in AANAT and HIOMT activities and melatonin production

<table>
<thead>
<tr>
<th>Inbred strain</th>
<th>AANAT activity</th>
<th>HIOMT activity</th>
<th>Melatonin</th>
<th>Ref.</th>
</tr>
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<tr>
<td>Classical laboratory mouse</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>129/Sv</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>(3)</td>
</tr>
<tr>
<td>AKR/J</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>(2)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>(2)</td>
</tr>
<tr>
<td>C3H/He</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(3)</td>
</tr>
<tr>
<td>C57BL/6J</td>
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<td>−</td>
<td>−</td>
<td>(1)</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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<td>(3)</td>
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<td>−</td>
<td>(1)</td>
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<td>(1)</td>
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<tr>
<td>SK/CamEi</td>
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</tr>
<tr>
<td>SF/CamEi</td>
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<td>(+)</td>
<td>+</td>
<td>(1)</td>
</tr>
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<td>+</td>
<td>(1)</td>
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<td>CAST/Ei</td>
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<td>−</td>
<td>−</td>
<td>(1)</td>
</tr>
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<td>M. m. molossinus-derived</td>
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<tr>
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<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(3)</td>
</tr>
<tr>
<td>Mol-Nis</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(3)</td>
</tr>
<tr>
<td>Mol-A</td>
<td>(+)</td>
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<td>+</td>
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<tr>
<td>MSM/Ms</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(Current study)</td>
</tr>
</tbody>
</table>

+ Active/proficient, experimentally validated; (+) active, inferred from melatonin productivity; − inactive/deficient.


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**Table S2.** List of SNPs/deletion in *Hiomt* CDS of different mouse strains

| Nucleotide number | G | C | C | C | T | A | T | G | G | G | G | G | C | C | C | C | C | C | C | C | C | T |
| 18                |   |   |   |   |   |   |   | T | T | A |   |   |   |   |   |   |   |   |   |   |   |   |   | del10 |
| 27                |   |   |   |   |   |   |   |   | T | T | C |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 30                |   |   |   |   |   |   |   |   | T | T | C | T |   |   |   |   |   |   |   |   |   |   |   |   |
| 69                |   |   |   |   |   |   |   |   |   | T | C |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 90                |   |   |   |   |   |   |   |   |   |   | T | T |   |   |   |   |   |   |   |   |   |   |   |   |
| 105               |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |   |
| 114               |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   | T |   |   |   |   |

Amino acid substitution

| R78G | T91S | A127S | T179A | A226V | R242C | V274L | R344C | G358D | D370E | S381R |

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* Nucleotides different from the C3H-type sequence are shown in boldface type. Nonsynonymous variations are shown in red, and the substituted amino acid residues are indicated on the lowest row.

† C3H/He, CBA/J, DBA/1J, and SM/J have the identical sequence.

‡ C57BL/6J, 129/Sv, AKR/N, BALB/cN, RFM/Ms, NC/Nga, CF1/Sgn, FVB/N, and NZB/N have the identical sequence.

§ There is a 10-bp deletion (del10) causing a frameshift (FS).
Table S3. Comparison of two mouse PAR genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>HIOMT/ASMT</th>
<th>STS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme encoded</td>
<td>Hydroxyindole O-methyltransferase</td>
<td>Steroid sulfatase</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td>Pineal gland and retina</td>
<td>Ubiquitous</td>
</tr>
</tbody>
</table>

**Human**
- **Chromosomal location**: PAR1 (Xp 22, Yp 11)
- **Disorders**: (Autistic spectrum disorders)
- **Gene size**: 28 kb
- **GC content in CDS**: 56%
- **GC3**: 68%
- **Human Chromosomal location**: Xp 22 (proximal to PAR1)
- ** GC content in CDS**: 52%
- **GC3**: 61%
- **Disorders**: X-linked ichthyosis (1, 2)

**Rat**
- **Chromosomal location**: 12q 11
- **Gene size**: ~5–6 kb
- **GC content in CDS**: 65%
- **GC3**: 74%
- **Rat Chromosomal location**: Xq 12–14
- **GC content in CDS**: 63%
- **GC3**: 75%

**Mouse**
- **Chromosomal location**: PAR
- **Gene size**: 5.4–5.7 kb
- **GC content in CDS**: 76%
- **GC3**: 95%
- **Activity diversity*: C3H/He + C57BL/6J +++ (3, 4)
- **Mouse Chromosomal location**: PAR
- **Gene size**: 9 kb
- **GC content in CDS**: 75%
- **GC3**: 97%
- **Activity diversity*: CBA/Ms ++ C3H/HeJ – C3H/An –

*Symbols represent relative levels of the enzymatic activity: +++ > ++ > + > – (almost no activity).