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30 July 1985; accepted 21 October 1985

Genetic Control of Melatonin Synthesis in the Pineal Gland of the Mouse

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Pineal melatonin may play an important role in regulation of vertebrate circadian rhythms and in human affective disorders. In some mammals, such as hamsters and sheep, melatonin is involved in photoperiodic time measurement and in control of reproduction. Although wild mice (*Mus domesticus*) and some wild-derived inbred strains of mice have melatonin in their pineal glands, several inbred strains of laboratory mice (for example, C57BL/6J) were found not to have detectable melatonin in their pineal glands. Genetic analysis suggests that melatonin deficiency in C57BL/6J mice results from mutations in two independently segregating, autosomal recessive genes. Synthesis of melatonin from serotonin in the pineal gland requires the enzymes *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT). Pineal glands from C57BL/6J mice have neither NAT nor HIOMT activity. These results suggest that the two genes involved in melatonin deficiency are responsible for the absence of normal NAT and HIOMT enzyme activity.

MELATONIN IS SYNTHESIZED IN the pineal gland from serotonin by a well-known pathway: serotonin is first acetylated to form *N*-acetylserotonin (NAS) by the enzyme *N*-acetyltransferase (NAT), and then NAS is methylated by the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) to form melatonin. NAT activity is cyclic, with high levels at night and lower levels in the daytime (*1*). This cyclic modulation of NAT activity level is under the control of a circadian pacemaker, which in mammals includes the suprachiasmatic nucleus (*2*). HIOMT activity is nearly constant throughout the day. Rhythmic NAT activity results in robustly rhythmic melatonin synthesis (*1*). All vertebrate species so far examined show a daily rhythm of pineal melatonin content (*1, 3*). We report here that some domesticated inbred strains of mice (for example, C57BL/6J) have no pineal melatonin at any time of day or night, whereas wild mice of the same species (*Mus domesticus*) synthesize melatonin with normal rhythmicity. Our data strongly suggest that two independently assorting mutant genes, which affect the activity of NAT and HIOMT, are responsible for the melatonin deficiency in C57BL/6J mice.

We have measured pineal melatonin content in a large number of mice of several different strains (*4*). These can be divided into three groups: (i) strains that were established as inbred over 40 years ago [C57BL/6J, AKR/J, BALB/c, and NZB/BLNJ (*5*)]; (ii) wild-derived strains inbred for less than 30 years [IS/CamEi, SK/CamEi, SF/CamEi, PERU-Atteck/CamEi, and one strain of *Mus casteneus*, the Asian house mouse, CAST/Ei (*5, 6*)]; and (iii) mice (*Mus domesticus*) trapped in Alberta, Canada, and bred by random mating for about five generations over 5 years in the laboratory of F. Bronson [referred to here as the field-derived strain (FDS)].

We have assayed pineal melatonin content at several times during the day and night (at hours 6, 14, 18, and 22, where hour 0 = lights on and hour 12 = lights off) in male and female mice of the strains in the first group. There was no detectable melatonin in any of these strains at any of the times

Table 1. Segregation of progeny in various types of matings. The expected ratios are calculated on the assumption that two independently segregating genes are responsible for melatonin deficiency in C57BL mice. The lower limit of melatonin detection in our assay is approximately 10 pg per gland (*4*). It is difficult to accurately estimate very low levels of melatonin, especially given the problems of nonzero cross-reactivity with other compounds such as NAS and variable nonzero blanks. For these reasons we have arbitrarily chosen a measured level below 20 pg per gland to indicate lack of melatonin synthesis. If this level were set at 15 pg, then the χ^2 values would be 13.65 in $F_1 \times$ C57BL and 8.49 in $F_1 \times F_1$. These χ^2 values would allow us to reject the null hypothesis (*8*). However, χ^2 values obtained if the level were set at 25 or 30 pg would not allow us to reject the null hypothesis. The best χ^2 values for our model are obtained when the threshold level is set at 25 pg ($\chi^2 = 0.317$, $F_1 \times$ C57BL; $\chi^2 = 0.423$, $F_1 \times F_1$).

Ratio	No melatonin	Melatonin	χ^2
	$F_1 \times$ C57BL		
Observed	60	25	
Expected (of 85 subjects)	63.75	21.25	0.88
	$F_1 \times F_1$		
Observed	21	33	
Expected (of 54 subjects)	23.625	30.375	0.52
	$F_1 \times$ FDS		
Observed	0	26	
Expected (of 26 subjects)	0	26	0

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examined [the mean values were below 10 pg per gland, the lower limit of detection in our assay procedure (*4*)]. Of the strains in the second group, strains SK/CamEi, SF/CamEi, and PERU-Atteck/CamEi did have pineal melatonin at hour 22 (the only time at which measurements were made) [means ± SEM were as follows: SK/CamEi, 143.5 ± 16.4 pg per gland (n = 8); SF/CamEi, 137.7 ± 17.7 (n = 6); and PERU-Atteck/CamEi, 240.8 ± 22.6 (n = 6); there were no significant sex differences]. However, there was no detectable melatonin in pineal glands of strain IS/CamEi at hours 6, 14, 18, and 22 (n = 2 to 5) or in CAST/Ei at hour 22 (n = 5). Pineal melatonin content of FDS mice measured at 4-hour intervals is compared with pineal melatonin content in C57BL mice in Fig. 1.

To test the hypothesis that the observed differences in pineal melatonin content in the FDS and C57BL mice have a genetic basis, we measured pineal melatonin content in F_1 hybrids of the two strains. Figure 2 shows the distribution of pineal melatonin values obtained at hour 22 from C57BL mice, FDS mice, and F_1 hybrids. The presence of melatonin in the pineal glands of the F_1 hybrids suggests that genetic factors, absent in C57BL mice, are essential for synthesis of melatonin.

In order to determine the number of genes involved in melatonin deficiency, we examined segregation patterns of melatonin content in three crosses, $F_1 \times F_1$, $F_1 \times$ C57BL, and $F_1 \times$ FDS. If we assume that there exist two independent loci that control two essential steps in the melatonin synthesis pathway and that there is incomplete dominance at these two loci, we can make explicit predictions about segregation ratios and amounts of melatonin expected from these crosses. We expect to find melatonin in those animals that have significant activity at both synthetic steps and little or no melatonin in those animals that lack activity at one or the other step. The $F_1 \times$ C57BL mating should produce progeny with a segregation ratio of 3 (without melatonin) to 1 (with melatonin). Melatonin levels in these animals should be the same as those in the F_1 hybrids, because F_1's and those backcross progeny that are able to synthesize melatonin will have the same genotype. The F_2 progeny should have a ratio of 7 (without melatonin) to 9 (with melatonin). Melatonin content in these animals is expected to show a wide distribution, because 1/16 of the progeny would have the same genotype as FDS, 1/4 would have the same genotype as the F_1's, and the remaining 1/4 would be heterozygous at one locus and homozygous at the other. All progeny of the $F_1 \times$ FDS mating would be expected to have melatonin; lower levels than those found in FDS might be expected in some mice because of heterozygosity at either locus. The data from these crosses are shown in Fig. 2 and are analyzed in Table 1. The results strongly support the hypothesis that two independently segregating genes are responsible for the melatonin deficiency and suggest incomplete dominance of the wild-type FDS alleles.

These data led us to look for the site or sites of the genetic defects in the melatonin synthesis pathway. Using high-performance liquid chromatography with electrochemical detection (HPLC-EC), we were able to measure normal levels of serotonin in the pineal glands of C57BL mice (*7*). This indicates that the genetic defect does not involve a reduced level of serotonin synthesis or uptake. Using HPLC-EC, we were unable to detect 6-hydroxymelatonin (the major metabolite of melatonin) in the pineal glands of C57BL mice, an indication that

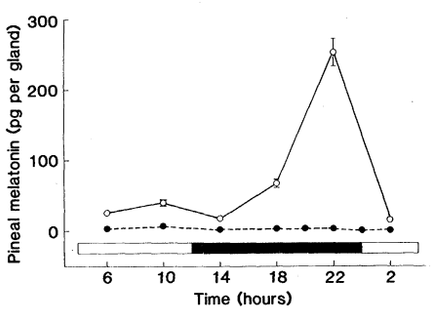

Fig. 1. Pineal melatonin levels in C57BL (closed circles) and FDS mice (open circles). Timing of the light/dark cycle is shown by the bar. Each point is the mean for at least ten males and ten females at a single time; the SEM values are shown when they exceed the size of the symbol. There were no significant sex differences except in FDS mice at hour 10 [males: 54.7 ± 3.7 (n = 6); females: 31.2 ± 4.2 (n = 9)]; $P < 0.01$.

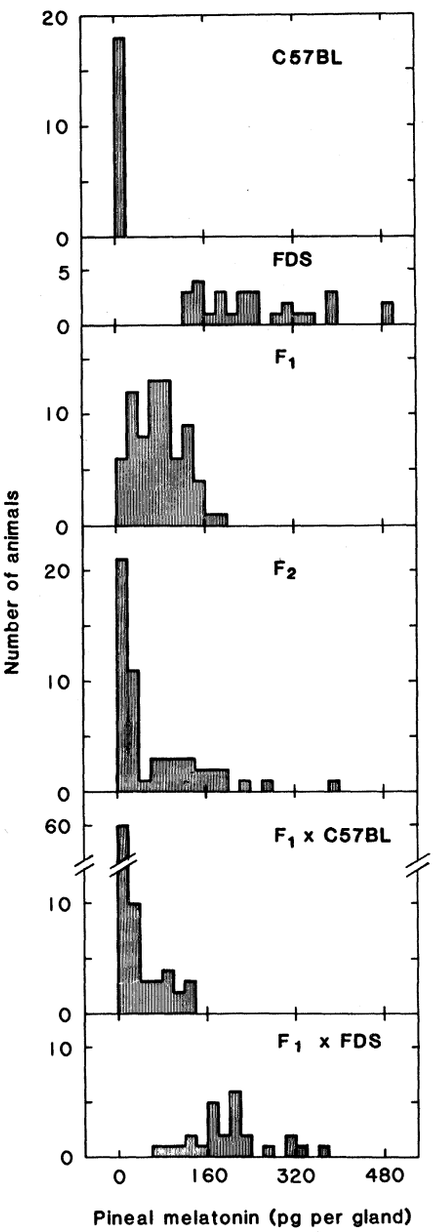

Fig. 2. Distribution of pineal melatonin content for C57BL and FDS mice and for the offspring of specific matings (see text). All data were obtained at hour 22 (see Fig. 1). We obtained data shown for the F_1 mice by making reciprocal crosses C57BL female × FDS male and FDS female × C57BL males. There were no significant differences between the offspring in these two groups, nor was there a sex difference in either F_1 ($P > 0.05$). This suggests that there are no maternal effects on melatonin production and that the genes responsible are autosomal. Therefore, the data from males and females have been combined for each of the groups.

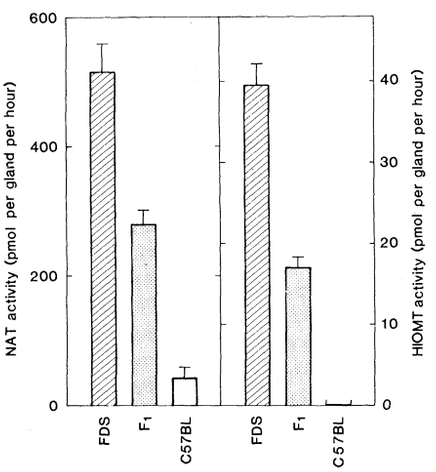

Fig. 3. *N*-acetyltransferase activity (NAT) and hydroxyindole-*O*-methyltransferase activity (HIOMT) measured at hour 22 for C57BL mice, FDS mice, and F_1 hybrids. Because there were no significant sex differences in any of the groups examined ($P > 0.05$), data from the two sexes have been pooled. Each bar represents the mean ± SEM of 7 to 15 determinations (two to four pineal glands per determination).

our inability to detect melatonin was probably not due to its being produced and then rapidly degraded in the pineal. It thus seemed that the melatonin deficiency in the pineal glands of C57BL mice might be due to defects in one or both of the enzymatic steps in the synthesis of melatonin from serotonin.

Because two to four pineal glands must be pooled to make reliable enzyme measurements, we were not able to examine directly the segregation pattern of enzyme activities in F_2 animals. The activities of the two enzymes (NAT and HIOMT) involved in the synthesis of melatonin from serotonin at hour 22 in C57BL mice, FDS mice, and F_1 hybrids are shown in Fig. 3. The segregation experiments, in which melatonin was measured, suggested that two genes were involved in melatonin deficiency. Our measurements of NAT and HIOMT enzyme activity suggest that mutant alleles of these two genes are responsible for NAT deficiency and HIOMT deficiency. The observed intermediate enzyme levels in the F_1 hybrids may reflect heterozygosity at both loci.

We hypothesize that the melatonin deficiency observed in C57BL mice is due to the presence of recessive mutant alleles at two independently segregating autosomal loci, one of which controls NAT activity and the other HIOMT activity. One additional observation supports this hypothesis. Although pineal glands of NZB mice do not contain melatonin, we have been able to measure NAT activity (241.1 ± 21.8 pmole per gland per hour, $n = 7$) and the presence of its product *N*-acetylserotonin (by HPLC-EC) in their pineal glands at hour 22. We were unable to detect HIOMT activity. This suggests that the two enzymes are independently regulated and that there may exist three distinct forms of melatonin deficiency involving these loci: (i) the genes for both NAT and HIOMT may be defective, as we suspect in C57BL mice; (ii) only the gene for HIOMT may be defective, as we suspect in NZB mice; and (iii) only the gene for NAT may be defective (no example yet found).

Melatonin can act as an antigonadal factor regulating reproductive responses of some mammals (*3*). Its role, if any, in regulating reproduction in the mouse is unknown. Domesticated mice have been selected to breed in unusual, laboratory environments, and vigorous inbreeding is known to reduce fecundity. Under such conditions the presence of melatonin might exert a negative effect on reproductive success. If that were so, pineal melatonin synthesis might well have been inadvertently eliminated in the course of selection for inbred strains that breed well in the laboratory.

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4. Mice (50 to 120 days of age) were decapitated at various times of the day or night; the skull caps with attached pineal glands were then cut away and immediately placed on dry ice. Samples were frozen (−20°C) and used within 30 days for melatonin assay or within 4 days for enzyme assays. An infrared image converter (FJW Industries) was used for all manipulations in darkness. Melatonin was extracted and measured by radioimmunoassay in a modification of the method of M. Rollag and G. Niswender [*Endocrinology* **98**, 482 (1976)], and NAT and HIOMT activity were measured by a modification of the method of D. Sugden *et al.* [in *Methods in Biogenic Amine Research*, S. Parvez *et al.*, Eds. (Elsevier, New York, 1983), p. 567]. Mice were housed on wood shavings in plastic cages with unlimited food and water available under a 12:12 light-dark regime. The room temperature was kept at 21° ± 1°C. C57BL/6J mice were bred in our colony, BALB/c were obtained from J. Weston at the University of Oregon, and wild-derived inbred strains were obtained from E. M. Eicher, Jackson Laboratory, Bar Harbor, ME. FDS mice were shipped from the laboratory colony of F. H. Bronson at the University of Texas, and other mice were purchased from the Jackson Laboratory.
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8. Even though melatonin synthesis might be completely blocked by the absence of either enzyme, activity of the other enzyme could result in the synthesis of indoles other than melatonin which cross-react with the melatonin antibody. For example, in $F_1 \times C57BL$ matings, two-thirds of those progeny which, according to our hypothesis, should not be able to synthesize melatonin, bear a dominant allele for either NAT or HIOMT; in $F_1 \times F_1$ matings six-sevenths of the mice not able to synthesize melatonin bear such an allele and therefore might produce cross-reacting indoles. It may also be that all mice have low levels of NAT (see NAT levels for C57BL mice in Fig. 3) and that, when HIOMT is present, low levels of authentic melatonin are made.

9. We thank G. Cahill and J. Postlethwait for helpful comments on the preliminary manuscript, E. M. Eicher for supplying wild-derived inbred strains, and F. H. Bronson for his gift of the FDS mice and for valuable discussion. This work was supported by NIH grant 13162 and NSF grant DCB-8409010 to M.M. and by grants from the Fogarty International Fellowship (FO5TW03377) and Medical Research Foundation of Oregon to S.E.

24 June 1985; accepted 24 October 1985

Bacterial Grazing by Planktonic Lake Algae

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Six common species of lake algae were found to ingest bacteria. The ingestion rates measured were of the same magnitude as those recorded for marine microflagellates totally dependent on external sources of carbon. A large biomass of *Dinobryon* species removed more bacteria from the water column of a lake than crustaceans, rotifers, and ciliates combined.

THE VIEW THAT PHYTOPLANKTON receive all their energy through photosynthesis was first placed in doubt when it was shown that some algae supplement their carbon supply by taking up dissolved organic carbon (*1*). The phytoplankton could no longer, therefore, be viewed as a strictly autotrophic community, even though this uptake is normally modest and provides only a small fraction of the total carbon acquired (*1*). We now provide evidence that at least some natural phytoplankton are phagotrophic and apparently obtain a substantial fraction of their energy and nutrients by ingesting bacteria at rates very similar to those measured for nonphotosynthetic microflagellates.

The study was carried out in Lac Cromwell, Quebec, on 7 to 8 July 1984. Tracer quantities of bacteria-sized fluorescent latex beads (diameter, 0.6 μm), were released into the plankton caught in a Haney in situ grazing chamber (*2*) at a depth of 3 m. After 1, 4, 7, 10, 13, or 17 minutes the chamber was retrieved and the plankton were preserved and stained (*3*). Sample aliquots were poured onto Nuclepore filters (pore size, 10 μm) for epifluorescence counting of beads ingested by the plankton. We confirmed that the bead uptake rate was representative of bacterial uptake by performing experiments in which algae were exposed to mixtures of beads and tritium-labeled bacteria (*4, 5*).

Four species of the common planktonic alga *Dinobryon* were major consumers of bacteria in Lac Cromwell: *D. sertularia*, *D. sociale* v. *americanum*, *D. cylindricum* (Fig. 1a), and *D. bavaricum*. Other members of the Chrysophyceae, *Uroglena americana* (Fig. 1b) and *Uroglena conradii*, also ingested particles. The "grazing" algal community was found to be most concentrated in a thin layer within the thermocline; there *Dino-*

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