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Melatonin Content of the Pineal Gland in Different Mouse Strains

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Pineal melatonin content at several times during the day and night was measured in 36 inbred strains of mice *(Mus musculus)* kept under LD 12:12 cycles. The results have indicated that only five inbred strains have pineal melatonin content, with higher levels during the night and lower levels during the day; the other 31 strains do not contain detectable melatonin in their pineal gland at any of times examined. The former group includes two commonly used strains (C3H/He and CBA/Ms) and three wild-derived strains (Mol-A, Mol-Nis, MOM). C3H and CBA mice showed a similar pattern of pineal melatonin rhythm with a peak at 2 hours before lights on. The peak levels were about 150 pg/gland in both strains. The rhythmic patterns of melatonin content in Mol-A, Mol-Nis, and MOM were slightly different from those in CBA and C3H. In the wild-derived strains, the peak of melatonin content did not occur at 2 hours before lights on but tended to occur at midnight. The peak levels were 67–91 pg/gland at the highest point in these strains.

Key words: melatonin, circadian rhythm, mouse, strain difference

INTRODUCTION

Pineal melatonin may play an important role for the photoperiodic time measurement and the regulation of circadian rhythms in several vertebrates species [Tamarkin et al., 1985]. Melatonin is synthesized from serotonin by a well-known pathway: serotonin is first acetylated to form N-acetylserotonin (NAS) by the enzyme serotonin N-acetyltransferase (SNAT), and then NAS is methylated by the enzyme hydroxyindole-O-methltransferase (HIOMT) to form melatonin. SNAT activity shows daily changes with higher levels at night and lower levels in the daytime, whereas HIOMT activity is almost constant throughout the 24 h light:dark cycle. Thus, it is thought that a daily rhythm of melatonin synthesis is caused by rhythmic SNAT activity [Axelrod, 1974; Klein, 1985].

All vertebrate species examined have been shown to have pineal melato-

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nin. However, recently it has been shown that although wild mice and some wild-derived inbred strains of mice (SK/CamEi, SF/CamEi, PERU-Atteck/CamEi) synthesize pineal melatonin with normal rhythmicity, some inbred strains of laboratory mice (for example, C57BL/6J) do not have pineal melatonin [Ebihara et al., 1986]. The domestic pig *(Sus scrofa),* although the pineal contains NAT and HIOMT, also lacks a pineal melatonin rhythm under certain photoperiods [Reiter et al., 1987]. The results of genetic analysis using C57BL and wild mice have indicated that melatonin deficiency in C57BL mice is caused by mutations in two independently segregating, autosomal recessive genes, which control the activity of SNAT and HIOMT. These mutant genes may spread in many laboratory mice. Indeed, C57BL/6J, AKR/J, and BALB/c mice, which have different ancestral backgrounds, have neither SNAT nor HIOMT activity, and the NZB stain whose origin is quite different from that of others also does not have HIOMT but does have SNAT activity [Ebihara et al., 1987].

All the strains so far examined for pineal melatonin originate in Europe or the United States. Therefore, in the present study, we examined pineal melatonin content in a variety of inbred strains including strains bred from Japanese "fancy mice" [Festing and Lovell, 1981] and Japanese wild mice so as to obtain more information about melatonin deficiency in laboratory mice.

MATERIALS AND METHODS

Animals

Thirty-six inbred strains of mice (both sexes, 1.5–10 months of age) were used in the present study. These can be divided into six groups according to origin: 1) developed from domesticated mice in Europe or the United States (AKR/J, BALB/c, CBA/Ms, C57BL/6, C57BL/10, C3H/He, HTG, DBA/2, HTH, HTI, RFM, 129/Sv, DDN, CF#1); 2) developed from Japanese "fancy mice" (KR, KR/C, NC, OZB, OZC, OZD, OZH, OZK, CRN); 3) derived from a mating of several mice belonging to groups 1 and 2 (BS, CS, DCR/c, IMV, PONY, WN, IIITES, IXBL); 4) developed from Japanese wild mice *(Mus musculus molossinus)* (Mol-A, Mol-Nis, MOM); 5) developed from wild mice trapped in Europe (SK/Nga); 6) derived from a mating of several mice belonging to groups 1, 2, and 4 (AWB).

C57BL/6 and C3H/He were purchased from the dealer (Shizuoka Laboratory Animal Center, Japan), and the others were obtained from the Department of Animal Genetics, Faculty of Agriculture, Nagoya University. Mice were maintained on wood shavings in plastic cages under a 12:12 h light:dark cycle (LD 12:12, lights on 09:00 h). Food (Labo MR stock, Nihon Nousan Kougyou Co., Japan) and water were provided ad libitum, and the room temperature was kept at 24 ± 1°C.

Sampling of Pineal Glands

Pineal glands were collected at one point (15:00) in the light phase and three points (23:00, 03:00, 07:00) in the dark phase. In the strains in which we found melatonin in their pineal glands, two additional points (11:00, 19:00) in the light period were examined for pineal melatonin. Consequently, the daily

rhythm of pineal melatonin content was shown as changes of every 4 hours. Mice were decapitated, and the skull caps with attached pineal glands were cut away and immediately placed on dry ice. Sampling of one pineal was performed within 1 min, and all of the samplings of pineals were performed within 15 min before and after each sampling time. All manipulations during the dark phase were conducted under a dim red light (640–700 nm, <2 lux). Samples were stored at −20°C until homogenization. Before the assay, each pineal gland was removed from the skull cap and homogenized for 10 s by a sonicator (Handy Sonic model UR-20P, TOMY SEIKO CO., LTD.) in 400 μl of phosphate-buffered saline-gel (PBS-gel; 0.01 M sodium phosphate buffer/0.14 M NaCl/0.1% gelatin, pH 7.4).

Melatonin Radioimmunoassay

The double antibody radioimmunoassay (RIA) was used to measure pineal melatonin content. The rabbit antiserum to melatonin was generously supplied by Dr. Kawashima (Kyoritsu College of Pharmacy, Japan), and caprine anti-rabbit γ-globulin serum was obtained from Nihon Shibayagi-Center (Gunma, Japan).

[^{3}H]melatonin (77 Ci/mmol) was obtained from Amersham, and crystalline melatonin was purchased from Sigma. Each sample was assayed in duplicate, and the standard curve was obtained from measurements in triplicate. One hundred microliters of pineal homogenate was dispensed into each assay tube (MILL-3, LUMAC/3M b.v., Netherlands), and 50 μl of antiserum (1:12,500 dilution in the PBS containing 0.05 M EDTA and 1% normal rabbit serum) was added. Then 50 μl (2,500 cpm) of ^{3}H-labeled melatonin was added to each assay tube. The tubes were mixed by vortex when all additions had been made. After incubation at 4°C for 48 h, 200 μl of goat anti-rabbit γ-globulin serum (1:90 dilution of PBS containing 0.05 M EDTA) was added to each tube. Then the tubes were mixed and allowed to incubate at 4°C for 48 h. Bound and free melatonin were separated by centrifugation (3,000*g*, 30 min, 4°C), and the radioactivity of the precipitate was counted. The inhibition curve was linearized using a logit transformation, and the slope was determined for least-squares fit. The limits of sensitivity was 1.0–1.5 pg/tube for the standard curve. Usually the minimum detection level for the assay of the pineal homogenate was 4–5 pg/tube (16–20 pg/gland). The intraassay coefficient of variation for control samples containing 13.1 pg/tube (n = 7) was 8.4%. The interassay coefficient of variation was 15.7% at the concentration of 12.4 pg/tube (n = 18).

Specificity. The antiserum was highly specific for melatonin, and its cross-reactivities with 12 precursors and metabolites of melatonin have been previously determined to be less than 0.01% except for the slight crossreaction (2.6%) with 6-hydroxymelatonin [Kawashima et al., 1984].

Quantitative recovery. Ten pineal glands of C57BL/6 mice collected during the light in LD 12:12 cycles were homogenized in 2 ml PBS-gel. Melatonin (1.56–100 pg/tube) was added to 50 μl pooled mouse pineal homogenate and quantitatively recovered (Fig. 1). The slope of the least-squares regression line fitted to the data was 1.03, and the correlation coefficient between the amounts added and those assayed was 0.99. The y intercept value represents the

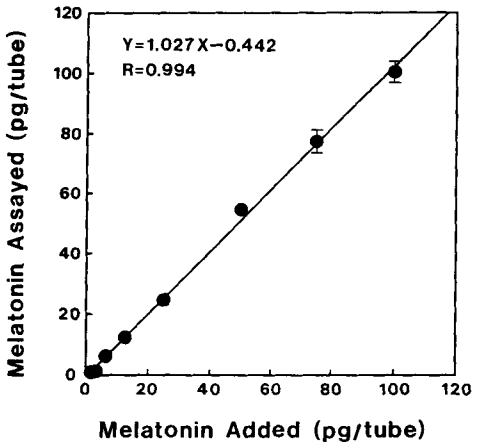

Fig. 1. Quantitative recovery of melatonin added to pooled mouse (C57BL/6) pineal homogenates. Values are means of four to five determinations. The S.E.M. values are shown when they exceed the size of the symbol.

endogenous melatonin level. But the endogenous melatonin was not measured because C57BL/6 mice lack pineal melatonin.

Parallelism. Six pineals of C3H/He mice collected during the dark (2 h before light on) in LD 12:12 cycles were homogenized in 600 μl PBS-gel. Inhibition curves were generated for serial dilution of mouse pineal homogenate and for melatonin standards (Fig. 2). The data were linearized by using a logit transformation, and the slopes were determined by least-squares regression lines. The slopes of the regression lines were −2.37(pineal homogenate) and −2.30(melatonin standard). These were not significantly different ($P < .05$, the analysis of covariance).

Statistics

The data were initially subjected to a one-way analysis of variance (ANOVA). Significant differences between means were determined by the Student-Newman-Keuls test.

RESULTS

Strain Differences

Since no sex differences in results were observed, the male and female results were combined [Ebihara et al., 1986]. In our assay, the minimum melatonin detection level of the pineal homogenate was 16–20 pg per gland. It was difficult to estimate accurately very low levels of melatonin. We had the problem such as nonzero crossreactivity with other compounds. For these reasons we have arbitrarily chosen a level of 24 pg per gland (6 pg per tube) to classify the strains into two groups, one with melatonin and the other with nondetectable melatonin [Ebihara et al., 1986](Table 1). The strains in which the mean value of melatonin content was below 24 pg per gland at each of the times examined (for each strain, more than three animals per each hour were

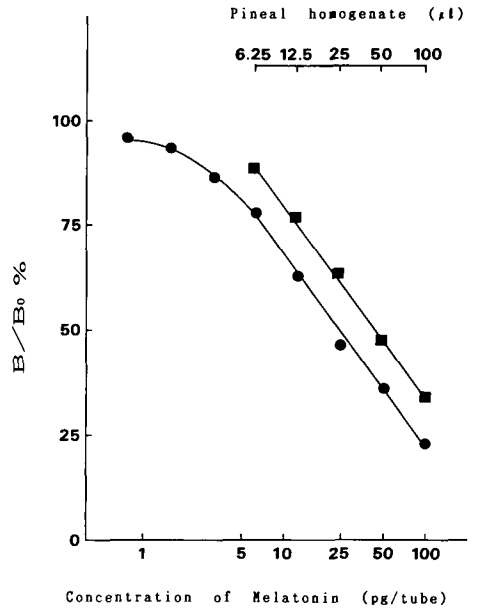

Fig. 2. Parallel inhibition curves obtained with varying quantities of melatonin (●) and serially diluted mouse (C3H/He) pineal homogenates (■). Values are means of four to five determinations.

TABLE 1. Classification of the Mouse Strains Based on the Level of Pineal Melatonin Content[1]

Melatonin	CBA/Ms, C3H/He, Mol-A, Mol-Nis, MOM
Nondetectable melatoin	AKR/J, BALB/c, C57BL/6, C57BL/10, HTG, DBA/2, HTH, HTI, RFM, 129/Sv, DDN,CF#1, KR, KR/C, NC, OZB, OZC, OZD, OZH, OZK, CRN, BS, CS, DCR/c, IMV, PONY, WN, IIITES, IXBL, SK/Nga, AWB

[1]The strains in which the mean value of pineal melatonin content was below 24 pg/gland at any of the times examined were determined to belong to the no melatonin group. The mice maintained under the 12L:12D cycle (light 09:00–21:00) were killed at one point (15:00) in the light phase and three points (23:00, 03:00, 07:00) in the dark phase. More than three animals per each point were examined in each strain.

used) were categorized as a nondetectable melatonin group. In this group, 31 strains were included. In the other five strains that were classified as a melatonin group, pineal melatonin content showed a daily rhythm, and its value was 67–151 pg/gland at the peak level (C3H/He, CBA/Ms, MOM, Mol-Nis, and Mol-A). The differences in pineal melatonin content between the nondetectable melatonin group and the melatonin group were distinctive, and no intermediate strains were observed.

Melatonin Rhythms

Daily changes of pineal melatonin content in the mice with melatonin measured at 4 h intervals are shown in Figures 3 and 4. It was characteristic of

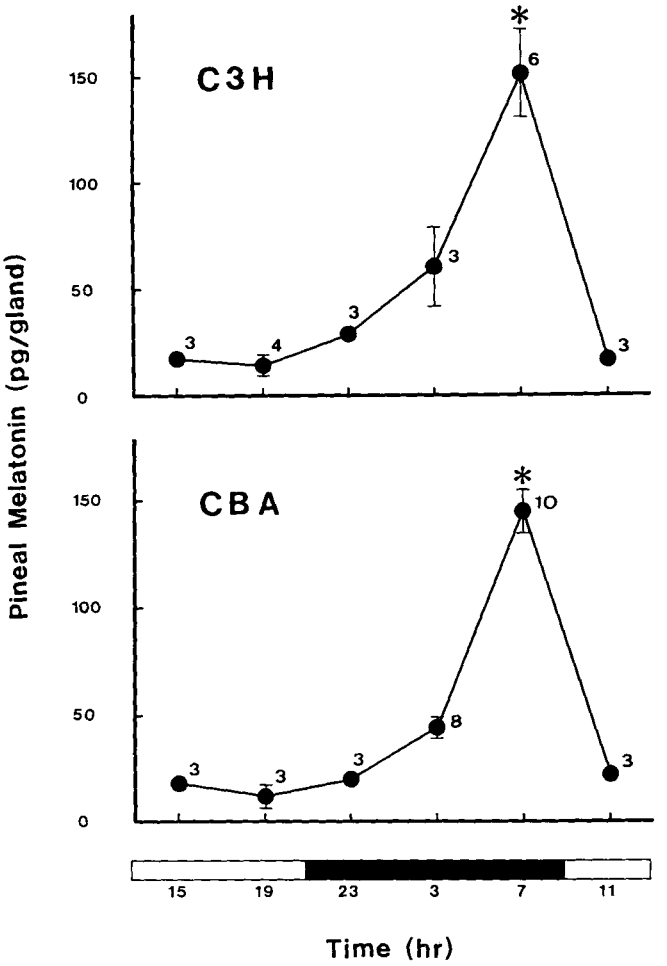

Fig. 3. Daily changes of pineal melatonin content in C3H/He and CBA/Ms mice exposed to LD 12:12. The points represent the means ± S.E.M. The number adjacent to each point is the number of the animals examined in each time. Timing of the light/dark cycle is shown by the horizontal bar. Asterisks indicate significance relative to the data of other times ($^*P < .01$).

all the strains in the melatonin group that the level of melatonin was very low during the light, and the low level persisted until 2 h after lights off. The peak level of melatonin content was significantly higher than all other times in both C3H and CBA mice ($P < .01$). CBA and C3H mice showed a similar pattern of daily melatonin rhythms with a peak at 2 h before lights on (07:00). The peak level was about 150 pg/gland in both strains.

MOM, Mol-Nis, and Mol-A, which are derived from Japanese wild mice, showed a slightly different pattern of pineal melatonin content from that of C3H and CBA. In MOM mice, melatonin levels increased at midnight and decreased thereafter. In Mol-Nis mice, melatonin levels increased at midnight, and these levels continued at least for 4 h. Mol-A mice also showed an increase in pineal melatonin content at midnight and tended to keep the level at 07:00 h. The

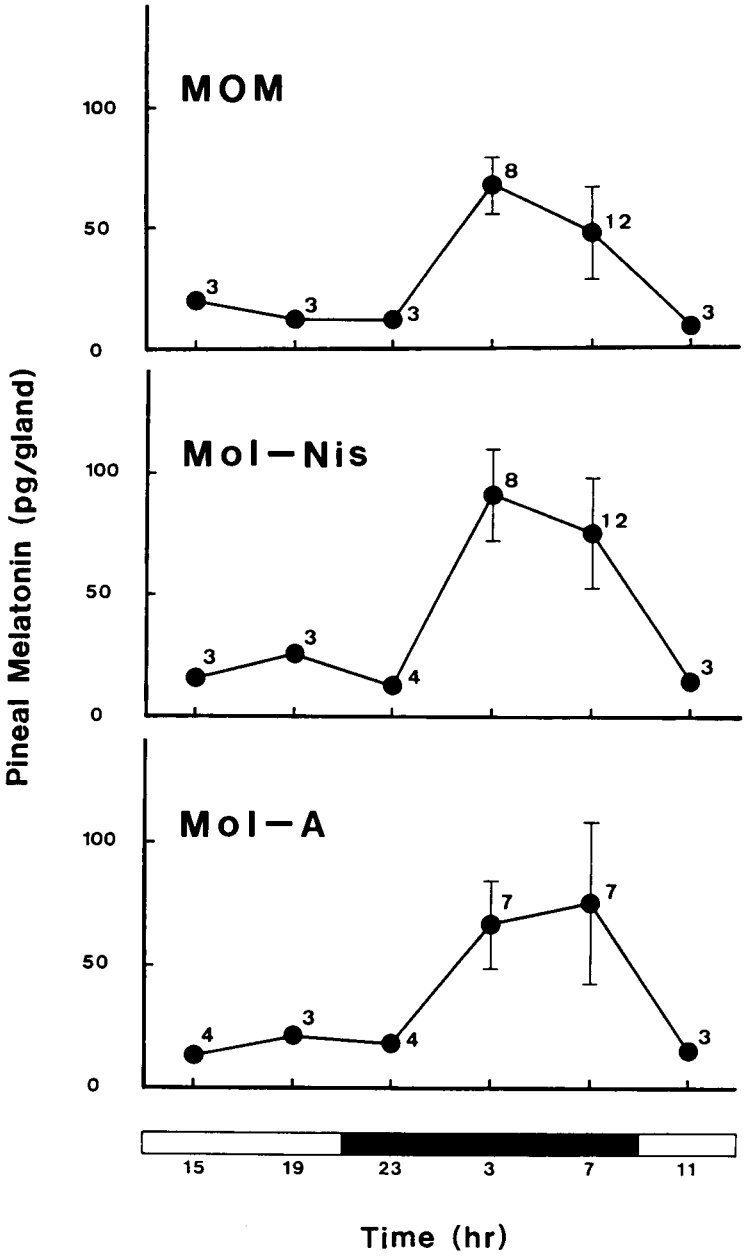

Fig. 4. Daily changes of pineal melatonin content in MOM, Mol-Nis, and Mol-A mice exposed to LD 12:12.

highest level of pineal melatonin content in these wild-derived strains was about 80 pg/gland. However, there were no significant differences in the melatonin levels in any comparison among times ($P > .05$) because of a large variation of pineal melatonin content at 03:00 and 07:00 h.

DISCUSSION

The results obtained in parallel inhibition and quantitative recovery tests have shown that our RIA procedure is sufficient for measuring pineal melatonin content in the mice. The antiserum in the present study has been successfully used for detection of plasma melatonin concentration in goats [Maeda et al., 1984], pigeons [Oshima et al., 1987], and rats and pineal melatonin content in rats [Kawashima et al., 1984]. Although we could not compare the present RIA method with the previous one directly using the same strains, the level of pineal melatonin obtained in the present study seems to be consistent with the previous study [Ebihara et al., 1986].

We have measured pineal melatonin content in 36 strains of mice. Of these, only five strains were found to have pineal melatonin in their pineal gland, but other strains were not found to have reliable melatonin (<24 pg/gland). We have arbitrarily chosen a measured level of 24 pg per gland to classify the strains into the two groups. All the strains categorized as the melatonin group show daily changes of their pineal melatonin content with 67–151 pg per gland at the peak level during the dark, but the measured values in other strains of the nondetectable melatonin group are below 24 pg per gland throughout the day. Using the criteria, a measured level below 24 pg per gland, we could categorize the strains into two groups distinctly. However, we cannot say yet that the strains showing the value below 24 pg per gland are not able to synthesize melatonin in their pineal gland. This would require the demonstration that activities of the enzymes involved in melatonin synthesis such as SNAT are absent in their pineal gland. In addition, more time points and varying photoperiods may be necessary if certain strains have very short duration rise in pineal melatonin during the dark phase. We used mice with different ages of from 1.5 to 10 months. Since the circadian rhythm of melatonin is suppressed in advanced age in many species [Reiter, 1988], it may be possible that nondetectable melatonin in certain strains is caused by aging factor. However, it is unlikely, because C3H mice aged 9 months have melatonin at 07:00 h and most of the 7–10 month old mice are used only during the day.

MOM, Mol-Nis, and Mol-A were developed from Japanese wild mice *(Mus musculus molossinus)* trapped in Japan in 1967–1980, whereas both C3H and CBA were developed in 1920 from a cross of a Bagg albino female and a DBA male [Festing, 1979]. The patterns of pineal melatonin rhythms in the wild-derived strains are similar, and those in C3H and CBA are almost the same, suggesting that genetic factors may be important in determining the pineal melatonin rhythm. The differences in the pattern of pineal melatonin rhythm between the wild-derived strains *(M. musculus molossinus)* and other strains developed mainly from *M. musculus domesticus* may be due to differences of subspecies [Festing and Lovell, 1981]. Although it is unknown why melatonin deficiency occurred in many inbred strains, two possibilities should be considered.

One possibility is that mutations may occur in the early stage of the domestication of the laboratory mouse and spread throughout many inbred lines. It is known that many of the strains are related, having come from the same outbred colony or having some other form of common ancestry [Festing, 1979].

Another possibility is that genes controlling melatonin synthesis may be easy to change. Ebihara et al. [1986] have reported that SK/Cam mice have pineal melatonin (about 140 pg/gland) at 2 h before lights on in LD 12:12 cycles. In the present study, however, melatonin is not found at any of the times examined in the pineal gland of SK/Nga mice, which is the subline of SK/Cam. SK/Nga mice have been bred from SK/Cam introduced from the Jackson Laboratory in 1975, and a brother × sister mating has been carried on over 20 generations. These results may support the latter possibility, but if so, the question arises why old strains such as C3H and CBA still keep pineal melatonin. In any case, using molecular techniques it should be possible to discover the specific sites of mutation in the various inbred strains.

It is well known that C3H mice possess the mutant gene (rd), which causes the selective degeneration of photoreceptor cells [Tansley, 1951, 1954]. According to Tansley, the retinae of C3H mice appear to develop normally until about 13 days of age. By day 19, most of the cells in the outer nuclear layer are dead, and at 4 weeks there is no sign of the rods or their nuclei. In spite of this histological view, pineal melatonin rhythm is C3H mice entrains to LD cycles with the same pattern as CBA mice with normal retina. This suggests that photoreceptors other than rods may be involved in the mediation of light information to the pineal gland in C3H mice. It is known that wheel-running rhythms also entrain to LD cycles in C3H mice [Ebihara and Tsuji, 1980]. The comparison of the light sensitivity or the spectral properties influencing pineal melatonin synthesis between C3H and CBA mice may be important for understanding photoreceptive mechanisms for pineal melatonin synthesis.

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LITERATURE CITED

Axelrod, J. (1974) The pineal gland: A neurochemical transducer. Science 184:1341–1348.

Ebihara, S., D.J. Hudson, T. Marks, M. Menaker (1987) Pineal indole metabolism in the mouse. Brain Res. 416:136–140.

Ebihara, S., T. Marks, D.J. Hudson, M. Menaker (1986) Genetic control of melatonin synthesis in the pineal gland of the mouse. Science 231:491–493.

Ebihara, S., K. Tsuji (1980) Entrainment of the circadian activity rhythm to the light cycle: Effective light intensity for a zeitgeber in the retinal degenerate C3H mouse and the normal C57BL mouse. Physiol. Behav. 24:523–527.

Festing, M.F.W. (1979) Inbred Strains in Biomedical Research. The Macmillan Press Ltd., London.

Festing, M.F.W., D.P. Lovell (1981) Domestication and development of the mouse as a laboratory animal. In : Biology of the House Mouse. R.J. Berry, ed. Academic Press, London, pp. 43–62.

Kawashima, K., A. Nagakura, R.J. Wurzburger, S. Spector (1984) Melatonin in serum and the pineal of spontaneously hypertensive rats. Clin. Exp. Theory Pract. A6(8):1517–1528.

Klein, D.C. (1985) Photoneural regulation of the mammalian pineal gland. In: Photoperiodism, Melatonin and the Pineal. D. Evered, S. Clark, eds. Pitman, London, pp. 38–56.

Maeda, K., Y. Mori, T. Sawasaki, Y. Kano (1984) Diurnal changes in peripheral melatonin concentration in goats and effects of light or dark interruption. Jpn. J. Vet. Res. 46(6): 837–842.

Oshima, I., H. Yamada, K. Sato, S. Ebihara (1987) The phase relationship between the circadian rhythms of locomotor activity and circulating melatonin in the pigeon *(Columba livia)*. Gen. Comp. Endocrinol. 67:409–414.

Reiter, R.J. (1988) Intrinsic rhythms of the pineal gland and associated hormone cycles in body fluids. Annu. Rev. Chronopharmacol. 4:77–105.

Reiter, R.J., J.H. Britt, J.D. Armstrong (1987) Absence of a nocturnal rise in either norepinephrine, N-acetyltransferase, hydroxyindole-O-methyltransferase or melatonin in the pineal gland of the domestic pig kept under natural environment photoperiods. Neurosci. Lett. 81:171–176.

Tamarkin, L., C.J. Baird, O.F.X. Almeida (1985) Melatonin: A coordinating signal for mammalian reproduction? Science 227:714–720.

Tansley, K. (1951) Hereditary degeneration of the mouse retina. Br. J. Ophthalmol. 35:573–582.

Tansley, K. (1954) An inherited retinal degeneration in the mouse. J. Hered. 45:123–127.