# The Pineal Control of Aging

## The Effects of Melatonin and Pineal Grafting on the Survival of Older Mice

WALTER PIERPAOLI,[a] ANTONELLA DALL'ARA,[a]
ENNIO PEDRINIS,[b] AND WILLIAM REGELSON[c]

[a]*Institute for Biomedical Research*
*Via Luserte, 2*
*6572 Quartino-Magadino, Switzerland*

[b]*Istituto Cantonale di Patologia*
*6604 Locarno, Switzerland*

[c]*Medical College of Virginia*
*Virginia Commonwealth University*
*Box 273, MCV Station*
*Richmond, Virginia 23298*

Melatonin modulates the seasonal "Zeitgeber" which is largely affected by day length and/or temperature and which governs avian and mammalian nesting, fat deposition, molting and sexual cycling. Melatonin production may govern sexual maturity, and it has been observed that levels of melatonin decline with age.[1,2] Melatonin secretion is largely circadian and produced and derived from the pineal gland during the dark (scotophase) circadian cycle.[3,4]

As melatonin production may govern sexual maturity and declines clinically with age,[1,2] and as recent data suggests that it may also have an immunoregulatory role,[5,6] we felt that its multiple roles may govern the pattern of aging and senescence. For this reason, melatonin was given in drinking water to syngeneic mice during the dark cycle to see if it would influence patterns of survival or disease. In initial studies, we found in C3H/He female mice, 12 months of age, that melatonin *shortened* survival by inducing ovarian cancer. In contrast, initial results in older mice showed enhancement of longevity by 20% as compared to age-matched controls.[5–7] Based on these early results, experiments with circadian (night) administration of melatonin were replicated. We also homologously transplanted the pineal gland, the primary source of melatonin, from young to older mice to determine if there would be effects on mouse longevity when a youthful intact pineal was grafted into older mice.

We used the thymus as the graft recipient site, as the thymus and the pineal gland share a common adrenergic innervation via the superior cervical ganglion.[8] Pineal function is also associated with thyrotropin releasing hormone (TRH) production, and we have shown that TRH restores thymic function.[9]

For these reasons, as well as for surgical anatomic convenience, a thymic placement of the "young" (3–4 months) pineal grafts was thought to provide the best approach for homologous pineal engraftment.

We also examined survival, changes in thyroid production (T3, T4), immune response and lipid levels in treated and untreated mice in an attempt to derive insights into mechanisms of melatonin and pineal-modulated response in aging mice.

Both models used resulted in a significant enhancement of survival, suggesting that both melatonin and pineal function may play a role in mouse longevity.

## METHODS

### *Melatonin Exposure*

Mice were fed ad libitum using commercial mouse chow (NAFAG 890, 10 mm, Gossau, Switzerland). Darkness and light exposure were controlled by a fixed timer governing 2 standard fluorescent fixtures (Philips TLD 36W/84) 7 pm light-off, 7 am light-on. Melatonin (10 $\mu$g per ml tap water) was administered in the drinking water with a fixed darkness cycle and the control and melatonin-containing, opaque bottles were removed from 8:30 am to 6:00 pm. We examined survival, and the mice were individually weighed monthly to determine if the effects seen related to dietary intake. Mice were housed 4–10 to a cage.

The mouse strains studied and the ages at which melatonin was administered are presented below in FIGURES 1–3.

### *Pineal Implantation into the Thymus*

Donor mice were 3- to 4-month-old, post pubertal BALB/c or C57BL/6. Recipients were groups of aging, BALB/c, C57BL/6 or C57BL/6 × BALB/c F1 hybrid female mice, 16 to 22 months old, depending on the experiment. The donor mice were killed by cervical dislocation and the skull fragment to which the pineal gland adheres was removed and immersed in cooled TC 199 medium with antibiotics (penicillin-streptomycin). The three main radial ligaments were dissected under a dissection microscope and the pineal was carefully displaced with fine scissors and removed, *in situ,* contained in its original membranes. The maintenance of original supporting membranes around the pineal appears to aid the engraftment with vascularization.

The aging graft recipient was anesthetized by ip injection of barbiturate (Vetanarcol, Veterinaria Inc., Zürich, Switzerland). The shaven chest was sterilized with Merfen and the skin above the jugulum was cut for 5–8 mm. The sternum was medially excised from the jugulum for a length of 2–3 mm by using bent scissors. After cutting the muscles, the mediastinal tissue was exposed and the residual, generally atrophic or involuted thymus was exposed by exerting moderate pressure on the abdomen. A single pineal gland in its membranes was positioned on the tip of a needle and introduced into it by gentle aspiration with a one-ml syringe under the dissection microscope. The pineal gland was rapidly injected into a lobe of the exposed thymus after introduction of the needle for 1–2 mm under the capsule. Occasionally, when a successful transplantation of the pineal was doubtful because of displacement of the pineal from the thymus, a second pineal gland was injected. The sternum, muscles and skin were then sutured and a protective plastic film (Nobecutan, Bofors, Sweden) was sprayed on the wound. Postoperative mortality was negligible, but in a few cases the operation produced the immediate rapid death of the mouse due to hemorrhage or pneumothorax.

The recipient mice were females of uniform age, housed 3 to 7 per cage. They were prepared for surgery and studied in groups, as indicated in TABLE 1. Weight changes of control and pineal-transplanted animals were also recorded monthly.

**TABLE 1.** Implantation of a Pineal Gland from Young 3- to 4-Month-Old Donors into the Thymus of Old Aging, Strain- and Sex-Matched Mice Postpones Aging and/or Prolongs the Life of the Pineal-Implanted Recipients

| Groups | Strain and Treatment | Age at Implant or S.O. (Months) | No. of Mice[b] | No. of Surviving Mice (Months of Age) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 |
| A | Implanted C57BL/6 | 16 | 7 | 7 | 7 | 7 | 7 | 7 | 6 | 6 | 5 | 4 | 3 | 3 | 2 | 1 | 1 | 1 | 0 | |
| B | Control C57BL/6 | 16 | 7 | 6 | 6 | 6 | 4 | 4 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| C | Implanted hybrids[a] | 19 | 5 | — | — | — | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 2 | 1 |
| D | Control hybrids | 19 | 6 | — | — | — | 6 | 6 | 4 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | | | |
| E | Implanted BALB/cJ | 22 | 3 | — | — | — | — | — | — | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | 0 | |
| F | Control BALB/cJ | 22 | 5 | — | — | — | — | — | — | 5 | 5 | 5 | 2 | 0 | 0 | 0 | 0 | 0 | | |

[a] C57BL/6XBALB/cJ female hybrids.

[b] All donor and recipient mice used were inbred females. For details on the method and technique, see text.

A versus B: $p < 0.05$ (Mann-Whitney "U" test).

C versus D: $p < 0.01$ (Mann-Whitney "U" test).

E versus F: $p < 0.05$ (Mann-Whitney "U" test).

### *Pinealectomy*

Pinealectomy was performed in young, 3- to 4-month-old C57BL/6 female mice by transcranial galvanocauterization under barbiturate anaesthesia (Vetanarcol).

### *Determination of Thyroid Hormones*

For triiodothyronine (T3) or thyroxin (T4) determinations, the mice were bled from the retroorbital plexus under acute ether anaesthesia at 1 am under dim red light illumination.

Sera from individual mice were kept separate and frozen at −30°C until the hormones were measured by radioimmunoassay (T4-Amerlex-M and T3-Bridge, Serono).

### *Assessment of Delayed-Type Hypersensitivity (DTH) Response*

DTH response to oxazolone was assessed by application of 4 $\mu$l of 5% oxazolone (Aldrich Chem. Co., Milwaukee, WI) dissolved in acetone/oil (1/1) to the clipped skin of the chest and upper abdomen. Four days later, the mice were challenged by topical application of 25 $\mu$l 0.5% oxazolone on both sides of the right ear. DTH response was assessed by measuring the increase in ear thickness of oxazolone-sensitized mice 48 hours after challenge, with a modified micrometer dial gauge (Verdict Gauge Ltd, Dartford, Kent, UK).

### *Determination of Lipids in Plasma*

The mice under investigation were selected randomly and bled under acute ether anaesthesia from the retroorbital plexus between 0:30 and 1:30 am. The plasma from individual mice was kept separate. Cholesterol, phospholipids and triglycerides were measured with an Hitachi 737 Analyser.

### *Light Microscopy*

Fresh specimens for histological examination were fixed in 8% buffered formalin, embedded in paraffin and stained with haematoxylin-eosin.

## RESULTS

### *Chronic Administration of Circadian (Night) Melatonin to One-Year-Old Mice Does Not Postpone Aging but Induces a High Incidence of Tumors*

In preliminary studies, exogenous, night administration of melatonin prolonged the life of mice when the treatment started at the age of 18–20 months.[5–7] In order to verify whether the onset of melatonin treatment at an *earlier* age in mice might affect aging and thus prolong their life span beyond that observed when treatment was started in older mice, two identical experiments were per-

formed in which melatonin treatment was started in one-year-old C3H/He female mice. The results illustrated in FIGURE 1 show that melatonin not only failed to prolong the life span of the mice, but, on the contrary, induced a high number of tumors primarily affecting the reproductive tract (lympho- or reticulosarcoma, carcinoma of ovarian origin; histology not shown here) and thus adversely affected the health and survival of melatonin-treated mice. These data suggest that at concentrations of 10 $\mu$g per ml in the drinking water, melatonin administration by the oral route in relatively "younger" female mice may produce derangements of the neuroendocrine pineal-piloted regulation of sexual organs, this resulting in onset of tumors of the reproductive tract.

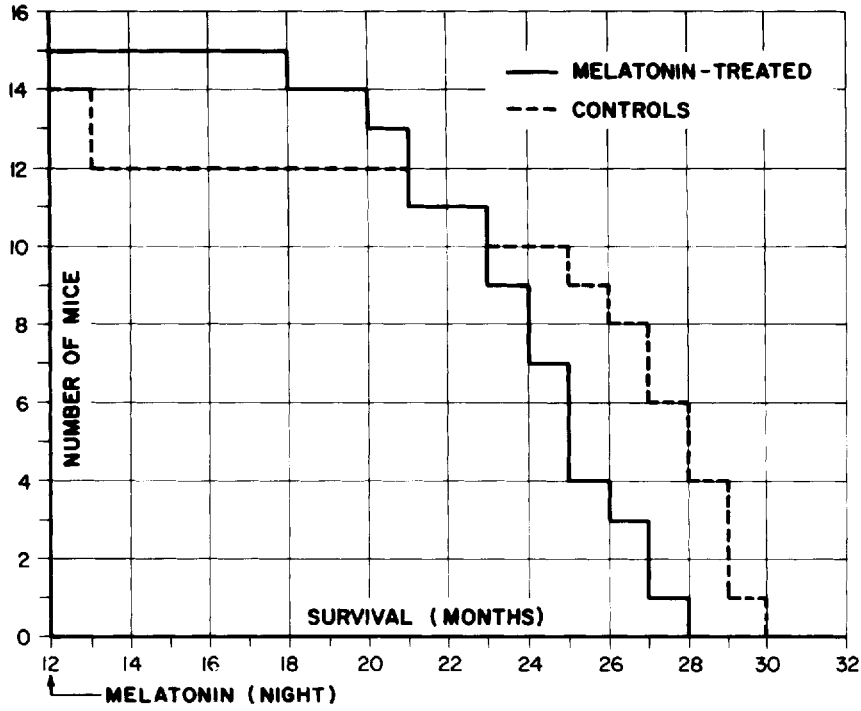


**FIGURE 1.** Chronic administration of exogenous (night) melatonin to one-year-old female C3H/He mice shortens their life span.

### *Chronic Administration of Circadian (Night) Melatonin to Young, Autoimmune Disease-Prone New Zealand Black (NZB) Female Mice Prolongs Their Life*

As shown in FIGURE 2, a remarkable prolongation of life was seen when NZB mice were chronically given melatonin in the drinking water at night, while no effect was seen when melatonin was given during the day. In spite of the effect of melatonin, the common causes of death in all melatonin-treated or control NZB

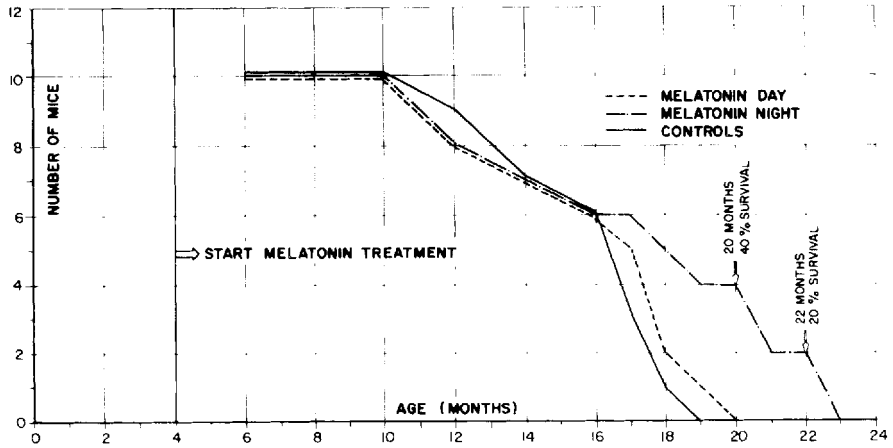


**FIGURE 2.** Circadian (night) administration of melatonin prolongs life of New Zealand black (NZB) female mice.

mice were autoimmune haemolytic anemia, nephrosclerosis and development of systemic or localized type A or B reticulum cell neoplasia.

### *Night Administration of Melatonin to Old, Aging C57BL/6 Mice Retards Their Senescence*

A repetition of our experiments by night administration of melatonin in older, aging C57BL/6 male mice resulted again in a significant prolongation of their survival and confirmed thus our earlier preliminary findings (FIG. 3). Melatonin treatment starting at 19 months of age prolonged the absolute duration of their life by 6 months when compared to untreated controls. There was no significant weight loss or gain in the melatonin-treated mice as compared with controls. Therefore, a decreased food intake or anorexia, with resultant caloric restriction to explain the improvement in survival of the treated mice, does not explain the survival prolongation.

### *Implantation of a Pineal from Young Donors into the Thymus of Aging Recipients Greatly Prolongs Their Survival and Maintains Juvenile Conditions*

TABLE 1 shows the pattern of survival in pineal-implanted C57BL/6, BALB/c × C57BL/6 hybrids and BALB/c females, pineal-engrafted at 16, 19 and 22 months. There was a striking difference in survival between controls and pineal homografted animals. All untreated animal controls were dead at 26 months while several pineal/thymus-transplanted animals were still alive at 31 months. No significant weight loss was seen in pineal-grafted mice. It is significant that this procedure improves survival in mice well along in their life cycle and beyond their reproductive estrous cycle. As seen in FIGURE 4, pineal implantation from young to older mice resulted in a remarkable prolongation of juvenile body conditions (pelage, skin, activity).

In order to ascertain whether this new method permitted proper, clear engraftment of the whole, intact pineal gland into the thymus, and whether the engrafted gland was vascularized and accepted with no visible alterations of its structure and cell (pinealocyte) function (nuclear or cytoplasmatic changes), a few pineal-implanted mice were sacrificed 3, 4 and 6 weeks after implantation. Serial 5 $\mu$m sections of all the thymuses were prepared and examined. As can be seen in the example of FIGURE 5, the pineal gland was clearly found in the thymic cortex. The pineals appeared in excellent condition, apparently intact and functioning, with no significant signs of cellular alterations. However, in spite of the clear life-prolonging effects of pineal implantation in old mice (TABLE 1), it is still unknown whether or not the grafted pineal is also innervated and able to produce and secrete melatonin in the blood circulation.

A more detailed investigation of cell viability and function of the engrafted pineal gland in the thymus of aging, surviving mice is in progress, combined with a study on the effect of pineal engraftment on the preservation of thymus size and cellularity.

### *Melatonin Treatment Modifies Night Levels of Thyroid Hormones and Preserves Cell-Mediated Immunity in Aging Mice*

As shown in TABLE 2, in surviving mice at 19 and 23 months, melatonin treatment resulted in a significant decrease in night levels of T3 and T4 after 7 months and maintained an efficient cellular immune response to oxazolone sensitization.

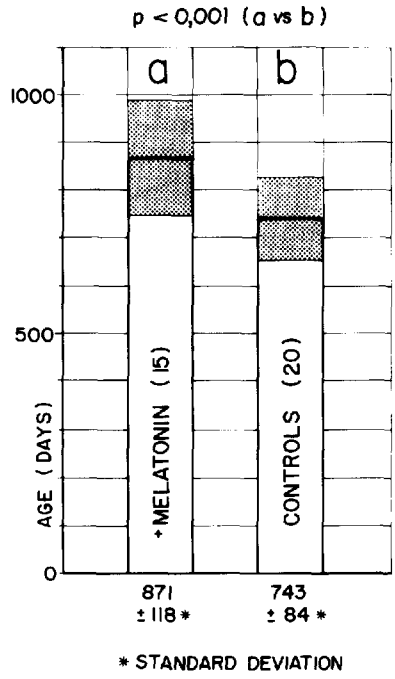


**FIGURE 3.** Aging postponement and/or life prolongation in C57BL/6 male mice consequent to night administration of melatonin.

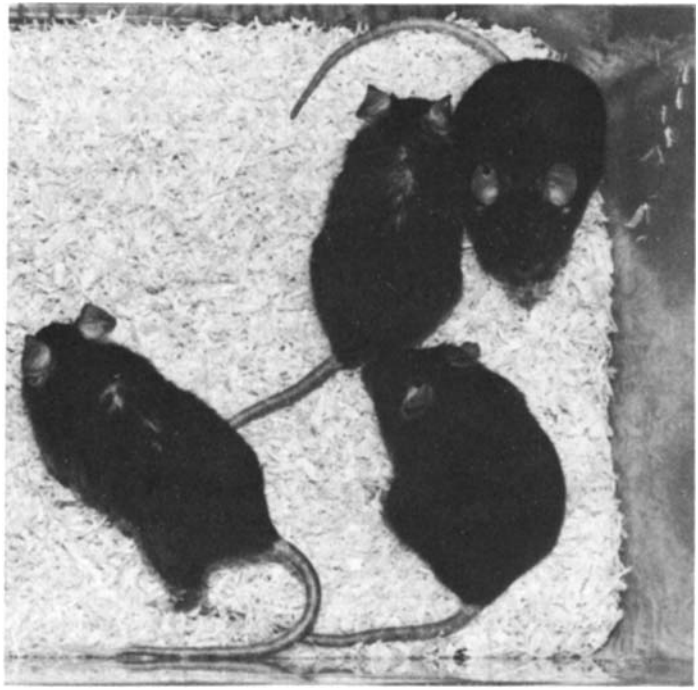

**FIGURE 4.** Young-to-old pineal implantation into the thymus delays aging and prolongs a juvenile status in mice. In this group of C57BL/6, 20-month-old female mice, the two mice on the *right-hand side* have been implanted with a pineal gland from a 3-month-old strain- and sex-matched donor at the age of 16 months. Notice maintenance of a healthy and luxuriant fur coat and youthful conditions in these two pineal-grafted mice. The mice in the picture correspond to Groups A and B of TABLE 1. One pineal-grafted mouse is still alive (31 months old).

### *Early Pinealectomy in Mice Results in Increased Levels of Lipids in Blood*

As seen in TABLE 3, removal of the pineal gland in 4-month-old C57BL/6 mice resulted in an alteration of lipid metabolism. Pinealectomized mice, in contrast to sham-operated animals, showed a rise in cholesterol, triglycerides and circulating phospholipids.

## DISCUSSION

We have shown that in early ontogeny the developing thymic, hypothalamic-pituitary and thyroid functions are functionally interdependent.[10] The thymus programs the immature neuroendocrine system by affecting the maturation of still modifiable hormonal feedback mechanisms.[11] The thymus is thus functionally linked to the thyroid gland. On the other hand, we have suggested and partially demonstrated that the melatonin-pineal effects observed in aging mice may be

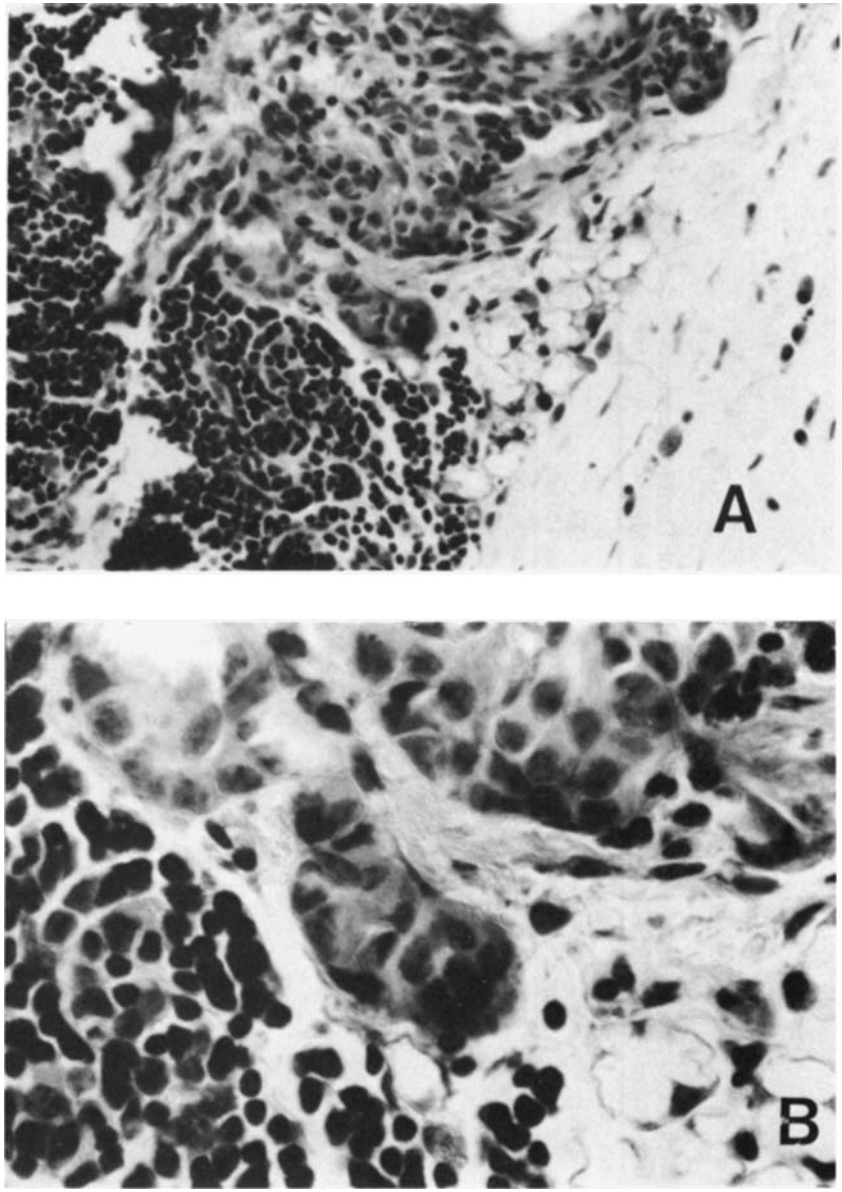


**FIGURE 5.** Young-to-old pineal implantation in the thymus: pineal gland of a 3-month-old donor mouse grafted in the thymic cortex of a 6-month-old recipient, at four weeks after transplantation; **(A)** × 400, **(B)** × 1000. Donor and recipient were inbred, histocompatible C57BL/6 mice. Typical, normal and viable clusters of pinealocytes are assembled in the context of the intact, transplanted pineal gland, which closely maintains its original structure. On the *left-hand and lower side* of the picture, packed, normal thymocytes of the thymic cortex are visible. Haematoxylin-eosin.

**TABLE 2.** Chronic (Night) Treatment with Melatonin Modifies Night Levels of Thyroid Hormones in Serum and Maintains the Delayed-Type Hypersensitivity (DTH) Response of Aging C57BL/6 Male Mice

| Groups | No. of Surviving Mice | Age (M) | Melatonin (M of Treatment) | T3 (ng/ml) | T4 ($\mu$g/100 ml) | Oxazolone Response Before or After Challenge |
|---|---|---|---|---|---|---|
| A. Untreated | 10 | 19 | — | 0.854 ± 0.165 | 5.48 ± 1.09 | — |
| B. Melatonin-treated | 10 | 19 | 3 | 0.873 ± 0.160 | 5.46 ± 1.51 | — |
| C. Untreated | 4 | 23 | — | 0.850 ± 0.028 | 4.94 ± 1.10 | 32.44 ± 4.52[c] / 34.72 ± 3.21 (+7%)[a] |
| D. Melatonin-treated | 8 | 23 | 7 | 0.682 ± 0.049[e] (−25%) | 3.79 ± 1.37[d] (−30%) | 27.75 ± 0.99 / 33.33 ± 4.00 (+16%)[b] |

[a] Not significant (after challenge compared with before challenge).
[b] $p < 0.005$ (analysis of variance; two-tailed $t$ test for unpaired normal samples).
[c] Mean ear thickness ($mm^{-2}$) ± SD.
[d] Not significant (D versus C).
[e] $p < 0.001$ (D versus C).

**TABLE 3.** Early Pinealectomy Results in an Increase of Lipid Levels in the Peripheral Blood of Aging C57BL/6 Female Mice[a]

| Groups | No. of Mice | Age[b] (Months) | Months after Pinealectomy | Cholesterol (nMol/l) | Triglycerides (nMol/l) | Phospholipids (nMol/l) |
|---|---|---|---|---|---|---|
| Pinealectomy | 8 | 23 | 19 | 1.91 ± 0.35 (+30%)[c] | 1.03 ± 0.28 (+26%)[d] | 2.21 ± 0.27 (+21%)[e] |
| Sham-operated | 6 | 23 | 19 | 1.47 ± 0.11 | 0.82 ± 0.13 | 1.82 ± 0.60 |

[a] The mice were bled individually from the retroorbital plexus under light, acute ether anaesthesia and dim red light between 0.30 and 1.30 a.m.
[b] When bleeding and measurements of lipids were performed.
[c] $p < 0.01$.
[d] Not significant.
[e] Not significant.

mediated via the thyrotropin-releasing hormone-TSH-thyroid-thymus axis.[9] In regard to the above, there may also be a true anatomical connection between these apparently unrelated organs and tissues. It has been shown in similar fashion to the thymus, that the innervation of the pineal gland is derived mainly from the superior cervical ganglia, although direct innervation from the CNS also exists.[12] The pineal gland also contains a large number of lymphocytes closely linked to its rich perivascular, perifollicular and intrafollicular nerves and terminals. From this point of view, the pineal gland closely resembles the thymus, which is also richly innervated by, and directly connected with, the superior cervical ganglion.[8,13] Thus, independent of their functional significance, the thymus, the pineal gland and the thyroid[8] share common adrenergic innervation, possibly for short-range communication. However, the presence of lymphocytes within the pineal as a neuroendocrine organ is still an enigma. These functional and anatomical connections suggested that the thymus could be a suitable transplantation site for the pineal gland, offering the possibility of rich and rapid vascularization, and, thus, the growth of viable pineal glandular tissue with such transplantation.

If aging is a programmed event governed by a neuroendocrine clock, the role of the pineal in governing circadian and circannual rhythms, pubertal development and seasonal sexual cycling suggests that it may have a place in the programming or prevention of senescence. This is supported by our results as can be seen in FIGURE 3 and TABLE 1. Most importantly, pineal engraftment was performed in aging and in 16-, 19- and 22-month-old mice. The engrafted mice, in some cases, lived for an increased life span of 5 to 6 months, with a median of 4.2, 4.5 and over 6.5 months longer than controls (TABLE 1). The effect of pineal engraftment from young to old resulted in a 17, 21 and 27% increase in absolute survival suggesting the possibility that engraftment of a young pineal may have a rejuvenating effect, reversing patterns of intrinsic pathology associated with aging.

Our interest in pineal engraftment was based on our studies reported here using melatonin, in which melatonin given during the dark cycle of circadian rhythm in BALB/c female (data not shown here) and C57BL/6 male mice prolonged survival in treated animals when administered in the drinking water beginning at 15 and 19 months throughout their remaining life. The gain in average survival is from 743 to 871 days (TABLE 3). In contrast, when melatonin was given to one-year-old female C3H/He mice, the results were calamitous, with premature death due to the development of ovarian tumors in the melatonin-treated mice. It was not surprising, in this study, that ovarian tumors developed following chronic melatonin administration, as Kikuchi *et al.*[14] found that melatonin stimulated *in vitro* proliferation of a human ovarian KF cell line. It has been shown that human and rat ovaries contain receptors for melatonin which can modulate ovarian function[15] and/or steroidogenesis[16] and, in turn, ovarian function can affect melatonin levels.[17]

Our data on the development of ovarian tumors in C3H/He mice on melatonin feeding, beginning at 12 months, contradicts studies in which chronic melatonin administration has been shown to inhibit hormonally dependent prostatic and breast cancer.[18,19] In other experiments (data not shown here), either because of strain differences or because melatonin administration was begun at 15 months in BALB/c and at 18 months in C57BL/6 female mice, late in the sexual cycling of these female mice, early induction of tumors was not observed and there was a 20% prolongation in survival. Experiments must be carried out in which melatonin treatment starts later at 18–20 months of age in female C3H/He mice.

Although the role of the pineal and its major hormone melatonin are not fully defined,[1–3] if aging is a programmed event governed by a neuroendocrine clock,

the mechanism of action may relate to the reported binding action of melatonin to the suprachiasmatic nucleus.[20,21] Our pineal engraftment and melatonin observations logically relate to the key role of the pineal and its hormones in governing circadian rhythm, pubertal development and seasonal sexual cycling, suggesting that the pineal may have an important place in the programming or prevention of senescence.[22] Certainly, there is evidence that the length of sexual life in a species appears to govern the median and absolute levels of survival. This is seen in animals as diverse as nematodes, drosophila, rodents and primates.

Related to the above, the age of melatonin administration is critical, as neonatal melatonin administration can stimulate sexual maturity in the rat.[23] Thus, melatonin must be viewed as a time-keeping hormone[24] wherein its major source, the pineal, functions as a neuroendocrine transducer interacting with the suprachiasmatic nuclei of the hypothalamus[21,22,25] and the reproductive system.[26,27]

In regard to physiologic mechanisms, the pineal gland (epiphysis cerebri), as a vertebrate endocrine organ, receives impulses from neural sympathetic sources which govern melatonin production. Melatonin (N-acetyl-methoxy-tryptamine) is derived primarily from the pineal gland, although the retina, the GI tract and, in rodents, the harderian glands contribute.

As mentioned previously, the timekeeping role for the pineal is seen in the delay in sexual maturation induced by melatonin in male rats, where it decreases pituitary binding and the hypothalamic synthesis of gonadotrophin-releasing hormone.[28] It would be of interest if this phenomenon were one key to melatonin's antiaging effect. However, pinealectomy, which reduces melatonin production, produces varied results on sexual maturity depending on the photoperiod governing the sexual response in the animal.[29]

The major physiologic role for the pineal is in governing seasonal adaptation, critical for species survival, particularly in seasonal breeders and in small rodents subject to harsh winters where short day length modulates winter adjustment. Pineal function modulates weight loss, molting of winter coat and can induce torpor, changes in thermogenic capacity and reproductive regression. In the clinic, aspects of depression seen with age have been related to melatonin circadian levels or decreased pineal sensitivity with exposure to light.[30] In this regard, total light deprivation reduces serum thyroxin (T4). This effect is prevented by pinealectomy. In contrast, pinealectomy induces thyroid hypertrophy, an effect reversed by melatonin.[31,32] A progressive derangement of overall thyroid function and thyroid-mediated adaptation mechanisms (*e.g.*, motor activity, thermoregulation, sleep, decline of cell-mediated immunity) are typical of the aging syndrome.[33] In evaluating the aging-postponing effects of circadian melatonin, we considered the "thyroid system" as a main route by which melatonin may exert its correcting immunopotentiating and antidistress effects.[5,9] We therefore measured night levels of thyroid hormones in the blood of aging, melatonin-treated mice. We also measured a parameter which expresses impairment or decrease of thyroid function and its liver-related oxidative mechanisms, namely, lipid levels in the peripheral blood of pinealectomized mice. It can be seen that chronic night treatment with melatonin in the drinking water in aging mice significantly lowers night levels of T3 and T4 in peripheral blood (TABLE 2) and thus affects aging-related thyroid dysfunction by a mechanism yet to be elucidated. However, as thyroid hormones are involved in the synthesis and degradation of cholesterol and in the detoxication processes of the liver, removal of the main source of circadian (night) melatonin through pinealectomy may affect both production of thyroid hormones and levels of lipids in peripheral blood. In fact, as shown in TABLE 3, levels of cholesterol, triglycerides and phospholipids are remarkably increased in

the peripheral blood of 23-month-old, aging C57BL/6 mice which had been pinealectomized at the age of four months. In contrast, chronic treatment with night melatonin in aging mice corrects age-related increase of lipids in blood (data not shown here) even though thyroid hormonal production is decreased (TABLE 2). The above effects of melatonin on thyroid function may be pertinent to Denckla's observations regarding the rejuvenating action of hypophysectomy in rat models. In Denckla's studies, hypophysectomy enhances thyroid hormonal action[33] with apparent evidence of functional reversal of age-related physiologic changes, including reversal of nephrosclerosis, restoration of immune response and return to a youthful hepatic functional profile. In support of possible melatonin or pineal influence in thyroid function, recent work by Puig-Domingo *et al.*[34] in the Richardson Ground Squirrel has shown that thyroxine 5'-deiodinase is found in the frontal cortex, cerebellum, pineal gland and brown adipose tissue (BAT). In these hibernators, melatonin administration produced a 7-fold increase in BAT thyroxine deiodinase with an enhancing effect for both BAT and the pineal deiodinase upon exposure to cold. The modulation of the thermogenic capacity of BAT by melatonin suggests that melatonin may be pertinent to both temperature and energy utilization changes that occur with age. Of interest as to this and to the effect of dietary restriction on life extension, there is a loss of thermogenic response with overfeeding beyond 26 weeks of age in rats.[35]

Supporting the validity of our studies are data showing the decline of melatonin circadian values with age in rats and hamsters.[1,2,36,37] Although response to light-dark cycling remains intact, there is an age-related decrease in melatonin values in the pineal itself and in the circulating levels of melatonin.[38]

Clinically, Touitou *et al.*[39] found that levels of plasma melatonin in elderly institutionalized patients showed a decline. Clinical correlates with disease or pathology have been attempted but have not been significantly defined. Similarly, Grinevich and Labunetz[40] found an age-related decrease in 6-oxymelatonin excretion in 140 normal male subjects over 30 years of age, although this was not seen in women. Of interest to our pineal thymic grafting, this study described an age-related decrease in thymic serum factor (TSF) while blood cortisol levels rose with age.

Nair *et al.*[41] and Sack *et al.*[42] have shown a clinical age-related 24-hour decrease in serum melatonin as measured by radioimmunoassay and a lag in circadian melatonin peaking. They suggest that this could be used as a clinical biomarker of aging. Waldhauser *et al.*[43] confirmed a nocturnal decline with lowest serum levels in the 70–90 year old group.

In a search for mechanisms of age-related melatonin effects, Sharma *et al.*[44] reviewed the literature regarding melatonin and corticosteroid changes with age. Their data, from normal individuals (44 men, 27 women) divided into three age groupings, show an age-related decline in melatonin and corticosteroid production. With age, there was a later diurnal onset of melatonin production with an earlier daily output of cortisol. These observations have been confirmed in a smaller series by Thomas & Miles.[45] Pertinent to this, Gupta[46] found an inverse relationship between growth hormone releasing factor (GRF) and melatonin levels in male rats. Similar clinical results, which may be pertinent to aging, were also found in children and adults.

Waldhauser & Wurtman[47] reviewed the literature regarding pineal modulation of hypothalamic function. Recent Soviet data suggest that a pineal polypeptide may reduce sensitivity to dexamethasone.[48] This pineal product was involved in stimulating increased transcortin binding of corticosteroids[49] which decreases with age. The action of hydrocortisone in neonatal rats delays maturation of

pineal noradrenergic uptake and Yuwiler[50] suggests that steroids directly affect the pineal's response to noradrenergic stimulation by actions distal to $\beta$-receptor response. Chronic steroid exposure reduces noradrenergic stimulation of pineal N-acetyltransferase (NAT) activity and the formation of N-acetylated indols.

Rivest *et al.*[51] have shown clinical melatonin values to be highest when corticosteroid values were lowest. Rebuffat *et al.*[52] report that long-term melatonin administration causes hypertrophy of adrenal zona glomerulosa with a rise in serum aldosterone in rats. Wurtman *et al.*[53] also found adrenal enlargement subsequent to surgical pinealectomy or increased exposure to darkness. This makes sense, as Demisch *et al.*[54] have found that as little as 1 mg of evening dexamethasone suppresses nocturnal melatonin production.

Of interest to distress-mediated injury and the presence of melatonin in the GI tract, melatonin protects the rat stomach from serotonin and ethanol-induced ulceration which is thought to relate, in turn, to a reduced gastrointestinal glandular mucosal flow.[55]

In regard to the above, De Fronzo & Roth[56] have also suggested that there was a relationship between pineal function and the adrenals. Troiani *et al.*[57] found that saline injection in rats inhibited melatonin synthesis, probably via enhanced corticosteroid output, inasmuch as hypophysectomy blocked this response. Again, these results are important to Denckla's observation, and others', that hypophysectomy can reverse or delay aging in mice and rats.[33] In that regard, Denckla postulated that hypophysectomy removed "DECO", a "death hormone" that governs aging. What may have been involved in Denckla's observations was the action of a block to distress-mediated pineal inhibition of melatonin production.

In the search for an explanation pertinent to our effects on prolonging survival in mice, Maestroni & Pierpaoli[58] showed that functional (constant light) or pharmacological $\beta$-adrenergic blockage produced not only impairment of body growth but caused thymolymphatic atrophy and a decline in antibody production. In further support of this, Csaba *et al.*[59,60] had earlier shown that pinealectomy exerted profound effects on thymic morphology and function. In this regard, melatonin seems to upregulate immune response and antagonize the immunosuppressive effects of acute distress.[5]

Distinct from melatonin, Pierpaoli and Yi[9] have shown that TRH has remarkable thymus stimulating and immunoenhancing effects. However, in the case of TRH, its upregulating action is reported to antagonize endorphin effects[61] distinguishing it from melatonin. Furthermore, pineal function seems to be responsible for TRH production. In fact TRH is nocturnally elevated in a circadian fashion similar to melatonin.[62] TRH, as a tripeptide, is found in porcine, ovine and rodent pinealocytes. In addition, B and T lymphocytes are found in pineal parenchyma of the 3–4-week-old chicken.[13] This indicates that the pineal may have lymphoproliferative capacity. Vede *et al.*[63] reported that T cells increase with age in the rat pineal. Olath and Glick[64] suggest that the ectodermal origin of pinealocytes indicate that the pineal may influence thymic function in similar fashion to thymic keratinocytes.[65,66] Hume *et al.*[67] found that endocrine glands contain significant macrophage populations and that the pineal contains sinusoidal perivascular F4/80, antigenically identifiable phagocytic cells which could process antigen to modulate immune responses.[67]

The modulating effects of pineal engraftment in our study may also relate to the fact that the pineal contains peptide hormones found in the hypothalamic-neurohypophyseal system, *i.e.*, oxytocin, vasopressin and vasotocin-like peptides in addition to melatonin or serotonin-related indoles. Prechel *et al.*[68] showed that these neuropeptides are not seasonally completely dependent on superior cervical

ganglion sympathetic control and Schroder *et al.*[69] showed that vasopressin inhibits nocturnal melatonin production.

In a search for mechanisms of age-related melatonin decline, Tang *et al.*[70] showed that there was a decline in mid-dark pineal serotonin, norepinephrine and dopamine production in 18-month-old rats. As an explanation, they suggested that sympathetic activity enhanced conversion of serotonin to melatonin in the pineal.[70] An age-related decline in pineal response might reflect a pineal functional decline in sympathetic tone and, in that regard, there is delayed recovery of $\beta$-adrenergic downregulation induced by desmethylimipramine in 20–26-month-old rats.[71] This was confirmed by Greenberg[72] who showed that aged (24-month-old) mice lose their capacity to develop $\beta$-adrenergic hypersensitivity during the light cycle or following reserpine administration, where noradrenaline production is decreased or inhibited. As reported above, the pineal loses its capacity to upregulate both $\beta$ and $\alpha$ receptors as a function of age because of delayed recovery from adrenergic downregulation.[72] In support of this, *in vivo* studies show that noradrenergic sensitivity declines with age and that there is an inverse relationship between corticosteroids and melatonin present in clinical depression.[73] In this regard, reduced melatonin production is found in elderly patients suffering from orthostatic hypotension.[74] This indicates a possible association between melatonin synthesis and sympathetic tone. In support of this, cold immobilization stress in rats causes a marked rise in pineal melatonin. This is accompanied by inhibition of monoamine oxidase which is ascribed to a brain-associated peptide, identified as "tribulin."[75]

Wu *et al.*[76] report that swimming stress enhances melatonin output at night or in the presence of desmethylimipramine, a norepinephrine blocker. As mentioned previously, the pineal contains high concentrations of monoamine oxidase. Agents like clorgyline and deprenyl, which inhibit the decrease of norepinephrine[77] induced by monoamine oxidase degradation, enhance melatonin production,[78] and there is recent work suggesting that deprenyl may enhance longevity and sexual performance in male rodents.

In another related area, Wright *et al.*[79] showed an age-related decline in superior cervical ganglion and pineal nerve growth factor (NGF) content. This change was sex related, NGF being higher in males or following testosterone treatment. Reuss *et al.*[80] also showed an age-related decline in pineal night-time melatonin excretion in 18-month-old rats correlating with decreased spontaneous pineal electrical activity in older animals.

As discussed earlier, in our study the thymus was used as the graft site, as both the innervation of the pineal gland and the thymus is derived mainly from the superior cervical ganglia,[8] although there is additional direct innervation from the CNS to the pineal.[12]

In the search for age-related mechanisms, Dax and Sugden[81] suggest that the major reason for decline in pineal melatonin synthesis with age is not receptor related in the Wistar rat, but due to a decline in pineal hydroxyindole-o-methyltransferase.

In another aspect of mechanisms of aging, one action of melatonin is the regulation of forebrain dopaminergic function.[82] L-dopa given to rats increases melatonin pineal content,[83] and there is a mixed clinical literature regarding the benefits of melatonin in Parkinson's disease and other movement disorders. As L-dopa administration enhances the life expectancy and motor responses of mice, it would be of interest if, in our melatonin or pineal transplantation studies, the results seen were mediated through the dopaminergic system.

In support of the above, Zisapel and Laudon[84] have shown that melatonin

blocks the stimulated release of 3H-dopamine from the rat hypothalamus *in vitro*. Inhibition of dopamine release by melatonin has been observed in the ventral hippocampus, medulla-pons preoptic area and median and posterior hypothalamus, but not in the striatum or other areas.[85] In view of the above and the dopaminergic inhibition of prolactin secretion, it is of interest that melatonin causes a decrease in prolactin secretion in pituitary-grafted male rats. In addition, melatonin is reported to increase serotonin and decrease dopamine turnover in intact rats. Friedman *et al.*[86] have shown pineal indoles to decrease with age in old rats that were sensitive to imipramine $\beta$-adrenergic receptor, and also found serotonin receptor reduction with age.

The action of pinealectomy on prolactin production has been reviewed by Stanisiewski *et al.*[87] and Boissin-Agasse *et al.*[88] In mink, prolactin production following pinealectomy varies independent of its yearly photoperiods, and Kennaway *et al.*[89] have observed a heightened prolactin level in pinealectomized ewes. It would be of interest to investigate whether the rise in prolactin with age is a function of pineal changes with age, related to declining nocturnal melatonin production. In this regard, the role of melatonin in the induction of adrenarche is confused, as is the role of prolactin: dopaminergic blockade can hasten puberty in dogs.[90]

In another area, Haldar-Misra and Pevet[91] developed a concept that 5-methoxy indoles, including melatonin produced by the pineal, can affect the secretion of protein/peptides by the pineal. This would explain the potential value of pineal engraftment from young to old mice as a method of maintaining pineal secretory integrity in older animals.

One of the biomarkers of aging is a change in polyamine ratios, and there is an increase in spermine over polyamines. In this regard, pinealectomy induces a decrease in ornithine decarboxylase.[92] Polyamines are key intracellular cations that are modulators of DNA and RNA synthesis.

Kahan[93] cites his work with pineal transplantation into the anterior eye chamber of the "rd" rat which develops spontaneous retinal degeneration. Pineal transplantation from normal animals prevented retinal dystrophy in these animals by supplying melatonin. He states in his review that patients with retinitis pigmentosa were "transitorily improved by melatonin injections" but provides no data.

One clinical potential of melatonin reflects upon its capacity to suppress ventral prostatic hyperplasia in rats.[94] These effects depend upon dosage. High dosages inhibited acinar proliferation, while lower dosages resulted in reduced stroma and epithelium. Based on this, one must ask if benign prostatic hypertrophy might relate to declining clinical melatonin levels with age.

As discussed previously, melatonin has direct inhibitory effects on human breast cancer cells in tissue culture which relates to the observation of a direct lethal action on this estrogen-sensitive cell line.[19] In addition, melatonin exerts direct inhibitory action on the Dunning prostatic adenocarcinoma.[18]

Our results in prolonging survival in aging mice did not concern dietary deprivation. No relevant difference in body weight was observed between control and melatonin-treated mice. It is in fact known that daily melatonin injections in male Syrian hamsters increase body weight.[95,96] Feeding melatonin increases plasma concentration of melatonin and increases fat deposition in the carcass of heifers.[97] Melatonin peaks at a point of lowest body temperature. The role of dietary deprivation in extending life expectancy has been related to a fall in body temperature. The decline in thyroid hormone production on nocturnal administration of melatonin (TABLE 2) suggests that this may be an important parallel effect to caloric restriction. In this regard, short-term fasting has been associated with a rise in

norepinephrine and serum melatonin.[98,99] Others[100] describe underfeeding as a pineal-function-potentiating-factor in the rat. In addition, there is evidence that melatonin can modulate magnesium and zinc cationic levels.[101] This is of interest because of evidence that $Mg^{++}$ enhances melatonin synthesis.[102] In this regard, Morton[101,102] suggests the possibility, based on work of Zaboni *et al.*[103] and Karppanen *et al.*[104] that pineal function could be a factor in hypertension. Alternatively, Landfield[105] has shown that $Mg^{++}$ supplementation enhances CNS behaviour in aging mice, which may be due to a melatonin-magnesium relationship. If zinc levels are modulated by melatonin, this could be a factor in thyroid function and aging as discussed by Fabris *et al.*[106] and Travaglini *et al.*[107]

In summary, the two most important factors for species survival and maintenance of "identity" are gonadal and immunologic.[11] Based on this, we felt that the pineal is the developmental pacemaker which in the course of neuroendocrine ontogeny plays a major role in translating and modulating environmental cues to govern the developmental and functional integration of the hypothalamic-pituitary-gonadal-adrenal-TRH-thyroid system.[5,7,9] The pineal is constantly translating external stimuli (light, temperature, magnetism, pheromones, antigens) as well as internal messages (neuroendocrine, autonomic, psychic) into circadian and seasonal responses necessary for individual and species survival.

We feel the results in this study, which suggest that melatonin nocturnal administration or youthful pineal transplantation into older mice prolongs median and absolute survival, indicate that we must explore the mechanisms involved which may include trophic factors related to pineal peptides or indoles.

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