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Pineal control of aging: Effect of melatonin and pineal grafting on aging mice

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ABSTRACT **Dark-cycle, night administration of the pineal hormone melatonin in drinking water to aging mice (15 months of age) prolongs survival of BALB/c females from 23.8 to 28.1 months and preserves aspects of their youthful state. Similar results were seen in New Zealand Black females beginning at 5 months and C57BL/6 males beginning at 19 months. As melatonin is produced in circadian fashion from the pineal, we grafted pineals from young 3- to 4-month-old donors into the thymus of 20-month-old syngeneic C57BL/6 male recipients, and a 12% increase in survival was induced. Prolongation of survival was also seen on pineal transplant to the thymus in C57BL/6, BALB/cJ, and hybrid female mice at 16, 19, and 22 months. In all studies, the endogenous pineal of grafted mice was left *in situ*. Pineal grafted aged mice display a remarkable maintenance of thymic structure and cellularity. Preservation of T-cell-mediated function, despite age, as measured by response to oxazolone is seen. Other evidence suggests that melatonin and/or pineal-related factors could produce their effects through an influence on thyroid function. These data indicate that pineal influences have a place in the physiologic regulation of aging.**

The pineal hormone melatonin is secreted in all mammals during the dark phase of the circadian cycle (1), but, even more importantly, there are indications that it is a key regulator of aging and senescence (2, 3). The role of melatonin in controlling sexual maturity, sexual cycling, cancer, stress, and the immune response makes it likely that the pineal may be a factor in the syndrome of aging (4–6). With this in mind, we have administered exogenous melatonin in the drinking water of mice during a fixed circadian dark cycle—i.e., when melatonin is normally produced—in order to ascertain its influence on patterns of survival.

In addition, as the pineal gland is the prime source of melatonin, we transplanted the pineal gland from young to syngeneic histocompatible older recipients. We have utilized the thymus as the graft recipient site inasmuch as the thymus and the pineal gland share a common adrenergic innervation via the superior cervical ganglion (7, 8). This common innervation is of importance as melatonin synthesis is inhibited by pharmacologic sympathetic blockade, which also modulates the immune response (9). Moreover, the pineal morphologically contains lymphocytes and it has been likened in its embryologic developmental origin to the thymus (10).

In our studies, exogenous nocturnal circadian administration of melatonin or engraftment of young homologous (3–4 months) pineals to old (18–22 months) syngeneic mice adjacent to the thymus, leaving the recipient's pineal *in situ*, resulted in a significant enhancement of survival independent of significant weight loss. These results suggest that the pineal may act as an endogenous clock governing aging.

MATERIALS AND METHODS

Melatonin Administration. Mice were fed ad libitum using NAFAG pellets (Gossau, Switzerland) and housed 4–10 to a cage in air-conditioned quarters at 22°C. Light exposure was controlled by a fixed timer that governs a standard fluorescent fixture (Philips TLD 36W/84). Melatonin, solubilized in ethanol, was given in the drinking water during a fixed darkness cycle from 6 p.m. to 8:30 a.m. (10 μg/ml of tap water, 0.01% ethanol). The control (only ethanol) and melatonin-containing water bottles were removed from 8:30 a.m. to 6 p.m. No drinking water was given during that period. The mice were individually weighed at intervals to determine whether the effects seen were related to dietary intake.

Pineal Grafting. In young-to-old, pineal-to-thymus grafting, the "young" pineals were obtained from 3- to 4-month-old, postpubertal mice. Syngeneic recipients were groups of "aging" mice. The recipient mice were uniform as to sex and age, housed 3–7 per cage. They were prepared for surgery and studied in groups, as indicated in Tables 1 and 2. Weight changes of control and pineal-transplanted animals were recorded monthly.

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 containing penicillin and streptomycin. The pineal was carefully separated with fine scissors and removed *in situ* within its original supporting membranes, maintenance of which aids graft vascularization.

Grafts recipients were anesthetized by i.p. injection of a barbiturate (Vetanarcol; Veterinaria, Zurich). Thereafter, the shaven chest was prepped with 70% ethanol and a 5- to 8-mm-long midline skin incision was made commencing just below the neck. A 2- to 3-mm length of the thorax was opened and the mediastinal tissue was exposed and the native thymus became available *in situ* by exerting moderate pressure on the abdomen. A single donor pineal gland was positioned on the tip of a hollow needle and introduced into the needle by gentle aspiration. The pineal graft was injected slowly into the right or left lobe of the thymus with rotation of the needle. Occasionally at surgery, when a successful transplantation of the pineal was in doubt, a second pineal gland was used. The sternum, muscles, and skin were then sutured and a protective plastic film (Nobecutan, Bofors, Sweden) was sprayed on the wound. In a few instances the operation led to immediate death of the mice due to hemorrhage or pneumothorax. Control groups were similarly transplanted into the thymus with a pineal-size matched fragment from the donor brain cortex.

Immune response was measured by the delayed-type hypersensitivity (DTH) response to oxazolone (2). Statistics used equal variance *t* test for unpaired normal samples (two tailed).

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Abbreviations: NZB, New Zealand Black; DTH, delayed-type hypersensitivity.

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Light Microscopy. Serial sections (5 μm) of thymic pineal grafts and thyroids were obtained from 21-month-old BALB/c female mice at sacrifice 3 months after pineal grafting in 5–10 animals from control and treated groups. The sections were stained with hematoxylin/eosin and examined microscopically in blinded fashion without knowledge of which experimental group they belonged to.

RESULTS

Oral Administration of Melatonin. Fig. 1 depicts a survival comparison between normal controls and melatonin-treated BALB/c female mice. The average survival of controls was 715 days vs. 843 days for melatonin-treated mice. In the controls (Fig. 1) median survival was 23.8 months, whereas the median melatonin-treated survival was 28.1 months, with an absolute upper limit of survival on melatonin administration of 29.4 months as compared to 27.2 months for controls. Equal variance *t* test for unpaired normal samples treated vs. controls showed $P < 0.001$. There were no significant differences in weight between the two groups at any time.

Fig. 2 shows prolongation of life when NZB mice were given melatonin in the drinking water, daily, at night, with no effect seen when melatonin was given during day light hours. Using log-rank values comparing control with night-administered melatonin showed a *P* value of 0.059. The common causes of death in all melatonin-treated or control NZB mice were autoimmune hemolytic anemia, nephrosclerosis, and development of a systemic or localized type A or B reticulum cell neoplasia, which characterizes end-stage disease in these aging mice.

Fig. 3 shows results of melatonin treatment, starting at 19 months of age in C57BL/6 male mice. Melatonin in drinking water prolonged the absolute duration of life by up to 6 months when compared to untreated controls. The average weight changes of the melatonin-treated mice as compared with controls were not a factor in survival.

Implantation of a Pineal from Young Donors into the Thymus of Aging Recipients. 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, respectively. All untreated controls were dead at 26 months, whereas several pineal/thymus-transplanted animals were still alive at 31 months, and there was a significant prolongation of life in all three grafted groups. As seen in Table 1, *P* values ranged from <0.01 to <0.05 in comparing control vs. pineal-transplanted groups, and weight loss was not a factor.

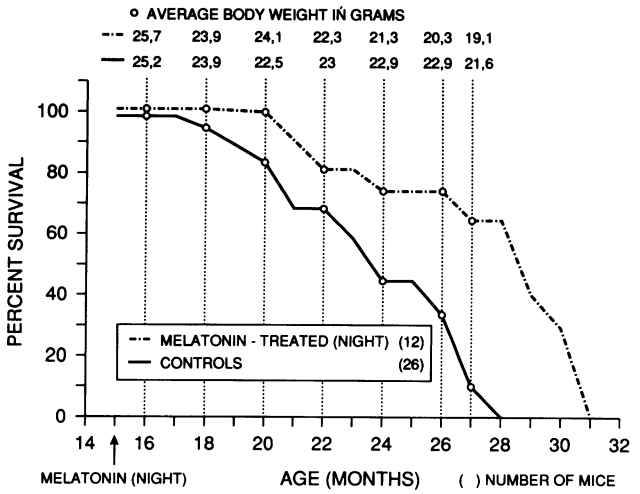


FIG. 1. Aging postponement and/or life prolongation in BALB/c female mice consequent to night administration of melatonin.

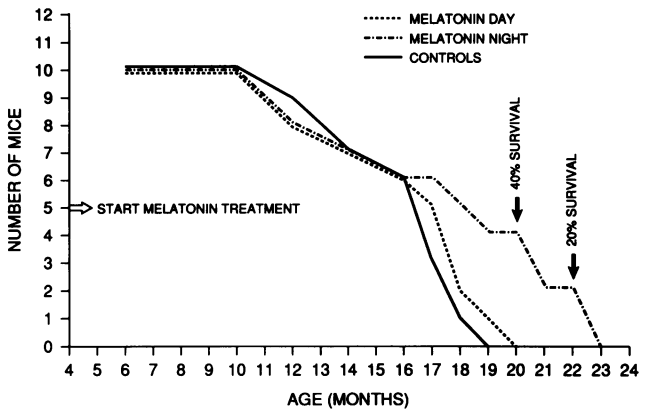


FIG. 2. Survival in New Zealand Black (NZB) female mice given melatonin in drinking water. Day versus night. [Reprinted with permission from ref. 2 (copyright New York Academy of Sciences).]

The effect of pineal engraftment into the thymus (Fig. 4) showed prolonged survival in the 20-month-old C57BL/6 male mice grafted with a pineal from 3-month-old syngeneic donors. In this study, although the significant difference between pineal-grafted and controls resulted in only a 12% enhancement of survival, the absolute range of survival in the treated animals is >810 days, with a single animal (not counted in final evaluation) surviving 1035 days as compared to 747 days for control animals. The standard error is indicated in Fig. 4. Body weight changes did not contribute to survival between pineal-grafted and control groups. No life-prolonging effect was seen in the control group implanted into the thymus with a pineal-size fragment of brain cortex.

These results fully confirmed our observations in C57BL/6, BALB/cJ, and hybrid females as can be seen in Table 1. Most important, pineal engraftment was performed in different aged groups: 16-, 19-, and 22-month-old mice. The engrafted mice, in some cases, lived for an increased life-span of 4–6 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 ($P < 0.01$ or < 0.05, Table 1).

Fig. 5 shows the typical morphology of a residual pineal gland from a 3-month-old donor mouse grafted to the thymus of an 18-month-old recipient, at 3 months after transplantation. Donor and recipient were inbred, histocompatible BALB/cJ mice. Identical results were seen in grafting the pineal to 6-month-old and 20-month-old recipients.

As can be seen, typical normal and viable clusters of pinealocytes are still assembled within the intact, transplanted pineal gland, which closely maintains its original structure. Perhaps the most remarkable finding was that in all

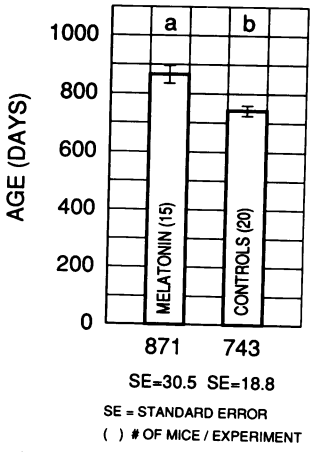


FIG. 3. Survival in C57BL/6 male mice consequent to melatonin, during dark cycle, beginning at 19 months when mice show onset of age-related death. [Reprinted (with modification) with permission from ref. 2 (copyright New York Academy of Sciences).]

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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

Group	Strain and treatment	Age at implant or sham-operated, months	No.	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*	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		

*C57BL/6 × BALB/cJ female hybrids. All donor and recipient mice used were inbred females. For details on the method and technique, see text. Data have been published in ref. 2. A vs. B: $P < 0.05$ (Mann–Whitney U test). C vs. D: $P < 0.01$ (Mann–Whitney U test). E vs. F: $P < 0.05$ (Mann–Whitney U test).

sacrificed animals at advanced age (21 months), the thymic structure of the pineal-grafted mice is still maintained (Fig. 5 *A* and *A1*), whereas in control samples the thymus contained no residual thymic lymphocytes (Fig. 5*B*).

As illustrated in Table 2, melatonin treatment and pineal grafting resulted in a significant maintenance of a vigorous immunological response expressing cell-mediated transplantation immunity, as measured by DTH response to oxazolone. Significance is indicated in the legend to Table 2, where the immune reactivity, present in pineal-grafted mice as compared to controls, confirmed the histologic observation of repopulation of the thymus seen in pineal-grafted mice (Fig. 5).

Recent observations on thyroid function in melatonin-treated mice (2) prompted us to examine thyroid morphology in pineal-grafted senescent mice. Light microscopy of the thyroid gland of pineal-grafted and control aging mice in our "blinded" histologic study showed a very remarkable maintenance of a youthful thyroid morphology, as compared to control (Fig. 6).

DISCUSSION

If aging is a neuroendocrine programed event, 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 programing or prevention of senescence (1–4). In support of this there is an age-related decrease in melatonin values in the pineal itself and in the circulating levels of melatonin (11). Clinically, Touitou *et al.* (12) and others (2) have found that clinical levels of plasma melatonin in elderly patients show a significant decline.

Melatonin treatment in C57BL/6 male mice beginning at 19 months prolonged absolute duration of survival by 6 months. Similar results were seen in BALB/c female mice and in NZB mice, with melatonin treatment onset at 15 and at 5 months, respectively. These NZB female treated mice given melatonin during the dark cycle showed a 20% increase in survival at 22 months, with all controls dead at 19 months.

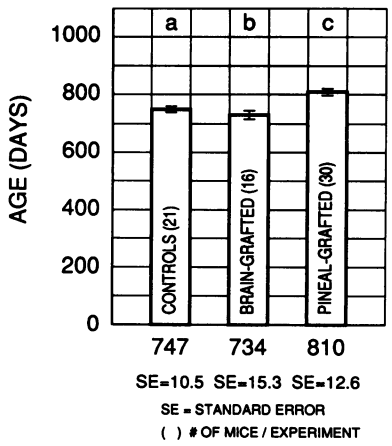


FIG. 4. Survival following pineal transplantation into 20-month-old C57BL/6 male recipients from 3-month-old donors.

Most important, when youthful pineals were engrafted into aging 16-, 19-, and 22-month-old mice, the engrafted mice, in some cases, lived for an increased life-span of 4 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. This is further confirmed by a 12% increase in absolute survival on pineal to thymus engraftment from young mice to 20-month-old C57BL/6 mice (Fig. 4). In these studies, melatonin-treated and pineal to thymus-grafted mice, despite the advanced age of recipients, show preservation of T-cell-mediated immune function as measured by the DTH response to oxazolone (Table 2).

In the pineal-grafted animals, survival data are reinforced by the apparent juvenile morphologic state of the thymus and thyroid and the immune status of recipients despite their age (Figs. 5 and 6). The maintenance of thymic function is not surprising as melatonin and the pineal are known to enhance the immune response (9, 10, 13–18). This may well delay the appearance of tumors (19) and autoimmune disease (20) as factors in age-related pathology.

Melatonin has been shown to block ovulation and is now being evaluated as a clinical contraceptive (21). In that regard, the pineal adapts the internal neuroendocrine environment to changes in external variables that can involve not only exposure to light but also humidity, magnetism, temperature, antigens, pheromones, hunger, sexual drive, fear, and distress (1, 2, 4, 22). Pineal function may have a complementary modulating role against stress-mediated corticosteroid action (2–3, 4, 6, 14–16, 23–28). Russian data suggests that a pineal polypeptide may reduce sensitivity to dexamethasone (26). Anisimov *et al.* (29) have found a pineal peptide that delays aging, which may be a factor in preventing oncogenesis (30).

Apart from melatonin and as yet undefined pineal peptides (26, 29, 30), the pineal also contains thyrotropin-releasing hormone (TRH) (31, 32) and modulates the 5′ thyroid deiodinase (33). TRH and melatonin can block steroid- or stress-related thymic involution (9, 16, 34, 35) and restore immunocompetence in nude (athymic) mice (16). TRH and melatonin have receptors in the preoptic hypothalamic areas of the brain important to thyroid and thymus regulation (22, 24, 34, 35).

The pineal graft may act directly on its thymic neighbor via melatonin or possibly via other pineal hormones that diffuse into the thymus. The grafted pineal may receive sympathetic fibers from the superior cervical ganglion, which normally innervates the thymus, and thus may have a normal pattern of nocturnal melatonin release that acts on the thymus to rejuvenate the gland.

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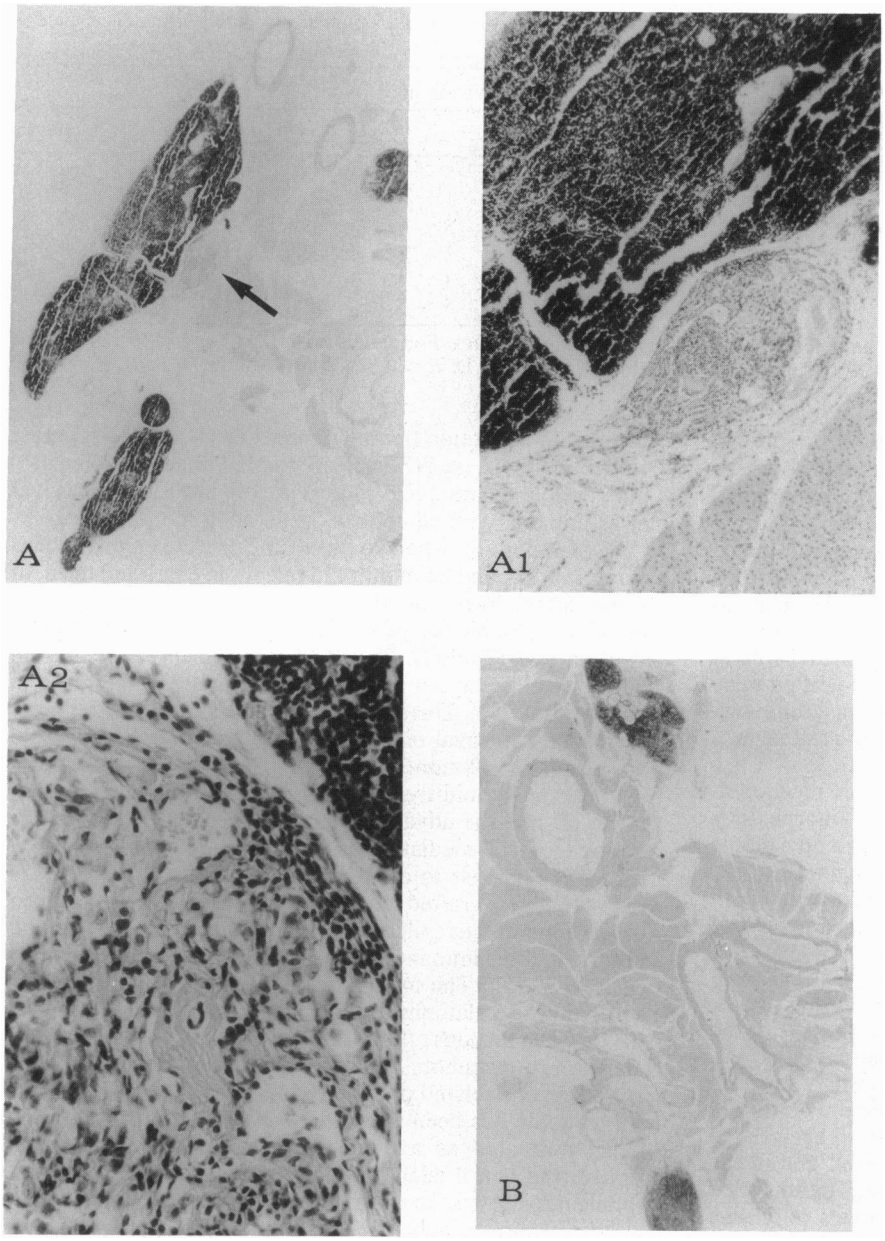


FIG. 5. Viable pineal gland in the thymus of a 21-month-old BALB/c female mouse at 3 months after grafting. (*A*) The arrow indicates the site of implantation, close to a thymic lobe, whose cellularity and structure are largely maintained. (*A1*) Notice the remarkable maintenance of a typical thymic cortex, with densely packed thymocytes. (*A2*) Clusters of viable pinealocytes can be seen in the grafted pineal. (*B*) Residual thymic rudiment of a 21-month-old BALB/c female mouse. Notice the presence of two atrophic small lymph nodes. (Hematoxylin/eosin; *A* and *B*, ×20; *A1*, ×70; *A2*, ×260.)

Whatever the mechanism, maintenance of immune responsiveness follows youthful pineal engraftment, and thymic and thyroid morphologic restoration occurs at a time when the normal involution of age is demonstrable. Our exogenous use of circadian melatonin and pineal engraftment of young pineals to the site of the thymus in aged mice suggests that there may be a firm relationship between the pineal, its products, and the thymus, providing a homeo-

Table 2. Dark cycle treatment with melatonin or transplantation of pineal glands from young donors maintains DTH response and retards aging in old mice

Group	Treatment	Strain	Sex	No.	Age, months	Melatonin, months of treatment	DTH response to oxazolone[a]		Survival, days
							Before challenge	After challenge	
A	Untreated	BALB/cJ	♀	9	26	—	28.25 ± 4.57	31.62 ± 7.11 (+12%)[b]	716 ± 101
B	Melatonin	BALB/cJ	♀	15	26	10	25.69 ± 1.96	31.06 ± 4.22 (+21%)[c]	843 ± 39 (+18%)[j]
C	Pineal[d]	BALB/cJ	♀	6	26	—	26.00 ± 3.60	33.67 ± 3.06 (+30%)[e]	902 ± 35 (+26%)[i]
D	Untreated	C57BL/6	♀	22	25	—	24.38 ± 2.14	29.62 ± 3.11 (+22%)[f]	773 ± 121
E	Melatonin	C57BL/6	♀	22	25	7	23.26 ± 1.06	29.93 ± 3.76 (+29%)[g]	826 ± 110 (+7%)[k]
F	Untreated	C57BL/6	♂	8	23	—	32.44 ± 4.52	34.72 ± 3.21 (+7%)[b]	743 ± 84
G	Melatonin	C57BL/6	♂	10	23	7	27.75 ± 0.99	33.33 ± 4.00 (+20%)[h]	871 ± 118 (+17%)[l]

Analysis of variance: after challenge compared with before challenge (see *Materials and Methods* and *Results*). [a]Data are presented as mean ± SD. [b]Not significant (NS). [c]$P < 0.005$. [d]Five months after implantation into the thymus of a pineal from a young, 3-month-old donor. [e]$P < 0.048$. [f]$P < 0.001$. [g]$P < 0.0001$. [h]$P < 0.005$. Statistics of survival (days, Mann–Whitney U test): [i]$P < 0.05$, C vs. A; [j]$P < 0.001$, B vs. A; [k]NS, E vs. D; [l]$P < 0.001$, G vs. F.

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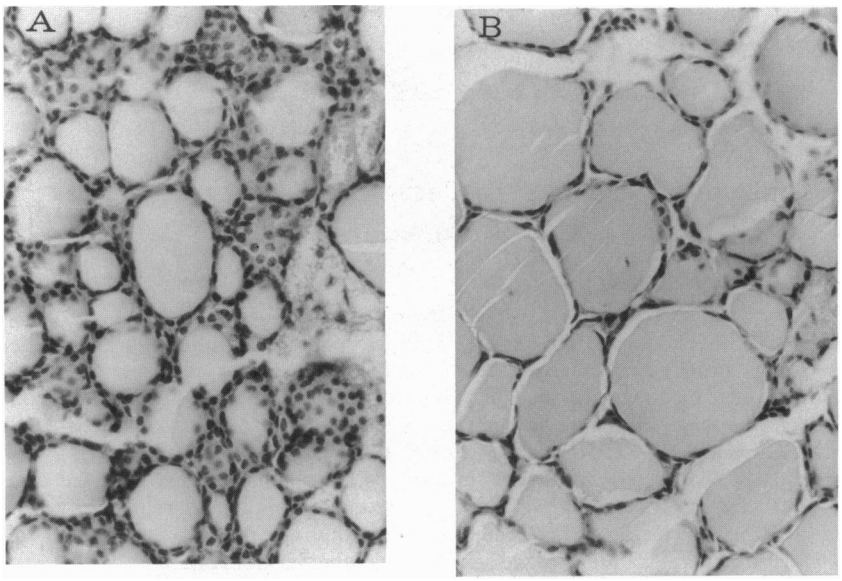


FIG. 6. Maintenance of juvenile structure and function in the thyroid gland of aging BALB/c mice grafted into the thymus with a pineal gland from young donors. (*A*) Pineal-grafted: Notice normal cellular and follicle size and structure. (*B*) Control: Notice flattened epithelium and distended follicles as a manifestation of hypofunction. (Hematoxylin/eosin; ×130.)

static control mechanism of significance for aging and survival.

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