The Pineal Aging and Death Program

Life Prolongation in Pre-aging Pinealectomized Mice

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ABSTRACT: A precise temporal program for growth, fertility, aging, and death exists in the "pineal complex" of the brain. It tracks, like a "clock," the ontogenetic phases of our life program. Transplantation of a very old pineal gland into the thymus or under the kidney capsule of a young mouse produces acceleration of aging and early death. We investigated the existence of such an inner biological clock on the assumption that a time exists in the pineal program when the pineal gland actively starts to deliver aging and death "signals" to the body, thus accomplishing its genetically inscribed sequence. Groups of BALB/c male or female mice were surgically pinealectomized (PX) at the age of 3, 5, 7, 9, 14, and 18 months, and their life span was evaluated. Periodical measurements of blood and hormonal and metabolic parameters were taken. Results showed that while PX at the age of 3 and 5 months promotes acceleration of aging, no relevant effect of PX is observed in mice PX at 7 or 9 months of age. On the contrary, a remarkable life prolongation was observed when mice were PX at the age of 14 months. No effects were seen when the mice were PX at 18 months of age. The same aging-promoting or -delaying effects were confirmed in the hematological and hormonal-metabolic values measured. The findings demonstrate the existence of an evolutionary–developmental role for the pineal complex during growth, fertility, and aging. The dominant role of the pineal in the initiation and progression of aging as a death signal is clear, but its nature and mechanism are totally unknown. In fact new experiments showed that an additional pineal gland from a young donor, when grafted into a young mouse, induces acceleration of aging. The significance of these intriguing findings is discussed.

KEYWORDS: pinealectomy; aging; aging clock; aging program; death program; hormone cyclicity; pineal grafting; zinc; thyroxin

INTRODUCTION

We have repeatedly demonstrated the existence of a precise life, aging, and death "clock" in the pineal network of the brain, in those neural structures controlling and modulating our genetically inherited life "program."[1–4] In a previous work we observed the remarkable life-shortening effect of grafting very old pineals into young

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hosts.[5] Later we conducted experiments in which we tried to determine, again in rodents, the time when the pineal gland starts to deliver the so-called aging and death signals to the body. As hinted in the previous work,[5] if the aging pineal gland actively promotes aging at the end of the adult and fertile life, it must be possible to establish the time when removal of the aging pineal would interfere with the aging process and somehow prolong life.

Our experiments were divided into two groups. One group of experiments were carried out over the course of three years and used groups of BALB/c female or male inbred mice, pinealectomized (PX) or sham-operated (SO) surgically by using a stereotaxis equipment. The mice were 3, 5, 7, 9, 14, and 18 months of age at the time of the surgery. They were kept under observation as long as they lived. Peripheral blood was taken periodically to evaluate hormonal, hematologic, and metabolic changes. A second set of experiments involved similar groups of 4-month-old young mice of the same strain. Pineal gland from young donors of the same age were implanted under the kidney capsule. This unusual experiment was suggested by the idea that an additional exogenous young pineal may influence the function (and aging) of the endogenous young pineal.

MATERIALS AND METHODS

Animals

Balb/c, inbred, genetically H-2–compatible male and female mice, bred and maintained in air-conditioned rooms under conventional conditions in our laboratory, were used as donors and recipients. Donors and recipients of a pineal gland were of the age indicated in the experiments. The mice were housed in plastic cages (4–6 mice per cage) and fed with standard maintenance pellets (NAFAG, Gossau, Switzerland) and tap water *ad libitum*. Room temperature was 20°C. Illumination was 7 P.M. lights off and 7 A.M. lights on.

Pineal Grafting

The pineal glands were removed from 4-month-old donors sacrificed by rapid cervical dislocation, and implanted into the thymus of the 4-month-old recipients. The mice were implanted (PG) or sham-operated (SO) with the procedure described in detail in previous reports.[3] Briefly, the mice were anesthetized and surgery was performed between 9 and 11 A.M. The animals were housed in groups of five and controlled daily in the first week, and then weekly for mortality rates and body weight.

Pinealectomy

Pinealectomy was performed in a manner similar to the method described in detail previously.[4] Briefly, anesthetized (Hexenal, 200 mg/kg) mice were fastened in a stereotaxic apparatus.[4] After shaving, the head skin was cut sagittally along the midline, and the aponeurosis and other soft tissues were removed to make visible the fissures of the calvarium. A triangle-shaped skull fragment located at the intersection of the sagittal and occipital fissures was cut with the help of a dental drilling ma-

chine. The next steps of the operation were carried out with of a dissection stereomicroscope. After turning the animal's head around its midline horizontal axis, we lifted the skull fragment, including its adherent pineal. The pineal ligaments connected with dura mater were cut with fine scissors, and the pineal gland was removed. Bleeding from the brain vessels was stopped by repeated rinsing with saline and drying with tissue pads. The skull fragment without the pineal gland was then cemented with polymer. After drying, the scalp skin was sutured with silk stitches, and the sealed operation field was sprayed with antibiotic powder. Sham-operated mice underwent the same procedure except that the pineal was exposed but not removed.

Plasma Zinc Determination

Zinc was determined by atomic absorption spectrophotometry (AAS) from individual plasma, previously frozen at −70°C, according to the method of Fernandez and Kahn[6] and the reference standard procedure suggested by Evenson and Warren.[7]

Blood Cell Counts

All mice were bled from the retroorbital venous plexus under rapid ether anesthesia. White blood cell (WBC) numbers were evaluated by direct counting in a Bürker chamber. In addition, blood smears were stained with May-Grünwald-Giemsa for differential counting of white cells. Absolute leukocyte and lymphocyte number and relative lymphocyte number were measured.

Lipid Measurement

The assays were performed with 10-μL plasma samples obtained from peripheral blood of individual mice by using enzymatic methods and a Johnson & Johnson Vitros 250 Dry-Chemistry Analyzer.

Thyroid Hormone Measurement

Thyroid hormones (total T3, total T4) were measured in plasma samples from individual mice. T3 and T4 values were determined by the MEIA-method (microparticle-enzyme-immunoassay).

Statistical Analysis

Results are expressed as mean ± SD. Statistical significance was determined by using analysis of variance (ANOVA). When significant differences were found, statistical analysis was made by paired Student's *t*-test. Log rank test was used to evaluate the difference of survival rates between pineal grafted or pinealectomized mice and sham-operated mice. Differences were considered statistically significant when $P < 0.05$.

TABLE 1. Effect of surgical pinealectomy in 3- to 5-month-old Balb/cJ male mice on plasma zinc level, peripheral blood leukocytes, lymphocytes, thyroid hormones (T3 and T4), and plasma triglycerides

Age of mice (mo)	11		15		19	
Mo after pinealectomy:	7		11		15	
Parameter measured	Pinealectomized ($N = 14$)	Sham operated ($N = 14$)	Pinealectomized ($N = 10$)	Sham operated ($N = 13$)	Pinealectomized ($N = 6$)	Sham operated ($N = 6$)
Blood leukocytes ($No./mm^3 \times 10^3$)	9.25 ± 15.4	99.4 ± 18.6	76.2 ± 20.2	96.1 ± 28.7	98.8 ± 22.8	81.4 ± 9.5
% Lymphocytes	56.8 ± 8.5***	69.9 ± 8.4	55.3 ± 3.6***	68.5 ± 8.8	44.5 ± 3.4*	55.2 ± 8.6
Blood lymphocytes ($No./mm^3 \times 10^3$)	51.3 ± 10.7**	84.0 ± 15.5	42.9 ± 13.4*	67.5 ± 25.5	42.4 ± 16.3	42.2 ± 11.5
Zn plasma level (μg/dL)	67.2 ± 4.7*	72.1 ± 7.5	68.2 ± 14.9	69.3 ± 17.6	ND	ND
T3 (mmol/L)	ND	ND	0.51 ± 0.12	0.45 ± 0.09	0.95 ± 0.55	0.92 ± 0.46
T4 (nmol/L)	ND	ND	50.4 ± 13.6	54.9 ± 15.3	27.7 ± 14.2*	58.5 ± 15.7
Triglycerides (mmol/L)	ND	ND	2.94 ± 0.57*	2.38 ± 0.53	ND	ND

NOTE: Mean ± SD. *$P < 0.05$ when compared to sham operated; **$P < 0.01$ when compared to sham operated; ***$P < 0.001$ when compared to sham operated (Student's *t*-test). Balb/cJ male mice were pinealectomized at 3–5 months of age.

RESULTS

Pinealectomy in 3- and 5-Month-Old Mice

The results illustrated in FIGURE 1 and on TABLE 1 show that PX performed in 3- and 5-month-old mice significantly accelerated aging and consequently shortened their life span. The aging-accelerating effects of PX were also clearly expressed by the drop of lymphocyte counts in the peripheral blood, by the consistent decrease of zinc levels, by the negative effects on thyroxin (T4) production, and by the increased level of triglycerides (TABLE 1).

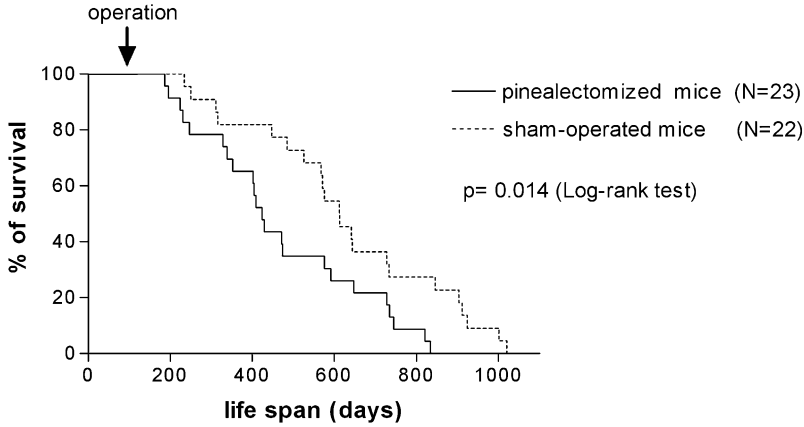

FIGURE 1. Pinealectomy in 3–5-month-old Balb/cJ male mice induces an earlier onset of aging. Survival curves (Kaplan-Meier) are different; $P < 0.05$ by log-rank test.

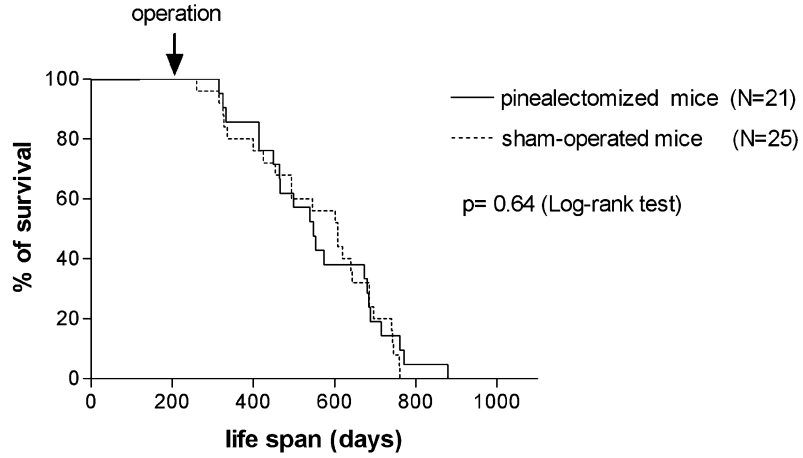

FIGURE 2. Pinealectomy in 7–9-month-old Balb/cJ male mice does not affect their life span. Survival curves (Kaplan-Meier) are not different; $P > 0.05$ by log-rank test.

TABLE 2. Effect of surgical pinealectomy in 7-month-old Balb/cJ male mice on plasma zinc level, peripheral blood leukocytes, lymphocytes, thyroid hormones (T3 and T4), and plasma triglycerides

Age of mice (mo):	14		18		22	
Mo after pinealectomy:	7		11		15	
Parameter measured	Pinealectomized ($N = 8$)	Sham operated ($N = 7$)	Pinealectomized ($N = 6$)	Sham operated ($N = 6$)	Pinealectomized ($N = 4$)	Sham operated ($N = 4$)
Blood leukocytes ($No./mm^3 \times 10^3$)	76.0 ± 14.9	91.3 ± 10.8	84.5 ± 29.8	99.3 ± 27.7	98.5 ± 25.0	79.5 ± 27.2
% Lymphocytes	52.5 ± 5.2*	59.3 ± 6.5	62.7 ± 9.6	56.9 ± 5.0	40.5 ± 10.6	38.7 ± 8.7
Blood lymphocytes ($No./mm^3 \times 10^3$)	39.7 ± 7.2**	54.3 ± 9.7	46.0 ± 10.3	54.0 ± 13.0	37.8 ± 5.9	30.8 ± 12.9
Zn plasma level (µg/dL)	59.0 ± 9.6*	73.6 ± 7.9	56.8 ± 13.4	49.7 ± 6.4	ND	ND
T3 (mmol/L)	ND	ND	0.59 ± 0.34	0.67 ± 0.17	ND	ND
T4 (nmol/L)	ND	ND	43.7 ± 13.7	35.3 ± 6.3	ND	ND
Triglycerides (mmol/L)	ND	ND	3.07 ± 0.58	3.72 ± 0.69	ND	ND

NOTE: Mean ± SD. *$P < 0.05$ when compared to sham operated; **$P < 0.01$ when compared to sham operated (Student's t-test). Balb/cJ male mice were pinealectomized at 7 months of age.

TABLE 3. Effect of surgical pinealectomy in 9-month-old Balb/cJ male mice on plasma zinc level, peripheral blood leukocytes, lymphocytes, thyroid hormones (T3 and T4), and plasma triglycerides

Age of mice (mo):	13		16	
Mo after pinealectomy:	4		7	
Parameter measured	Pinealectomized ($N = 7$)	Sham operated ($N = 7$)	Pinealectomized ($N = 6$)	Sham operated ($N = 6$)
Blood leukocytes (No./mm^3×10^3)	100.0 ± 20.5	95.1 ± 28.6	105.5 ± 20.4	97.2 ± 20.7
% Lymphocytes	59.0 ± 7.8	64.9 ± 9.7	54.8 ± 8.0	63.5 ± 13.4
Blood lymphocytes (No./mm^3×10^3)	59.0 ± 14.0	60.6 ± 19.5	58.2 ± 15.3	62.8 ± 20.0
Zn plasma level (µg/dL)	62.3 ± 4.6*	71.8 ± 4.5	ND	ND
T3 (mmol/L)	ND	ND	0.97 ± 0.45	0.97 ± 0.21
T4 (nmol/L)	ND	ND	50.2 ± 14.2	53.6 ± 18.6
Triglycerides (mmol/L)	ND	ND	2.74 ± 0.33	2.59 ± 0.44

NOTE: Mean ± SD. *$P < 0.01$ when compared to sham operated; **$P < 0.01$ when compared to sham operated (Student's *t*-test). Balb/cJ male mice were pinealectomized at 9 months of age.

Pinealectomy in 7- and 9-Month-Old Mice

PX in 7- and 9-month-old mice did not significantly affect their longevity and life span (FIG. 2). As shown on TABLES 2 and 3, no relevant and durable effects were also observed in their peripheral blood.

Pinealectomy in 14-Month-Old Mice

PX in 14-month-old mice produced a considerable delay of aging and/or a life prolongation (FIG. 3). Also PX at that age resulted in highly significant and positive effects on peripheral blood parameters, such as lymphocyte counts, plasma zinc level, and thyroxin (T4) production (TABLE 4). In contrast and unexpectedly, four months after PX an increased level of triglycerides was observed, but they consistently decreased at 8 months after PX (TABLE 4). The positive effects of PX were particularly evident at 4 months after PX (TABLE 4).

Pinealectomy in 18-Month-Old Aging Mice

In spite of some transitory early and late positive effects on the peripheral blood values measured (lymphocytes and zinc in TABLE 5), and on survival rate (FIG. 4), no significant prolongation of their life span was observed in PX 18-month-old mice.

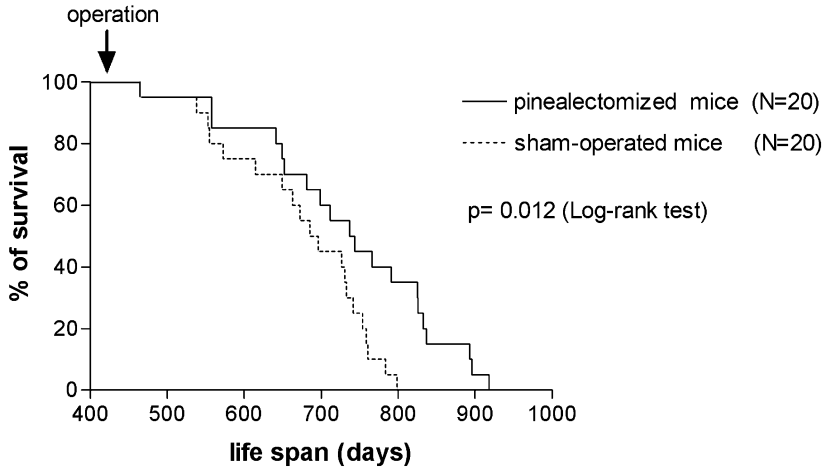

FIGURE 3. Pinealectomy in 14-month-old Balb/cJ male mice induces a delay of their aging and a consequent prolongation of their life. Survival curves (Kaplan-Meier) are different; $P < 0.05$ by log-rank test.

TABLE 4. Effect of surgical pinealectomy in 14-month-old Balb/cJ female mice on plasma zinc level, peripheral blood leukocytes, lymphocytes, thyroid hormones (T3 and T4), and plasma triglycerides

Age of mice (mo):	18		22	
Mo after pinealectomy:	4		8	
Parameter measured	Pinealectomized ($N = 9$)	Sham operated ($N = 10$)	Pinealectomized ($N = 6$)	Sham operated ($N = 6$)
Blood leukocytes (No./mm^3×10^3)	48.5 ± 7.0	47.1 ± 7.7	61.5 ± 14.4	44.8 ± 15.2
% Lymphocytes	67.9 ± 4.7***	55.3 ± 6.8	55.6 ± 4.2	53.0 ± 6.7
Blood lymphocytes (No./mm^3×10^3)	33.1 ± 5.9*	26.0 ± 4.7	34.1 ± 8.4	25.2 ± 9.8
Zn plasma level (μg/dL)	78.7 ± 17.6*	57.8 ± 11.0	ND	ND
T3 (mmol/L)	0.79 ± 0.26	0.60 ± 0.14	1.20 ± 0.41	0.94 ± 0.57
T4 (nmol/L)	71.3 ± 19.0**	48.1 ± 10.6	66.0 ± 18.2	56.6 ± 9.9
Triglycerides (mmol/L)	3.47 ± 0.50**	2.42 ± 0.41	1.95 ± 0.30	2.26 ± 0.30

NOTE: Mean ± SD. *$P < 0.05$ when compared to sham operated; **$P < 0.01$ when compared to sham operated; ***$P < 0.001$ when compared to sham operated (Student's t-test). Balb/cJ female mice were pinealectomized at 14 months of age.

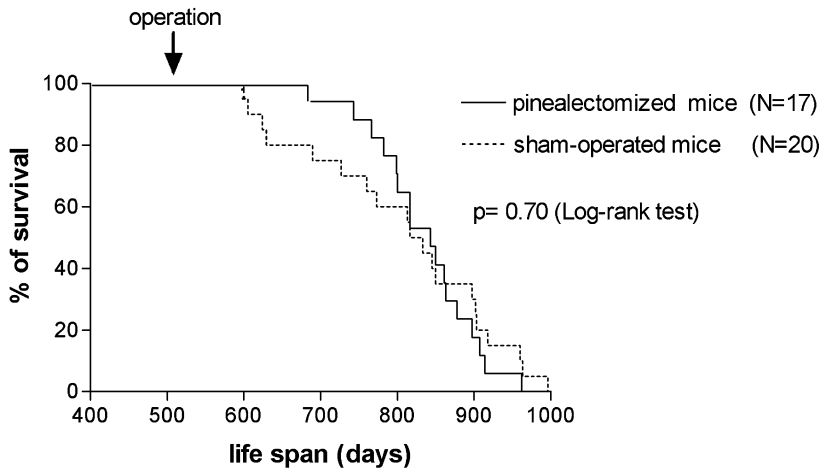

FIGURE 4. Pinealectomy in 18-month-old Balb/cJ male mice does not affect their life span. Survival curves (Kaplan-Meier) are not different; $P > 0.05$ by log-rank test.

TABLE 5. Effect of surgical pinealectomy in old mice on plasma zinc level, peripheral blood leukocytes, and lymphocytes

Age of mice (mo):	22		25	
Mo after pinealectomy:	4		7	
Parameter measured	Pinealectomized ($N = 8$)	Sham operated ($N = 9$)	Pinealectomized ($N = 8$)	Sham operated ($N = 9$)
Blood leukocytes (No./mm^3×10^3)	103.1 ± 11.5	94.1 ± 25.5	95.0 ± 15.0	92.4 ± 16.2
% Lymphocytes	62.5 ± 5.1**	41.9 ± 1.7	57.1 ± 2.9**	46.9 ± 4.8
Blood lymphocytes (No./mm^3×10^3)	62.2 ± 6.8**	39.4 ± 10.7	54.4 ± 9.8*	43.4 ± 9.3
Zn plasma level (μg/dL)	86.1 ± 14.9**	64.6 ± 9.1	76.0 ± 10.0	65.5 ± 10.0

NOTE: Mean ± SD. *$P < 0.05$ when compared to sham operated; **$P < 0.01$ when compared to sham operated (Student's t-test). Balb/cJ male mice were pinealectomized at 18 months of age.

TABLE 6. Effect of young pineal grafting into young recipients on plasma zinc level, peripheral blood leukocytes, lymphocytes, thyroid hormones (T3 and T4), and plasma triglycerides

Age of mice (mo):	8		12	
Mo after pineal grafting:	4		8	
Parameter measured	Young+young pineal ($N = 6$)	Young sham operated ($N = 6$)	Young+young pineal ($N = 7$)	Young sham operated ($N = 7$)
Blood leukocytes (No./mm^3×10^3)	56.1 ± 5.8	59.0 ± 10.1	57.3 ± 8.0	57.6 ± 13.8
% Lymphocytes	66.0 ± 13.2	80.5 ± 5.2*	66.4 ± 8.4	70.2 ± 5.9
Blood lymphocytes (No./mm^3×10^3)	36.5 ± 4.5	47.2 ± 6.3*	37.3 ± 8.2	40.6 ± 6.7
Zn plasma level (μg/dL)	54.7 ± 3.3*	67.7 ± 9.4	ND	ND
T3 (mmol/L)	0.71 ± 0.13	0.66 ± 0.10	0.73 ± 0.25	0.60 ± 0.14
T4 (nmol/L)	82.8 ± 12.6**	60.7 ± 5.2	72.0 ± 8.3*	60.8 ± 10.2
Triglycerides (mmol/L)	3.28 ± 0.51*	2.59 ± 0.15	2.65 ± 0.50	2.16 ± 0.43

NOTE: Mean ± SD. *$P < 0.05$ when compared to sham operated; **$P < 0.01$ when compared to sham operated (Student's *t*-test). A pineal gland from 4-month-old donors was implanted into the thymus of 4-month-old Balb/cJ recipients. Donor and recipient mice were female.

Pineal Grafting from Young Donors into Young Recipients

As shown in FIGURE 5, transplantation of a "young" pineal gland into the thymus or under the kidney capsule of "young" recipients of the same age resulted into a significant early onset of aging in the grafted mice and into a shortening of their life span. The aging-accelerating effects of young-pineal grafting into young recipients was confirmed by the measurement of several parameters in their peripheral blood. At 4 months after pineal grafting, lymphocyte counts and plasma zinc were remarkably reduced, triglycerides were significantly increased, while T4 and T3 were increased, hinting to a durable hyperthyroidism condition (TABLE 6). The effects were still visible but less evident at 8 months after pineal grafting (TABLE 6).

DISCUSSION

The findings shown above have demonstrated once more the existence of an "aging program" in the pineal gland and that a very precise time exists when the aging pineal actively promotes aging. In the mouse strain used, we identified this age to be 14 months. Pinealectomy at this age significantly prolonged the life span of the mice (FIG. 3). Evidence from the blood measurements showed that removal of the pineal in mice at the age of 14 months resulted in maintenance of more juvenile hormonal and metabolic values at 4 and 8 months after PX (TABLE 4). On the contrary, a dele-

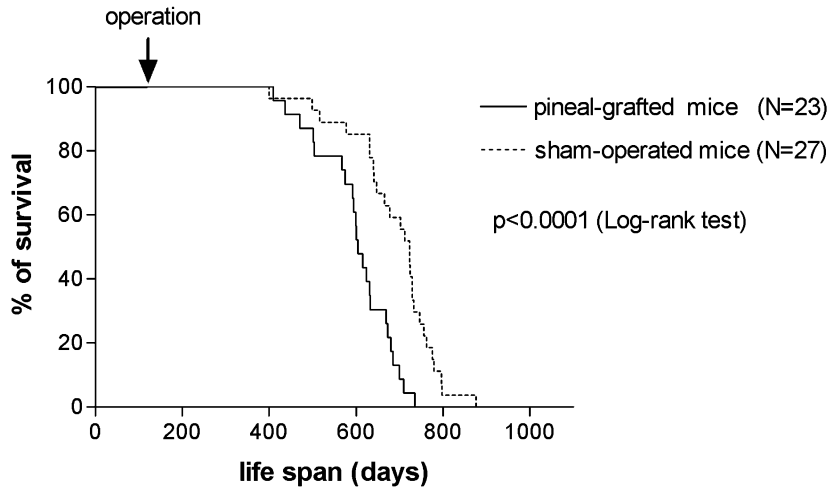

FIGURE 5. Transplantation of a young pineal gland (pineal gland from 4-month-old Balb/cJ female mice) into normal young, 4-month-old Balb/cJ female mice induces an earlier onset of aging. Survival curves (Kaplan-Meier) are different; $P < 0.0001$ by log-rank test.

terious effect of pinealectomy was seen in mice PX at the age of 3 and 5 months, with acceleration of aging parameters and an earlier death (FIG. 1). This observation confirms the developmental role of the pineal gland in early life and that its "program" includes several steps ranging from growth, to fertility, and on to aging and death.[1–5] Our experiments also demonstrate that PX at an age greater than 14 months does not affect the life span of the mice. Apparently this is the time when the pineal gland has accomplished its "aging program" and prevention of and/or recovery from aging becomes impossible (FIG. 4). The experiments with young mice grafted with a pineal from young mice of the same age were most intriguing. Young pineal–grafted young mice showed a shortened life span, as if the grafted young pineal gland promoted aging in the young mouse (FIG. 5). We attribute this amazing observation to accelerated aging of the grafted pineal gland, obviously deprived of its neural connections. However different interpretations are possible for this phenomenon.

Altogether these data clearly confirm once more the undeniable existence of a "clock" for aging and also of a signal for dying in the pineal gland; and they confirm that the "program" can be studied, interpreted, and possibly modified. The cellular and molecular mechanisms underlying this most fundamental aspect of our life program deserves intensive investigations in different species of mammals, man included.

[*Competing interests*: The author declares that he has no competing financial interests.]

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