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Thyrotropin-Releasing Hormone Accelerates and Enhances the Age-Postponing Effects of Melatonin

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ABSTRACT

Studies over a period of several years have suggested an age-postponing effect of circadian nocturnal administration of melatonin and of young-to-old pineal grafting in rodents. Of the two procedures, the effect of pineal grafting was significantly more pronounced. Also, old-to-young and young-to-old pineal transplantation in normal or pinealectomized recipients suggested that the pineal itself contains the capacity to prevent or to accelerate the course of aging depending on the age of the donor and/or of a recipient when the pineal is transplanted. This observation prompted the idea that the "program of aging" might be governed by the capacity of the pineal to maintain the control of central neuroendocrine functions and to constantly synchronize the synthesis and release of hormones according to a strict circadian periodicity and seasonal rhythmicity. This report deals with the experimental evidence that, while melatonin alone exerts a low-level age-postponing activity, its age-delaying effects are greatly enhanced and accelerated when given in combination with a pineal peptide, thyrotropin-releasing hormone (TRH). This peptide may be a key element in the mechanism by which both melatonin and pineal grafting might postpone aging. In fact, as suggested by our data here, TRH could be one of the basic mediators in the brain (pineal-hypothalamic-hypophyseal axis) and in peripheral endocrine glands (e.g., the β, insulin-producing cells in the pancreas). TRH may directly translate the light and temperature-mediated environmental stimuli into rapid energy-adapting biochemical processes which constantly monitor cell functions relating to energy production, in particular those required for thermoregulation. We show here that this energy-monitoring action of TRH is not thyroid mediated. We also show that TRH is not itself a toxic agent even when administered daily for long periods at a very high pharmacological dosage.

INTRODUCTION

WE HAVE PREVIOUSLY REPORTED that the pineal and hypothalamic tripeptide thyrotropin-releasing hormone (TRH) possesses remarkable immunoreconstituting and antiviral activity and may be the main vehicle and key agent for the potential anti-aging effects of chronic, nocturnal administration of melatonin.[1] Subsequent studies showed that TRH can forestall thymic involution and maintain peripheral blood lymphocyte counts in anterior

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hypothalamic area (AHA)–lesioned mice.[2] On the basis of these findings, we postulated that the ubiquitous tripeptide TRH—as a result of its cellular and anatomical localization in the brain, its varied effects on hormone release, and its role in the activation of tyrosine kinase activity—could be a key effector molecule for potential anti-aging effects of melatonin and of young-to-old pineal grafting.[3,4] In other words, we suggested that TRH may be the true amplifier and synchronizer of hormone synthesis and release, generating a precise circadian and seasonal cyclicity, in relation to thermoregulation and other adaptive processes (e.g., reproduction, immunity).[3–5]

In order to evaluate whether or not TRH is functionally linked *in vivo* to the age-delaying properties of circadian melatonin, as suggested by our previous data,[1,2] we devised an experiment in which groups of old mice were treated chronically with melatonin and/or TRH singly or in combination. The purpose of the experiment was to study whether or not the two agents may act synergistically to improve age-postponing interventions, thus prolonging longevity.

MATERIALS AND METHODS

Animals

Inbred female young (3–4-month-old) and senescent (20-month-old) BALB/c mice bred and maintained under conventional conditions at our laboratory were used. Room temperature was 20–22°C, illumination was 7 a.m. light-on, 7 p.m. light-off. The animals were kept in groups of five per cage and checked twice daily. Also, 9-month-old, male, inbred C57BL/ 6 mice were used for initial testing of TRH effects.

Substances

Melatonin was a gift from Helsinn Chemicals (Biasca, Switzerland). Chemical purity was over 99.9. Synthetic TRH-tartrate was a gift from Senn Chemicals (Dielsdorf, Switzerland).

Solutions of active agents and their oral administration

Fresh water solutions of TRH were prepared twice weekly and given in the drinking water at a concentration of 100 μg/ml. Melatonin was dissolved as described previously.[4,5] It was administered at the final dose of 10 μg/ml in the drinking water and changed twice weekly. Some groups of mice were given TRH and melatonin simultaneously. The water bottles were painted black to avoid direct illumination of the solutions, and the darkened bottles were removed from all cages at 8 a.m., kept in a refrigerator at 5°C, and put back at 6 p.m., including the bottles containing only water to which the same amount of ethylalcohol used to dissolve melatonin was added.

Blood samples

All blood draws for blood counts, lipid and thyroid hormone measurement were performed strictly between 8 and 10 a.m.

Leukocyte and lymphocyte counts

All mice were bled from the retroorbital venous plexus under rapid ether anaesthesia. Blood smears were prepared. Absolute leukocyte and lymphocyte number and relative lymphocyte number were measured.

Lipid measurements

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 measurements

Thyroid hormones—T3, T4, and thyroid-stimulating hormone (TSH)—were measured in plasma samples from individual mice. T3 and T4 values were determined by microparticle enzyme immunoassay (MEIA). Thyroid-stimulating hormone (TSH) was measured by using an Ultrasensitive II Kit based on the MEIA technique, with an Abbott Axsym-Analyzer.

RESULTS

Effect of increasing doses of TRH on thyroid hormone levels

In order to determine if chronic injection of different, increasing doses of TRH produces a

TABLE 1. EFFECT OF 20 DAYS' TREATMENT WITH TRH ON BLOOD LEVELS OF T3, T4, AND TSH IN MICE

Groups (G)	*Treatment*	n	*T3 (nmol/L)*	*T4 (nmol/L)*	*TSH (μU/mL)*
1	Controls (diluent)	4	0.76 ± 0.06	47.2 ± 8.1	0.011 ± 0.005
2	TRH morning, 100 μg	4	0.84 ± 0.02	74.9 ± 13.3	0.007 ± 0.005
3	TRH evening, 100 μg	3	0.91 ± 0.02	68.7 ± 13.1	0.004 ± 0.005
4	TRH morning, 10 μg	4	1.03 ± 0.07	82.0 ± 1.9	0.009 ± 0.006
5	TRH evening, 10 μg	4	0.82 ± 0.08	65.7 ± 4.2	0.005 ± 0.004

Mean ± SD for T3: G2 versus G4 = $p < 0.01$; G3 versus G5 = NS. T4: G2 versus G4 = NS; G3 versus G5 = NS. TSH: G2 versus G4 = NS; G3 versus G5 = NS (Student's *t* test).

C57BL/6 male mice aged 9 months were injected intraperitoneally for 20 days at 6 p.m. (evening) or at 8 a.m., (morning) with 0.5 ml volume containing 10 μg or 100 μg of TRH in bidistilled water. Controls were injected with the same amount of diluent. The mice were bled from the retroorbital venus plexus under rapid acute ether anaesthesia at 12 a.m. T3, T4, and TSH were measured by radioimmunoassay in the serum of individual mice.

progressive increase of thyroid hormone levels, depending on the dose of TRH used, five groups of four 9-month-old C57BL/6 male mice were injected daily intraperitoneally for 20 days at 8 a.m. or at 6 p.m. with 10 or 100 μg TRH. After 20 days, the mice were tested individually and thyroid hormones were measured in their plasma. The results shown in Table 1 demonstrate that increasing dosages of TRH beyond 10 μg/day do not significantly enhance synthesis or release of thyroid hormones, and no additive thyroid stimulating effects are produced by very high doses of TRH. On the basis of this observation, we decided to add 100 μg/ml TRH to the drinking water, assuming that mice drink about 5 ml water/day and that the dose of TRH by oral route must be 20–50 times higher than the parenteral route in order to achieve similar pharmacological effects.[6]

Effects of TRH, melatonin, or TRH plus melatonin

Survival rate and longevity. The results in which groups of 20-month-old, presenescent BALB/c female mice were given chronically in the drinking water melatonin TRH, or melatonin plus TRH combined are shown in Table 2. It can be seen that, while TRH or melatonin, as predicted from our previous findings,[1–5] each significantly increased survival and prolonged longevity, the combination of both of them together remarkably improves the survival rate. The longevity of the senescent mice was prolonged far beyond that observed with each agent alone. This was also reflected in their motile activity, fur conditions, and body weight, as gleaned from our daily observations. Therefore, melatonin and TRH act synergistically and induce a significantly amplified effect.

Peripheral blood counts. It can be seen in Tables 3 and 4 that, at 2 months, but especially at 4 months after initiation of the treatment, melatonin affects postively the number of peripheral blood lymphocytes. TRH has an even greater effect. However, a greater effect is achieved by their administration in combination. Prolongation of treatment for an additional 2 months results in an additional increase of blood lymphocytes, maintaining a remarkable and statistically highly significant juvenile value with both melatonin and TRH combined

TABLE 2. EFFECT OF TREATMENT WITH MELATONIN, TRH, AND MELATONIN + TRH ON LIFE SPAN IN OLD MICE

Groups	*Treatment*	*No. of mice*	*Survival (days)*
A	Old control	15	764 ± 54
B	Old + melatonin	14	810 ± 50
C	Old + TRH	15	804 ± 80
D	Old + melatonin + TRH	16	861 ± 70

Mean ± SD for A versus B = $p < 0.05$; A versus C = NS; A versus D = $p < 0.05$; B versus D = $p < 0.05$; C versus D = $p < 0.05$ (Student's *t* test).

Treatment with melatonin, TRH, and melatonin + TRH was started in 20-month-old BALB/cJ female mice.

TABLE 3. EFFECT OF 2 MONTHS' TREATMENT WITH MELATONIN, TRH, AND MELATONIN + TRH ON BLOOD LEUCOCYTES AND LYMPHOCYTES IN OLD MICE

Parameters measured	*(Y) Young*[a] *(n = 8)*	*(A) Old (n = 10)*	*(B) Old + melatonin (n = 10)*	*(C) Old + TRH (n = 10)*	*(D) Old + melatonin + TRH (n = 10)*
Blood leucocytes (no./mm^3 × 10^3)	102.0 ± 7.2	112.7 ± 13.3	110.4 ± 10.0	113.8 ± 6.4	115.3 ± 11.8
Percentage lymphocytes (I)	84.1 ± 3.2	46.7 ± 5.4	59.8 ± 7.2	65.1 ± 8.8	66.8 ± 6.5
Blood lymphocytes (II) (no./mm^3 × 10^3)	85.7 ± 6.6	52.2 ± 5.7	65.9 ± 8.3	74.2 ± 11.9	76.5 ± 6.3

[a]3–4-month-old mice.

Mean ± SD for I: A versus B, C and D = $p < 0.01$; B versus C = NS; B versus D = $p < 0.05$; C versus D = NS; Y versus D = $p < 0.01$. II: A versus B, C and D = $p < 0.01$; B versus C = NS; B versus D = $p < 0.01$; C versus D = NS; Y versus D = $p < 0.01$ (Student's t test).

Treatment with melatonin, TRH, and melatonin + TRH was started in 20-month-old BALB/cJ female mice.

(Table 4). The synergistic effect of melatonin and TRH thus results in an increased number of peripheral blood lymphocytes, a number much more pronounced than that produced by melatonin or TRH alone, and closer to the values in young mice (Table 4).

Lipid levels. When cholesterol and triglycerides were measured in the peripheral blood of the mice at 2 months after the initiation of the treatment, it was found that the combination of melatonin and TRH completely reversed the typical aging-related increase of blood cholesterol and triglycerides of the old mice, and they returned to fully juvenile levels (Table 5).

DISCUSSION

The findings reported above indicate that the combination of melatonin and TRH administered chronically via the nocturnal drinking water in senescent mice prolongs their longevity beyond that observed when melatonin or TRH is given alone.[3,4] This is not surprising if we consider the remarkable and rapid metabolic and immunoenhancing activities reported for TRH in several studies.[1–3] The positive and rapid effects of acute administration of TRH previously observed in immunodeficient athymic nude, corticosteroid-treated, immunized, and virus-infected mice do not depend at all on an increased secretion of thyroid

TABLE 4. EFFECT OF 4 MONTHS' TREATMENT WITH MELATONIN, TRH, AND MELATONIN + TRH ON BLOOD LEUCOCYTES AND LYMPHOCYTES IN OLD MICE

Parameters measured	*(Y) Young*[a] *(n = 6)*	*(A) Old (n = 7)*	*(B) Old + melatonin (n = 5)*	*(C) Old + TRH (n = 5)*	*(D) Old + melatonin + TRH (n = 8)*
Blood leucocytes (no./mm^3 × 10^3)	121.8 ± 16.4	129.1 ± 8.8	128.2 ± 15.1	135.4 ± 12.7	138.0 ± 11.9
Percentage lymphocytes (I)	85.2 ± 4.3	44.4 ± 4.6	55.4 ± 6.3	52.4 ± 5.2	65.1 ± 4.0
Blood lymphocytes (II) (no./mm^3 × 10^3)	100.9 ± 9.4	57.2 ± 6.4	70.6 ± 9.0	70.8 ± 8.5	90.4 ± 9.8

[a]3–4-month-old mice.

Mean ± SD for I: A versus B, C and D = $p < 0.01$; B versus C = NS; B versus D = $p < 0.01$; C versus D = $p < 0.01$; Y versus D = $p < 0.01$. II: A versus B, C and D = $p < 0.01$; B versus C = NS; B versus D = $p < 0.01$; C versus D = $p < 0.01$; Y versus D = NS (Student's t test).

Treatment with melatonin, TRH, and melatonin + TRH was started in 20-month-old BALB/cJ female mice.

TABLE 5. EFFECT OF 2 MONTHS' TREATMENT WITH MELATONIN, TRH, AND MELATONIN + TRH ON BLOOD LEVELS OF CHOLESTEROL AND TRIGLYCERIDES IN OLD MICE

Parameters measured	*Young*[a] (n = 8)	*Old* (n = 10)	*Old + melatonin* (n = 10)	*Old + TRH* (n = 10)	*Old + melatonin + TRH* (n = 10)
Cholesterol (mmol/L)	1.47 ± 0.21***	1.81 ± 0.71	1.80 ± 0.32	1.65 ± 0.29	1.26 ± 0.13*
Triglycerides (mmol/L)	0.96 ± 0.18 NS	1.17 ± 0.29	1.15 ± 0.29	1.19 ± 0.46	0.84 ± 0.18**

[a]3–4-month-old mice.

Mean ± SD, *$p < 0.05$ when compared to old controls; **$p < 0.0001$ when compared to old controls; ***$p < 0.05$ when compared to old + melatonin + TRH; NS when compared to old + melatonin + TRH (Student's t test).

Treatment with melatonin, TRH, and melatonin + TRH was started in 20-month-old BALB/cJ female mice.

hormones and are thymus-mediated.[1] They also seem to follow a circadian variability, although this effect may be masked by the dose of TRH given.[1] Therefore, we postulated that TRH is in fact the key vehicle for the age-postponing effects of melatonin and the most relevant element of the pineal network.[1,2] Given the observation that the age-postponing effects of young-to-old pineal grafting by far exceed those of melatonin, and that the chronic administration of increasing doses of melatonin does not further prolong longevity (Pierpaoli et al., unpublished data), we suggest here that the mechanism of action of melatonin may be based simply on exogenous melatonin saturation in the pineal, which, through negative feedback inhibition (when given nocturnally), blocks melatonin synthesis and secretion in the pineal gland. In other words, we think that nocturnal melatonin administration puts the pineal "to sleep" and thereby helps to retain juvenile function and allows the maintenance of active pineal peptide production, including TRH especially. This apparently simple interpretation is supported by the fact that the striking age-delaying effects of young-to-old pineal grafting in mice and rats[3–5,7] cannot depend on a restoration of melatonin production in the pineal gland, because transplanted pineal glands are not reinnervated and do not reacquire β-adrenergic–mediated synthetic function. The observed phenomenon must therefore be due to some pineal product other than melatonin. This interpretation is also consistent with the observation that, while the implantation of a very old pineal into a young mouse accelerates aging,[8] the implantation of a young pineal gland into an intact nonpinealectomized animal will prolong its longevity.[4,5] This surprising observation means that it is the pineal itself and its secretory products such as TRH that cause any putative life-prolonging effects of young-to-old pineal grafting, and not melatonin itself. We therefore strongly support the claim that TRH is at least one of the key pineal peptides whose action can mimic and even surpass the effects of pineal grafting. We further suggest that the "melatonin hype" is simply the outcome of a "TRH high."[9]

We suggest that the simultaneous administration of melatonin and TRH will enhance any age-postponing effects because it protects pineal function, thus enhancing and maintaining the pineal's capacity to produce TRH. Additionally, the juvenile pineal will maintain the ability to produce endogenous levels of TRH when the administration of melatonin and/or TRH are eventually discontinued. However, the suggestion that all the positive effects observed in our melatonin and pineal-grafting models are simply due to maintenance of TRH secretion from the pineal gland needs experimental confirmation.

The hemopoietic and the metabolic effects of the combination of melatonin and TRH in aging mice are also evidence for a rapid metabolic effect of TRH, which restores peripheral lymphocyte and lipid levels, as reported in our previous work.[2] The key role of TRH (a ubiquitous peptide, highly concentrated in the pineal gland[10,11]) in oxidative and metabolic pathways may serve to explain how it may restore hemopoietic and immune functions as well as normalizing lipid metabolism. In spite of its

semantic, historical definition as a "hormone," TRH, even when given chronically by endogenous route at huge doses, has no clear deleterious effects.[12,13] Low oral doses of TRH appear to be reasonably safe for use on long-term studies of possible antiaging effects in humans. Our findings are also consistent with the TRH hypothesis of pineal function.[14]

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