

# Nighttime Changes in Norepinephrine and Melatonin Content And Serotonin Turnover in Pineal Glands of Young and Old Rats Injected with Freund's Adjuvant

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## Abstract

**OBJECTIVES:** This study was performed to search for changes in rat pineal function attributed to age and immunization with Freund's adjuvant.

**METHODS:** Young (2 months) and old (18-20 months) Wistar rats were injected s.c. with Freund's adjuvant or its vehicle. Eighteen days later, at the acute phase of arthritis, pineal concentration of serotonin (5-HT), 5-hydroxy-indoleacetic acid (5-HIAA), norepinephrine (NE) and melatonin was measured by high pressure liquid chromatography at 4 different time intervals throughout the nocturnal activity span.

**RESULTS:** Old rats had the lowest pineal 5-HT and 5-HIAA content, the decrease in 5-HIAA exceeding that of 5-HT; consequently, old rats had the lowest 5-HIAA/5-HT ratio, an index of pineal 5-HT turnover. Although immunization did not affect globally pineal 5-HT or 5-HIAA levels, significant interactions "immunization x age" and "immunization x time" were found, i.e., immunization augmented pineal 5-HT content at the beginning of the activity span in young rats and at second half of the activity span in young and old rats, and increased pineal 5-HIAA concentration in young rats at the second part of the activity span only. Freund's adjuvant treatment increased pineal 5-HT turnover exclusively in old rats, an effect mainly seen during the second part of the activity span. Old rats exhibited the lowest pineal NE and melatonin levels, immunization further depressing them.

**CONCLUSION:** The effect of immunization with Freund's adjuvant on a number of pineal pre- and postsynaptic parameters are age-dependent.

## Introduction

Adjuvant arthritis in rats is usually induced by injection of mycobacterium tubercle cell walls suspended in incomplete Freund's adjuvant [1]. A parallel clinical and behavioral study of adjuvant-induced arthritis in the rat showed four stages in the time-course of the disease: preclinical (first week), acute (weeks 2–4), post-acute (weeks 5–8) and recovery (weeks 9–11) [2]. Adjuvant arthritis is widely employed as an experimental paradigm to examine the relationships between the brain and the immune system. In this model it was shown that cytokines like interleukins 1, 2 or 6, granulocyte-macrophage colony-stimulating factor or interferon- $\alpha$  are responsible for many of the symptoms associated with inflammation ("sickness behavior") [3,4].

In a previous study we examined young (50 days old) and old (18 months old) Sprague-Dawley rats injected with mycobacterial Freund's adjuvant at preclinical and acute phases of adjuvant's arthritis [5]. At every post-injection interval (6, 12 and 18 days after injection) old rats had significantly lower nocturnal pineal melatonin levels. On day 18 of arthritis, decreased levels of pineal melatonin were also seen in young rats.

The present study was undertaken to further analyze pineal pre- and postsynaptic parameters during pineal nocturnal activation in young and old arthritic Wistar rats. Pineal serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured by high pressure liquid chromatography (HPLC) at 4 time intervals during the nocturnal activity span. In addition, pineal concentrations of norepinephrine (NE) and melatonin were also measured by HPLC.

## Material and Methods

Experiments were carried out in adult male Wistar rats, kept under light between 0900 and 2100 h daily. Rats had access to food and water ad libitum. Adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in European Communities Council Directives (86/609/EEC).

Groups of young (2 months) and aged (18–20 months) rats were injected s.c. with Freund's complete adjuvant (0.5 mg heat-killed *Mycobacterium butyricum*/rat) or its vehicle (0.5 ml paraffin oil containing 15% mannide monooleate) at 11:00 h. Although arthritis is induced most easily in inbred Lewis or Sprague-Dawley rats, it is also produced, to a milder extent, in Wistar rats [6–9]. Rats injected with Freund's adjuvant vehicle were included as a control of any inflammatory reaction the adjuvant's oil alone might cause [10–12]. The course of adjuvant-induced arthritis was followed by behavioral observation including spontaneous behavior-mobility, exploring,

rearing and scratching [1,2]. Eighteen days after Freund's adjuvant injection a general lack of mobility and exploring behavior, an increase in scratching behavior and signs of hyperalgesia were established in young and old rats as compared with their respective adjuvant's vehicle-injected groups. As reported previously by using plethysmography [5], old rats exhibited less behavioral signs of inflammation (spontaneous behavior-mobility, exploring, scratching) than young rats. At this time after injection, groups of 6–8 rats were killed by decapitation at 4 h-intervals throughout the night, starting at 2100 h. The brains were quickly removed and the pineal gland was taken out, weighed and homogenized in chilled (0–1 °C) 2 M acetic acid. After centrifugation (at 15 000 x g for 30 min, at 5 °C), the samples were analyzed for NE, 5-HIAA and 5-HT by high performance liquid chromatography (HPLC), using electrochemical detection (Coulchem, 5100A, ESA; USA). A C-18 reverse phase column eluted with a mobile phase (pH 4, 0.1 M sodium acetate, 0.1 M citric acid, 0.7 mM sodium octylsulphate and 0.57 mM EDTA containing 10% methanol, v/v), was employed. Flow rate was 1 ml/min, at a pressure of 2200 psi. Fixed potentials against H<sub>2</sub>/H<sup>+</sup> reference electrode were: conditioning electrode: –0.4 V; preoxidation electrode: +0.10 V; working electrode: +0.35 V. The linearity of the detector response was tested within the concentration ranges found in pineal supernatants. Results were expressed as pg/ $\mu$ g supernatant protein. NE, 5-HT and 5-HIAA were calculated from the chromatographic peak heights by using external standards. The turnover of 5-HT was assessed from 5-HIAA/5-HT ratios [13].

Pineal melatonin content was assayed by HPLC as previously described [5]. A reverse phase LC 40 HPLC system with electrochemical detection (Bioanalytical System, West Lafayette, IN, USA) was used. The electrochemical detector employed was an LC 3 amperometric controller with a TL 8A glass carbon electrode and a column Biophase ODS 5  $\mu$ m, 25x4.6 mm i.d. The solvent system employed was 0.1 M sodium acetate, 0.1 M citric acid, 50 mg/l EDTA and 37% methanol, at pH 4.2. Applied potential was +0.9 V. Melatonin concentrations were calculated from the chromatographic peak heights by using external standards. Results were expressed as ng/pineal.

Statistical analysis of results was performed by a two-way factorial analysis of variance (ANOVA) or by a one-way ANOVA followed by Student – Newman – Keuls test, as stated. P values lower than 0.05 were considered evidence for statistical significance.

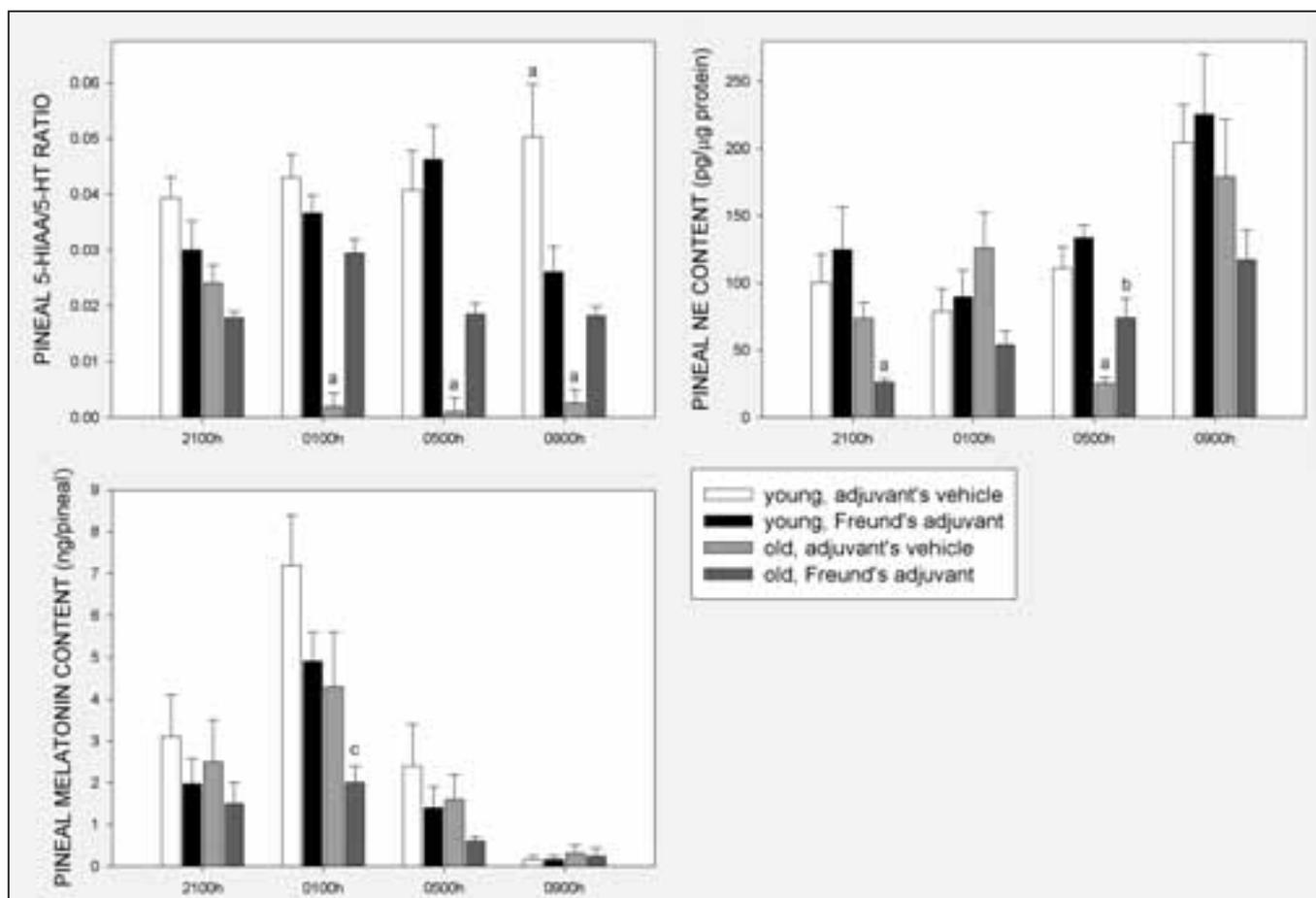
## Results

Table 1 summarizes the changes in nighttime pineal 5-HT and 5-HIAA content found in young and old rats injected with Freund's adjuvant or its vehicle

**Table 1.** Pineal 5-HT and 5-HIAA content in young and old rats injected with Freund's adjuvant or its vehicle 18 days earlier.

	Young, adjuvant's vehicle	Young, Freund's adjuvant	Old, adjuvant's vehicle	Old, Freund's adjuvant
<b>5-HT content</b>				
2100 h	5896 ± 926	10928 ± 1610 <sup>a</sup>	4200 ± 707	4580 ± 986
0100 h	8819 ± 1756	6942 ± 1507	7084 ± 1598	4773 ± 1301
0500 h	2626 ± 236 <sup>b</sup>	4821 ± 1224	2791 ± 487 <sup>b</sup>	6505 ± 1477
0900 h	10074 ± 2878	8603 ± 2184	8112 ± 1476	5154 ± 1280
<b>5-HIAA content</b>				
2100 h	224 ± 22	272 ± 25	93 ± 12 <sup>c</sup>	69 ± 6 <sup>c</sup>
0100 h	316 ± 71	246 ± 56	180 ± 28	146 ± 26
0500 h	108 ± 21	243 ± 27 <sup>a</sup>	45 ± 4	90 ± 13
0900 h	323 ± 39 <sup>a</sup>	208 ± 49	126 ± 9	94 ± 25

Groups of 6–8 rats were killed by decapitation at 4 different time intervals throughout the activity span (i.e., at the beginning, and on the 4<sup>th</sup>, 8<sup>th</sup> and 12<sup>th</sup> h of scotophase). Shown are the means ± SEM. Letters designate significant differences in a one-way ANOVA followed by a Student – Newman – Keuls test within each time interval, as follows: <sup>a</sup> p<0.05 as compared to the remaining groups; <sup>b</sup> p<0.05 as compared to their respective group receiving Freund's adjuvant injection; <sup>c</sup> p<0.05 as compared to young rats injected with Freund's adjuvant or its vehicle.



**Figure 1.** Pineal 5-HT turnover (as assessed from 5-HIAA/5-HT ratio), and pineal NE and melatonin content, in young and old rats injected with Freund's adjuvant or its vehicle 18 days earlier. Groups of 6–8 rats were killed by decapitation at 4 different time intervals throughout the activity span (i.e., at the beginning, and on the 4<sup>th</sup>, 8<sup>th</sup> and 12<sup>th</sup> h of scotophase). Shown are the means ± SEM. Letters designate significant differences in a one-way ANOVA followed by a Student – Newman – Keuls test within each time interval, as follows: <sup>a</sup> p<0.05 as compared to the remaining groups; <sup>b</sup> p<0.05 as compared to young rats injected with Freund's adjuvant; <sup>c</sup> p<0.05 as compared to young rats injected with Freund's adjuvant or its vehicle. For further statistical analysis, see text.

18 days earlier. A factorial ANOVA taking age as a global factor revealed significantly lower pineal 5-HT and 5-HIAA content in aged rats ( $F_{1,97}=15.7$  and  $57.7$ ,  $p<0.00001$ , respectively). Although immunization did not affect globally pineal 5-HT or 5-HIAA levels, significant interactions “immunization x age” and “immunization x time” were found, i.e., immunization augmented pineal 5-HT content at the beginning of the activity span in young rats, and at second half of the activity span in young and old rats ( $F_{3,97}=3.65$ ,  $p<0.01$ ) while it increased pineal 5-HIAA concentration in young rats only and at the second part of the activity span ( $F_{3,97}=4.12$ ,  $p<0.01$ , factorial ANOVA).

Generally, the decrease in pineal 5-HIAA concentration exceeded that of 5-HT; hence, 5-HT turnover (as assessed from 5-HIAA/5-HT ratio) was lower in aged rats. This is shown in Fig. 1, upper left panel. A factorial ANOVA indicated a significantly lower pineal 5-HIAA/5-HT ratio in old rats ( $F_{1,97}=54.7$ ,  $p<0.00001$ ). Old rats receiving adjuvant's vehicle showed the lowest 5-HIAA/5-HT ratio, which was increased by Freund's adjuvant administration at every time interval examined after 0100 h, an effect not seen in young rats (Fig. 1, upper left panel). This was statistically indicated by the significant interactions “immunization x age” (Freund's adjuvant increased pineal 5-HT turnover in old rats only,  $F_{1,97}=4.95$ ,  $p<0.03$ ) and “immunization x time” (the effect of Freund's adjuvant was seen after the 4<sup>th</sup> h of activity,  $F_{3,97}=2.86$ ,  $p<0.05$ ) found in a factorial ANOVA.

Figure 1, upper right panel, depicts the nighttime changes of pineal NE content. A factorial ANOVA taking age as a main factor revealed a significantly lower NE content in the pineal gland of aged rats ( $F_{1,97}=15.7$ ,  $p<0.0001$ ) and significant time-dependent effects with higher values at the end of the activity span ( $F_{3,97}=15.9$ ,  $p<0.00001$ ). A significant interaction “age x immunization” was found, i.e. Freund's adjuvant treatment decreased pineal NE content in aged rats only ( $F_{1,97}=5.17$ ,  $p<0.02$ ) (Fig.1, upper right panel).

Old rats had the lowest pineal melatonin levels when analyzed as a main factor in a factorial ANOVA ( $F_{1,97}=2.78$ ,  $p<0.05$ , Fig. 1, lower panel). In addition, a global depressive effect of immunization on pineal melatonin content was found ( $F_{1,97}=6.23$ ,  $p<0.01$ ). Significant time-dependent effects, with higher values at the first half of activity span were detected ( $F_{3,97}=11.1$ ,  $p<0.0001$ ) (Fig.1, lower panel).

## Discussion

An age-related reduction in immune function associated with cell-mediated immunity occurs in both experimental animals and humans (for references see [14]). In advancing age alterations are mainly observed in T cell mediated immunity including decreased proliferative responsiveness of T cells to mi-

togens, decreased T cell-dependent humoral immune responses, lowered resistance to tumor cell challenge, decreased graft-vs.-host reactivity, delayed skin allograft rejection time, impaired delayed hypersensitivity, reduced cytolytic immune response, altered cytokine production after stimulation, and decreased natural killer cell activity [14].

In the present study, we employed the injection of complete Freund's adjuvant as an activator of cell-mediated immune responses in young and old rats. During the acute phase or Freund's adjuvant arthritis (on day 18 after injection) a decrease of pineal NE content occurred in aged rats only. The treatment also depressed pineal melatonin content, maximally in old rats. Pineal 5-HT content augmented at the beginning of the activity span in Freund's adjuvant-treated young rats only, while pineal 5-HT and 5-HIAA augmented in Freund's adjuvant-treated young and old rats at second half of the activity span. Since the increase in 5-HT exceeded that of 5-HIAA, pineal 5-HT turnover globally decreased in old rats. Old rats receiving adjuvant's vehicle showed the lowest pineal 5-HT turnover, which was increased by Freund's adjuvant administration at every time interval examined after 4 h of scotophase. In contrast, this effect of Freund's adjuvant was not seen in young rats. Collectively, the results indicate that the effect of immunization on a number of pineal pre- and postsynaptic parameters, remarkably 5-HT turnover and melatonin content, are aged-dependent.

Both in aged rodents [15–18] and humans [19–25] decreases in pineal melatonin secretion have been documented. Neuronal degeneration at the major circadian oscillator, the suprachiasmatic nuclei [26] and a decrease in number of pinealocytes [27] have been proposed as relevant phenomena for age-related decreases in melatonin levels. Age-associated decreases in pineal catecholamine and indoleamine levels have also been documented [16,18].

Our present study, including 4 time intervals throughout the nocturnal activity span, agrees with the existence of age-dependent decreases in pineal 5-HT and 5-HIAA content. Since, the decrease in 5-HIAA concentration generally exceeded that of 5-HT, pineal 5-HT turnover also decreased in old rats. Whether the changes observed are presynaptic or depended on intrapineal mechanisms cannot be presently ascertained. Age-dependent decreases in amplitude and mesor of 24-h rhythms of sympathetic nervous system activity, as assessed in selected autonomic ganglia and their innervating territories in rats by measuring tyrosine hydroxylase activity, were previously reported [28].

Summarizing, bilateral interactions between pineal gland and the immune system seem to occur. Both *in vivo* and *in vitro* experiments have showed that the

pineal gland, via melatonin, enhances immune function [29]. The immune system may, via synthesis and secretion of cytokines, influence pineal gland function, thereby closing an information loop probable relevant to homeostasis. In mammals, immune signals like gamma-interferon [30] or tumor necrosis factor-alpha [29] are involved in regulating pineal function. Therefore, the changes in pineal function reported herein in adjuvant arthritis are probably a part of the widespread systemic disease associated with inflammation ("sickness behavior") [3,4].

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