COSMOS 2044
EXPERIMENT K-7-19

PINEAL PHYSIOLOGY IN MICROGRAVITY: RELATION TO RAT GONADAL FUNCTION

NASA-AMES RESEARCH CENTER
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INTRODUCTION

Relative to other endocrine organs, research on the physiology of the pineal has been a rather recent endeavor, and discoveries relative to pineal physiology have proceeded rather slowly. Considerable advances in this area have occurred over the past two decades and many textbooks are now espousing it as a true endocrine gland (Hadley, 1988). It is now known that the pineal organ can interact with many endocrine and nonendocrine tissues in a regulatory fashion. It is well established that an antigonadal hormone of pineal origin (most likely the indoleamine melatonin) is involved in the photoperiodic regulation of reproduction in seasonally breeding mammalian species, and some nonseasonal breeding species (possibly also humans). Recent reviews implicate the pineal in the functioning of other organs and systems besides those involved in reproduction including: temperature regulation, thyroid, growth hormone, adrenal glucocorticoid synthesis, behavior (arousal/depression), circadian system (activity/rest), and skin pigmentation (in lower animals). Several excellent journal reviews and books on pineal physiology have appeared over the past ten years (See Reiter, 1981a; Reiter, 1981b; Vollrath, 1981; Reiter, 1982; Brinkley, 1983; Axelrod, et al. 1983; Preslock, 1984; Reiter, 1984; Brown and Wainwright, 1985; 1985; O'Brien and Klein, 1986). In view of the fact that the pineal is an important link to the environment (Reiter, 1986), it is conceivable that exposure to microgravity and spaceflight might alter the function of this gland and, in turn, affect various physiological functions including the circadian timing system and reproduction.

Primary control of pineal function is mediated by the photoenvironment. Light impinging upon the retina influences the pineal via the following pathway: retino-hypothalamic tract, suprachiasmatic nuclei, median forebrain bundle, superior cervical ganglia, sympathetic efferents. Adrenergic receptor activation results in stimulation of N-Acetyltransferase (NAT) activity with resulting production of melatonin from the precursor serotonin (5-hydroxytryptamine, 5-HT). Indeed, the serotonin concentration of the pineal gland exceeds that of any other organ and is fifty times that of any other brain area (Quay, 1963). Melatonin is probably the most important pineal secretory product in terms of distant regulatory responses (e.g. antigonadal effects). However, several other non-indole hormones are reputed to be synthesized there, (e.g., arginine vasotocin, oxytocin, arginine vasopressin, an alpha-MSH like peptide, GnRh, TRH, renin, and angiotensin I). The pineal melatonin content fluctuates with a pronounced circadian rhythm (amplitude of about 20-plus orders of magnitude) and it is rapidly inhibited when the animal is exposed to light. Wurtman and Ozaki (1978) suggested that the availability of serotonin may be involved in regulating the synthesis of melatonin in the pineal. Chan and Ebadi (1980) provided evidence that under certain experimental conditions serotonin may inhibit the activity of NAT, a key enzyme in the synthesis of melatonin. Serotonin also exhibits circadian rhythmicity (amplitude of approximately 2-3 orders of magnitude) and this rhythm persists even in blinded weanling rats. Given its key role in the regulation of melatonin synthesis, its high concentration, and
that its levels may persist longer than the more rapidly changing melatonin, we felt that serotonin might give a more accurate assessment of the effects of microgravity on pineal function following recovery of the animals from the flight. We also measured 5-hydroxyindole acetic acid (5-HIAA), a major metabolite of serotonin, hoping that we might be able to assess an effect on serotonin metabolism (turnover).

One of the most interesting concomitants to spaceflight and exposure to microgravity has been the disturbing alteration in calcium metabolism and resulting skeletal effects. It was recognized as early as 1985 (cited in Kitay and Altschule, 1954) that the pineal of humans calcified with age. However, little can be found in the literature relating calcification and pineal function. Given the link between exposure to microgravity and pertubation of calcium metabolism and the fact that the pineal is apparently one of the only "soft tissues" to calcify, we examined pineal calcium content following the spaceflight.

MATERIALS AND METHODS

Cosmos 2044 animal groups

A) Flight animals

Pineals were obtained from 4(#6, #8, #9 and #10) of the 10 male rats (Czechoslovakian - Western origin, Institute of Experimental Endocrinology, Bratislave, Czechoslovakia) that were flown aboard the Soviet Biosatellite Cosmos 2044. The flight launched on September 15, 1989 at 10:30 and landed at 05:45 (Moscow time) on September 29, 1989 for a flight of 13 days and 19+ hours. Except for a slight rise in cabin temperature the last two days, the mission went flawlessly. Starting on August 25, 1989, the rats were fed the flight diet (70% water). The animals were adapted to flight cages prior to the loading of the flight rats on to the vehicle at 15:00 on September 13, 1989. Fourteen gram boluses of food (total 55 g/rat/day) were provided at 2:00, 8:00, 14:00 and 20:00 hrs each day. Food consumption averaged 45 g per day. Rats had free access to water and their consumption was 203 ml per day. The lights were on from 8:00 to 24:00 hr each day. The partial pressure of oxygen was 140-160 mm Hg and 2 mm or less for carbon dioxide. Ambient temperatures varied as follows: Days 1-3, 21-25°C and days 13-14, 26-29°C (rising occasionally to 30°C). After recovery, the rats were sacrificed on September 29, 1989 starting at 11:00 hr.

B) Basal Control Animals.

They were put into flight-type cages and had a flight (paste) diet supplied in a single bolus of 55 g for 14 days before sacrifice on September 15, 1989. Temperature, humidity, and lighting were similar to in-flight conditions.

C) Synchronous Control Animals.

These rats were maintained in flight-type cages on a flight (paste) diet. They were exposed to the launch G forces and vibration and exposed to the same lighting regimen and temperature as flight rats. The feeding schedule was similar to the flight rats (food consumption averaged...
50 g/rat/day). They were sacrificed on October 3, 1989, starting at 11:00 hr.

D) Vivarium Control Animals.

These animals were kept in cages of the same size as the flight cages with environmental conditions similar to those in flight. They were fed the same quantity of food per day (55 g), but in only one feeding. They were sacrificed on October 5, 1989, starting at 11:00 hr.

E) Tail Suspended.

These animals were caged individually and were given a single daily bolus of paste diet (55 g/day). They were sacrificed on October 7, 1989, starting at 11:00 hr.

Sample collection and Initial Tissue Extraction Step

Upon dissection, the pineals were immediately placed in prechilled cryovials (10 x 55 mm), placed into liquid nitrogen for quick-freeze, and shipped to Moscow in a liquid nitrogen biotransporter. The samples were shipped to the U.S.A. and stored until analysis at -70°C. Trunk blood was collected immediately following decapitation into heparinized tubes and centrifuged at 4°C. Plasma (1.5 ml) was rapidly frozen in liquid nitrogen and shipped to the U.S.A. in a biotransporter. In the U.S.A., this volume was thawed and aliquoted (100µl) for distribution to various investigators.

The pineal glands were kept on dry ice, weighed, and individually homogenized in 200 µl of perchloric acid. 100 µl of the crude homogenate was frozen and saved for melatonin and calcium analysis. The remaining aliquots (100 µl) were immediately used for the HPLC analysis of 5-HT and 5-HIAA.

HPLC analysis of pineal serotonin, 5-HIAA, and plasma 5-HT

Aliquots (100 µl) of the crude pineal homogenate were centrifuged using Centrex brand microfilters (0.45 µm pore size, Schleicher and Schuell, Inc. Keene, New Hampshire, U.S.A.). Homovanillyl alcohol was added to the homogenates to act as an internal standard at a final concentration of 2x10⁻⁶ M. Serotonin and 5-HIAA were analyzed in the filtered homogenates by HPLC using a modification of the method of Medford and Barchas (1980). The filtered homogenates were injected into a u-Bondapack brand C-18 reverse phase column (100 mm x 4.6 mm) of a high pressure liquid chromatograph (Bioanalytical Systems, Inc.). The mobile phase consisting of 0.1 M sodium acetate, 0.1 M citric acid and 25% v/v methanol (pH 4.1) was run through the column at a flow rate of 0.8 ml/min. The various peaks were detected using an electrochemical detector (Bioanalytical Systems, Inc.) mounted with a glassy carbon working electrode and Ag/AgCl reference electrode set at an oxidation potential of 0.85 V and sensitivity of 10 nAmps/V. Peaks were integrated and analyzed using the Baseline 810 program (Dynamic Solutions).
For analysis of plasma 20μl of 1.0 M perchloric acid was added to 100 μl of plasma. The mixture was filtered using micro-filter centrifuge tubes (Centrex brand, see above). The filtrate (10-15μl) was injected onto the HPLC column as above.

Radioimmunoassay of pineal melatonin content

The melatonin content of the pineal homogenates (75 μl aliquots) were determined by radioimmunoassay using "ultraspecific" melatonin antiserum and a procedure provided by Dr. Brown (CIDtech Research, Inc., Ontario, Canada) using "H-melatonin (Amersham Corp., Arlingon Heights, IL, U.S.A.). The melatonin for standard was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. (cat, #M5250). The assay performance characteristics in our laboratory were: sensitivity (defined as three standard deviations from the counts for the zero reference standard tube), 5 pg/ml; interassay coefficient of variation, 11.5%; and intraassay coefficient of variation, 6.27%.

Atomic absorption analysis of pineal calcium content

Total calcium content of the pineal homogenates was determined by atomic absorption spectrophotometry using an electrothermal atomizer equipped with a carbon rod. Aliquots (5 μl) of the homogenates were filtered with ultra-pure water (7 mohm resistance, Multi-Q Water System, Millipore, Corp., Bedfor, MA, U.S.A.). Volume added was 200 or 250 μl to achieve absorbance values in the range 0.1-0.5 absorbance units. Calcium reference standard was obtained from VWR, Inc., San Francisco, CA, U.S.A. (cat. #EM-CX0082-1). Assay sensitivity was approximately 4 pg/μl.

Statistical analysis

Given the experimental design, the groups were analyzed by one-way analysis of variance (ANOVA). If the ANOVA indicated a between group difference (p<0.05), then the data were further analyzed by one or more of the following statistical tests: Duncan's Multiple Range Test, Newman-Keul's Multiple Comparison Test, or Fisher's Least Significant Difference Test. In certain instances, Student's t-test for nonpaired samples was applied. The 95% confidence limit was considered significant in all tests.

RESULTS

The results of this study are summarized in Tables 1-3 and Figures 1-4.

A. Organ Weights

The data (Table 1) indicate that at the time of sacrifice, the flight, synchronous and tail suspended rats were smaller than the vivarium control animals (P<0.05). Due to this difference, pineal, testes and adrenal weights were normalized to percent of the body weight for group comparison. Although the average pineal weight of flight animals was not significantly different from that of vivarium or synchronous control animals, the relative pineal weight (weight/100 gm body weight) of the flight animals was significantly greater than that of the vivarium controls (P<0.05). On the other hand, both the average pineal weight and relative
pineal weight of tail suspended animals were significantly greater than those of vivarium control rats ($P<0.01$) (see also Fig. 1). In addition, the average testes weight as well as the relative testes weight of flight animals were statistically lower than those of the synchronous control rats ($P<0.05$). The testes weight and the relative testes weight of tail suspended rats were statistically lower than those of vivarium and synchronous controls ($P<0.01$). The relative adrenal weight of flight and tail suspended animals were significantly higher than that of vivarium control rats. The adrenal enlargement is consistent with chronic exposure to one or more environmental stressors. It should be noted also, that although there was no significant difference between the plasma corticosterone levels of tail suspended rats and those of vivarium controls at time of sacrifice, the plasma corticosterone levels of flight animals were significantly higher than those of vivarium control rats ($P=0.016$) (data courtesy of Dr. R. Grindeland and M. Vasques, NASA - Ames Research Center)

B Pineal Gland Analysis

Pineal melatonin content was determined for individual glands and the values normalized and reported as pg/milligram pineal tissue (See Table 2). There were no statistically significant differences among the test groups. The results are also summarized in Figure 2.

Serotonin (5-HT) and 5OHIAA content were also determined for individual glands and the values normalized and reported as ng/milligram pineal tissue (see Table 2). The pineal serotonin (5-HT) content of flight animals (expressed as ng/mg tissue) and of tail suspended rats (expressed as ng/gland) were significantly higher than those of synchronous controls ($P<0.05$). In addition, the pineal 5-HIAA content of flight rats was significantly higher than that of synchronous controls ($P<0.01$), while that of tail suspended animals was significantly higher than either vivarium or synchronous controls ($P<0.01$). (See Figure 3 and 4).

Table 2 summarizes the pineal calcium determinations. The pineal calcium content of flight and tail suspended rats (expressed as pg/gland) was significantly higher than that of synchronous controls ($P<0.01$).

C Plasma Serotonin (5-HT) and Testosterone Concentrations

The data shown in table 3 indicates that plasma testosterone concentration of the flight and tail suspended animals was significantly lower than that of synchronous, vivarium or basal rats.

As shown in table 3, plasma 5-HT concentration of flight animals was significantly lower than that of basal rats ($P<0.01$). In addition, plasma 5-HT concentration of tail suspended rats was significantly higher than that of synchronous controls ($P<0.05$). It should also be noted that plasma 5-HT concentration of synchronous controls was significantly lower than that of basal rats ($P<0.01$).
DISCUSSION

Considerable evidence supports the existence of a pineal humoral factor or factors (most likely melatonin) that can influence the hypothalamic-pituitary gonadal axis in many vertebrate species (Vollrath, 1981) including the rat (Binkley, 1983). If indeed the pineal is a major "link to the environment" (Reiter, 1986) it is, therefore, possible that gonadal function of rats flown in space might be altered via a mechanisms that includes involvement of the pineal.

Several pieces of evidence indicate that gonadal function of the rats aboard Comos 2044 spaceflight may have been compromised. The testes weight and relative testes weight of flight animals were statistically lower than those of synchronous control rats (Table 1). Plasma testosterone concentration of flight animals was significantly lower than that of synchronous, vivarium or basal rats (Table 3).

A similar compromise of gonadal function was observed in rats flown aboard Cosmos 1877 (Holley et al., 1989). It is also interesting to note, that tail suspended rats had lower testes weight and lower plasma testosterone concentration than synchronous, vivarium or basal rats (Table 3).

It is known that light has a dramatic and rather immediate inhibitory effect upon rat pineal N-acetylserotonin and pineal melatonin levels (Binkley, 1983). As a result we felt that to assess pineal function we would have to measure parameters that were less labile. Green, et. al. (1977) indicated that potent stressors (e.g. electroconvulsive shocks) could alter serotonin turnover for up to 6 days post exposure. Since serotonin is an important precursor to melatonin we felt that its measurement and relative inertia with respect to relative changes in concentration might be used as an indirect indicator of melatonin synthesis. To do this, however, we felt measurement of 5-HIAA, a major serotonin metabolite, would be necessary as an indication of serotonin turnover.

As suspected, the pineal melatonin levels were very low at time of sacrifice (Table 2 and Figure 1). Given that the rats were sacrificed about 3 hours after lights on, and that the major circadian drop in circulating melatonin is locked to the time of lights on (Binkley, 1983), it is not surprising that the pineal melatonin content was low in all groups. Given the limited amount of plasma available to us for the analysis of 5-HT (100 µl), and given the low pineal melatonin levels, we did not measure the plasma melatonin concentration.

Table 2 and Figures 3 and 4 summarize the pineal 5-HT and 5-HIAA determinations. Note that exposure to space environment resulted in a significant increase in the pineal content of these substances. A similar observation was noted in animals flown aboard Cosmos 1877 (Holley et al., 1989). The pineal gland profile was not reflected in the blood however (Table 3). It is known that the plasma 5-HT levels may reflect peripheral secretion and may not be an accurate indicator of central serotonergic mechanisms. The results indicate that exposure to the space environment had an effect on the pineal 5-HT content and its turn-over as indicated by concomitant increase in 5-HIAA. This would be consistent with increased melatonin secretion during the spaceflight which may have been involved in the antigonadal activity noted.
It has been suggested that melatonin may be a hormone of "stress" given its increased secretion during conditions such as insulin-induced hypoglycemia (Wurtman and Moskowitz, 1977). Yet the melatonin circadian rhythm is 180 degrees out of phase with the corticosterone circadian rhythm and there is evidence for a direct inhibitory role of the pineal and melatonin on adrenal glucocorticoid synthesis (Ogle and Kitay, 1978). The adrenal hypertrophy in flight animals as evidenced by a significant increase in the relative adrenal weight as compared to vivarium controls and the elevated plasma corticosterone levels of flight animals would indicate a chronic stress response.

It is interesting to note that alterations in pineal 5-HT and 5HIAA content as well as plasma testosterone levels observed in the present study in tail suspended rats are quite similar to those observed in flight rats.

The pineal of humans and some other mammalian species contain multi-layered hydroxyapatite concentrations called corpora arenacea, or brain sand. Although the degree of radiologically detectable calcification of the human pineal gland appears to increase with age, there is no indication that increased calcification is related to loss of cellular activity. Histological and biochemical studies have shown that the appearance of the pinealocyte cell type, pineal serotonin content and HIOMT enzyme activity do not change with age (Giarman, 1960; Rodin and Overall, 1967; Smith, et al. 1977; Wurtman, 1964). Lukaszyk and Reiter (1975), suggest that the deposition of calcium may be related to polypeptide secretion by the pineal gland, suggest that the deposition of calcium may be related to polypeptide secretion by the pineal gland, and may serve as an index of previous glandular activity rather than degeneration. In the present study, the pineal calcium content of flight and tail suspended rats was significantly higher that that of synchronous controls.

In summary, we conclude that the spaceflight resulted in a stress response as indicated by adrenal hypertrophy, that gonadal function was compromised, and that the pineal may be linked as part of the mechanisms of the responses noted.
Figure 1.

Pineal weight and pineal relative weight (percent body weight = mg/100 g body weight) of vivarium control (V), synchronous control (S), flight animals aboard Cosmos 2044 (F) and tail suspended rats (T). Values are means ± S.E.M. (a = versus V, P < 0.05; b = versus V, P < 0.01).

Figure 2.

Pineal melatonin expressed as pg/gland and pg/mg tissue in vivarium control (V), synchronous control (S), flight animals aboard Cosmos 2044 (F) and tail suspended rats (T), values are means ± S.E.M.

Figure 3.

Pineal serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) expressed as ng/gland in vivarium control (V), synchronous control (S), flight animals aboard Cosmos 2044 (F) and tail suspended rats (T). Values are means ± S.E.M. (a = versus S, P < 0.05; b = versus S, P < 0.01; c = versus V, P < 0.01).

Figure 4.

Pineal serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) expressed as ng/mg tissue in vivarium control V), synchronous control (S), flight animals aboard Cosmos 2044 (F) and tail suspended rats (T). Values are means ± S.E.M. (a = versus S, P < 0.05; b = versus S, P < 0.01; C = versus V, P < 0.01).
REFERENCES


Table 1.

Body Weight, Pineal Weight, Testes Weight and Adrenal Weight of Rats Flown Aboard Comsos 2044

<table>
<thead>
<tr>
<th>Group-Subj. #</th>
<th>Body Wt. (g)</th>
<th>Pineal Wt. (mg)</th>
<th>Pineal Wt. (%B.W)</th>
<th>Testes Wt. (%B.W)</th>
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F = Flight group  
S = Synchronous control group  
V = Vivarium control group  
T = Tail suspended group  
Wt = Weight  
B.W = Body weight  

a = Versus V, P < 0.05  
b = Versus V, P < 0.01  
c = Versus S, P < 0.05  
d = Versus S, P < 0.01
Table 2:

Pineal Content of Rats Flown Aboard Cosmos 2044: Melatonin (Mel), Serotonin (5-HT), 5-Hydroxyindoleacetic Acid (5-HIAA) and Calcium (Ca)

<table>
<thead>
<tr>
<th>Group-Subj #</th>
<th>Mel (Pg/gl)</th>
<th>Mel (Pg/mgt)</th>
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<th>5-HT (ng/mgt)</th>
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</table>

F = Flight group  
S = Synchronous control group  
V = Vivarium control group  
T = Tail suspended group  
Mel = Melatonin  
5-HT = Serotonin  
5-HIAA = 5-hydroxyindoleacetic acid  
Ca = Calcium  
1gl = 1 whole pineal gland  
1mgt = 1 milligram pineal tissue  
a = Versus S, P<0.05  
b = Versus S, P<0.01  
c = Versus V, P<0.01
Table 3.
Concentration of Plasma Serotonin (5-HT) and Testosterone
of Rats Flown Aboard Cosmos 2044

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<th>Group-Subj #</th>
<th>5HT (ng/ml)</th>
<th>Testosterone* (ng/ml)</th>
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<td><strong>0.27</strong></td>
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F = Flight  
S = Synchronous control group  
V = Vivarium control group  
B = Basal control group  
T = Tail suspended group  
* = Determined by RIA, data provided by Dr. R. Grindeland and M. Vasgues (NASA, Ames).

\[ a = Versus B, P<0.01 \]  
\[ b = Versus S, P<0.05 \]  
\[ c = Versus B, P<0.05 \]  
\[ d = Versus S, P<0.01 \]  
\[ e = Versus V, P<0.01 \]
PINEAL WEIGHT

PERCENT B.WT.

FIGURE 1

mg

% B.WT.
Figure 2

- Group T
- Group F
- Group S
- Group V

Melatonin Content

pe/mg tissue  pe/ gland

Legend:
CONTENT (ng/gland)

FIGURE 3

5-HT

5-HIAA
CONTENT (ng/mg tissue)

5-HT
5-HIAA

FIGURE 4