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Age-Dependent Changes in 24-Hour Rhythms of Thymic and Circulating Growth Hormone and Adrenocorticotropin in Rats Injected with Freund's Adjuvant

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Key Words

Thymus · Aging · Freund's adjuvant · Circadian rhythms · Growth hormone · ACTH · Aspartate · Glutamate · Taurine · GABA

Abstract

Objective: To analyze the 24-hour changes in thymic and serum concentration of growth hormone (GH) and adrenocorticotropin (ACTH) and their correlation with thymic concentrations of glutamate, aspartate, taurine and GABA in young and old rats during the acute phase of adjuvant's arthritis. ***Methods:*** Young (50-day-old) and old (18-month-old) rats were injected subcutaneously with Freund's adjuvant or its vehicle (paraffin oil containing 15% mannide monooleate). Eighteen days later, they were killed at six different time intervals throughout a 24-hour cycle. Serum and thymic levels of GH and ACTH were measured by radioimmunoassay. Thymic amino acid concentration was measured by HPLC. A quantitative assessment of arthritis was made in an independent group of rats by plethysmography. ***Results:*** Old rats injected with Freund's adjuvant exhibited fewer clinical signs of inflammation than young rats. Significant 24-hour changes in thymic and serum GH occurred, except for serum GH in adjuvant's vehicle-treated old rats. Aging augmented thymic GH and decreased serum GH. Immunization with Freund's adjuvant did not modify GH concentration. Thymic and serum concentration of GH correlated negatively. Thymic ACTH varied significantly over 24 h with maxima during the dark phase, except in Freund's adjuvant-treated young rats. Maximal serum ACTH levels occurred in the late afternoon except in Freund's adjuvant-treated old rats which showed maxima at night. Immunization with Freund's adjuvant augmented thymic and circulating concentrations of ACTH. Thymic and serum concentration of ACTH correlated positively. Thymic concentration of glutamate, aspartate and taurine decreased in aged rats and correlated significantly with thymic ACTH. ***Conclusion:*** The results support the existence of a thymic compartment of GH and ACTH that may be independently regulated.

Introduction

The thymus gland is a primary lymphoid organ. Within it, bone-marrow-derived T-cell precursors undergo maturation, a complex process including selection of the

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T-cell repertoire, with positively selected cells migrating to the peripheral lymphoid organs and further expanding. This process is influenced by a thymic microenvironment comprised by thymocytes, thymic epithelial cells and thymic stromal cells, as well as by the neuroendocrine milieu provided by several hormones and neurotransmitters [for references, see 1].

Extrathymic hormonal influences include pituitary-derived hormones, like growth hormone (GH) or adrenocorticotropin (ACTH), the latter acting both directly and via the secretion of adrenal corticosteroids [1–4]. Besides, a possible thymic production of pituitary hormones has been documented, as revealed by the detection of immunoreactivity for GH, ACTH, prolactin, thyrotropin (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), oxytocin and vasopressin, and the specific messenger RNAs for some of these hormones, in thymic cells [1, 5–10].

Aging brings about a progressive disruption in the integration of this network [11]. Involution of the thymus parallels the somatopause that accompanies the decrease in plasma GH in aging. Treatment with GH restores the architecture of involuted thymus by reversing the loss of immature cortical thymocytes and by preventing the decline in thymulin synthesis in old or GH-deficient animals and humans [12–15]. The data strongly support the dependence of age-related processes in the thymus on circulating hormone levels. In addition, aging is characterized by changes in circadian rhythms, the most marked modification being the attenuation of amplitude, together with an advance of phase, a shortening of period and a desynchronization of rhythms [16–19].

Since aging is associated with declines in multiple areas of immune function, it seemed feasible that differences in circadian response to an immune challenge may occur with age [20–22]. However, no information was available on the correlation of aging of the thymus with the thymic concentration of pituitary hormones, nor on the effect of aging on 24-hour rhythms in intrathymic hormone concentration after an immune challenge.

This prompted us to carry out the present study, whose objective was to analyze, in young and old rats, the 24-hour changes in thymic and serum levels of GH and ACTH 18 days after an immune challenge given by injecting Freund's adjuvant. Adjuvant arthritis in rats is usually induced by injection of *Mycobacterium* tubercle cell walls suspended in incomplete Freund's adjuvant [23]. This inflammatory disease has been widely employed as an experimental paradigm to examine the relationships between the brain and the immune system, one feature of mycobacterial adjuvant arthritis less often addressed being alteration of the circadian time structure during the immune reaction. We previously reported changes in the 24-hour rhythms of adenohypophysial hormone release and hypothalamic monoamine and neuropeptide content during Freund's adjuvant arthritis in rats. Our results were compatible with significant effects of immune-mediated inflammatory response on hormone secretory processes, which were partially sensitive to immunosuppression by cyclosporine [24–26]. These as well as other studies indicated that significant changes in 24-hour rhythms did occur during experimental arthritis in rats [27–35]. As a continuation of those experiments, we hereby describe the 24-hour changes in thymic and serum concentration of GH and ACTH and their correlation with thymic concentrations of glutamate, aspartate, taurine and GABA (as indexes of thymic metabolism) in young and old rats during the acute phase of adjuvant's arthritis. Rats injected with Freund's adjuvant vehicle (15% mannide monooleate in paraffin oil) were included as a control of any inflammatory reaction the adjuvant's oil alone might cause [36–38]. A quantitative assessment of arthritis was made plethysmographically in an independent group of rats.

Materials and Methods

Chemicals

O-Phthalaldehyde (OPA), 2-mercaptoethanol and amino acid standards were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Freund's complete adjuvant was obtained from Difco, Detroit, Ill., USA. Double-distilled deionized water was used for preparation of solutions and buffers.

Animals and Experimental Design

Experiments were carried out in male Wistar rats of two ages, i.e. young (50 days old) and old (18 months old), kept under light between 08:00 and 20:00 h daily and having access to food and water ad libitum. Light intensity at the level of the animal cages was about 200 lx. 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).

Rats were subcutaneously injected 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. Assessment of arthritis development was made clinically [23, 39]. Although arthritis is induced most easily in inbred Lewis rats, it is also produced, to a milder extent, in Wistar rats [40–43]. The course of adjuvant-induced arthritis was followed by behavioral observations including those of spontaneous behavior-mobility, exploring, rearing and scratching. On day 18 after injection, groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-hour cycle. Care was taken to avoid any major pre-sacrifice

stress by submitting the animals to daily 1-min sessions of gentle manipulation for a week before sacrifice. Serum from the trunk blood was collected and kept frozen for hormone measurements while the thymus was readily dissected out, wiped out of blood, weighed and frozen at −80°C.

A quantitative assessment of arthritis was made in an independent group of rats treated in a similar way to that described above, on day 18 after injection. The hindpaws of the animals were submerged in a plethysmometer bath (Ugo Basile, Milano, Italy) to the level of the lateral malleolus to register variations in size, as previously described [44]. After this, animals were killed as described above and the thymus was quickly dissected out, weighed and put on balanced salt solution, the cells being gently teased apart and counted after removing the clumps by centrifugation.

Tissue Preparation

To measure the thymic concentration on GH and ACTH, the tissue was homogenized (1:2, w/v) in 0.01 *M* phosphate buffer containing 1% bovine serum albumin. After centrifugation at 1,000 *g* for 15 min, the supernatant was removed and kept frozen at −80°C until further analysis. To measure thymic amino acid concentration, the tissue was homogenized in cold 2 *M* acetic acid (1–4°C), heated for 5 min at 100°C and centrifuged at 2,000 *g* for 10 min, at 4°C. The supernatant was removed and kept frozen at −80°C until further analysis.

High-Pressure Liquid Chromatography (HPLC)

Amino acid concentration was measured by HPLC using fluorescence detection after pre-column derivatization with OPA as described elsewhere [45]. An aliquot of tissue supernatant containing homoserine as an internal standard was neutralized with NaOH (4 *M*) and was reacted with OPA reagent (4 m*M* OPA, 10% methanol, 2.56 m*M* 2-mercaptoethanol, in 1.6 *M* potassium borate buffer, pH 9.5) for 1 min at room temperature. At the end of this period, the reaction was stopped by adding acetic acid (0.5% v/v). Samples were immediately loaded through a Rheodyne (Model 7125) injector system (50-µl loop) to reach a C-18 reverse-phase column (4.6 mm ID × 150 mm, Nucleosil 5, 100A). Elution was achieved by means of a mobile phase consisting of 0.1 *M* sodium acetate buffer (pH 6.5) containing 35% methanol, at a flow rate of 1 ml/min and a pressure of 140 bars. The column was subsequently washed with the same buffer containing 70% methanol and re-equilibrated with the elution buffer before re-use. The filter fluorometer was set at the following wavelengths: excitation: 340 nm, emission: 455 nm. The procedure allowed a distinct separation and resolution of the amino acids measured. Amino acid concentration was calculated from the chromatographic peak heights by using standard curves and the internal standard. The linearity of the detector response for aspartate, glutamate, taurine and GABA was tested within the concentration ranges found in thymic extracts.

Radioimmunoassay (RIA)

GH and ACTH levels were measured by homologous specific double antibody RIAs using material kindly supplied by the NIDDK's National Hormone and the Pituitary Program. The intra- and interassay coefficients of variation were 6–8%. Sensitivities of the RIAs were 200 and 195 pg/ml for GH and ACTH, using the NIDDK rat GH RP-2 and ACTH RP-1, respectively.

Table 1. Inflammation of hindpaws, as assessed plethysmographically, in young and old rats on day 18 after the subcutaneous injection of Freund's complete adjuvant or its vehicle

	Hindpaw volume ml
Young rats injected with adjuvant's vehicle	3.23 ± 0.31
Young rats injected with Freund's adjuvant	6.12 ± 0.65[a,b]
Old rats injected with adjuvant's vehicle	2.81 ± 0.29
Old rats injected with Freund's adjuvant	4.71 ± 0.34[a]

Shown are the means ± SEM, n = 8/group.

[a] $p < 0.01$ as compared to Freund's adjuvant-treated rats; [b] $p < 0.05$ as compared to Freund's adjuvant-treated old rats (ANOVA followed by a Student-Newman-Keuls test).

Statistical Analysis

Statistical analysis of results was performed by parametric or nonparametric analysis of variance (ANOVA), a factorial ANOVA or a regression analysis, as stated. Curve estimation regression was made by using SPSS software, version 10.1 (SPSS Inc., Chicago, Ill., USA). Mean values were considered significantly different if $p < 0.05$.

Results

Eighteen days after Freund's adjuvant injection, a lack of mobility and exploring behavior, an increase in scratching behavior and signs of hyperalgesia were clearly established in young and old rats as compared with their respective adjuvant's vehicle-injected groups. Old rats exhibited fewer behavioral signs of inflammation (spontaneous behavior-mobility, exploring, scratching) than young rats. The quantitative assessment of arthritis by plethysmography is summarized in table 1. Hindpaw volume in young rats injected with Freund's adjuvant was significantly larger than that of similarly treated old rats.

The weight of thymus (mg, mean ± SEM) in the four groups of rats studied was: 601 ± 13 (young rats injected with Freund's adjuvant), 600 ± 12 (young rats injected with adjuvant's vehicle), 100 ± 7 (old rats injected with Freund's adjuvant), and 101 ± 8 (old rats injected with adjuvant's vehicle). Cellularity, expressed as number of cells/mg thymus × 10^{-4}, in the same groups of animals was: 5.19 ± 0.54 (young rats injected with Freund's adjuvant), 4.62 ± 0.61 (young rats injected with adjuvant's vehicle), 1.63 ± 0.22 (old rats injected with Freund's adjuvant), and 1.55 ± 0.16 (old rats injected with adjuvant's vehicle).

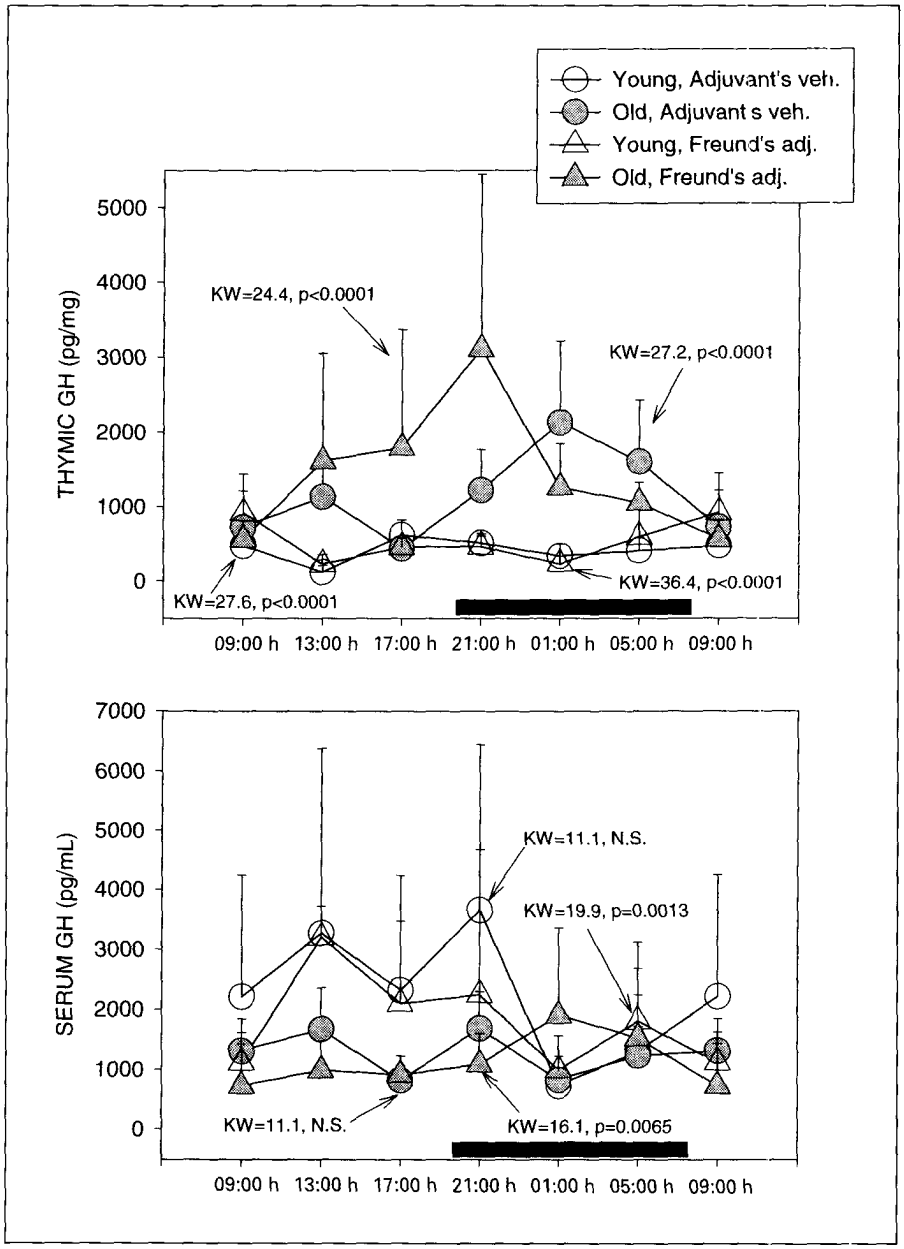

Fig. 1. Twenty-four-hour variations in thymic and serum concentration of GH in young and old rats injected with Freund's adjuvant or its vehicle 18 days earlier. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-hour cycle, as described in Methods. Shown are the means ± SD; the data of two independent experiments are pooled (n = 10–14 rats/group). Results were statistically analyzed by a nonparametric ANOVA. Kruskal-Wallis statistics and the approximate p value (as calculated by χ^2) are depicted in the figure. NS = Not significant. For further statistical analysis, see text.

Figure 1 shows the 24-hour changes in thymic and serum GH concentration. A nonparametric ANOVA indicated significant 24-hour changes in thymic and serum GH levels in the four groups of rats studied, except for the case of serum GH in old rats administered with adjuvant's vehicle. Maxima in circulating and thymic concentration of GH were unrelated. Analyzed as a main factor in a factorial ANOVA, aging augmented thymic GH ($F_{1,223} = 82.28$, $p < 0.00001$) and decreased serum GH ($F_{1,217} = 23.19$, $p < 0.00001$). The differences between rats receiving Freund's adjuvant and its vehicle did not attain significance.

Figure 2 depicts the 24-hour changes in thymic and serum ACTH concentration in the same group of animals. Except for young rats injected with Freund's adjuvant, significant 24-hour variations of thymic ACTH concentration with maxima during the dark phase were found. As far as circulating ACTH is concerned, young rats injected with Freund's adjuvant, and young and old rats receiving adjuvant's vehicle, showed maxima in the late

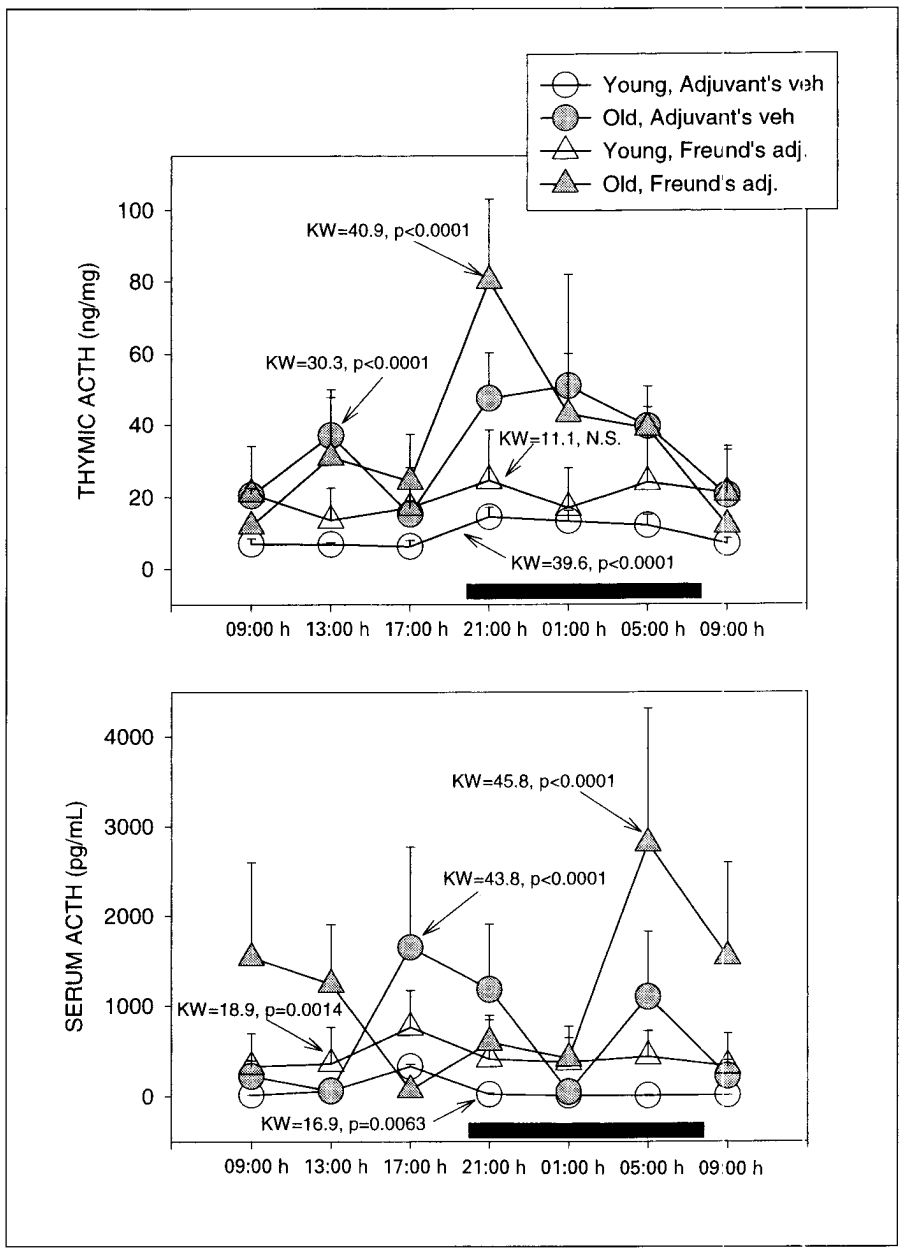

Fig. 2. Twenty-four-hour variations in thymic and serum concentration of ACTH in young and old rats injected with Freund's adjuvant or its vehicle 18 days earlier. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-hour cycle, as described in Methods. Shown are the means ± SD; the data of two independent experiments are pooled (n = 10–14 rats/group). Results were statistically analyzed by a nonparametric ANOVA. Kruskal-Wallis statistics and the approximate p value (as calculated by χ^2) are depicted in the figure. NS = Not significant. For further statistical analysis, see text.

afternoon while old rats injected with Freund's adjuvant showed maxima at night.

Main factor analysis in a factorial ANOVA indicated that old rats had higher values of ACTH than young rats, both in the thymus ($F_{1,226}$ = 113.89, $p < 0.00001$) and in circulation ($F_{1,233}$ = 80.45, $p < 0.00001$). Immunization with Freund's adjuvant augmented ACTH concentration ($F_{1,226}$ = 9.52, p = 0.0023, for thymus; $F_{1,233}$ = 27.76, $p < 0.00001$, for circulation). This was similarly observed in young and old rats (fig. 2).

The correlation between thymic and serum concentrations of GH and ACTH in individual rats are depicted in figure 3. A significant positive correlation was found between thymic and serum ACTH ($p < 0.00001$). Thymic and serum concentrations of GH showed a marginally significant negative correlation (p = 0.04) (fig. 3).

Figure 4 shows the 24-hour changes in thymic concentration of aspartate, glutamate, taurine and GABA. For every amino acid examined, the highest concentrations occurred at night. Analyzed as a main factor in a two-way

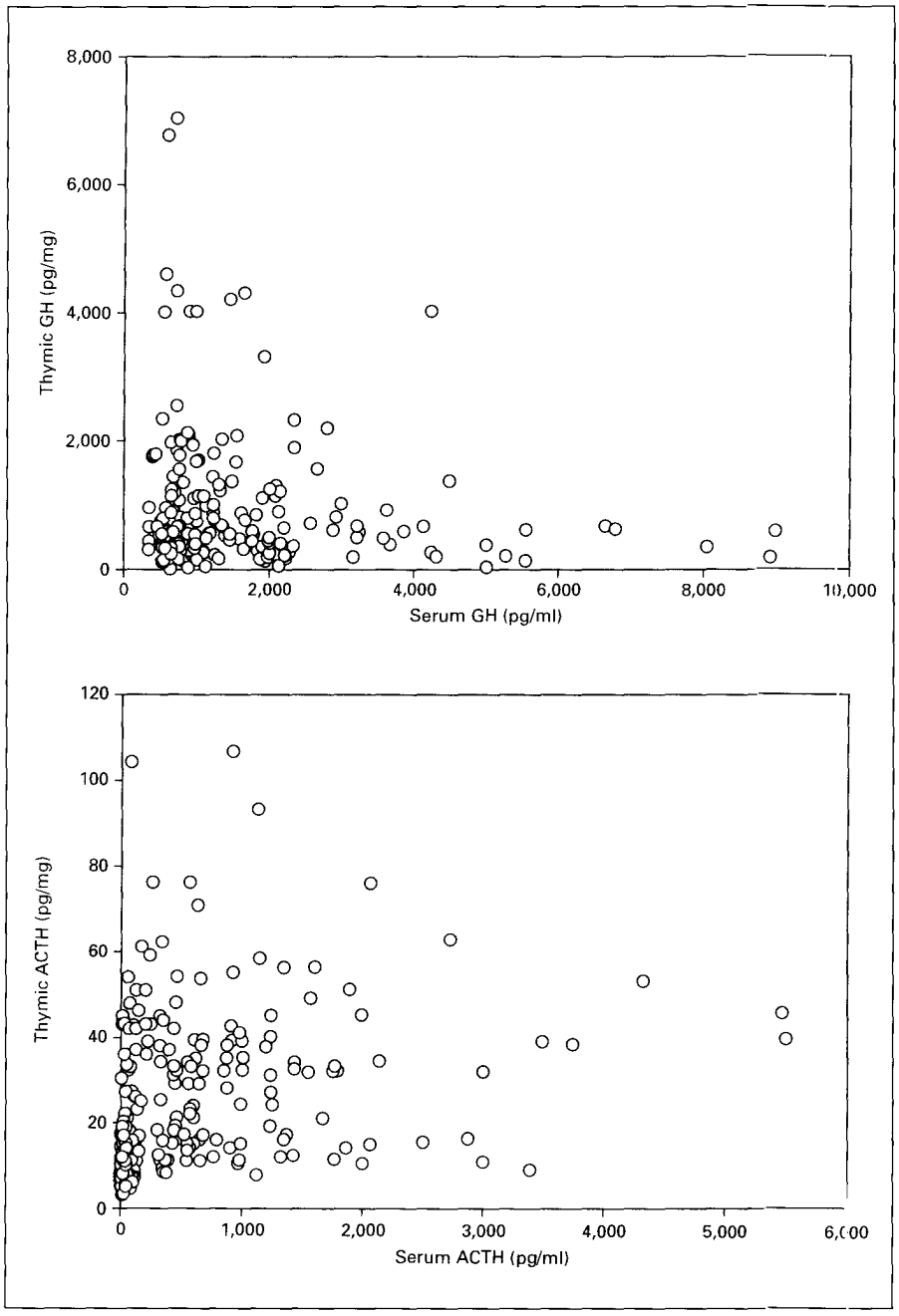

Fig. 3. Correlation between thymic and serum concentrations of GH (upper panel) and between thymic and serum concentrations of ACTH (lower panel). Curve estimation regression for the GH set of values indicated a best fit for a negative log correlation model with $r^2 = 0.262$ and $b_1 = -0.0901$ ($F_{1,224} = 4.20$, $p = 0.04$). Curve estimation regression for the ACTH set of values indicated a best fit for a positive power correlation model with $r^2 = 0.262$ and $b_1 = 0.19$ ($F_{1,241} = 85.74$, $p < 0.00001$).

Table 2. Correlation between thymic concentrations of amino acids and thymic levels of ACTH

	Model	r^2	d.f.	F	p value	b_0	b_1
Aspartate	power	0.112	107	13.45	<0.0001	16.7	0.316
Glutamate	linear	0.097	107	11.43	0.001	96.3	0.965
Taurine	power	0.067	107	7.74	0.006	67.2	0.156
GABA	power	0.174	107	22.53	<0.0001	19.6	0.467

d.f. = Degrees of freedom.

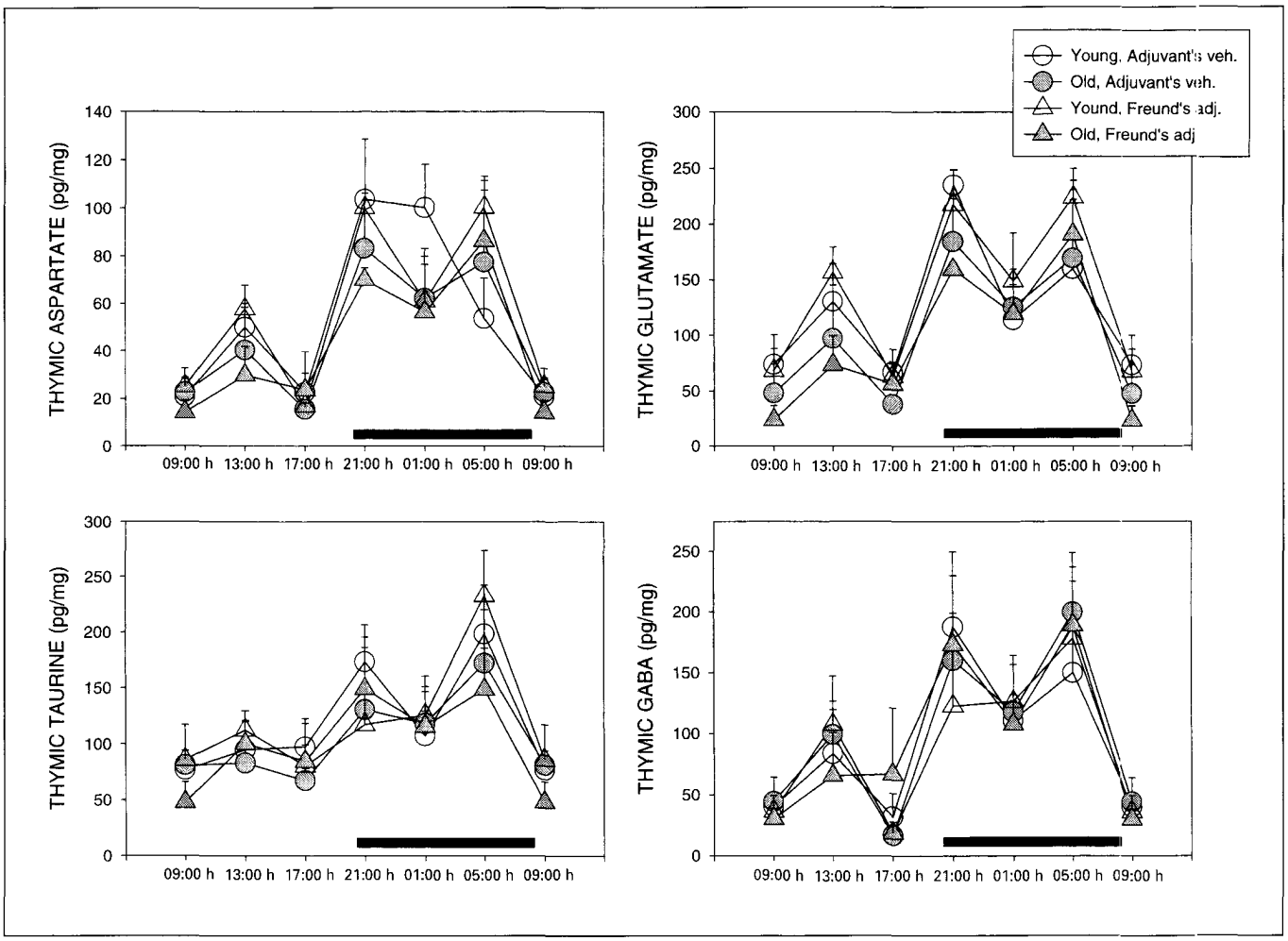

Fig. 4. Twenty-four-hour variations in thymic concentration of aspartate, glutamate, taurine and GABA in young and old rats injected with Freund's adjuvant or its vehicle 18 days earlier. Groups of 5–7 rats were killed by decapitation at six different time intervals throughout a 24-hour cycle, as described in Methods. Shown are the means ± SD. The changes observed as a function of time of day were significant in all examined groups as indicated by individual nonparametric ANOVAs. For further statistical analysis, see text.

ANOVA, aging decreased thymic concentration of aspartate ($F_{1,112} = 7.56$, $p = 0.0072$), glutamate ($F_{1,112} = 19.12$, $p < 0.00001$) and taurine ($F_{1,103} = 6.16$, $p = 0.0147$), but not those of GABA ($F_{1,117} = 0.72$, $p = 0.41$). The differences between rats receiving Freund's adjuvant and its vehicle did not attain significance for any of the amino acids studied (factorial ANOVA).

Table 2 shows the correlation between the thymic concentrations of amino acids and thymic concentration of ACTH. A significant positive correlation of ACTH with every amino acid examined was found. In the case of thymic GH, such a correlation did not exist (results not shown).

Discussion

Injection of Freund's complete adjuvant induces an inflammatory disease of the joints that includes four stages: preclinical (first week), acute (weeks 2–4), postacute (weeks 5–8) and recovery (weeks 9–11) [23, 39]. The acute stage (weeks 2–4) is defined by signs of hyperalgesia, lack of mobility and a pause in body weight gain; during the acute period, hindpaw and forepaw joint diameters increase [39]. All these signs were present in the groups of rats hereby described.

In Freund's adjuvant-injected rats, the 24-hour organization of the biologic responses is altered. Changes in cir-

cadian rhythms are apparent at an early phase of experimental arthritis in rats and persist thereafter [29–32, 46]. In previous studies we reported significant effects of immune-mediated inflammation on 24-hour variations of ACTH, GH, LH, testosterone, prolactin and TSH release after Freund's adjuvant injection to young adult [24, 25] and old rats [33]. In old rats, 24-hour rhythms of circulating TSH, LH and testosterone became blunted. Aged rats exhibited significantly higher circulating levels of prolactin, and lower levels of GH, TSH, FSH and testosterone [33].

The present results confirm that circulating GH levels decreased in aged rats regardless of the administration of mycobacterial adjuvant. In contrast, thymic concentrations of GH augmented in aged rats and showed 24-hour variations that were unrelated to those found in circulation, thus underlying dissociation between extrathymic and thymic synthesis of the peptide. Indeed, a marginally significant negative correlation occurred between serum and thymic GH levels when the individual pair of values of the whole set of rats was computed.

In view of the fact that circadian changes in aging include reduction of amplitude of rhythms [16–19], it is remarkable that the opposite was found as far as thymic GH levels are concerned. Morphological studies aimed to define the location (thymocytes, thymic epithelial cells, stromal cells) would be helpful to delineate the changes in intrathymic GH occurring during aging. In addition, studies including hypophysectomized or mutant (Snell) animals that lack GH can be useful to further examine this subject. Collectively, the results suggest that mechanisms other than the hypothalamic-pituitary axis may be involved in the regulation of thymic GH.

In old rats, increasing levels of pituitary hormones restore thymic functions [2, 11–13, 47–50]. Among the pituitary hormones involved, GH is relevant. For example, changes in thymic microenvironment produced by GH modulate T-cell differentiation. GH stimulates the secretion of thymulin and conversely, low levels of circulating thymulin parallel hypopituitary states [for references, see 10]. The influence of GH on thymic epithelium is pleiotropic: GH enhances in vivo the expression of cytokeratins, stimulates in vitro proliferation and enhances extracellular matrix-mediated thymic epithelial cells/thymocyte interactions [10]. Receptors for GH are detectable on both thymocytes and thymic epithelial cells [51] and several studies indicate that both thymocytes and thymic epithelial cells produce GH [10]. Indeed, the results strongly indicate that the thymus is physiologically under control of both intra- and extramural GH [4].

In the present study, the age-related decrease of circulating GH coexisted with an augmented thymic concentration of the hormone. Moreover, a negative correlation was found between serum and thymic GH. To what extent intramural GH-producing thymic cells are negatively influenced by circulating GH levels awaits further examination.

As far as ACTH is concerned, proopiomelanocortin-related molecules and cytokines occurred in thymic cells from 4-day-old chicken, and their number increased with age [52]. Gap junction-mediated cell coupling among thymic epithelial cells showed up-regulation after ACTH exposure [10]. Our present results indicate that significant levels of ACTH are present in the thymus, and that these levels increased in aged rats. As in the case of GH, 24-hour changes in thymic ACTH in aged rats tended to be higher in amplitude than in young rats. In contrast to circulating GH levels, serum ACTH in old rats was also augmented. Indeed, a positive correlation was found between circulating and thymic concentrations of ACTH, suggesting the dependence of thymic ACTH levels from pituitary ACTH secretion.

It must be noted that the one cannot rule out at present whether the positive correlation of serum and thymic ACTH was due to the presence of blood in the thymus, since no attempt was made to remove blood hormone contaminants from tissue. However, the negative correlation between serum and thymic GH could be interpreted to indicate that GH in blood did not significantly contaminate the thymus. Whether changes in hormone receptors, or in the amount of fat in the aged thymus tissue, explain the differential behavior of GH and ACTH concerning their serum/thymus ratios, awaits further investigation. Indeed, thymic tissue of aged rats was much smaller, had a smaller number of cells, and was composed of a substantial amount of fat tissue as compared to young rats.

In the present study, the 24-hour variations in the thymic concentration of several amino acids were measured as an index of thymic metabolism. Indeed, glucose is not the only quantitatively significant energy substrate in lymph cells, since glutamine at near-physiological concentration can be readily utilized by those cells [53]. As shown above, glutamate (the precursor of glutamine), aspartate, taurine and GABA displayed significant 24-hour rhythms in the thymus. The concentration of all the amino acids, except GABA, decreased in the thymus of aged rats. These results should be contrasted with a recent study indicating that aging augmented lymph node concentration of glutamate and taurine [45]. Therefore, aging appears to affect differentially amino acid metabolism in

the thymus and lymph nodes. The lack of age-related decrease in thymus GABA concentration was unexpected, in view of the decrease in GABA-transaminase activity found in the thymus of aged rats [54]. The four amino acids examined correlated positively with thymic ACTH concentration, but not with those of GH.

Summarizing, a possible reciprocal relationship between the pituitary and the thymus appears to occur for GH. The characteristics of this putative, paracrine-autocrine-regulated, thymic GH system and its involvement in the homeostatic mechanisms triggered by aging deserve to be further examined.

Acknowledgments

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