Endocrine, *vol. 25, no. 1, 41–48, October 2004* 0969–711X/04/25:41–48/$25.00

24-Hour Pattern of Circulating Prolactin and Growth Hormone Levels and Submaxillary Lymph Node Immune Responses in Growing Male Rats Subjected to Social Isolation

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To assess the effect of social isolation of growing rats on 24-h rhythmicity of circulating prolactin and growth hormone (GH) levels and submaxillary lymph node immune responses, male Wistar rats were either individually caged or kept in groups (4–5 animals per cage) for 30 d starting on d 35 of life. Plasma prolactin and GH levels, and submaxillary lymph node lymphocyte subset populations, interferon (IFN)-γ release and mitogenic responses to concanavalin A (Con A) and lipopolysaccharide (LPS) were determined at six time intervals during the 24 h span. Social isolation brought about changes in mean values and 24-h pattern of plasma prolactin and GH levels and lymph node immune responses. After isolation, prolactin and GH mean values decreased, and lymph node T, B, non T–non B, $CD8^+$, and $CD4^+$–$CD8^+$ cells augmented, whereas lymph node $CD4^+/CD8^+$ ratio, IFN-γ release and mitogenic responses decreased. Social isolation resulted in disruption of 24 h rhythmicity of every immune parameter tested. $CD4^+/CD8^+$ ratio, IFN-γ release and Concanavalin A (Con A) and lipopolysaccharide (LPS) responses correlated significantly with plasma prolactin or GH levels while T/B ratio correlated with plasma prolactin levels only. B, non T–non B, and $CD4^+$–$CD8^+$ cells correlated negatively with plasma prolactin. Modifications in mean value and 24-h rhythmicity of plasma prolactin and GH levels are presumably involved in the effect of social isolation on immune responsiveness.

Key Words: Prolactin; growth hormone; isolation; submaxillary lymph nodes; circadian rhythms; lymphocyte subsets; interferon-γ; concanavalin A; lipopolysaccharide.

Received July 13, 2004; Revised August 13, 2004; Accepted September 17, 2004.

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Introduction

It is well established that hypophysial hormones are important modulators of the immune system. Prolactin and growth hormone (GH) derive from a common ancestral molecule, and show modulatory effects on immune functions in a number of species, including humans *(1,2)*. The administration of GH enhances non-specific humoral defense systems and both prolactin and GH increase phagocytic activity of leukocytes. Production of immunoglobulins is also regulated by prolactin and GH. Prolactin and GH mainly influence the cellular arm of immune defense involving Th 1 cytokines *(3–6)*. On the other hand, significant immune-suppressive effects of prolactin have been noted, several investigators reporting that prolactin decreased natural killer cell migration and activity and reduced lymphocyte proliferative capacity and cytokine release in rodents *(1,2)*.

Social isolation, i.e., individual housing of laboratory animals, is a model for lack of social interactions among animals and to some extent for the situation of humans who feel isolated *(7)*. Several studies indicate that in this situation an immunodeficiency state arises *(8-12)*. In addition, decreases in plasma levels of prolactin occurred in subordinate hamsters after exposure to a social conflict *(13)* and in isolated male hamsters as compared to hamsters with a family *(14)*. In agreement with this, we recently reported decreased levels of plasma prolactin after a 1-mo isolation of growing male rats when assessed at six different time intervals during the 24 h span *(15)*. In view of the very strong link that exists between prolactin and the immune system, we wished to assess to what extent the changes in 24 h pattern of prolactin release correlated with the 24 h pattern of lymphocyte subset populations, interferon (IFN)-γ release, and lymphocyte mitogenic responses in submaxillary lymph nodes after social isolation in growing male rats. Plasma levels of growth hormone (GH) were also measured.

Results

Circulating prolactin and GH levels in group-caged and isolated rats are depicted in Fig. 1. Social isolation signifi-

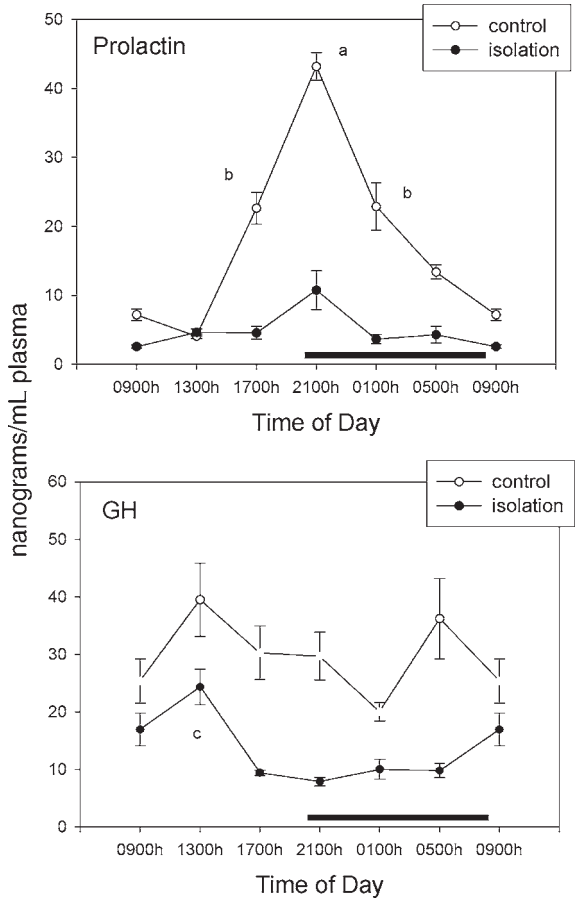

Fig. 1. Effect of isolation on 24-h changes in plasma prolactin and GH levels. Groups of six to eight rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Tukey–Kramer's multiple comparisons test, as follows: [a]$p < 0.01$ vs all time points; [b]$p < 0.01$ vs 0900, 1300, and 1500 h; [c]$p < 0.01$ vs 0100, 0500, 1700, and 2100 h. For further statistical analysis, see text.

cantly decreased prolactin and GH levels ($F_{1,77} = 279.1$ and 72.4, $p < 0.00001$, factorial analysis of variance, ANOVA) and distorted their 24 h pattern, with abolition of the prolactin peak ($F_{5,77} = 33.4$, $p < 0.00001$ for the interaction "time × manipulation" in the factorial ANOVA) and appearance of a GH maximum at the first half of the rest span (Fig. 1).

Figure 2 shows the changes in T, B, and non T–non B lymphocytes in submaxillary lymph nodes, and in T/B ratio, along the 24 h span. Social isolation augmented significantly T, B, and non T–non B cells, as indicated by the analysis of main factors in a factorial ANOVA ($F_{1,76} = 36.1$, 11.9 and 165.9, $p < 0.00001$, respectively). Significant interactions "time × grouping of animals" were detectable for T and non T–non B cells, and T/B ratio ($F_{5,76} = 7.3$, 7.6 and 4.3, respectively, $p < 0.002$). Specifically, the maximum in T cells observed at the second half of the activity phase was no longer found in isolated rats, while two rather than one maximum was apparent in non T–non B cells of isolated rats. A maximum of B cells at the beginning of resting phase was found only in isolated rats (Fig. 2).

Percentage of $CD4^+$, $CD8^+$, and $CD4^+$–$CD8^+$cells, and the ratio between $CD4^+$ and $CD8^+$ cells, are depicted in Fig. 3. Analyzed as a main factor in a factorial ANOVA, isolation augmented $CD8^+$ and $CD4^+$–$CD8^+$ cells ($F_{1,76} = 33.3$ and 40.7, $p < 0.00001$) and decreased $CD4^+/CD8^+$ ratio ($F_{1,76} = 74.8$, $p < 0.00001$). Significant interactions "time × grouping of animals" were detected in every case, isolated rats exhibiting two rather than one maximum of $CD4^+$ cells, one rather than two maxima of $CD8^+$ cells, a phase delay in maximum of $CD4^+$–$CD8^+$ cells, and suppression of daily maximum in $CD4^+/CD8^+$ ratio ($F_{5,76} = 10.9$, 9.7, 11.9 and 6.3, respectively, $p < 0.001$).

Figure 4 shows the vitro IFN-γ production by submaxillary lymph node cells in the two groups of rats. Isolation brought about a significant decrease of IFN-γ release ($F_{1,78} = 248.4$, $p < 0.00001$, factorial ANOVA) and modified significantly its 24-h pattern of release, i.e., the relatively high nocturnal concentration found in controls as compared to their diurnal values was no longer detectable in isolated rats ($F_{5,78} = 4.1$, $p < 0.01$ for the interaction "time × grouping of animals", factorial ANOVA).

Figure 5 depicts the 24-h changes in mitogenic responses to Con A and LPS of submaxillary lymph node from isolated and group-caged rats. A factorial ANOVA indicated significant decreases in mitogenic responses of lymph cells of isolated rats ($F_{1,75} = 120.4$ and 11.7, $p < 0.00001$, for Con A and LPS, respectively) as well as significant interactions between time of day and grouping of animals, i.e., isolated rats exhibited two maxima in mitogenic response to Con A, at the beginning of the resting and activity phases rather than the single maximum found in group-caged controls ($F_{5,75} = 7.4$, $p < 0.0001$) and a phase advance of about 4 h in the case of LPS response ($F_{5,75} = 15.9$, $p < 0.00001$).

As summarized in Table 1, significant positive correlations of prolactin with T/B ratio, $CD4^+/CD8^+$ ratio, IFN-γ release, and Con A and LPS mitogenic responses and significant negative correlations of prolactin with B cells, non T–non B cells and $CD4^+$–$CD8^+$ cells, were detected. GH levels correlated positively with $CD4^+/CD8^+$ ratio, IFN-γ release, and Con A and LPS mitogenic responses.

Discussion

Intermittent maternal separation and social isolation of rats during early postnatal life have been shown to induce profound and irreversible alterations in neuroendocrine and behavioral mechanisms *(16)*. Less is known about social isolation at a later stage of postnatal development, e.g., at the prepubertal age. Moreover, no study has addressed the question as to what extent the 24 h organization of the immune response is affected in individually housed growing rats. The foregoing results indicate that social isolation of prepu-

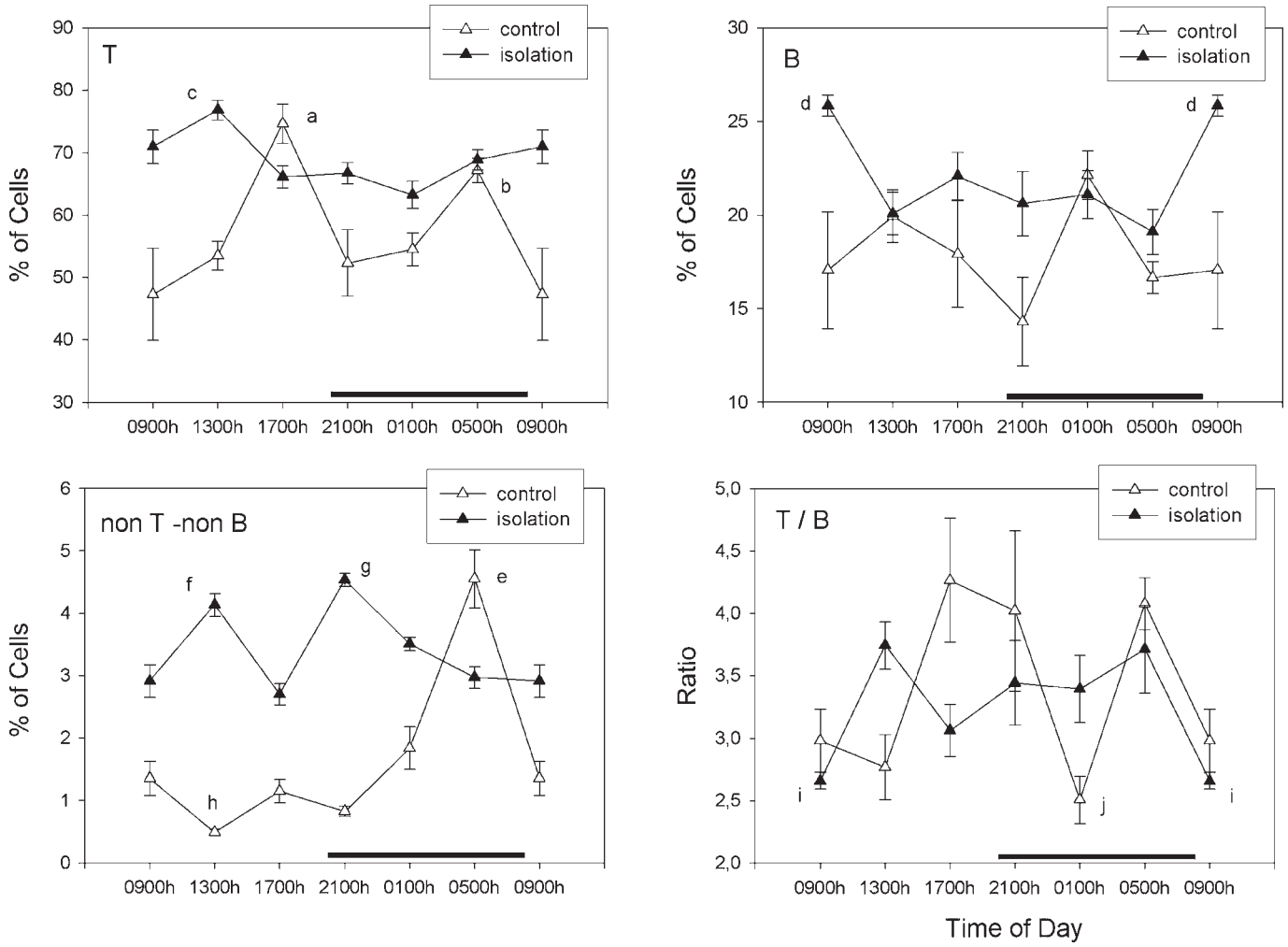

Fig. 2. Effect of isolation on 24-h changes of T, B, and non T–non B lymphocytes, and T/B ratio, in rat submaxillary lymph nodes. Groups of six to eight rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Tukey–Kramer's multiple comparisons test, as follows: [a]$p < 0.01$ vs 0100, 0900, 1300, and 2100 h; [b]$p < 0.01$ vs 0900 h; [c]$p < 0.01$ vs 1700 h; [d]$p < 0.01$ vs 0500 h; [e]$p < 0.01$ vs all time points; [f]$p < 0.01$ vs 0500, 0900, and 1700 h; [g]$p < 0.01$ vs 0500, 0900, 1700, and 2100 h. For further statistical analysis, see text.

bertal male rats brought about changes in mean values and 24-h pattern of plasma prolactin and GH levels and lymph node immune responses. After 30 d of social isolation, decreases in plasma prolactin and GH levels and distortion of their 24 h rhythmicity were detected. Lymph node T, B, non T–non B, $CD8^+$, and $CD4^+$–$CD8^+$ cells were augmented, and lymph node $CD4^+/CD8^+$ ratio, IFN-γ release and mitogenic responses decreased in isolated rats. Social isolation disrupted 24 h rhythmicity of every lymph node immune parameter tested. Plasma prolactin and GH levels correlated positively with $CD4^+/CD8^+$ ratio, IFN-γ release and Con A and LPS mitogenic response. T/B ratio correlated positively, and B, non T–non B cells and $CD4^+$–$CD8^+$ cells correlated negatively, with plasma prolactin.

Available information indicates that psychosocial events affect primarily T lymphocytes, and less B lymphocytes *(8–11)*. For example, social isolation of pigs in the first days of life caused a significant decrease in immune reactivity at d 12, as assessed by T-cell mitogenesis (Con A response) without affecting B-lymphocyte proliferation induced by LPS *(12)*. In the present study on social isolation at a later age in growing rats, both T and B cell activity (evaluated by the mitogenic responses to Con A and LPS) decreased in face of augmentation of T and B cell number. The increase in T cells appeared to be due to a shift in the $CD4^+$ (T helper) to $CD8^+$ (T suppressor/cytotoxic) lymphocyte ratio as demonstrated by the augmented number of $CD8^+$ cells and decreased $CD4^+/CD8^+$ ratio seen in isolated rats. Overall the increase in T or B cell number did not resulted in higher but rather lower mitogenic responses nor in the extent of IFN-γ release, which was very low in isolated rats. Further studies are needed to disclose the reasons for this discrepancy.

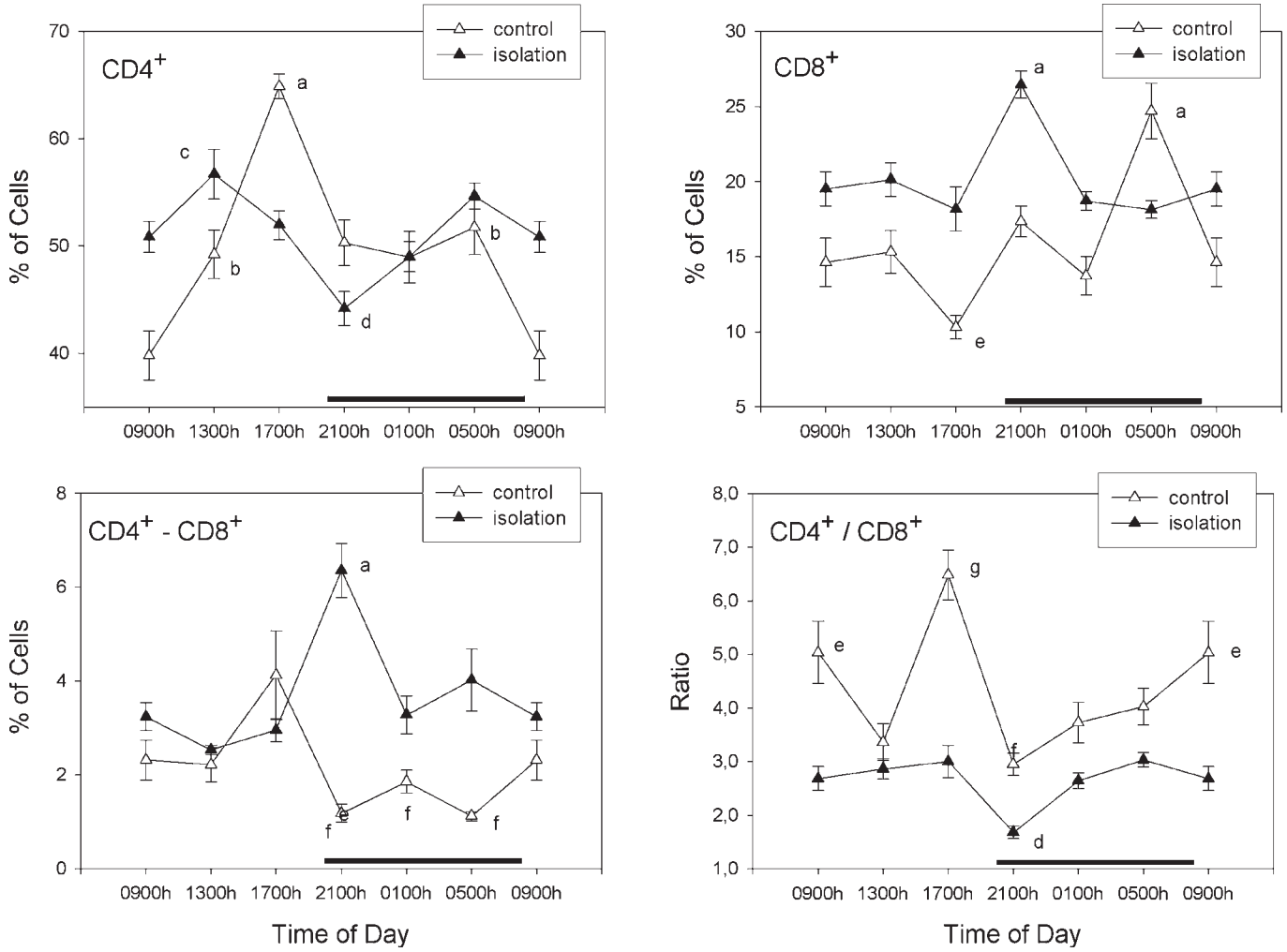

Fig. 3. Effect of isolation on 24-h changes of $CD4^+$, $CD8^+$, and $CD4^+$–$CD8^+$ cells, and $CD4^+/CD8^+$ in rat submaxillary lymph nodes. Groups of six to eight rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Tukey–Kramer's multiple comparisons test, as follows: $^{a}p < 0.01$ vs all time points; $^{b}p < 0.05$ vs 0900 h; $^{c}p < 0.01$ vs 0100 and 2100 h; $^{d}p < 0.01$ vs 0500, 1300, and 1700 h; $^{e}p < 0.01$ vs 2100 h; $^{f}p < 0.01$ vs 1700 h; $^{g}p < 0.01$ vs 0100, 0500, 1300, and 2100 h. For further statistical analysis, see text.

It is of interest to compare the present results in isolated growing rats with a recent study on the effect of social isolation/individual housing of adult mice on behavioral and immune responses *(17)*. The authors examined whether increasing duration of individual housing would result in immunoendocrine dysfunction. Then they assessed whether housing condition would affect the reaction to an acute mild psychological stress. The only difference detected between individually housed adult mice and those grouped was a reduction in cell proliferation and in the production of the Th 1 cytokine interleukin (IL)-2, thus resembling the decrease in Con A and LPS response and IFN-γ release (another Th 1 cytokine) found in the present study. When individually housed mice were exposed to a mild psychological stress like a forced exposure to a novel environment, they showed a lesser type 1 (IL-2) and type 2 (IL-4) cytokine production and splenocyte proliferation than grouped male mice *(17)*. Individually housed mice are more susceptible than group housed mice to experimentally induced illness such as tumors *(18–22)* and virus infection *(23)*.

It is of importance to note that a clinically relevant circadian component is the T helper 1 (Th1)/T helper 2 (Th2) balance. Both branches support different functions of defense. Th1 responses include cell-mediated reactions that are important for cellular pathogens, whereas Th2 responses regulate production of antibodies in response to extracellular pathogens and mediate allergic processes. Moreover, effects of IFN-γ, a major Th1 cytokine, and IL-4, a major Th2 cytokine, are antagonistic.

Petrovsky and Harrison *(24)* demonstrated that the early period of nocturnal sleep in humans is characterized by a substantial shift in this balance toward predominant Th1

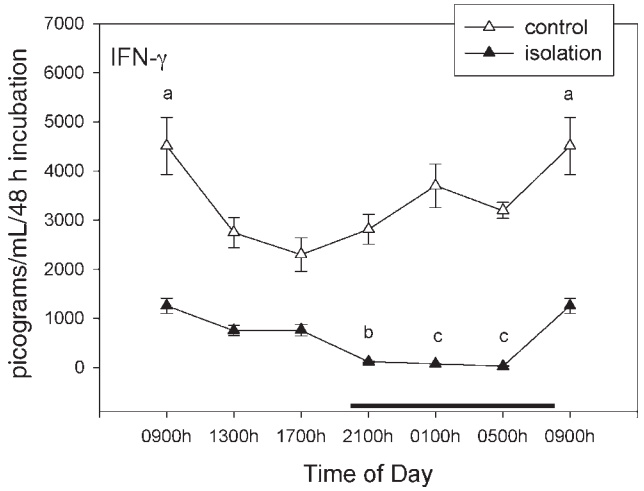

Fig. 4. Effect of isolation on 24-h changes in IFN-γ release from rat submaxillary lymph nodes. Groups of six to eight rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Tukey–Kramer's multiple comparisons test, as follows: [a]$p < 0.01$ vs 2300, 1700, and 2100 h; [b]$p < 0.01$ vs 0900 h; [c]$p < 0.01$ vs 0900, 1300, and 1700 h. For further statistical analysis, see text.

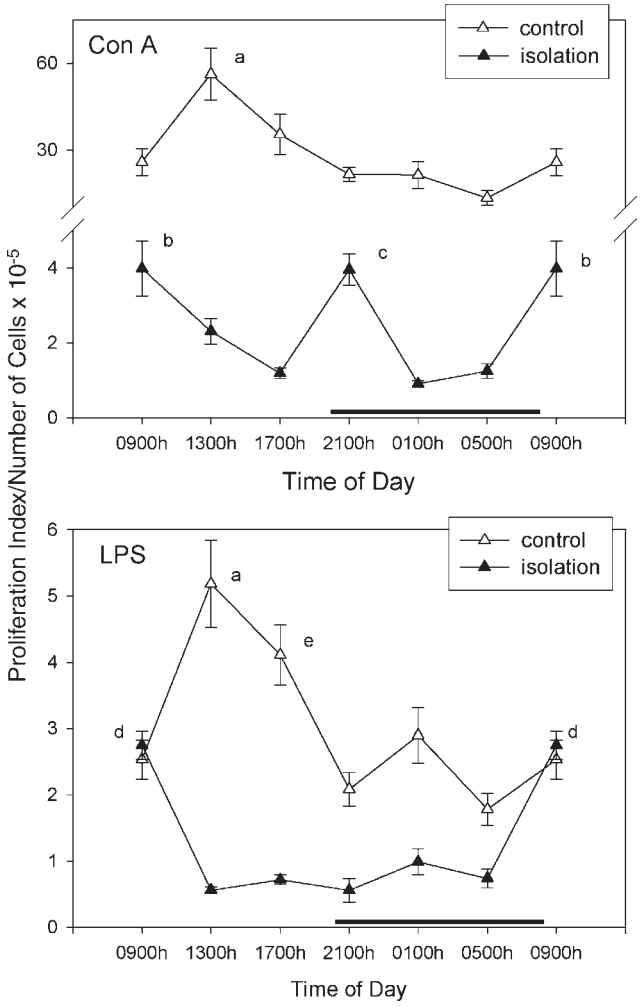

Fig. 5. Effect of isolation on 24-h changes in mitogenic responses to Con A and LPS in rat submaxillary lymph node cells. Groups of six to eight rats were killed by decapitation at six different time intervals throughout a 24 h cycle. Bar indicates scotophase duration. Shown are the means ± SEM. Letters indicate the existence of significant differences between time points within each group after a Tukey–Kramer's multiple comparisons test, as follows: [a]$p < 0.01$ vs 0100, 0500, 0900, and 2100 h; [b]$p < 0.01$ vs 0100, 0500, 1300, and 1700 h; [c]$p < 0.01$ vs 0100, 0500, and 1700 h; [d]$p < 0.01$ vs all time points. For further statistical analysis, see text.

cytokines. The early part of sleep, in which slow wave sleep predominates, itself can induce a significant shift toward enhanced Th1 cytokine activity *(25)*. The shift disappeared and was even replaced by an opposite shift toward Th2 cytokine activity during late sleep, which is dominated by rapid eye movement (REM) sleep and contains only negligible amounts of non-REM sleep.

Data from different groups showed that prolactin and GH specifically influence the cellular arm of immune defense involving Th1 cytokines *(3–6)*. This was uncovered in experiments on the effect of prolactin and anti-prolactin antibody in vitro that indicated a stimulating effect of the hormone on IFN-γ, IL-2, and tumor-necrosis factor (TNF)-α producing cells. Differential actions of prolactin and GH on lymphocyte functions occur. In mice, GH affects development of T cells particularly within the thymus, whereas prolactin augments peripheral T cell responses including helper function in antibody production *(26)*. The Th 1 enhancing effect of GH, although somewhat weaker, resembled that of prolactin, which is not unexpected as both hormones act via receptors of the same cytokine/hemopoietin receptor superfamily, sharing binding affinity and a similar intracellular protein cascade during transduction *(1,2)*.

The present results indicate a very strong correlation of plasma prolactin and GH levels with Th 1 response indicators like mitogenic activity, $CD4^+/CD8^+$ ratio and IFN-γ release. Overall, they suggest the possibility that social isolation affects the mechanisms that modulate immune responsiveness in part via modification of plasma prolactin and GH levels.

In rodents, social isolation resulted in significant physiological disturbances, including high levels of corticosterone and catecholamine in the serum, high levels of corticotrophin-releasing hormone, and behavioral disturbances such as increased aggressiveness and reduced pentobarbital-induced sleeping time *(27–32)*. However, in certain situations socially isolated animals exhibited a decrease in the electrical activity of neurons within the hypothalamus and had lower basal plasma corticosterone levels than did animals raised in social conditions *(27)*, suggesting less psychosocial stress in isolation. Further studies are needed to address the question as to what extent the hypophysial–adrenal axis is affected in a situation of social isolation like that described herein.

Table 1
Correlation of Plasma Prolactin and GH Levels and Immune Parameters in Submaxillary Lymph Nodes[a]

Dependent variable	Mathematical function	r^2	d.f.	F	p	b_0	b_1
			Prolactin				
Con A response	log	0.084	76	6.2	0.015	3.8566	6.0156
LPS response	exponential	0.078	76	5.67	0.02	1.2778	0.0184
B cells	linear	0.154	76	12.2	0.001	21.6720	–0.1544
non T–non B cells	linear	0.178	76	14.5	0.001	3.0906	–0.0495
T/B ratio	linear	0.099	76	7.4	0.008	3.0735	0.0228
$CD8^+$ cells	linear	0.058	76	4.0	0.047	18.7739	–0.0935
$CD4^+$–$CD8^+$ cells	linear	0.057	76	4.0	0.048	3.0810	–0.0292
$CD4^+/CD8^+$ ratio	log	0.105	76	7.8	0.007	2.5688	0.4803
IFN-γ	log	0.240	76	21.2	0.001	221.683	844.804
			GH				
Con A response	log	0.288	78	27.53	0.001	–32.941	16.7020
LPS response	log	0.228	78	20.11	0.001	–1.5221	1.2473
non T–non B cells	log	0.083	78	6.17	0.015	4.4952	–0.6800
$CD4^+$–$CD8^+$ cells	log	0.228	78	20.03	0.001	6.2088	–1.1809
$CD4^+/CD8^+$ ratio	log	0.137	78	10.83	0.002	1.1379	0.8241
IFN-γ	log	0.404	78	46.01	0.001	–2926.5	1655.46

[a]For experimental details see text.
d.f.: degrees of freedom.

Temporal organization is an important feature of the biological systems and its main function is to facilitate adaptation of the organism to the environment *(33)*. The daily variation of biological variables arises from an internal timekeeping system and the major action of the environment is to synchronize this internal clock to a period of exactly 24 h. Among several environmental synchronizers, social cues have been identified in rats *(33,34)*. Social isolation is capable of perturbing temporal organization by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself. In particular, social disruption in rodents has been found to alter body temperature, heart rate, and locomotor activity rhythms *(35–37)*. The present results demonstrate that social isolation of growing prepubertal rats causes concomitant effects on the mechanisms regulating the 24 h pattern of prolactin and GH release and that of immune reactivity in lymph nodes. Although the data do not allow definition of causal relationships between neuroendocrine and immune regulation, they strongly suggest that social isolation may use a common immune–brain circuitry. Further experiments are needed to assess whether the changes in amplitude as well in timing of 24-h rhythms seen in socially isolated rats can attributed either to an effect on the endogenous clock that modulates this circadian variation or to a masking effect on some output(s) of the clock.

Materials and Methods

Chemicals

Thymidine [methyl-^{3}H] (specific activity 20 Ci/mmol) was purchased from NEN Research Products, Boston, MA, USA. O-Phthalaldehyde (OPA), 2-mercaptoethanol, and amino acid standards were purchased from Sigma Chemical Co., St. Louis, MO, USA. Double-distilled deionized water was used for preparation of solutions and buffers.

Animals and Experimental Design

Thirty-five-day-old male Wistar rats were kept under standard conditions of controlled light (12:12 h light/dark schedule; lights on at 0800 h) and temperature (22 ± 2°C). In our colony, age of animals at weaning was 22–24 d. All experiments were conducted in middle spring (May). Rats were either put in individual cages (isolated group) or left in cages of four to five animals each in the same animal room. All animals had free access to food and water for the 30 d of the study. The experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science (ICLAS).

Groups of six to eight rats were killed by decapitation under conditions of minimal stress, at six different time intervals, every 4 h, throughout a 24-h cycle starting 1 h after lights on, at 0900 h. At night intervals, animals were killed under red dim light. Blood was collected from the trunk wound in heparinized tubes and was centrifuged at 1500*g* for 15 min. The plasma was collected and stored at –20°C. The submaxillary lymph nodes were removed aseptically from both sides, weighed, and placed in Petri disks containing balanced salt solution for further processing.

Hormone Assays

Plasma prolactin and GH levels were measured by a homologous specific double antibody RIA *(38)*, using materials

kindly supplied by the NIDDK's National Hormone and Pituitary Program. The intra- and interassay coefficients were 6–8%. Sensitivity of the RIAs was 0.04 ng/mL using the NIDDK rat prolactin RP-3 and r-GH-RP-2 standards. Results were expressed as ng/mL plasma.

Lymphocyte Subsets

The relative size distributions of lymph cells in submaxillary lymph nodes of rats were determined by FACS analysis, as previously described *(39)*. For these studies, we used the following monoclonal antibodies: Anti-rat LCA (OX-33) for B lymphocytes (Serotec, Oxford, UK), anti-rat TCR alpha/beta (R7.3) for T lymphocytes (Serotec, Oxford, UK), anti-rat CD4 (OX-35), which recognize a rat T helper cell differentiation antigen (Pharmingen, San Diego, CA, USA), and anti-rat CD8a (OX-8), which recognize the reactive antigen expressed on rat T cytotoxic/suppressor cells (Pharmingen, San Diego, CA, USA). Lymphocytes from submaxillary lymph nodes isolated as indicated above, were washed in cold PBS with 0.02% sodium azide and then incubated (3×10^5 cells/tube) with appropriate primary antibodies for 30 min at 4°C. Following two washes, the cells were incubated with 1 mL of PBS–BSA 1%, during 5 min at 4°C, washed three times, resuspended in 1% paraformaldehyde in PBS. Fluorescence intensity was analyzed by FACs (FACStarplus; Beckton Dickinson, Mountain View, CA). Dead cells were excluded by gating with propidium iodide.

IFN-γ Release

Submaxillary lymph node cells were suspended in sterile supplemented medium (RPMI 1640), containing 10% heat-inactivated, fetal bovine serum, 20 m*M* L-glutamine, 0.02 m*M* 2-mercaptoethanol, and gentamicin (50 mg/mL). Cells (10^5/100 µL) were incubated for 24 h, their media removed, and, after adding fresh media including all the components, they were incubated for 24 h more. Both media were collected and pooled for IFN-γ measurement. The incubations were performed in triplicates. IFN-γ concentration in media culture was measured after centrifugation to remove adherent cells. An ELISA commercial kit from Endogen (Woburn, MA, USA) previously validated in our laboratory was employed *(40)*. The assay was as follows: 100 µL of standards or unknown samples were added to each antibody-coated well, and the plates were incubated overnight at room temperature. The reaction was stopped by washing thrice with wash buffer (2% Tween 20 in 50 m*M* Tris, pH 3.6). The wells were incubated with 100 µL of biotinylated detecting antibody at the titer previously tested. After 1 h at room temperature the reaction was stopped by washing thrice with wash buffer. One hundred microliters of streptavidin–HRP solution (in Dulbecco's phosphate-buffered saline, pH 7.4) was then added and the samples were incubated for 30 min. The reaction was stopped by adding 100 µL of 0.18 *M* sulfuric acid. The plates were read within 30 min in an ELISA reader set at 450 nm and 550 nm. Values were obtained by subtracting the reading at 550 nm from the reading at 450 nm, to correct for any optical defect of microtiter plate. IFN-γ release was expressed as pg/mL/48 h incubation. Sensitivity of the assay was 100 pg/mL.

Mitogen Assays

Submaxillary lymph node cells were gently teased apart. After removing the clumps by centrifugation, the cells were suspended in sterile supplemented medium (RPMI 1640), containing 10% heat-inactivated, fetal bovine serum, 20 m*M* L-glutamine, 0.02 m*M* 2-mercaptoethanol, and gentamicin (50 mg/mL), and were counted. Mitogen assays were performed as described in detail elsewhere *(41)*. Submaxillary lymph node cells were used at a final number of cells/well (0.1 mL) of 5×10^5. Control and experimental cultures were run in triplicate. Mitogens (Con A or LPS) were added to the cultures at final supramaximal concentrations of 5 µg/mL. The cultures were incubated in a humidified 37°C incubator in an atmosphere of 5% CO_2. After 48 h incubation, ^{3}H-thymidine (0.2 µCi) was added to each well in a volume of 0.02 mL. Cells were harvested 5 h later using an automated sample harvester, and the filters were counted in a liquid scintillation spectrometer. The proliferation index was estimated as the ratio between stimulation in the presence of mitogens/controls. Results were expressed as proliferation index/number of cells.

Statistical Analysis

Statistical analysis of results was performed by a two-way factorial ANOVA. Generally, the analysis included assessment of the group effect (i.e., the occurrence of differences in mean values between isolated and control rats), of time-of-day effects (the occurrence of daily changes), and of the interaction between the two factors (grouping of animals and time, from which inference about differences in timing and amplitude could be obtained). *F* values together with their respective degrees of freedom as subscripts are shown. Post-hoc Tukey–Kramer's multiple comparisons tests were then employed to show which time points were significantly different within each experimental group to define existence of peaks. Curve estimation in regression analysis was made by using SPSS software, version 10.1 (SPSS Inc., Chicago, ILL, USA). *p* values lower than 0.05 were considered evidence for statistical significance.

Acknowledgments

This work was supported by grants from DGES, Spain, Fundación Rodríguez-Pascual, Spain, Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 6153) and Fundación Bunge y Born, Argentina. DPC is a Research Career Awardee from the Argentine Research Council (CONICET).

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