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Effect of social isolation on 24-h pattern of stress hormones and leptin in rats

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Abstract

This work analyzes the effect of social isolation of growing male rats on 24-h changes of plasma prolactin, growth hormone, ACTH and leptin, and on plasma and adrenal corticosterone concentrations. At 35 days of life, rats were either individually caged or kept in groups (6–8 animals per cage) under a 12:12 h light/dark schedule (lights on at 08:00 h). A significant arrest of body weight gain regardless of unchanged daily food intake was found in isolated rats after 2 weeks of isolation. On the 4th week, rats were killed at 6 time intervals during a 24-h cycle, beginning at 09:00 h. In isolated rats the 24-h pattern of all parameters tested became distorted, as assessed by Cosinor analysis. When analyzed as a main factor in a factorial analysis of variance, isolation decreased plasma prolactin and growth hormone, increased plasma leptin and corticosterone while decreased adrenal corticosterone. Plasma corticosterone levels correlated significantly with plasma ACTH and with adrenal corticosterone levels in group-caged rats only. These changes can be attributed to an effect of mild stress on the endogenous clock that modulates the circadian hormone release.

Keywords: Social isolation; Stress; Prolactin; GH; ACTH; Glucocorticoid; Leptin; Chronobiology

Introduction

Social isolation of laboratory animals is a model for the lack of social interactions among animals and to some extent, among humans (House, 2001). The most profound change that occurs with individual housing is an increase in aggression of males seen in rodents following even relatively brief periods of isolation (Brain, 1975; Baurmel et al., 1978). This indicates that isolation is a mild stress for rats. Individually housed animals are also hyperresponsive to stressors. For example, after 8 weeks of isolation, single-housed rats continued to spend more time apparently attempting to escape (sniffing and chewing at the bars and suddenly dashing around their cage) while those housed in groups spent more time sleeping and feeding (Hurst et al., 1999).

It is notable, however, that regardless of the stress produced, social isolated male hamsters (Matt et al., 1983) or rats (Esquifino et al., 2004) exhibited a decrease in plasma prolactin, a hormone whose release is triggered by stress. Moreover, in other studies, individual housing of male mice decreased plasma glucocorticoid levels and adrenaline turnover in the adrenal glands, these hormonal changes again being indicative of a decreased stress (Brain, 1975; Sayegh et al., 1990).

Taking into account the above considerations and considering that stress-related hormones are secreted in a circadian pattern (Esquifino et al., 1999; Garcia Bonacho et al., 2000; Castrillón et al., 2001), we undertook the present study to assess whether social isolation affects the mean levels and 24-h variations of plasma prolactin, growth hormone (GH), ACTH, corticosterone and leptin. Corticosterone concentration in adrenal gland was also measured. Specifically, we sought to answer whether or not individual housing could be a stressor for rats.

Materials and methods

Animals and experimental design

Five-week-old male Wistar were maintained under standard conditions with controlled light (12:12 h light/dark schedule;

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lights on at 08:00 h) and temperature (22±2 °C). Animals were divided into two groups: individually caged (isolated; ISO) and group-housed (6–8 rats per cage, controls; CTR); they were left undisturbed for a 30-day period with free access to food and water. At 65 days of life, the rats were sacrificed by decapitation under conditions of minimal stress at 6 different time intervals (6–8 rats per group), every 4 h throughout a 24-h cycle, starting at 09:00 h. All experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science (ICLAS). Trunk blood was collected and plasma samples were obtained by centrifugation of blood at 1500×*g* for 15 min and were stored at −20 °C until further analysis. Immediately after sacrifice, the adrenal glands were dissected out free of adipose tissue, and were collected in 0.1 M acetic acid for further measurement of corticosterone content.

Hormone measurements

Plasma prolactin, GH and ACTH levels were measured by a homologous specific double antibody radioimmunoassay (RIA), using materials kindly supplied by the NIDDK's National Hormone and Pituitary Program and by Dr. A. Parlow (Harbor UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90509, USA). The intra- and inter-assay coefficients of variation were 6–8% and 11–13%, respectively. Sensitivities of the RIAs were 50, 45 and 40 pg/ml, for prolactin, GH and ACTH, respectively, using the NIDDK rat prolactin RP-3, rat GH-RP-2 and rat-ACTH-RP-1, respectively (Esquifino et al., 1999; Garcia Bonacho et al., 2000; Castrillón et al., 2001).

Plasma and adrenal concentrations of corticosterone were evaluated by a specific RIA reported elsewhere (Spinedi et al., 1991) with a standard curve between 1 and 250 μg/dl and intra- and inter-assay coefficients of variation of 4–6% and 8–10%, respectively. Leptin levels were measured by a specific RIA developed in one of our laboratories (Giovambattista et al., 2000), the standard curve ranging between 0.4 and 50 ng/ml with intra- and inter-assay coefficients of variation of 5–8% and 10–13%, respectively.

Statistical analysis

Statistical analysis of results was performed by a Student's *t* test, a one-way analysis of variance (ANOVA), a two-way factorial ANOVA or a Cosinor, as stated. For the factorial ANOVA, the analysis included assessment of the group effect (i.e. the occurrence of differences in mean values between ISO and CTR rats), of time-of-day effects (the occurrence of daily changes) and of the interaction between the two factors (manipulation and time, from which inference about differences in timing and amplitude could be obtained). Post-hoc Tukey–Kramer's multiple comparisons tests in a one way ANOVA were then employed to show which time points were significantly different within each experimental group to define existence of peaks. Cosinor analysis was used to analyze general rhythmic parameters, i.e., acrophase (the maximum of the cosine function fit to the experimental data), mesor (the statistical estimate of the 24-h time series mean) and amplitude (half the difference between maximal and minimal values of the derived cosine curve). Percent of rhythm defined the part of variation that could be explained by a cosine function. Statistical analysis of Cosinor parameters was carried out by standard procedures (Nelson et al., 1979). Statistical significance of the derived cosine curves was tested against the null hypothesis (i.e., amplitude=0). Curve estimation in regression analysis was made by using SPSS software, version 10.1 (SPSS Inc., Chicago, IL, USA). *P* values lower than 0.05 were considered evidence for statistical significance.

Results

The effect of social isolation on daily secretory pattern of stress-related pituitary hormones is depicted in Fig. 1. Cosinor analysis of the data is summarized in Table 1.

The upper panel shows the 24-h changes in plasma prolactin in CTR and ISO rats. In a factorial ANOVA both treatment and time were identified as significant main factors ($F_{1,78}=213.1$ and $F_{5,78}=53.1$, $p<0.00001$, respectively), social isolation decreasing significantly prolactin levels. ISO also distorted the 24-h pattern of prolactin release, as shown by a significant interaction "time × manipulation" ($F_{5,78}=33.4$, $p<0.00001$). In CTR rats, the pattern of secretion was defined by trough values at the early light phase (09:00 and 13:00 h) and values that increased (at 17:00 h) to further reach peak values at the first half of the dark phase. In the Cosinor, acrophase was at 21:36 h (Table 1). Circulating prolactin levels decreased towards trough values between 01:00 and 05:00 h. Although ISO rats displayed a similar qualitative pattern of prolactin secretion (acrophase at 20:22 h), the amplitude of rhythm was significantly decreased by social isolation (Fig. 1, upper panel, and Table 1).

Fig. 1 (middle panel) shows daily pattern of GH release in CTR and ISO rats. In the factorial ANOVA, both treatment and time were identified as significant main factors ($F_{1,78}=21.4$, $p<0.001$ and $F_{5,78}=2.9$, $p<0.05$, respectively). ISO decreased significantly GH levels and distorted their 24-h pattern ($F_{5,78}=3.1$, $p<0.02$ for the interaction "time × manipulation" in the factorial ANOVA). In CTR rats, the pattern of secretion of GH was defined by peak values on the first part of the light phase, then decreasing to a plateau which lasted up to 21:00 h; thereafter, a significant decrease in GH circulating concentrations was found at both 01:00 and 05:00 h intervals. In a Cosinor analysis, only 46% of the variability in GH levels of CTR rats could be explained by the cosine function with acrophase at 14:01 h (Table 1), ISO decreased GH peak values and distorted its daily variations (Fig. 2, middle panel). However, the differences in acrophases did not attain statistical significance (Table 1).

Fig. 1 (lower panel) shows the 24-h pattern of ACTH release. In a factorial ANOVA only time of day was identified as a significant factor ($F_{5,78}=2.8$, $p<0.05$). In CTR rats, the pattern found was characterized by low levels during the morning, increasing to peak values at the afternoon (acrophase

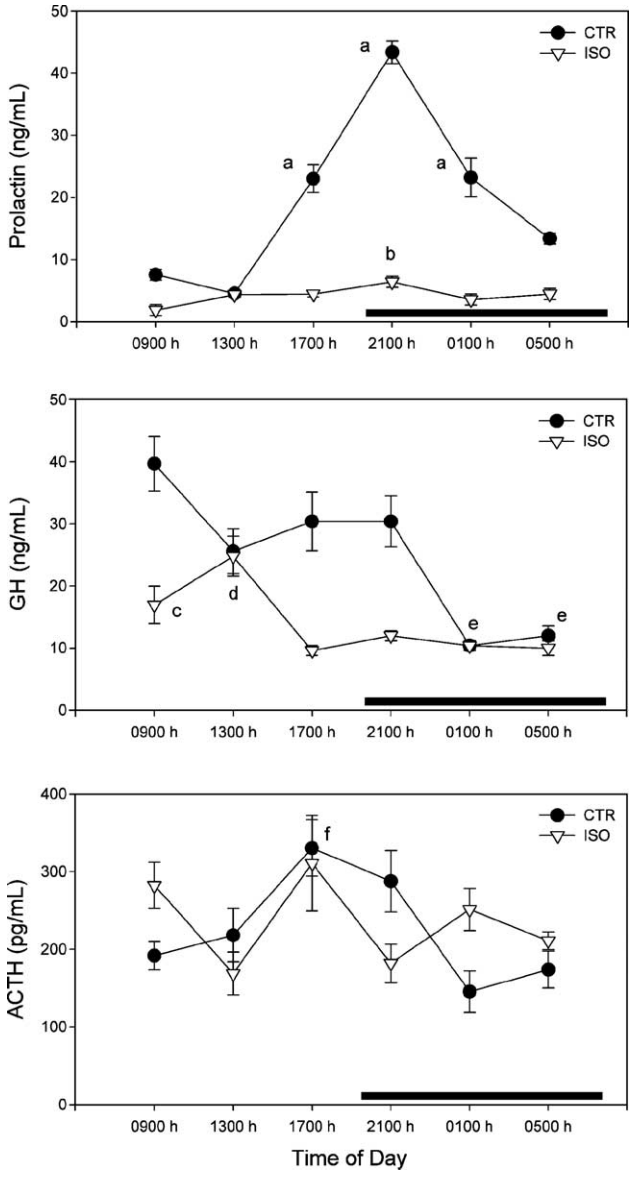

Fig. 1. Effect of isolation (ISO) on 24-h changes in plasma prolactin (upper panel), GH (middle panel) and ACTH (lower panel) concentrations in male rats. Groups of 6–8 rats were killed by decapitation at 6 different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. Letters indicate the existence of significant differences between time points within each group after a one-way ANOVA followed by a Tukey–Kramer's multiple comparisons test, as follows: [a] $p<0.01$ vs. all corresponding time points; [b] $p<0.01$ vs. 09:00 h; [c] $p<0.05$ vs. 13:00, 17:00, 01:00 and 05:00 h; [d] $p<0.05$ vs. 09:00 h, $p<0.01$ vs. the remaining time points; [e] $p<0.01$ vs. 09:00, 13:00, 17:00 and 21:00 h; [f] $p<0.05$ vs. 09:00, 13:00, 01:00 and 05:00 h. For further statistical analysis, see text.

at 17:19 h, as indicated by Cosinor analysis, Table 1), then followed by low levels during the whole dark phase. A one-way ANOVA indicated that time of day variations of ACTH in ISO rats were not statistically significant (Fig. 1, lower panel).

The effect of social isolation on the 24-h pattern of plasma and adrenal gland corticosterone levels is depicted in Fig. 2 (upper and middle panels). ISO enhanced adrenal glucocorticoid output, as shown by the analysis of plasma corticosterone as a main factor in the factorial ANOVA ($F_{1,78}=50{,}9$, $p<0.00001$; Fig. 2, upper panel) as well as by the significant difference in mesor after a Cosinor analysis (Table 1). Time of day changes were also significant in the factorial ANOVA ($F_{5,78}=4.4$, $p<0.002$). The characteristic pattern of CTR rats consisted in low plasma corticosterone values at the beginning of the light phase (09:00–13:00 h), and increases in corticosterone levels thereafter to attain peak values at the beginning of the scotophase. Acrophase in Cosinor analysis was at 22:19 h (Table 1). ISO rats displayed high plasma corticosterone levels at all times examined, with a maximum at 01:00 h, falling significantly to lower levels at 05:00 h ($p<0.05$) (Fig. 2, upper panel). These significant 24-h variations could not be explained by a cosine function (Table 1).

The daily pattern of adrenal gland corticosterone concentration of CTR rats mirrored that of blood (Fig. 2, middle panel), with acrophase at 24:00 h (Table 1). When analyzed as a main factor in a factorial ANOVA, ISO significantly ($p<0.00001$) reduced 24-h adrenal gland corticosterone levels ($F_{1,78}=181.1$). A significant interaction "treatment × time" was also uncovered ($F_{5,78}=3.8$, $p<002$), ISO rats losing the daily rhythm of adrenal gland corticosterone (Fig. 2, middle panel). Plasma corticosterone levels correlated significantly in a linear way with plasma ACTH and adrenal gland corticosterone in CTR rats only ($r^2=0.21$, $b^0=10.2$ and $b_1=0.007$, $F=8.5$, $p=0.001$, and $r^2=0.15$, $b^0=12.4$ and $b_1=2.01$, $F=6.5$, $p=0.01$, respectively).

Fig. 2 (lower panel) shows the effect of social isolation on daily secretory pattern of leptin. A factorial ANOVA indicated significant effects of treatment and time of day ($F_{1,78}=181.1$ and 10.4, $p<0.00001$, respectively) as well as a significant interaction "treatment × time", i.e., ISO augmented plasma

Table 1
Cosinor analysis of 24-h changes in plasma prolactin, GH, ACTH, corticosterone and leptin levels, and in adrenal gland corticosterone content, in control (CTR) and isolated (ISO) male rats

	Mesor	Amplitude	Acrophase (h, min)	Percent of rhythm (%)
Plasma prolactin				
CTR	19.2±2.1	16.7±3.3	21:36±01:24	85±4
ISO	4.2±0.5**	1.4±0.3**	20:22±02:05	54±9
Plasma GH				
CTR	24.7±2.1	10.1±2.4	14:01±02:34	46±9
ISO	13.9±3.3**	5.7±2.1*	11:58±02:45	56±7
Plasma ACTH				
CTR	224±23	80.6±11.0	17:19±01:23	77±4
ISO	234±45	n.s.	n.s.	n.s.
Plasma corticosterone				
CTR	20.6±3.2	8.2±1.1	22:19±02:45	92±4
ISO	36.3±4.5**	–	–	–
Adrenal corticosterone				
CTR	4.6±1.2	1.2±0.3	24:00±02:21	59±6
ISO	1.5±0.4**	n.s.	n.s.	n.s.
Plasma leptin				
CTR	3.7±0.4	2.3±0.4	04:12±00:56	76±10
ISO	9.2±2.1**	2.3±0.3	00:13±01:02*	66±8

Shown are the means ± S.E.M. ($n=6–8$/group). Mesor and amplitude values are expressed as ng/ml plasma or ng/mg adrenal gland. Percent of rhythm defines the part of variation that could be explained by a cosine function in Cosinor. Asterisks designate significant differences (*$p<0.05$, **$p<0.01$) as compared to the respective CTR, Student's t test. n.s. = not significant daily changes in a one-way ANOVA; (–) = not significant changes in Cosinor.

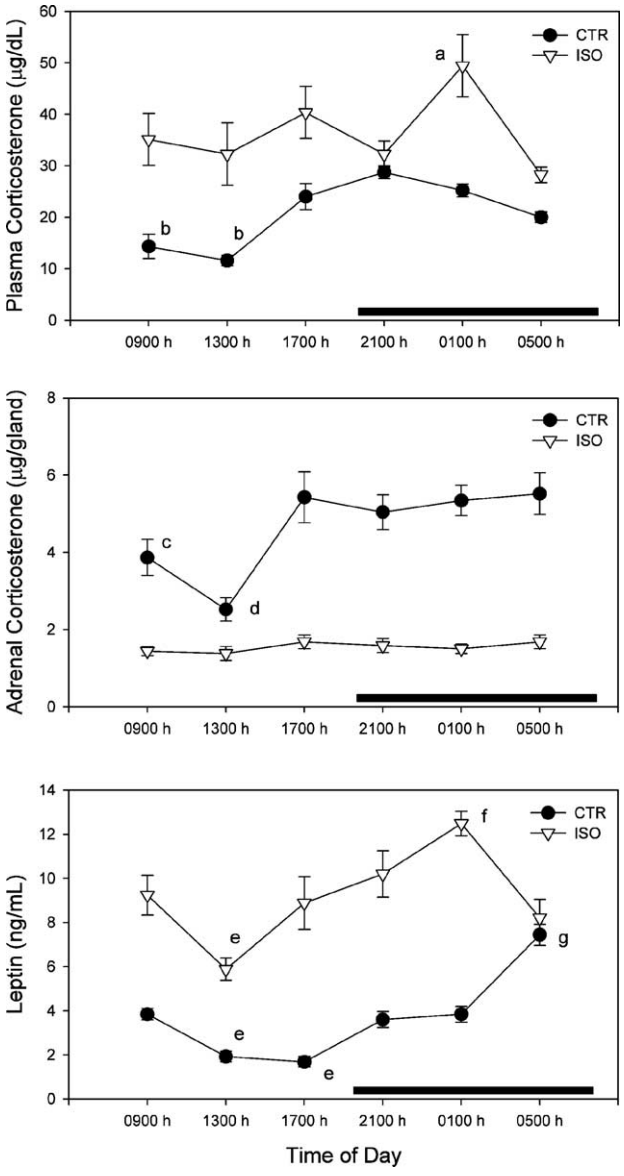

Fig. 2. Effect of isolation (ISO) on 24-h changes in plasma corticosterone levels (upper panel), adrenal gland corticosterone content (middle panel) and plasma leptin levels (lower panel) in male rats. Groups of 6–8 rats were killed by decapitation at 6 different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means±S.E.M. Letters indicate the existence of significant differences between time points within each group after a one-way ANOVA followed by Tukey–Kramer's multiple comparisons test, as follows: $^{a}p<0.05$ vs. 09:00 h; $^{b}p<0.01$ vs. 17:00, 21:00, 01:00 and 05:00 h; $^{c}p<0.05$ vs. 17:00, 21:00, 01:00 and 05:00 h; $^{d}p<0.05$ vs. 09:00 h, $p<0.01$ vs. the remaining time points; $^{e}p<0.01$ vs. 09:00, 21:00 and 01:00 h; $^{f}p<0.01$ vs. 05:00 and 17:00 h; $^{g}p<0.01$ vs. all corresponding time points. For further statistical analysis, see text.

leptin and changed its 24-h secretory pattern. The pattern in CTR rats was characterized by low levels during the entire light phase, with values increasing, after dark onset to attain the acrophase at 04:12 h (Table 1). ISO produced a significant phase-advance in leptin maximum (acrophase at 00:13 h, $p<0.05$, Table 1) decreasing towards trough levels at 05:00 h (Fig. 2, lower panel). Plasma leptin correlated significantly in a linear way with plasma corticosterone levels in CTR rats only ($r^2=1.61$, $b^0=5.81$ and $b_1=0.09$, $F=6.32$, $p=0.01$).

Discussion

The question posed in the Introduction may now be answered: the foregoing data indicate that isolation of young male rats for 4 weeks can be a stressful signal, since it augments corticosterone secretion and modifies its 24-h pattern. Although no substantial changes in 24-h profile or mean value of plasma ACTH were observed, adrenocortical function clearly augmented as a consequence of social isolation. Plasma corticosterone levels correlated significantly with plasma ACTH and adrenal gland corticosterone concentration in CTR rats only. On the other hand, the daily secretory patterns of two stress-related pituitary hormones, prolactin and GH, became distorted and their total output decreased after ISO. Four weeks of social isolation in growing rats induced a mild hyperleptinemia and disruption of the 24-h pattern of plasma leptin by phase-advancing its nocturnal peak about 4 h.

Challenging individuals to different (physical/psychological) stressors result in increased plasma glucocorticoid levels (Sachser, 1987). In the present study, ISO stress could be considered as mild in view of the unchanged circulating ACTH concentrations and increases in plasma corticosterone to a concentration (about 35 μg/dl) that was smaller than those reported after acute stress conditions, e.g. restrain (Akana and Dallman, 1997) or bacterial lipopolysaccharide injection (Giovambattista et al., 2000) (more than 70–80 μg/dl). It is of interest that the significant correlations between plasma ACTH and corticosterone, or between plasma and adrenal corticosterone levels, found in control rats, were no longer observed in isolated rats. Presumably, isolation stress affected adrenocortical cell function directly, either through circulating catecholamines or via the autonomic innervation to the adrenal gland (Kalsbeek et al., 2003).

With few exceptions (Grosvenor et al., 1965; Morishige and Rothchild, 1974; Morehead et al., 1990), increased plasma levels of prolactin are associated with acute exposure to a stressful stimulus (Matt et al., 1983; Huhman et al., 1991; Dijkstra et al., 1992). Although prolactin reliably increases in response to acute stress, prolactin responses to stressors often become inhibited over continuous exposure. Prolactin responses to footshock turn to inhibition across repeated exposures (Kant et al., 1983, 1985). A similar inhibition was seen in prolactin responses to chronic restraint (Kant et al., 1983, 1985; Raygada et al., 1992), chronic exposure to forced swimming (Kant et al., 1985), forced running (Kant et al., 1983), chronic noise (Armario et al., 1984) or repeated exposure to a novel environment (Yelvington et al., 1985). Social isolation decreased plasma levels of prolactin in hamsters (Matt et al., 1983) and rats (Esquifino et al., 2004). The present results further support the conclusion that solitary housing for several weeks of young male rats brought about the inhibition of prolactin secretion. Although this response could be interpreted as an adaptive mechanism to the novel environment, male ISO animals develop increased aggression (Brain, 1975), hyperresponsiveness to several stressors (Giralt and Armario, 1989) and a behavior characterized, among others, by spending more time to escape and less sleeping time

(Hurst et al., 1999). As far as the pattern of GH levels in ISO male rats, they are in agreement with data obtained after exposure of rats to different stress paradigms, such as abolition of GH responses (Seggie and Brown, 1976; Rice et al., 1978) and on circadian pattern of secretion (Marti et al., 1993).

It must be noted that circulating ACTH and corticosterone levels in group-housed rats were higher during the whole 24-h period than those measured in single-housed animals after either decapitation (Perello et al., 2003) or bled intravenously (Spinedi and Gaillard, 1998). This was presumably due to the fact that blood samples were collected after decapitation, a procedure known to produce acute stress responsiveness in rats particularly when kept in groups. The relatively low variance observed within groups tend to rule out the possible contribution of social hierarchy differences (i.e. the coexistence of a dominant male and many chronically stressed subordinates) as a cause for the high PRL, ACTH and corticosterone levels observed.

To our knowledge, this is the first observation showing an enhanced leptin secretion in ISO rats. As a hypothesis, the increased circulating levels of leptin in ISO rats decrease adrenal leptin-receptor (Ob-Rb) expression (Perello et al., 2004) and cause adrenal refractoriness to leptin inhibition of glucocorticoid production (Perello et al., 2003). Once installed, long-term increase in circulating glucocorticoid levels could cooperate to stimulate adipocyte leptin production (Vidal-Puig et al., 1997; Perello et al., 2003) in ISO rats, therefore perpetuating the effect. Further experiments are needed to determine the primary mechanism triggered by ISO in rats.

Indeed, the direct correlation between leptin and corticosterone circulating levels found in CTR rats became lost in ISO rats. Several studies have revealed an important role for leptin as a stress-related hormone (Ahima et al., 1996; Bornstein, 1997). It seems feasible that the mild stress caused by social isolation in rats resulted in enhanced glucocorticoid and leptin levels as stress-related responses.

Temporal organization is an important feature of the biological systems and its main function is to facilitate adaptation of the organism to the environment (Moore-Ede, 1986; Hastings et al., 2003). Stress is capable of perturbing this temporal organization by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself. In particular, social stress in rodents has been found to cause disruptions of the circadian rhythms of body temperature, heart rate and locomotor activity (Greco et al., 1989; Sgoifo et al., 2002; Spani et al., 2003). Further experiments are needed to assess whether the changes in amplitude as well in timing of 24-h rhythms of stress-related hormones seen in socially isolated rats can be attributed either to an effect on the endogenous clock that modulates the circadian variation of hormone release or to a masking effect on some output(s) of the clock.

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