

Altered Sleep Patterns and Physiologic Characteristics in Spontaneous Dwarf Rats

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Spontaneous dwarf rats are a useful experimental model for studying various biologic events associated with pituitary dwarfism. Dwarf rats occurred serendipitously in our colony of Wistar rats during experimental breeding. This study aimed to describe the sleep pattern and physiologic characteristics of these rats compared with normal-sized adult rats. Because growth hormone can attenuate the upregulation of ceruloplasmin expression caused by acute inflammation, we also assessed the basal levels of serum ceruloplasmin in these animals. At 90 d of age, body weight and length were significantly lower in dwarf rats relative to normal rats. Dwarf rats had lower concentrations of serum testosterone and growth hormone, but progesterone was unchanged. Corticosterone levels did not differ between groups. During the light period, the percentage of sleep time recorded and duration of slow-wave sleep did not differ between groups. However, compared with controls, dwarf rats had marked fragmentation of sleep and less paradoxical sleep. During the dark phase, sleep patterns in dwarf rats were within the normal range. Immunoblotting data showed that the levels of ceruloplasmin in serum were lower in dwarf rats. Our findings provide insight into pathologic processes related to growth hormone deficiency.

Abbreviations: GH, growth hormone; PS, paradoxical sleep; SWS, slow-wave sleep; sleep.

The number of studies on dwarf rats has increased over the past several years. Some dwarf rats have a selective absence of growth hormone (GH) due to a point mutation in the GH gene that creates a premature, in-phase stop codon.³⁶ The pituitary GH mRNA levels in these rats are less than 3% of levels in normal rats, and they have no immune-detectable GH in the pituitary gland or in the systemic circulation.³⁶ Because these features mimic GH deficiency in human, these rats have been used extensively to study GH-dependent physiologic processes and the general features of GH deficiency.

An important feature of GH is that it is released according to a circadian rhythm. Sleep patterns are altered in dwarf rats, suggesting that GH deficiency impairs sleep.²⁹ Previous descriptions of dwarf rats show less rapid-eye-movement sleep [REM or paradoxical sleep (PS)] and more nonREM sleep [NREM or slow-wave sleep (SWS)] than control rats during the light phase²⁹, the period during which rats sleep most. Altered sleep patterns, in turn, can affect the endocrine system, as we consistently have found in our studies evaluating the effect of lack of paradoxical sleep on hormone profiles.^{1-4,6-9} In addition, dwarf animals genetically deficient in GH may be immunocompromised.^{11,17,18,20,27} Some of immunologic deficiencies may be reversed by administration of either GH or a combination of GH and thyroxine.²⁰

Ceruloplasmin (Cp) is a copper-containing glycoprotein enzyme that can catalyze the oxidation of several substrates.²² Although its physiologic function is not understood fully, Cp has been suggested to be an 'acute phase' protein. Plasma concentrations of Cp increase as much as 3-fold during pregnancy and

various pathologic processes, including trauma and inflammation.¹⁶ Moreover, significant increases in plasma Cp occur after hypophysectomy, adrenalectomy, and thyroidectomy, suggesting an inflammatory response to the surgical procedure.^{19,39} Interestingly, in rainbow trout, treatment with GH attenuates Cp levels, suggesting that GH participates in the modulation of Cp expression.³⁹

In the current study, we examined the hormone profile, sleep pattern, and serum Cp levels of a newly identified spontaneous dwarf rat and normal rats to better characterize the physiopathologic consequences of the low GH level that occurs in these rats. To our knowledge, no study has addressed these parameters collectively in a single paper.

Materials and Methods

Animals. We recently found spontaneous dwarf rats in an experimental colony of rats derived from a Wistar rat strain (*Rattus norvegicus albinus*) originally purchased from (Charles River, Wilmington, MA). A single pair of male dwarf rats provided foundation stock for the mutant strain; approximately 20 generations have been obtained since 2000. We did not identify the mutation because it was not the aim of this study. In subsequent generations, we maintained the dwarf phenotype colony by breeding female normal with male dwarf rats. Male adult rats were housed 4 or 5 to an acrylic cage and maintained under standard laboratory conditions (23 ± 2 °C, 12:12 h light:dark cycle, lights on at 0700) with standard rat chow (Nuvilab, Nuvital, Colombo, Brazil) and water provided ad libitum. All procedures used in the present study complied with the *Guide for the Care and Use of Laboratory Animals*.²³ The study was conducted in accordance with the *Ethical and Practical Principles of the Use of Laboratory Animals*,⁵ and maximal effort was used to reduce the number of animals used in

Received: 09 Feb 2009. Revision requested: 23 Feb 2009. Accepted: 27 May 2009.
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the experiments while ensuring unambiguous and reliable statistical analysis and data interpretation.

Anthropometric measurements, blood sampling and hormone analysis. Before blood sampling, rats (control, $n = 12$; dwarf, $n = 8$) were weighed, and body length was measured (in millimeters from nose tip to the last hairs of the tail base). Rats underwent euthanasia between 0900 and 1200 by decapitation in an adjacent room less than 60 s after opening the cage. Blood was collected in glass tubes or those containing EDTA and centrifuged to obtain samples of serum or plasma, respectively.

For hormone measurement, 9 to 10 normal rats and 6 dwarf rats were used. For testosterone and progesterone analysis, blood was centrifuged at $3018.4 \times g$ for 10 min at room temperature and then frozen at -20°C until assayed. Testosterone (intraassay coefficient of variation, 7.7%) and progesterone (9.8%) were measured by chemiluminescent enzyme immunoassay (Advia Centaur, Bayer Corporation, Tarrytown, NY). Corticosterone (10.3%) concentrations were assayed by a double-antibody radioimmunoassay method specific for rats and mice by using a commercial kit (ICN Biomedicals, Costa Mesa, CA). The sensitivity of the assay is 0.25 g/dL. Serum levels of GH were assayed by using an immunoenzymometric assay (Tosoh, Kyobashi, Tokyo, Japan) and expressed as ng/mL; the intra- and interassay coefficients of variation were 5.4% and 2.6%, respectively, and the sensitivity of the assay was 0.1 ng/mL.

Ceruloplasmin. A separate set of rats (3 controls and 4 dwarves) was used for blood collection and assessment of serum Cp level. Blood was collected from the tail veins of conscious rats into microtubes without anticoagulant. Serum was recovered after centrifugation ($1,500 \times g$ 15min), and protein concentration was determined by using Bradford reagent (Sigma, St Louis, MO). Samples of 5 μg total serum protein underwent immunoblotting analysis by using a primary antibody against Cp (Sigma) and secondary antibodies conjugated with Alexa fluor 647 (Invitrogen, Carlsbad, CA) or CY3 (General Electric Healthcare, Fairfield, CT). As a loading control, total protein was stained with SYPRO-Ruby (Invitrogen). The membranes were scanned (Typhoon 9200, General Electric Healthcare) by using appropriate filter sets, and band intensities were quantified by using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Surgical preparation, electrocorticography, and electromyography. Normal and dwarf rats were implanted according to our well-established procedure with electrodes for electrocorticography and electromyography for assessment of sleep-wake cycles. Anesthesia was induced with diazepam (10 mg/kg, IP) and ketamine (90 mg/kg, IP). To record cortical activity with minimal theta component, 2 screw electrodes were placed ipsilaterally through the skull by using the coordinates 1 mm posterior to bregma, 3 mm lateral to the central suture for 1 screw and 1 mm anterior to lambda, 4 mm lateral to the central suture for the other. For nuchal electromyography, 2 wires were placed on the surface of the dorsal neck muscles and soldered to a 6-pin socket and covered with dental acrylic cement. Body temperature was maintained at 37°C by using an electric heating pad (Harvard Apparatus, Holliston, MA). Prophylactic treatment with antibiotics (0.5 mL IM; Veterinary Pentabiotic, Fort Dodge Animal Health, Fort Dodge, IA) and with sodium diclofenac (0.1 mL PO, Novafarma, Anapolis, Goias, Brazil) were done to prevent postsurgical infection. Rats were placed individually in rounded transparent

plastic cages and allowed a 10-d recovery period, followed by a 4-d adaptation period during which the cable was connected.

Cortical (2 channels) and heat-muscle (1 channel) activity were recorded (model QP 223A, Nihon Koden, Tokyo, Japan) for each animal. Electrocorticographic signals were amplified and low-pass-filtered at 0.1 s (1.6 Hz), and electromyographic activity was low-pass-filtered at 0.03 s (5.3 Hz). To ensure reproducibility, a single research scored all recordings, which were analyzed manually in a blinded fashion by using the Polysmith program (Neurotronic, Gainesville, FL). The analysis was based on the predominant amplitude and frequency of the tracing³⁷ and considered 30-s epochs, which were classified according to the dominant (that is, present for more than 50% of the epoch) state (that is, arousal, SWS, or PSJ) state of each epoch. The use of 30-s epochs reflects the parameters of the available system, which was designed for human sleep studies.

The following sleep parameters were assessed: percentage of sleep time (total time spent in sleep during the recording session); PS latency (time lag from the onset of sleep to the onset of the first PS episode); 3) total SWS (total percentage of all periods of deep sleep throughout the recording session); 4) total PS (total percentage of all periods of PS throughout the recording session); and 5) arousal bouts (events at least 15 s in duration, with abrupt modification of the baseline electrocortical frequency, high-amplitude electromyographic activity, and subsequent SWS). For clarity in presentation of the data, sleep, total PS, and SWS times were expressed as percentages of total recording time; PS bouts were expressed as an absolute number.

Statistical analysis. Data were analyzed by using SPSS (version 15, SPSS, Chicago, IL). Because data for sleep, hormonal, and Cp parameters did not meet conditions for normal distribution, dwarf and control groups were compared by using nonparametric Mann-Whitney tests for 2 independent samples. Data are expressed as mean \pm SEM, and the threshold for significance level was established at a P value of < 0.05 .

Results

Morphometric measurements. At 90 d of age, the body weight of dwarf male rats (mean \pm SEM, 155.6 ± 9.1 g) was approximately 38% that of age- and sex-matched normal male rats (411.6 ± 7.6 g). The body length of dwarf rats (14.6 ± 0.3 cm) was shorter than that of normal rats (25.7 ± 0.3 cm; Table 1). Overall, the dwarf rats showed proportional dwarfism (Figure 1).

Hormone concentrations. Dwarf rats had significantly ($P < 0.01$) lower concentrations of GH than did controls (9.8 ± 2.8 versus 2.1 ± 1.0 ng/mL). Testosterone levels (Table 1) were significantly ($P <$

Table 1. Morphometric measures and hormonal concentrations in control and spontaneous dwarf rats

	Control	Dwarf
Body weight (g)	411.6 ± 7.6	155.6 ± 9.1^a
Body length (cm)	25.7 ± 0.3	14.6 ± 0.3^a
Testosterone (ng/dL)	328.3 ± 77	41.9 ± 11.2^a
Progesterone (ng/mL)	1.5 ± 0.3	3.0 ± 1.1
Corticosterone (ng/mL)	105 ± 16.5	173.9 ± 32.2
Growth hormone (ng/mL)	9.8 ± 2.8	2.1 ± 1.0^a

Values are expressed as mean \pm SEM for 6 to 10 rats.

^aValue significantly ($P < 0.05$, Student t test) from that for control rats.

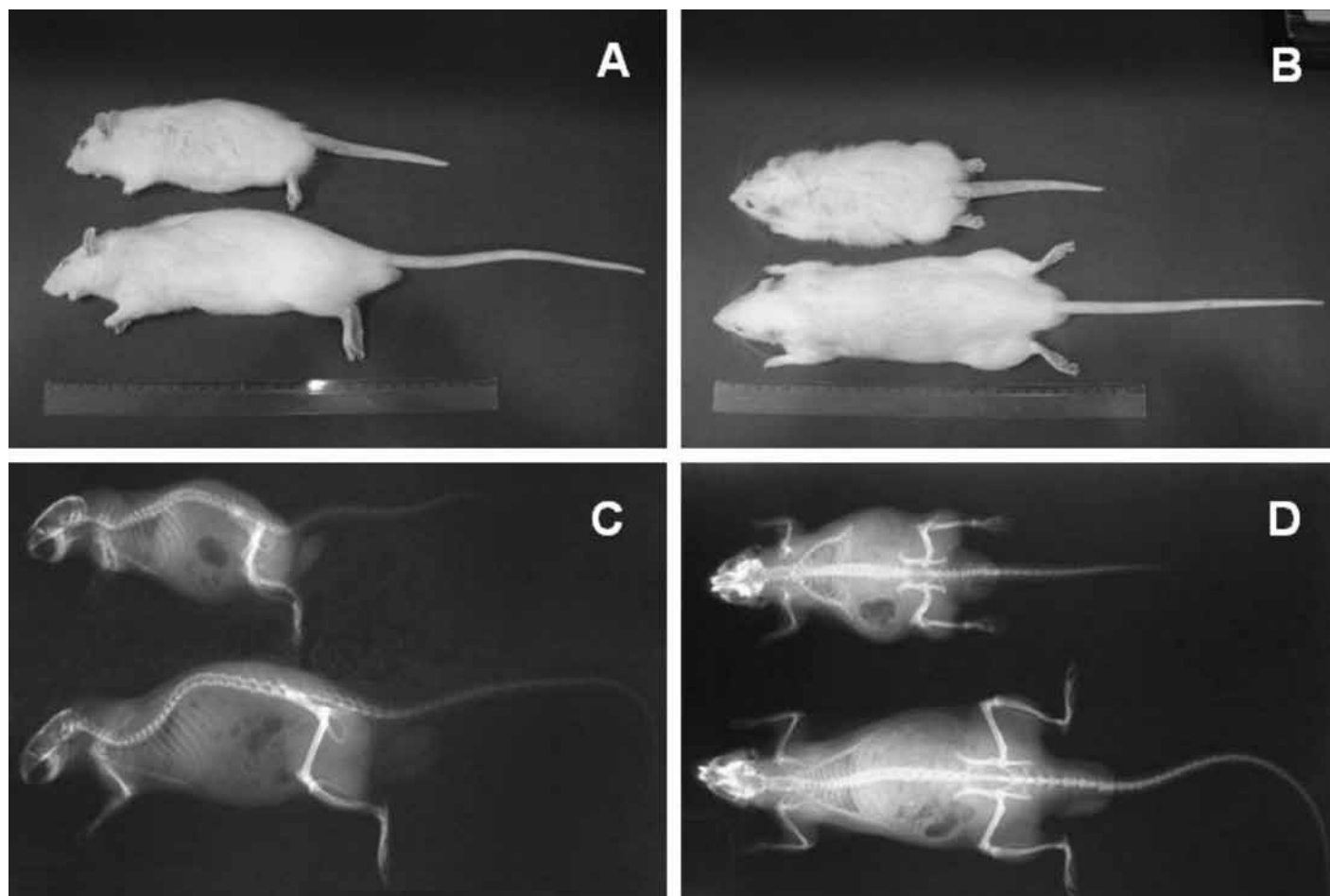


Figure 1. (A) Lateral and (B) dorsoventral photographs and (C) lateral and (D) dorsoventral radiographs of adult normal and dwarf rats. Note the small body size of the mutant rat compared with the normal rat.

0.001) different among groups, with dwarf rats (328.3 ± 77 ng/dL) showing an 87.2% reduction relative to control rats (41.9 ± 11.2 ng/dL). In contrast, levels of progesterone and corticosterone did not differ between groups.

Sleep parameters. Light period. Analyses of sleep patterns revealed that total sleep and SWS times were similar between the groups. The mean amounts of time spent sleeping during recording were 62.6% in controls and 67.5% in dwarf rats and did not differ significantly, despite a statistical trend ($P < 0.06$) toward more total sleep time in dwarf rats. Throughout the light period, dwarf rats showed significantly less PS time than did control rats ($14.7\% \pm 1.0\%$ versus $10.7\% \pm 0.7\%$; $P < 0.01$); Figure 2). Dwarf rats also exhibited approximately 20% more arousals during sleep than did control rats ($P < 0.01$), indicating marked fragmentation of sleep. The duration of the arousal bouts was 6.0 ± 0.6 min in the dwarf group compared with 13.4 ± 0.8 min in control rats ($P < 0.0001$, data not shown).

Dark period. Data recorded for all sleep parameters during the dark period did not differ significantly between groups (Figure 2).

Serum ceruplasmin level. Serum Cp levels were evaluated by immunoblotting, and the band intensity of each sample was normalized against the total protein loaded in each lane. Dwarf rats had significantly ($P = 0.03$) lower levels of serum Cp than did

normal controls (relative intensity, 8.27 ± 3.25 versus 5.23 ± 0.57 ; Figure 3).

Discussion

Dwarf rats are a useful model for investigating the physiologic role of GH.^{14,15,28,29} Studies of dwarf rats has yielded information about the role of GH in somatic growth,³² cardiac structure and function,¹⁵ and glucose metabolism related to hippocampus-dependent learning and memory.³³

As in previous reports, the spontaneous dwarf rats investigated in the present study showed lower GH and testosterone levels than did normal rats (Table 1). In a previous report, dwarf rats had lower testicular weight relative to body weight at 28 d of age, accompanied by lower testosterone levels, as compared with controls.³⁴ Growth hormone has been reported to affect testicular function by modulating gonadal steroid synthesis and gametogenesis.^{30,40}

Serum Cp is a multifunctional protein whose expression increases during the acute phase of inflammation. We found lower Cp levels in dwarf rats compared with age-matched controls. The lower Cp levels in the dwarf rats supports the importance of GH in the modulation of Cp expression.³⁹ A recent study showed that deprivation of PS increased susceptibility to lipopolysaccharide

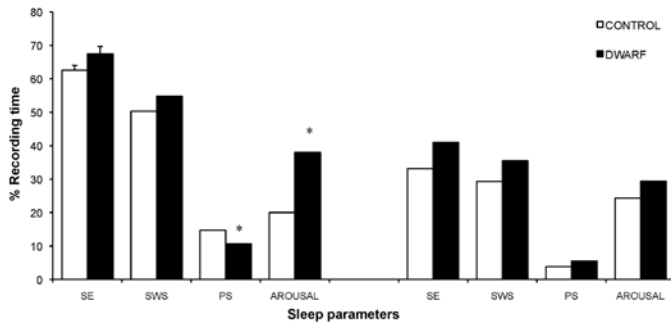


Figure 2. Percentage of total recording time (mean \pm SEM) in sleep efficiency (SE), slow-wave sleep (SWS), paradoxical sleep (PS), and arousal in control ($n = 6$) and dwarf ($n = 4$) rats during the light (left) and dark (right) periods. *, Value significantly ($P < 0.05$, Mann-Whitney test) from that of controls.

and behavioral alterations.⁴¹ In particular, treating animals with lipopolysaccharide after selective sleep loss increased group mortality rates by approximately 40%. This finding suggests that lack of PS increases susceptibility to septic shock and supports the hypothesis that sleep promotes an effective immune response. Be-

cause dwarf rats in the current study showed significantly less PS and increased sleep fragmentation (represented by an increased number of arousals; Figure 2), we speculate that these animals would show increased susceptibility to immunologic challenge such as from lipopolysaccharide. Research addressing the effects of lipopolysaccharide in GH-deficient animal models would contribute to our understanding of the neuroimmunoendocrinologic consequences of dwarfism.

Several animal models with mutation or genetic manipulation of the somatotrophic axis have been used to investigate the role of GH-releasing hormone and GH in sleep regulation. Similar to our findings, another study²⁹ has shown that spontaneous dwarf rats had decreased PS. However, in contrast to the previous study,²⁹ we found no significant change in SWS duration in dwarf rats, with a nonsignificant trend toward higher duration. These discrepancies could be related to the different methodologies used for sleep recording or the lower number of animals used in our study. In addition, the Sprague-Dawley control rats used previously²⁹ spent 47% of the 24 h cycle in SWS, whereas we measured 50% in our Wistar control rats. Therefore, different background strains might also have contributed to the discrepant results between the two studies.

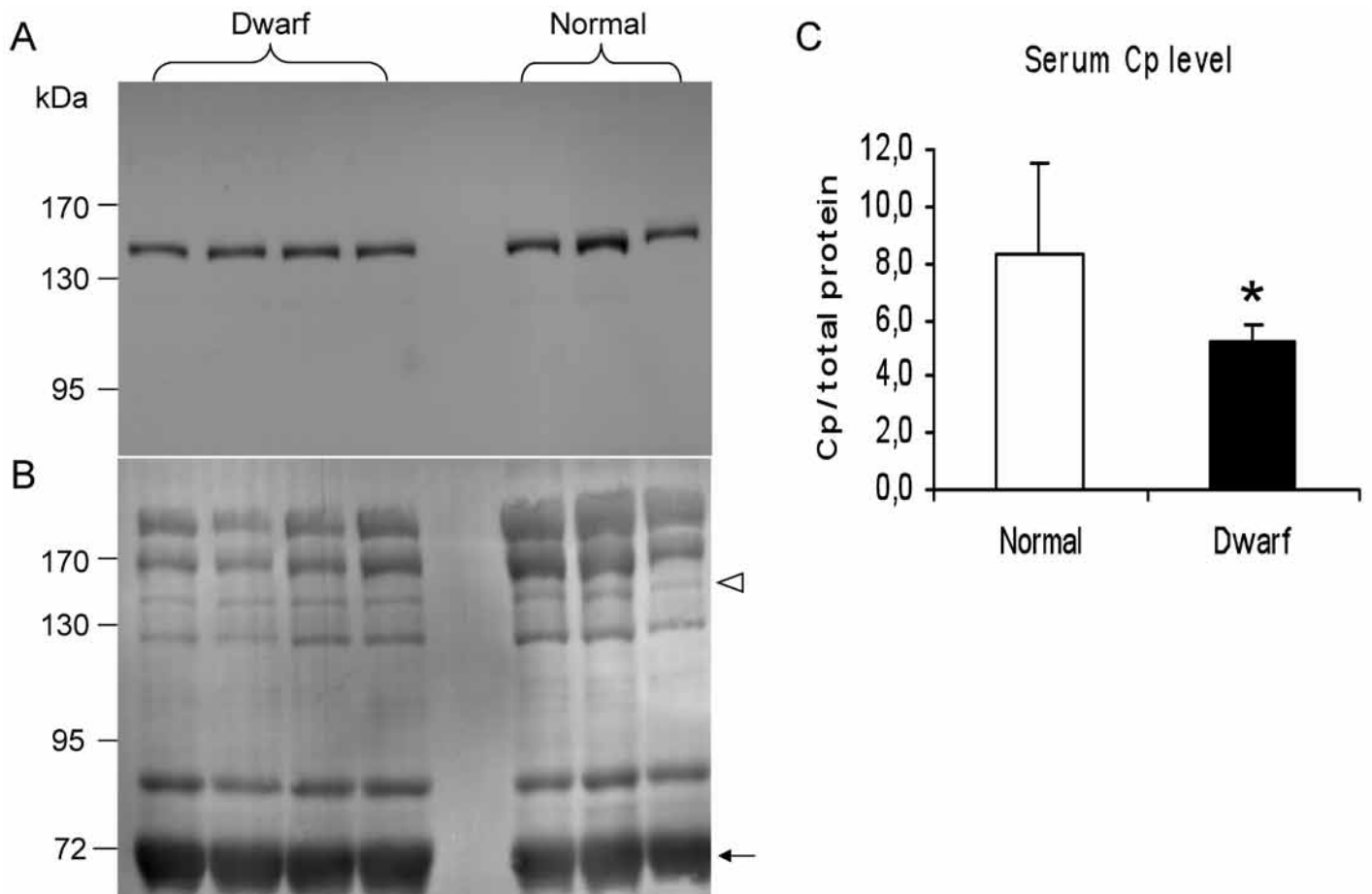


Figure 3. (A) Immunoblotting analysis of serum ceruloplasmin (Cp) in 4 dwarf and 3 normal rats. (B) The intensity of the Cp band was normalized to the total protein level. The bands indicated by the arrowhead were not used for normalization because they are similar in molecular weight to Cp. The bands indicated by the arrow were not included in the normalization due to signal saturation. (C) Intensity (mean \pm 1 SD) of serum Cp relative to total serum protein. *, Value significantly ($P < 0.05$, Mann-Whitney test) from that of controls.

The role of GH in physiologic sleep regulation (reviewed in reference 25) is controversial. GH-deficient humans show distinct changes in the duration of SWS (NREM)¹⁰ as well as decreased total sleep time and increased sleep fragmentation.⁴³ In hypophysectomized animals, findings of no alteration,³¹ increased SWS, and decreased PS⁴² have all been reported. In a more recent study,²⁶ hypophysectomized rats and dwarf Lewis rats showed marked decreases in the mRNA levels of GH-releasing hormone in the paraventricular nucleus of the hypothalamus.

Here we report that our spontaneous dwarf rats show sleep disturbances, hormone alterations, and decreased levels of Cp. Collectively, the current data provide further evidence of the complex integration of sleep, neuroendocrine, and immune functions related to GH deficiency and dwarfism.

Acknowledgments

We express our sincere thanks to the technicians from the Department of Psychobiology who found the first 3 dwarf rats among thousands of rats. The capable help of Fernanda Gonzalez in X-ray analysis is appreciated. This study was supported by the Associação Fundo de Incentivo à Psicofarmacologia (AFIP) and Fundação de Amparo à Pesquisa do Estado de São Paulo (CEPID 98/14303-3 to ST). ST and MLA are recipients of fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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