Neonatal handling and reproductive function in female rats

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Abstract

Neonatal handling induces anovulatory estrous cycles and decreases sexual receptivity in female rats. The synchronous secretion of hormones from the gonads (estradiol (E_2) and progesterone (P)), pituitary (luteinizing (LH) and follicle-stimulating (FSH) hormones) and hypothalamus (LH-releasing hormone (LHRH)) are essential for the reproductive functions in female rats. The present study aimed to describe the plasma levels of E_2 and P throughout the estrous cycle and LH, FSH and prolactin (PRL) in the afternoon of the proestrus, and the LHRH content in the medial preoptic area (MPOA), median eminence (ME) and medial septal area (MSA) in the proestrus, in the neonatal handled rats. Wistar pup rats were handled for 1 min during the first 10 days after delivery (neonatal handled group) or left undisturbed (nonhandled group). When they reached adulthood, blood samples were collected through a jugular cannula and the MPOA, ME and MSA were microdissected. Plasma levels of the hormones and the content of LHRH were determined by RIA. The number of oocytes counted in the morning of the estrus day in the handled rats was significantly lower than in the nonhandled ones. Neonatal handling reduces E_2 levels only on the proestrus day while P levels decreased in metestrus and estrus. Handled females also showed reduced plasma levels of LH, FSH and PRL in the afternoon of the proestrus. The LHRH content in the MPOA was significantly higher than in the nonhandled group. The reduced secretion of E_2, LH, FSH and LHRH on the proestrus day may explain the anovulatory estrous cycle in neonatal handled rats. The reduced secretion of PRL in the proestrus may be related to the decreased sexual receptiveness in handled females. In conclusion, early-life environmental stimulation can induce long-lasting effects on the hypothalamus-pituitary-gonad axis.

Journal of Endocrinology (2005) **184,** 435–445

Introduction

Environmental stimulation during the postnatal period, disturbing the mother–infant relationship, can induce long-lasting effects upon a variety of behavioral and neuroendocrine systems (Levine *et al.* 1967, Francis *et al.* 1999, Meerlo *et al.* 1999). Neonatal handling permanently alters hypothalamus-pituitary-adrenal axis responses to stressful stimuli. As adults, postnatal handled rats synthesize and secrete less corticotrophin-releasing hormone, adrenocorticotrophin and corticosterone in response to a wide variety of stressors, and show a faster return to basal levels following termination of the stimulus (Levine *et al.* 1967, Plotsky & Meaney 1993, Liu *et al.* 2000). Neonatal handling also induces behavioral alterations. Handled animals show decreased emotional reactivity, defecate less and explore more when placed in a novel environment (Levine *et al.* 1967, Núñez *et al.* 1996, Meerlo *et al.* 1999, Padoin *et al.* 2001, Severino *et al.* 2004).

Besides the decreased behavioral inhibition in novel and averse environments, previous study showed that daily handling of the offspring during the first 10 days of life reduced the sexual behavior of male and female rats (Padoin *et al.* 2001). Moreover, neonatal handling induced a significant reduction in ovulation, with most handled females showing anovulatory estrous cycles (Gomes *et al.* 1999). Although neonatal handling prevented the induction of constant diestrus by an unpredictable chronic stress (Gonzalez *et al.* 1994), the reduction of sexual receptiveness and ovulation raises doubts concerning the proposed positive effects of the neonatal handling procedure (Gonzalez *et al.* 1994, Costela *et al.* 1995). Nevertheless, independently of the consequences, these results show that neonatal handling may affect the activity of the

0022–0795/05/0184–435
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Printed in Great Britain
DOI: 10.1677/joe.1.05907
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hypothalamus–pituitary–gonad axis, in addition to the activity of the hypothalamus–pituitary–adrenal axis.

The reproductive cycle in the female is a recurring set of events that culminates in the ability to reproduce; that is, to ovulate eggs, mate, achieve fertilization, proceed through pregnancy and then deliver and nurse the pups (Schwartz 2000). In order to achieve success in these events it is necessary that the neuroendocrine structures participating in those processes act harmoniously. Estradiol (E_2) and progesterone (P) play a crucial role in the regulation of several mechanisms of the female reproductive system. Together they act on the hypothalamus, pituitary and ovaries to coordinate the cyclic neuroendocrine gonadotrophin production and ovulatory activity (Mahesh & Brann 1998, Conneely 2001).

In female rats, the increase in plasma luteinizing hormone (LH) in the proestrus is a key event for ovulation. The preovulatory surge of LH depends on hypothalamic LH-releasing hormone (LHRH) secretion from nerve terminals into the median eminence (ME; Levine *et al.* 1991, Ishikawa 1992). This process depends on both the positive-feedback actions of preovulatory E_2 secretions and specific neural signals to initiate the surge (Levine 1997).

The elevated E_2 level during the preovulatory period appears to provide a permissive signal for the LHRH and LH surges (Levine 1997, Herbison 1998, Mahesh & Brann 1998). Moreover, the ovarian steroid hormones E_2 and P have profound modulatory influences on neural circuits that regulate sexual behavior. During the estrous cycle, the sexual behavior depends on an increase in serum E_2 followed by an increase in P concentration. Sexual behavior is abolished by ovariectomy, and reinstated by injections of E_2 followed by P. Treatment with E_2 alone can induce some aspects of sexual behavior, such as receptivity; however, administration of E_2 followed by P induces the full complement of proceptive behaviors that occur during normal estrus (Auger 2001, Flanagan-Cato *et al.* 2001, Mani 2001).

Considering that neonatal handling reduces ovulation (Gomes *et al.* 1999) and sexual receptiveness in female rats (Padoin *et al.* 2001), the present study aimed at analyzing the pattern of E_2 and P secretion throughout the 4 day estrous cycle in neonatal handled rats. Moreover, we studied the effects of neonatal handling on the plasma concentrations of E_2, LH, follicle-stimulating hormone (FSH) and prolactin (PRL), and the contents of LHRH in the medial septal area (MSA), medial preoptic area (MPOA) and ME in female rats in the afternoon of the proestrus.

Materials and Methods

Animals

Pregnant female Wistar rats were brought from the colony of the Federal University of Rio Grande do Sul (Porto Alegre, Brazil) to the animal room in our laboratory. Approximately 7 days before delivery, the females were housed individually, and the presence of pups was checked twice a day. On the day of birth (day 0), the number of pups was culled to eight per female by randomly removing some of them, ensuring minimal contact with the remaining pups and the female. After weaning (postnatal day 21), the pups were housed in same-sex groups of between three and five per cage (41 cm long × 34 cm wide × 17 cm high) according to body weight. All rats were maintained on a 12 h light: 12 h darkness cycle (lights on from 0600 to 1800 h), the room temperature was 22 ± 1 °C, and water and food (Rodent chow; Nutrilab, Colombo, Brazil) were available at all times. The females were tested at the age of 80–120 days. Experiments were performed in accordance with the National Institutes of Health (NIH) guidelines and were approved by the University Research Committee of the Federal University of Rio Grande do Sul.

Neonatal handling

In all experiments, the rats were divided into two groups: nonhandled and handled. The nonhandled pups were not manipulated by either the researchers or the caretakers during the first 10 postnatal days. The handling procedure consisted of first removing the litter and the mother in their home cage to a quiet room next to the animal facility, with the same light period and temperature. First, the mother was placed in another cage next to the home cage, and all of the pups handled gently at the same time using both hands, covered with fine latex gloves, for 1 min. After handling, all pups were taken to the nest at the same time and then the mother was placed back in the home cage. This procedure was repeated from the first to the tenth postnatal days, during the light period of the daily photoperiod cycle (Gomes *et al.* 1999, Padoin *et al.* 2001, Severino *et al.* 2004).

Estrous cycle

Vaginal smears were taken daily after the 70th day of age and only those rats showing at least three consecutive regular 4 day estrous cycles were used for surgical procedures.

Jugular cannulation and blood sampling

The animals were anesthetized with tribromoethanol (Aldrich; 1 ml of a 2·5% solution/100 g body weight i.p.; Poletini *et al.* 2003) and a silastic cannula was inserted through the external jugular vein into the right atrium according to the technique of Harms & Ojeda (1974). Blood samples (0·4–0·6 ml) were collected in heparinized syringes with an equivalent volume of 0·9% NaCl (saline)

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solution replaced after each bleeding. Plasma was separated by centrifugation and stored at −80 °C until assay.

Experiment 1: effects of neonatal handling on E_2 and P throughout the estrous cycle

Neonatal handled and nonhandled female rats at the age of 90–110 days with three regular estrous cycles were submitted to jugular vein cannulation at 1400 h on the day before the blood sampling. In all cases, the vaginal smear to verify the phase of the cycle was obtained 1 h before the first blood sampling. In each phase (metestrus, diestrus, proestrus and estrus), different animals were used. Blood samples (0·4 ml) for E_2 measurement were collected every 3 h during 24 h, starting at 0800 h. In a different set of animals, blood was collected for P measurement in the same manner as for E_2, except in the proestrus–estrus period, during which blood samples were collected at 0800, 1100, 1400, 1600, 1800, 2000, 2200, 2400, 0200 and 0500 h. After collecting each blood sample, 0·4 ml 0·9% NaCl was injected to replace the volume withdrawn. During the dark period, the blood was collected under dim red light. Blood samples were centrifuged and plasma separated and stored at −80 °C until assayed for E_2 and P.

Experiment 2: effects of neonatal handling on E_2, LH, FSH and PRL plasma concentrations and number of oocytes

On the morning of the proestrus, between 1100 and 1200 h, the animals of both groups (nonhandled and neonatal handled) with three regular estrous cycles were submitted to jugular vein cannulation. At time of the experiment, a length of polyethylene tubing (PE-50) was connected to the jugular catheter, filled with heparinized saline (200 I.U. heparin/ml) and the rats allowed to remain undisturbed in their cages for an additional 30 min until the beginning of the experiment. Blood samples (0·6 ml) were collected every hour (1300–2000 h) in the afternoon of the proestrus in plastic heparinized syringes. After each blood sample was taken, 0·6 ml 0·9% NaCl was injected to replace the volume removed. The blood samples were centrifuged at 3000 ***g***. Plasma was separated and stored frozen at −80 °C until assayed for E_2, LH, FSH and PRL. On the estrus morning (0900 h), animals that had their blood collected for hormone assay on the previous day were decapitated, the ovaries removed and the oviduct dissected and squashed between two microscope slides. The number of oocytes of both oviduct ampullae was counted under the microscope (Zeiss, Goettingen, Germany) with a 2·5 × lens.

Experiment 3: effects of neonatal handling on LHRH content in MPOA, MSA and ME

In this experiment female rats were divided into two groups (nonhandled and neonatal handled). The animals were decapitated at 1800 h of proestrus. The brains were removed and frozen to −80 °C until the punches were processed. The anterior part of the brain was fixed in a cryostat with the temperature kept at −20 °C and the MPOA, MSA and ME were removed by the punch technique (Palkovits 1973). The punches were obtained according to the coordinates taken from the rat brain atlas (Paxinos & Watson 1986). The punches were homogenized in 100 µl 0·1 M HCl with a micro-ultrasonic cell disrupter. A 20 µl aliquot of the homogenate was separated to determine protein content using the Bradford dye-binding micromethod (Bradford 1976). After centrifugation at 3500 ***g*** for 10 min the supernatant was separated and stored at −80 °C until being assayed for LHRH.

To punch out the MSA, a frontal cut 9·7 mm anterior to the interaural line was performed and an 800 µm thick slice was obtained caudally from there. Two consecutive sections of 1000 µm (for MPOA) and 2000 µm (for ME) were sliced beginning 0·26 mm posterior to the bregma. The MPOA was microdissected with a 1·3 mm diameter circular needle and the ME with a 2 mm square needle.

RIA

Plasma E_2 and P concentrations were determined by double-antibody RIA using specific MAIA kits (BioChem ImmunoSystems, Bologna, Italy). The lower limit for detection was 7·5 pg/ml for E_2 and 0·075 ng/ml for P. RIAs for LH, FSH and PRL were performed using specific kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK; Baltimore, MD, USA). The antibodies used were anti-rat LH-S10, FSH-S11 and PRL-S9; the standards were LH-RP3, FSH-RP2 and PRL-RP3. The lowest limit for detection was 0·04 ng/ml for LH, 0·09 ng/ml for FSH, and 0·19 ng/ml for PRL. LHRH was determined by double-antibody RIA using specific antibody provided by the NIDDK.

Statistical analysis

Plasma levels of E_2, P, LH, FSH, PRL and LHRH content in the areas studied were expressed as means ± S.E.M. In each phase of the estrous cycle, the significance of differences of E_2 and P between groups (nonhandled and handled) and among the blood samples collected throughout the day was determined by two-way ANOVA with repeated measures followed by the Newman–Keuls test for multiple comparisons. Plasma levels of E_2, LH, FSH and PRL were measured in the afternoon of the proestrus and were analyzed as described for E_2 and P. The areas under the curve (AUCs), expressed as means ± S.E.M., for plasma E_2 and P in the four phases of estrous cycle and for plasma E_2, LH, FSH and PRL in proestrus were calculated and

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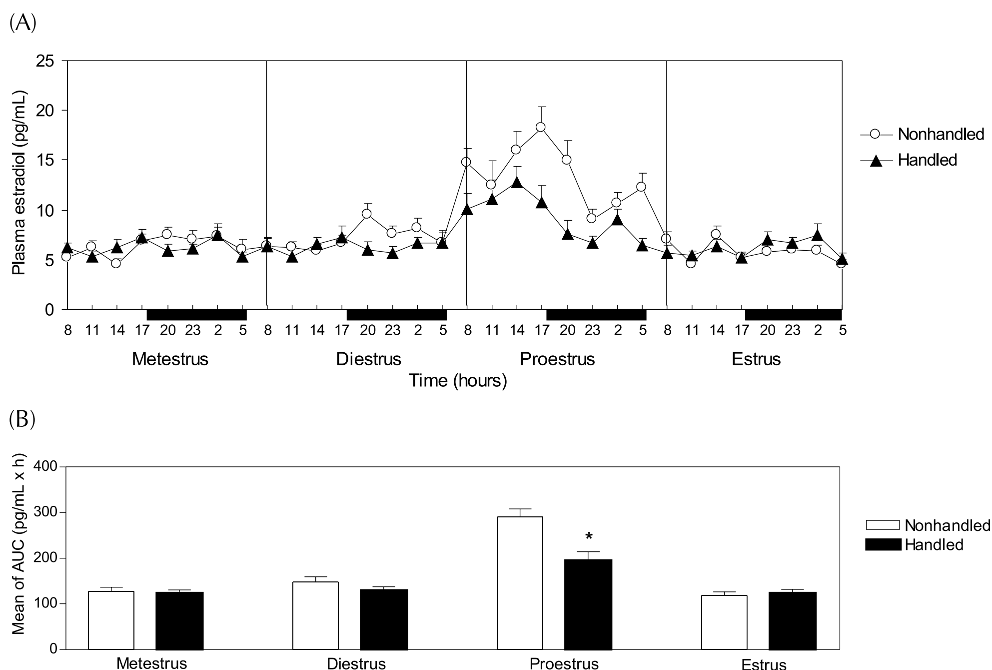

Figure 1 Effects of neonatal handling on the plasma E_2 levels (pg/ml) and AUC of E_2 in metestrus, diestrus, proestrus and estrus of female rats. The blood samples (0·4 ml) were collected every 3 h during each day of the estrous cycle from a jugular catheter, with an equivalent volume of 0·9% NaCl (saline) solution replaced after each bleeding. (A) Plasma hormone concentration (means ± S.E.M.); black bars represent the dark interval in the animal room (1800–0600 h). In all cases, n=12–16 for each time point. Data were analyzed using two-way ANOVA with repeated measures followed by the Newman–Keuls test for multiple comparisons. (B) Each bar represents the mean ± S.E.M. for AUC and the data were analyzed using Student's t-test. *P<0·001, significant difference from controls. In metestrus, diestrus and proestrus n=15 in each group; in estrus n=16 in each group.

the significance of differences between the groups was determined by the Student's t-test. The content of LHRH was compared between the groups by the Student's t-test. The number of oocytes was expressed as a median (with interquartile range) and compared between the two groups by the Mann–Whitney U test. In all cases, the level of significance was set at $P \leq 0·05$.

Results

Experiment 1: effects of neonatal handling on E_2 and P throughout the estrous cycle

Figure 1 shows the plasma concentrations of E_2 throughout the 4 day estrous cycle of the neonatal handling rats and the AUC values that express the total amount of E_2 released within each phase of the estrous cycle. In non-handled female rats, E_2 levels remained low on the day of estrus and metestrus, began to increase in the afternoon of the diestrus, and increased further to reach a peak at 1700 h of proestrus.

In metestrus, no significant main effect for group ($F_{1,28}$=0·05) was detected. A significant main effect for the time of day (F_{7196}=2·72, P<0·01) was shown and the Newman–Keuls *post hoc* analysis revealed that E_2 concentration at 1400 h was lower than at 0200 h. No significant interaction among groups and time of day was detected (F_{7196}=1·36). In metestrus, no difference in the AUC was detected between the groups (t_{28}=0·37).

In diestrus, no significant main effects for group ($F_{1,27}$=1·45) or time of day (F_{7189}=1·79, P=0·09) were detected. The interaction among groups and time of day reached significance (F_{7189}=2·06, P=0·05). Newman–Keuls *post hoc* analysis revealed that E_2 in the handled group at 2000 h was lower than the nonhandled one at the same time. No difference was detected between groups in the AUC (t_{28}=1·37).

In proestrus, significant main effects for group ($F_{1,28}$=15·02, P<0·001) and time of day (F_{7196}=5·86, P<0·001) were detected. Newman–Keuls *post hoc* analysis revealed that E_2 at 0800 and 1100 h was lower than at 2300 h; and at 1400 and 1700 h, E_2 was lower than at 2300, 0200 and 0500 h. However, no significant interaction among groups and time of day was shown (F_{7196}=1·64). The AUC of the handled group is lower than the nonhandled (t_{28}=3·64, P<0·001).

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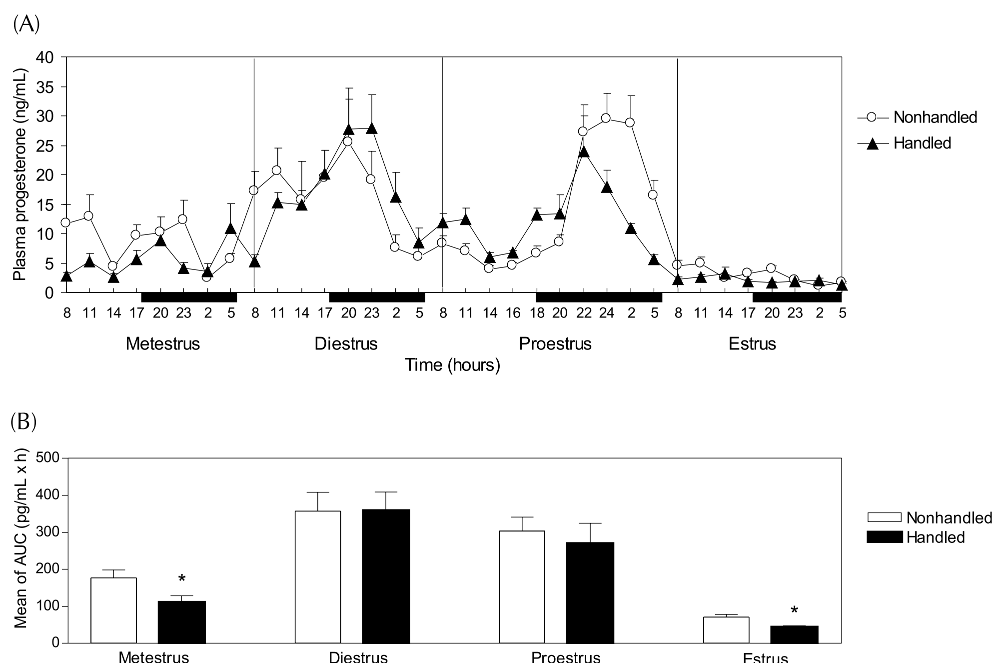

Figure 2 Effects of neonatal handling on the plasma progesterone levels (ng/ml) and AUC of progesterone in metestrus, diestrus, proestrus and estrus of female rats. The blood samples (0·4 ml) were collected every 3 h in the metestrus, diestrus and estrus. In the proestrus, blood samples were collected at 0800, 1100, 1400, 1600, 1800, 2000, 2200, 2400, 0200 and 0500 h from a jugular catheter, with an equivalent volume of 0·9% NaCl (saline) solution replaced after each bleeding. (A) Plasma hormone concentration (means ± S.E.M.); black bars represent the dark interval (1800–0600 h). In all cases, n=10–16 for each time point. Data were analyzed using two-way ANOVA with repeated measures followed by Newman–Keuls test. (B) Each bar represents the mean ± S.E.M. for AUC and the data were analyzed using Student's t-test. *P<0·001, significant difference from controls. In metestrus and diestrus n=15 in each group; in proestrus n=10; in estrus n=16 in each group.

In estrus, no significant main effect for group ($F_{1,30}$=1·65, P=0·21) was detected, but a significant main effect for time of day (F_{7210}=3·60, P<0·001) was shown. Newman–Keuls *post hoc* analysis revealed that E_2 at 1100 h was lower than at 1400 h; and at 1400 h, E_2 was higher than at 1700 and 0500 h. No significant interaction among groups and time of day was detected (F_{7210}=1·63). Moreover, the AUC did not reach a significant difference between groups (t_{30}=0·47).

Figure 2 shows the plasma concentrations of P throughout the 4 day estrous cycle of the neonatal handled and nonhandled rats and the AUC values that express the total amount of P released within each phase of the estrous cycle. In the nonhandled group, the pattern of P secretion during estrous cycle consisted of two major surges, one during diestrus and the other in the afternoon of the proestrus. The first surge began in metestrus and had a peak in diestrus. At this time, a precipitous drop in P level occurred. P values remained low until the afternoon of proestrus, when a surge in the night of proestrus occurred; then a new drop was observed, and P remained at baseline level on the day of estrus.

In metestrus, significant main effects for group ($F_{1,28}$=7·06, P<0·01) and time of day (F_{7196}=3·53, P<0·001) were detected. Significant interaction among groups and the time of day (F_{7196}=3·53, P=0·001) was also shown. Newman–Keuls *post hoc* analysis revealed that P concentration at 0800 h of the metestrus in neonatal handled rats was lower than the nonhandled ones. The AUC of the handled group is lower than the nonhandled (t_{28}=2·40, P<0·02).

In diestrus, no significant main effect for group ($F_{1,28}$=0·63) was detected, but a significant main effect for the time of day (F_{7196}=9·32, P<0·001) and a significant interaction among groups and the time of day (F_{7196}=2·63, P<0·01) were shown. Newman–Keuls *post hoc* analysis revealed that the P concentration in nonhandled rats at 1100 h was higher than at 1400 h; and at 2000 h, P was higher than at 1400, 0200 and 0500 h in same group. In handled rats, P concentration at 2000 h was higher than at 0800, 1100, 1400, 0200 and 0500 h; and at 2300 h, P was higher than at 0800 and 0500 h in same estrous cycle phase and group. The AUC analyses showed no difference between the groups (t_{28}=0·03).

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In proestrus, no significant main effect for group ($F_{1,18}$=0·65) was detected, but a significant main effect for time of day (F_{9162}=10·57, P<0·001) and a significant interaction among groups and the time of day (F_{9162}=3·85, P<0·001) were shown. Newman–Keuls *post hoc* analysis revealed that the P concentration at 0200 h of the proestrus in neonatal handled rats was lower than in the nonhandled ones. Moreover, in nonhandled rats at 0800, 1100, 1400, 1600, 1800 and 2000 h, P concentration was lower than at 2200, 2400 and 0500 h; in contrast in handled rats at 1400, 1600 and 0500 h, P concentration was lower than at 2200 h. However, no difference between groups in the AUC was detected (t_{18}=0·50).

In estrus, significant main effects for group ($F_{1,29}$=7·85, P<0·01) and time of day (F_{7203}=5·44, P<0·001) were detected. Significant interaction among groups and the time of day (F_{7203}=3·92, P<0·001) was shown. Newman–Keuls *post hoc* analysis revealed that the P concentration at 0800 and 1100 h of the estrus in the neonatal handled rats was lower than in the nonhandled ones. In the nonhandled group, P concentrations at 0800 and 1100 h were higher than at 1400, 2300, 0200 and 0500 h. In estrus, the AUC of the handled rats was also lower than the nonhandled (t_{30}=3·29, P<0·003).

Experiment 2: effects of neonatal handling on E_2, LH, FSH and PRL plasma concentrations and number of oocytes

Figure 3A shows E_2 plasma concentrations in the afternoon of the proestrus. Significant main effects for group ($F_{1,30}$=17·95, P=0·0002) and time of day (F_{7210}=29·37, P<0·0001) were detected. A significant interaction among groups and time of day was shown (F_{7210}=10·02, P<0·0001). *Post hoc* analysis revealed that there was an increase in E_2 plasma concentrations in the nonhandled group at 1400 h (compared with the subsequent times 1700, 1800, 1900 and 2000 h) and 1500 h (compared with the subsequent times 1600, 1700, 1800, 1900 and 2000 h) in the afternoon of the proestrus. From 1500 until 2000 h, plasma concentration of E_2 decreased. The E_2 plasma concentration in the handled group did not change from 1300 until 1900 h in the afternoon of the proestrus, but at 2000 h there was a decrease in E_2 plasma concentration compared at 1300, 1400, 1500, 1600, 1700, 1800 h in the afternoon of the proestrus. The AUC of E_2 is shown in Fig. 3B. The neonatal handled group had significantly smaller AUC (t_{30}=3·55, P=0·001) than the nonhandled group.

Figure 3C shows LH plasma concentrations during the afternoon of the proestrus. Significant main effects for group ($F_{1,30}$=34·81, P<0·0001) and time of day (F_{7210}=15·82, P<0·0001) were detected. Significant interaction among groups and time of day were detected (F_{7210}=6·25, P<0·0001). *Post hoc* analysis revealed that LH plasma concentration in the nonhandled group was higher from 1700 until 2000 h compared with the previous times but in the handled group there was no increase in LH plasma concentrations during afternoon of the proestrus. The AUC of LH is shown in Fig. 3D. The neonatal handled group had a significantly smaller AUC (t_{30}=5·60, P<0·0001) than the nonhandled group. When the handled group was divided into females that had oocytes and females that had no oocytes, the AUCs of LH in both handled groups (with and without oocytes) were not different. But the AUC of LH in the nonhandled group was higher ($F_{2,29}$=21·92, P<0.0001) compared with both handled groups.

Figure 3E shows the results of FSH plasma concentrations in the afternoon of the proestrus. Significant main effects for groups ($F_{1,30}$=7·36, P=0·01) and time of day (F_{7210}=13·10, P<0·0001) were detected. *Post hoc* analysis revealed that FSH plasma concentration at 2000 h was higher than from 1300 until 1900 h, and at 1800 and 1900 h were higher than at 1300, 1400, 1500 and 1600 h, and at 1700 h was higher than at 1300, 1400 and 1500 h. However, no significant interaction among groups and the time of day was detected (F_{7210}=1·44, P=0·19). The AUC of FSH in the handled group was significantly smaller (t_{30}=2·21, P=0·03) than that of the nonhandled group (Fig. 3F).

Figure 3G shows PRL plasma concentration during the afternoon of the proestrus. Significant main effect for groups was detected ($F_{1,30}$=42·74, P<0·0001) and significant main effect for time of day (F_{7210}=13·10, P<0·0001). Significant interaction among groups and the time of day were detected (F_{7210}=4·17, P=0·0002). *Post hoc* analysis revealed that PRL plasma concentration in the nonhandled group was higher from 1600 until 2000 h compared with the previous times (1300, 1400 and 1500 h), but in the handled group there was no increase in PRL plasma concentration during the afternoon of the proestrus. The AUC of PRL is shown in Fig. 3H. The neonatal handled group had a significantly smaller AUC (t_{30}=3·73, P=0·0008) compared with the nonhandled group.

Confirming previous results (Gomes *et al.* 1999), the median (with interquartile range) of the number of oocytes in neonatal handled female rats (1 (0–5·5)) was significantly lower than in the nonhandled ones (8·5 (7–9); U=37·50, P<0·001).

Experiment 3: effects of neonatal handling on LHRH content in MPOA, MSA and ME

Figure 4 shows the results of LHRH content in the MPOA, MSA and ME at 1800 h on the proestrus day. The LHRH content in the MPOA at 1800 h in the afternoon of the proestrus of the neonatal handled group was significantly higher than that in the nonhandled group (t_{38}=4·91, P=0·02). No significant differences were

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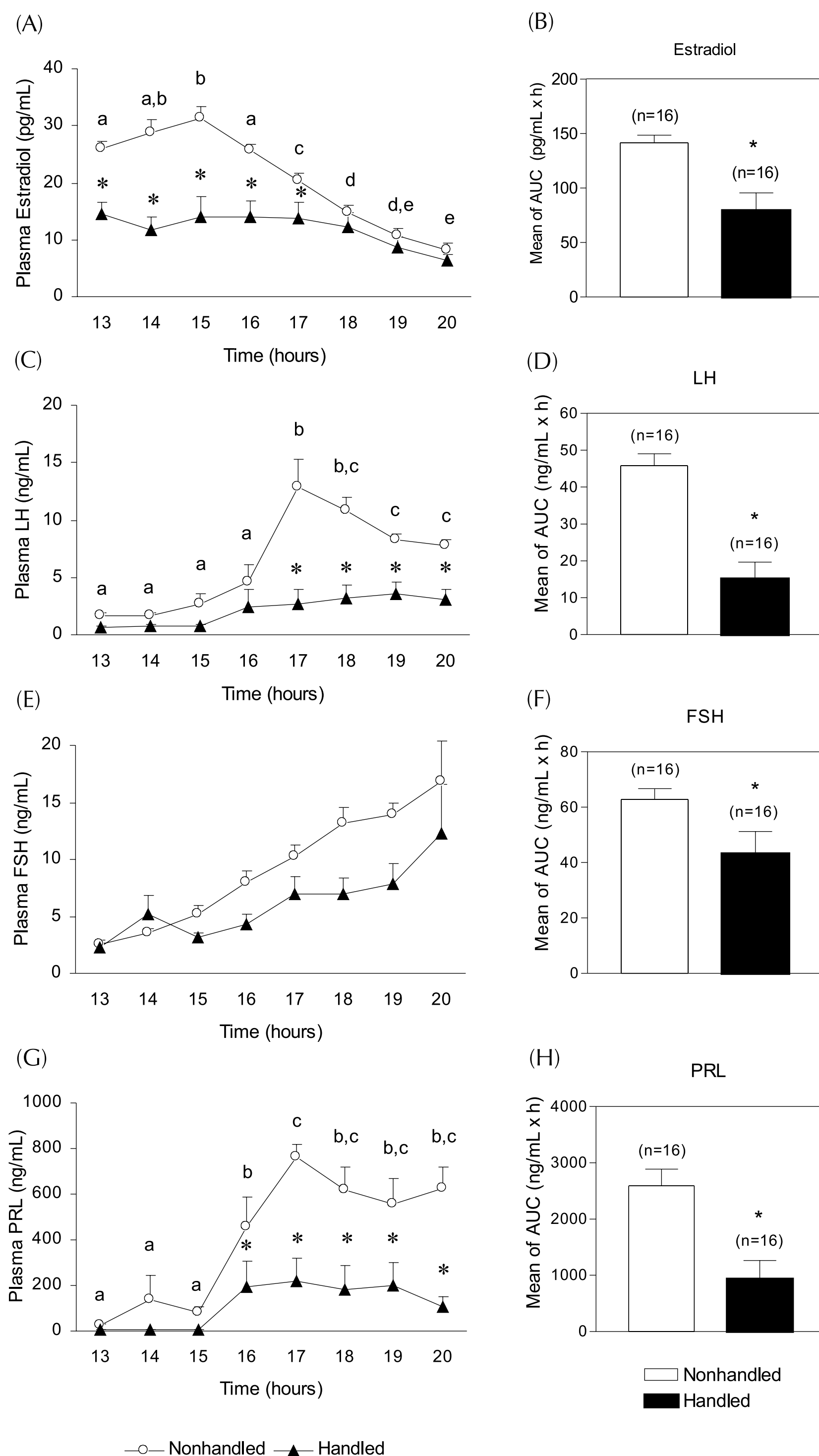

Figure 3 Effects of neonatal handling on the plasma concentrations of E_2 (pg/ml), LH, FSH and PRL (ng/ml) and AUC of E_2, LH, FSH and PRL in the afternoon of the proestrus in adult female rats. The blood samples (0·6 ml) were collected hourly from 1300 to 2000 h from a jugular catheter in proestrus, with an equivalent volume of 0·9% NaCl (saline) solution replaced after each bleeding. (A, C, E, G) Plasma hormone concentrations (means ± S.E.M.); data were analyzed using two-way ANOVA with repeated measures followed by the Newman–Keuls test (significance accepted at $P \leq 0{\cdot}05$). * Indicates a significant difference from controls at each time and the letters indicate significant differences within the same group. (B, D, F, H) Each bar represents the mean ± S.E.M. for AUC hormones. Data were analyzed using Student's *t*-test (significance accepted at $P \leq 0{\cdot}05$). * Indicates a significant difference from controls. The number of animals (*n*) is given in parentheses.

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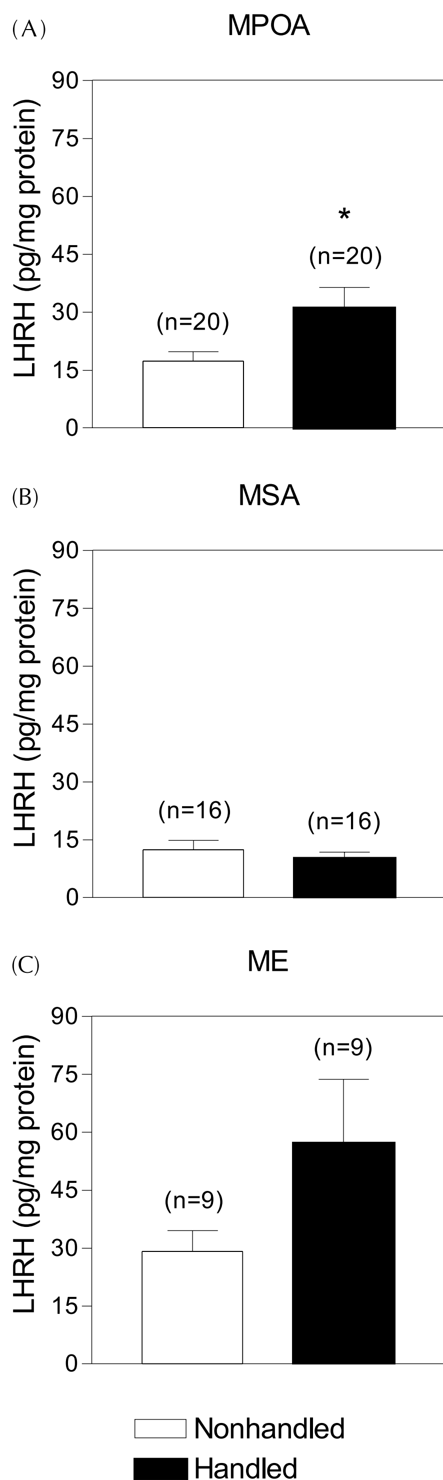

Figure 4 Effects of neonatal handling on LHRH content in the MPOA (A), MSA (B) and ME (C) in adult female rats decapitated at 1800 h in proestrus. Each bar represents the mean ± S.E.M. for LHRH content. The number of animals (*n*) is given in parentheses. Data were analyzed using Student's *t*-test (significance accepted at $P \leq 0{\cdot}05$). *Significant difference from controls.

detected in the LHRH content in the MSA ($t_{34}=0{\cdot}58$, $P=0{\cdot}56$) or in the ME ($t_{15}=1{\cdot}6$, $P=0{\cdot}11$).

Discussion

The decreased ovulation rate caused by the early-life environmental stimulation described previously (Gomes *et al.* 1999) was confirmed in the present study. Neonatal handling significantly reduced the number of oocytes in the morning of the estrus of most animals (approximately half of the handled females had no oocyte in the cycle phase analyzed). The reduced ovulation is probably due to changes in the hormonal profiles detected in the neonatal handled females.

In the nonhandled control rats, results showed that the plasma level of E_2 remained low on the day of estrus and metestrus, began to increase during the diestrus and increased further to reach a peak in the proestrus. On the other hand, plasma P showed two surges during the estrous cycle. The first surge represents the short functional life of the corpus luteum, while the second is the preovulatory surge of P in the evening of the proestrus. The patterns of E_2 and P secretion during the 4 day estrous cycle are in agreement with previous studies, although different blood-collection procedures were used (Butcher *et al.* 1974, Smith *et al.* 1975, Savoy-Moore *et al.* 1980). In previous studies, blood was collected by decapitation, while we used the same animal in each estrous cycle phase by sampling blood through jugular cannulation. Moreover, we used the Wistar strain while the other authors used Sprague–Dawley rats. Nevertheless, we were able to replicate the basic patterns of E_2 and P secretion in our control nonhandled females.

Ovulation is a complex process involving a sharp surge of steroid hormones, LH and FSH, in the preovulatory period (Freeman 1994). For the greater part of the estrous cycle (estrus, metestrus and diestrus), E_2 helps retrain LH and FSH secretion by negative feedback. But in the proestrus, E_2 also exhibits a positive-feedback influence upon the LHRH neurons and pituitary gonadotrophs to generate the preovulatory LH and FSH surges inducing ovulation (Levine 1997, Herbison 1998). Neonatal handling induced a prominent decrease in plasma E_2 on the proestrus day. The reduction in plasma E_2 could be one of the causes for the decrease in the number of oocytes in the handled females. Since the importance of LH in inducing ovulation is well recognized (Ishikawa 1992) and our results showed the absence of the LH surge in handled female rats, the reduction or absence of ovulation observed in the present and previous study (Gomes *et al.* 1999) could also be due to the alteration in the LH surge. The magnitude of the preovulatory LH surge is determined by the combined actions of the LHRH, E_2 (Karsch *et al.* 1997, Levine 1997, Herbison 1998) and ligand-independent activation of the P receptor in the

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anterior pituitary (Gonzáles *et al.* 2000). In the present work, the LHRH content in the MPOA and plasma E_2 were altered in the afternoon of the proestrus by neonatal handling, which could be the cause for the reduced LH surge.

The precise mechanism by which E_2 stimulates the surge of LHRH and LH remains unclear; however, several neurotransmitters appear to participate in that mechanism (Petersen *et al.* 2003). Noradrenaline (norepinephrine) from the locus coeruleus (LC) releases LHRH, as shown by noradrenergic-pathway ablation or the administration of α-adrenergic receptor antagonists that blocked the occurrence of the LH surge (Herbison 1998). Moreover, lesion of the LC in the morning of proestrus decreased the noradrenaline content in the MPOA and medial basal hypothalamus and blocked the preovulatory surge of LH and FSH and ovulation (Anselmo-Franci *et al.* 1997). Previous studies showed that neonatal handling increases the expression of the adrenergic α2 inhibitory autoreceptors in the LC in male rats (Liu *et al.* 2000) and induces a stable reduction in the number of cells in that noradrenergic nucleus in male and female rats (Lucion *et al.* 2003). These changes in the LC could have diminished the stimulatory effect of LC on the hypothalamus and consequently reduced the LHRH surge in the proestrus afternoon. In fact, our results showed increased LHRH content in the MPOA, which could be explained by alterations in the control mechanism of the LHRH neurons. This increase in LHRH content could represent a deficit in its release (Gerendai *et al.* 1980, Helena *et al.* 2002). The LC action on the LHRH neurons appears to be more closely related to the positive-feedback mechanism on the proestrus day than to the negative feedback that occur in the other phases of the cycle (Helena *et al.* 2002). This result probably explains why neonatal handling affected plasma E_2 on the proestrus day.

Another possible cause of the reduced ovulation in the neonatal handled females could be the deficit of the ovarian follicular growth, which is determined by the interaction of E_2, LH and FSH (Richards 1980, Tonetta & Dizerega 1989, Arai *et al.* 1998, Bao *et al.* 2000). Since neonatal handling reduces E_2 levels on the proestrus day, the FSH surge in the morning of estrus, which is stimulated by E_2 in proestrus and plays an important role in the selection of small antral follicles that will continue growth and potentially reach the preovulatory stage in the proestrus of the following cycle (Hirshfield & Midgley 1978, Szabo *et al.* 1998), could be reduced.

Neonatal handled female rats have shown reduced secretion of P in metestrus, but not in diestrus. From metestrus to diestrus, the P surge originates from the corpus luteum (Freeman 1994). The decreased P level on the metestrus day is probably due to the reduced ovulation induced by the handling procedure.

Besides affecting ovulation, neonatal handling also decreases sexual receptiveness in female rats (Padoin *et al.* 2001). The decreased plasma level of E_2 on the proestrus day could be one of the causes for the decrement in sexual behavior. Lordosis, the female receptive posture, requires coordinated actions of gonadal hormones with a descending neural pathway from the ventromedial hypothalamic nucleus (VMH; McEwen 1981, Etgen *et al.* 1999, Flanagan-Cato 2000). The action of E_2 in the VMH is crucial to the promotion of that behavior (Mathews & Edwards 1977, Pfaff & Sakuma 1979, Davis *et al.* 1982, Pleim *et al.* 1989). Receptor immunocytochemistry and *in situ* hybridization have demonstrated the presence of E_2 receptor in the VMH (Simerly *et al.* 1990, DonCarlos *et al.* 1991, Shughrue *et al.* 1997). On the other hand, there is strong *in vivo* evidence of genomic and membrane actions of P, as well as ligand-independent P receptor activation in the regulation of lordosis behavior and gonadotrophin release (Bellido *et al.* 1999, Schumacher *et al.* 1999, Chappell & Levine 2000, Auger 2001, Mani 2001). E_2 induces the expression of P receptors in the pituitary and hypothalamus, with the highest level in the proestrus (Guerra-Araiza *et al.* 2000, Scott *et al.* 2000, Turgeon & Waring 2000). We may infer that the reduction in E_2 secretion on the proestrus day decreased P receptor expression and thus it could have affected the lordosis response in the neonatal handled females.

The PRL surge also appears to be an important modulator for sexual behavior in female rats. In the afternoon of the proestrus, suppression of the spontaneous release of PRL with a dopamine agonist dramatically attenuates sexual receptivity (Witcher & Freeman 1985). The PRL can act centrally and/or peripherally through stimulation of adrenal P secretion to influence lordosis since ovariectomized and adrenalectomized rats treated with E_2 responded with lower levels of lordosis than did sham-operated controls (Witcher & Freeman 1985).

Previous work (Drago & Lissandrello 2000) suggests that an effective plasma concentration of PRL is important for the expression of the behavioral effects of this hormone because low doses of PRL (5 or 10 μg/kg s.c.) stimulate novelty-induced grooming in rats besides facilitating rather than inhibiting sexual behavior in male rats. According to this study the effects of acute injection of low doses of PRL or short-term hyperprolactinemia on female sexual behavior could possibly be explained by actions of this hormone on some neurotransmitter system other than the dopaminergic system.

E_2 affects PRL secretion at two levels. Directly at the pituitary lactotroph, E_2 controls PRL gene expression modifying its sensitivity to physiological stimulators and PRL secretion inhibitors. In the hypothalamus, E_2 modifies the activity of the neuroendocrine neurons known to control PRL secretion (Freeman *et al.* 2000).

In conclusion, the present results show that the profile of several hormones related to ovulation and sexual behavior has been altered by the neonatal handling procedure. In the proestrus, the expected plasma increases of E_2, LH and

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PRL were reduced; whereas the LHRH content in the MPOA was higher in the neonatal handled group compared with the nonhandled one. These hormonal changes are probably the cause of reduced ovulation and sexual receptiveness in female rats. We may conclude that early-life stimulation can induce long-lasting changes in the hypothalamus-pituitary-gonad axis.

Acknowledgements

The authors thank Sonia M Zanon and Ariana M Fernandes for technical assistance. FAPESP, CAPES and CNPq supported this research. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 2 October 2004
Accepted 3 November 2004

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