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Twenty-four hour rhythm of plasma prolactin in female rabbit pups Correlation with hypothalamic and adenohypophysial dopamine, serotonin, gamma-aminobutyric acid and taurine content

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Abstract

Lactation in the rabbit is a nocturnal activity, extremely short and regular, that can be a strong synchronizer for the development of circadian rhythmicity in the pups. In the present study, 24-h rhythmicity of plasma prolactin and median eminence and anterior pituitary content of dopamine (DA), serotonin (5HT), gamma-aminobutyric acid (GABA) and taurine were examined in 11 days old female pups kept under 16 h light:8 h dark photoperiods (lights on at 08:00 h). Groups of six to seven female rabbit pups were killed by decapitation at six different time points throughout a 24-h cycle, starting at 09:00 h. Plasma prolactin levels changed significantly throughout the day, showing two peaks, one at first half of rest span (at 13:00 h) and another one at the beginning of the scotophase (at 01:00 h), just preceding doe visit. Median eminence DA content changed in a bimodal way as a function of time of day, displaying two maxima, at the beginning of the rest span

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and of the activity phase. Median eminence DA and plasma prolactin correlated significantly in an inverse way. Two maxima in median eminence 5HT levels were found, about 4 h in advance to the prolactin peaks. Circulating prolactin correlated inversely with median eminence 5HT content and directly with adenohypophysial 5HT content. Median eminence GABA content reached its maximum at the beginning of the scotophase and correlated significantly with plasma prolactin concentration. A positive correlation between plasma prolactin and adenohypophysial taurine content was observed. These results show that the circadian rhythmicity in prolactin secretory mechanisms in female rabbit pups develops during the early neonatal life.

Keywords: Female rabbit pups; Prolactin; Dopamine; Serotonin; Gamma-aminobutyric acid; Taurine; Median eminence; Adenohypophysis; Circadian rhythms

1. Introduction

The rabbit has been employed as a laboratory animal since long. Due to its abundance, size and importance as an agricultural pest, the rabbit is one of the best-studied laboratory animals in the wild. In addition, as a member of the class lagomorpha, it provides a useful comparative model for the studies performed on commonly used laboratory rodent species (Manning et al., 1994; Thompson and King, 1994).

The rabbit is essentially nocturnal in behavior and displays a clear daily pattern of activity (Jilge and Hudson, 2001). For example, under both laboratory and natural conditions, the daily nursing visit of the doe is extremely short and regular, taking place typically in the dark (Broekhuizen and Mulder, 1983). The pups anticipate these visits with increased arousal and motor activity (Jilge, 1995). Despite the short duration of each nursing bout (around 3 min) per day, the altricial rabbit pups (which are blind for the first 10 days of life) can locate the mother's nipples and suckle milk due to the perception of an olfactory signal that is emitted by the mother's ventrum (Distel and Hudson, 1985).

This unusual pattern of maternal care and the demands it places on the litter provide an excellent opportunity for analyzing circadian rhythmicity during early ontogenic development (Jilge and Hudson, 2001). Indeed, many circadian rhythms in developing mammals are entrained by the rhythmicity of the mother (Davis and Gorski, 1988; Davis and Mannion, 1988). One of these rhythms is that of prolactin.

In rodents, the secretion of prolactin changes throughout the day, displaying a characteristic 24-h pattern with maximal values close to light–dark transition (Garcia-Bonacho et al., 2000; Castrillon et al., 2001). This pattern is not yet established in infantile rats before 21 days of age (Esquifino et al., 1998). As a continuation of our previous studies in rats, we wished to examine whether this lack of development of circadian rhythmicity in plasma prolactin was also apparent in rabbit pups. The hypothesis was that in view of the very restricted exposure of doe to lactation, it constitutes an effective "Zeitgeber" for plasma prolactin as it plays for many other circadian rhythms. Plasma prolactin levels and dopamine (DA), serotonin (5HT), gamma-aminobutyric acid (GABA) and taurine content of median eminence and adenohypophysis were examined in neonatal rabbits killed on day 11 of life at different time intervals during a 24-h period.

2. Material and methods

2.1. Animals

This study was performed with 24 multiparous, lactating Californian × New Zealand White crossbreed female doe rabbits. Animals were housed in research facilities of the Animal Production Department, Universidad Complutense. They were maintained under controlled light–dark cycles (16 h light:8 h dark; lights on at 08:00 h), housed in individual metal cages, fed ad libitum using a commercial pellet diet (Lab Rabbit Chow, Purina Mills, Torrejón de Ardoz, Madrid, Spain) and had free access to tap water. On day 1 after parturition, litter size was standardized to 8–9 by adding or removing kits to assure similar lactation conditions. Nursing visit of does under these conditions occurred during the dark phase of the photoperiod (around 02:00 a.m.) for 5 min (Hudson and Distel, 1989). This study was performed according to the CEE Council Directive (86/609, 1986) for the care of experimental animals. Groups of six to seven female rabbit pups were killed by decapitation on day 11 of life at six different time points every 4 h, throughout a 24-h cycle starting at 09:00 h. At night intervals, animals were killed under red dim light. Number of animals per time interval was: 09:00 h, 7; 13:00 h, 6; 17:00 h, 6; 21:00 h, 5; 01:00 h, 6; 05:00 h, 6. The brains were quickly removed, and the median eminence and the anterior pituitary were taken out. Tissues were weighed and stored at −80 °C until assay (within 2 weeks). The tissue was homogenized in chilled 2 M acetic acid and after centrifugation (at 15,000 × *g* for 30 min, at 5 °C), the samples were either analyzed for DA and 5HT or boiled for 10 min and further centrifuged at 5000 × *g* for 20 min to measure GABA and taurine, as described in detail elsewhere (Esquifino et al., 2000).

2.2. Prolactin assay

Plasma prolactin levels were measured by a specific radioimmunoassay method (Ubilla et al., 1992) using AFP-991086 antibody supplied by the National Institutes of Health (NIH, Bethesda, MD, USA) and Dr. A.F. Parlow (Harbour-UCLA Medical Center, CA, USA). The titer of antibody used was 1:62,500. The prolactin standard used was $RbPR_L$-RP-1. The hormone was labeled with ^{125}I by the chloramine-T method (Greenwood et al., 1963). The volume of plasma for prolactin determinations was 10 μl. *Staphylococcus aureus* (prepared by the Department of Plant Physiology, U.A.M., Madrid, Spain) was used to precipitate the bound fraction (Ubilla et al., 1992). All samples were measured in the same assay run to avoid inter-assay variations. The sensitivity of the assay for PRL was 0.125 ng/ml and the intra-assay coefficient of variation was <5%. The intra-assay coefficient was calculated using one pool of plasma measured 10 times in the same assay.

2.3. DA and 5HT analysis

DA and 5HT were measured by high performance liquid chromatography (HPLC) using electrochemical detection (Coulochem, 5100A, ESA, USA), as described elsewhere (Cano et al., 2001). A C-18 reverse phase column eluted with a mobile phase (pH 4. A 0.1 M sodium acetate, 0.1 M citric acid, 0.7 mM sodium octysulphate and 0.57 mM EDTA containing 10% methanol, v/v), was employed. Flow rate was 1 ml/min, at a pressure

of 2200 psi. Fixed potentials against H_2/H^+ reference electrode were: conditioning electrode: −0.4 V; preoxidation electrode: +0.10 V; working electrode: +0.35 V. Indoleamine and catecholamine concentrations were calculated from the chromatographic peak heights by using external standards and expressed as pg/μg protein. The linearity of the detector response for DA and 5HT was tested within the concentration ranges found in hypothalamic supernatants.

2.4. Amino acid analysis

Amino acids were separated and analyzed using HPLC with fluorescence detection after precolumn derivatization with OPA, as described elsewhere (Esquifino et al., 2000). An aliquot of the tissue supernatant containing homoserine as internal standard was neutralized with NaOH (4 M), and was then incubated at room temperature with OPA reagent (4 mM OPA, 10% methanol, 2.56 mM 2-mercaptoethanol, in 1.6 M potassium borate buffer, pH 9.5) for 1 min. At the end of this period, the reaction was stopped by adding acetic acid (0.5%, v/v). Samples were loaded through a Rheodyne Model 7125) injector system with a loop sampler of 20 μl to reach a C-18 reverse-phase column (4.6 mm i.d. × 150 mm, Nucleosil 5, 100 A). Elution was performed by using a mobile phase consisting of 0.1 M sodium acetate buffer (pH 6.5) containing 35% methanol, at a flow rate of 1 ml/min and at a pressure of 140 bars. The column was subsequently washed with the same buffer containing 70% methanol and was re-equilibrated with the elution buffer before re-use. The HPLC system comprised a solvent delivery system coupled to a filter fluorometer (excitation 340 nm, emission 455 nm). This procedure allows a clear separation and resolution of GABA and taurine.

Median eminence amino acid content was calculated from the chromatographic peak heights by using standard curves and appropriate internal standards. The linearity of the detector response for GABA and taurine was tested within the concentration range found in median eminence supernatants.

2.5. Statistics

Statistical analysis of results was performed by a one-way analysis of variance (ANOVA) followed by post hoc Tukey–Kramer's multiple comparisons tests. 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.

3. Results

Fig. 1 shows mean prolactin concentration throughout the day in female pups. Plasma prolactin levels changed significantly throughout the day ($p < 0.0001$), with two peaks, one at first half of rest span (at 13:00 h) and another at the beginning of the scotophase (at 01:00 h).

Figs. 2–5 depict the changes in median eminence and adenohypophysial content of DA, 5HT, GABA and taurine. Mean plasma prolactin concentration is plotted as a reference in every case.

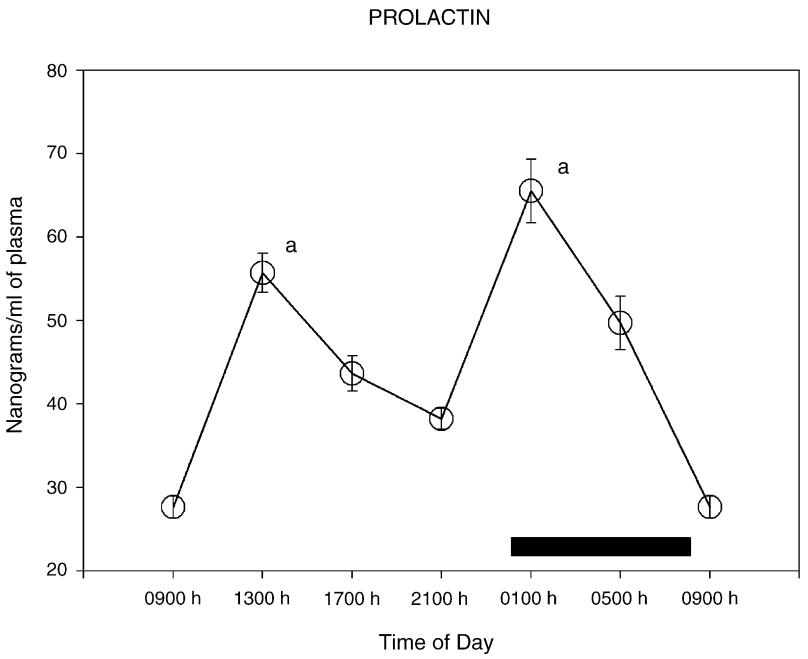

Fig. 1. Twenty-four hour changes in plasma prolactin levels of 11 days old female rabbit pups. Groups of six to seven pups were killed by decapitation at six different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. $^{a}p < 0.01$ vs. 09:00, 17:00 and 21:00 h, Tukey–Kramer's multiple comparisons test. For further statistical analysis, see text.

Resembling circulating prolactin, median eminence DA content changed in a bimodal way as a function of time of day, showing two maxima, at the beginning of the rest span (i.e. at 09:00 h) and of the activity phase (i.e. at 01:00 h) ($p = 0.001$, Fig. 2). In the case of adenohypophysial DA content, a single maximum occurred at the second half of scotophase (at 05:00 h) ($p < 0.001$). Plasma prolactin and median eminence DA content correlated in an inverse way, the correlation being described by a log model with $r^2 = 0.17$, $b_0 = 235.9$ and $b_1 = -2.12$ ($p = 0.04$).

As shown in Fig. 3, a bimodal pattern for 24-h changes of median eminence 5HT concentration was apparent in suckling pups ($p < 0.0001$). Two maxima of median eminence 5HT levels were found, at the beginning and at the end of the light phase (at 09:00 and 21:00 h, respectively), preceding by about 4 h the respective prolactin peaks. In the adenohypophysis, the 24 h pattern of 5HT content showed a peak at 13:00 h with somewhat constant, intermediate values during the scotophase ($p < 0.001$). Circulating prolactin correlated inversely with median eminence 5HT content and directly with adenohypophysial 5HT content. These correlations were best described by a log model with $r^2 = 0.57$, $b_0 = 1967.3$ and $b_1 = -429.8$ ($p = 0.00001$) (median eminence 5HT) and $r^2 = 0.17$, $b_0 = -133.3$ and $b_1 = 81.3$ ($p = 0.02$) (adenohypophysial 5HT).

Fig. 4 shows the changes in median eminence and adenohypophysial GABA content. In median eminence, GABA content attained its maximum at the beginning of the scotophase

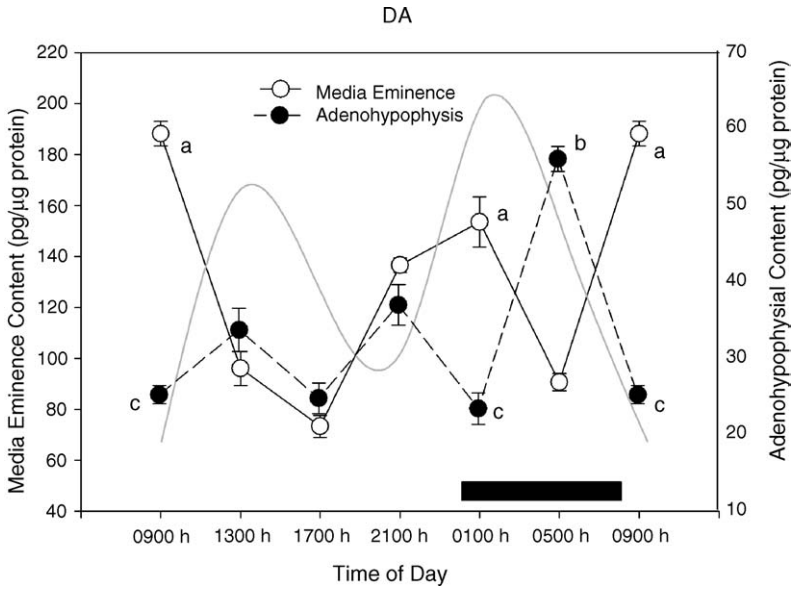

Fig. 2. Twenty-four hour changes in median eminence and adenohypophysial DA content in 11 days old female rabbit pups. Groups of six to seven pups were killed by decapitation at six different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. Circulating prolactin levels are shown in shaded line. Letters indicate the existence of significant differences between time points within each tissue after a Tukey–Kramer's multiple comparisons test, as follows: $^{a}p < 0.01$ vs. 13:00, 17:00 and 05:00 h; $^{b}p < 0.01$ vs. all time points; $^{c}p < 0.05$ vs. 21:00 h. For further statistical analysis, see text.

(at 01:00 h, $p < 0.001$). In the anterior pituitary, GABA content showed a peak between 13:00 and 17:00 h ($p = 0.0008$). Median eminence GABA levels correlated significantly with plasma prolactin, this correlation being described by a linear model with $r^2 = 0.19$, $b_0 = 33.7$ and $b_1 = 0.44$ ($p = 0.01$).

Fig. 5 depicts the 24 h changes in taurine content. Median eminence taurine concentration varied in a bimodal way showing a peak at 13:00 h, a nadir at 17:00 h, and high levels throughout the end of the light period and during the scotophase ($p = 0.0047$). Two peaks were seen for adenohypophysial taurine content ($p < 0.001$), one during the light phase of daily photoperiod (at 13:00 h), and another during the scotophase (at 05:00 h). A direct correlation between plasma prolactin and adenohypophysial taurine content, best described by a log model, occurred ($r^2 = 0.21$, $b_0 = -12.1$ and $b_1 = 4.79$ ($p = 0.01$).

4. Discussion

Foregoing results indicate that in 11 days old female rabbit pups, 24-h changes in plasma prolactin levels are detectable. The pattern displayed two maxima, a major one at the first half of the activity phase and a second one at the middle of the rest span. These findings differ substantially from what was found in rat pups of a comparable age, which did not

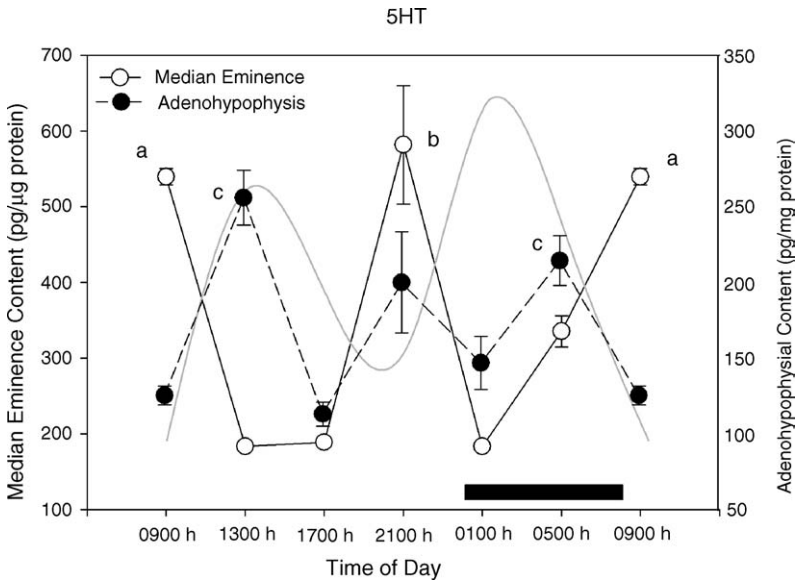

Fig. 3. Twenty-four hour changes in median eminence and adenohypophysial 5HT content in 11 days old female rabbit pups. Groups of six to seven pups were killed by decapitation at six different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. Circulating prolactin levels are shown in shaded line. Letters indicate the existence of significant differences between time points within each tissue after a Tukey–Kramer's multiple comparisons test, as follows: [a] $p < 0.01$ vs. 13:00, 17:00, 01:00 and 05:00 h; [b] $p < 0.01$ vs. 13:00, 17:00 and 01:00 h; [c] $p < 0.01$ vs. 09:00 and 17:00 h. For further statistical analysis, see text.

exhibit any prolactin circadian rhythm before weaning (Esquifino et al., 1998). Hence, a faster maturation of the circadian system seems to occur in the rabbit as compared to the rat.

The nocturnal maximum in plasma prolactin values measured in 11 days old female rabbit pups was attained 1 h after switching off the light, anticipating exposure to the daily nursing visit of the doe which is an extremely short and regular behavior (around 02:00 a.m., Hudson and Distel, 1989). Indeed, the anticipatory prolactin release was seen in all pups, supporting the conclusion that this daily rhythm in maternal behavior has a very strong "Zeitgeber" influence on the newborn. In adult animals, food availability acts as a "Zeitgeber" resulting in a "food anticipatory activity" that occurs only if feeding intervals are within the circadian range, circadian rhythms exhibiting a gradual resetting in response to mealtime shifts and a free-running during total food deprivation (Mistlberger, 1994). Since prolactin is readily released following stress (Ben-Jonathan and Hnasko, 2001), it seems feasible that food anticipatory activity acting through arousal mechanisms is responsible for the particular changes in plasma prolactin seen in rabbit pups.

It must be noted that rabbit pups are very immature at birth and their survival is clearly dependent on the doe's daily visit. The unusual limited maternal care pattern is under a tight circadian control (Jilge and Hudson, 2001). At 13–18 days of age, rabbit pups have grown, improved their motor and thermoregulatory capacity and are able to leave the nest (see for

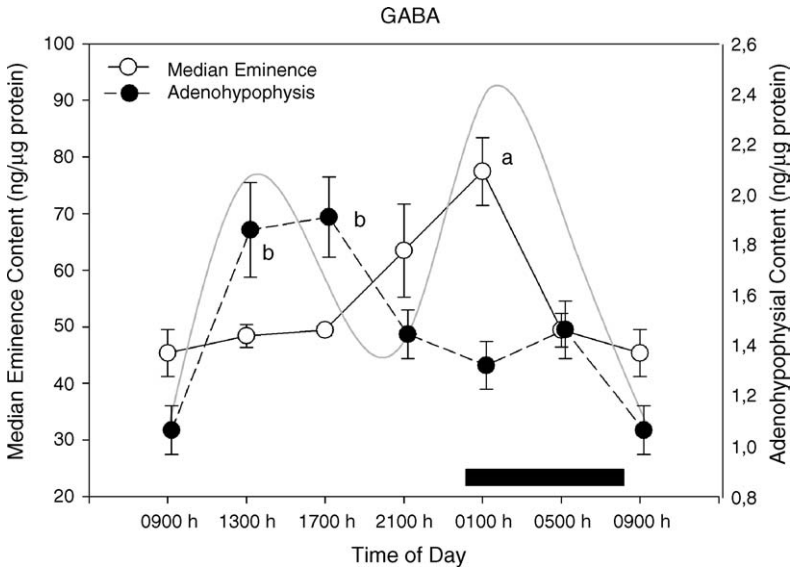

Fig. 4. Twenty-four hour changes in median eminence and adenohypophysial GABA content in 11 days old female rabbit pups. Groups of six to seven pups were killed by decapitation at six different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. Circulating prolactin levels are shown in shaded line. Letters indicate the existence of significant differences between time points within each tissue after a Tukey–Kramer's multiple comparisons test, as follows: [a] $p < 0.01$ vs. 09:00, 13:00, 17:00 and 05:00 h; [b] $p < 0.05$ vs. 09:00 h. For further statistical analysis, see text.

review (Hudson and Distel, 1986, 1987; Martinez-Gomez et al., 2004). Such a rapid growth is possible by a clear synchronization in behavior and physiology of the doe and the pups (Jilge and Hudson, 2001).

Prolactin is a versatile compound that has a dual function as a circulating hormone and as a cytokine. The prolactin receptor is a member of the cytokine receptor superfamily, linked to activation of signaling pathways that promote cell growth and survival. Through these mechanisms prolactin regulates diverse physiological functions via its effects on cellular processes such as proliferation and differentiation (Vera-Lastra et al., 2002; Yu-Lee, 2002). Prolactin secretion is under inhibitory control by hypothalamic DA (Ben-Jonathan and Hnasko, 2001). DA released from tuberoinfundibular dopaminergic neurons enters the hypophysial portal blood to reach the anterior pituitary and to inhibit prolactin secretion. In turn, prolactin stimulates DA secretion from dopaminergic neurons, closing a feedback loop. The foregoing results in 11 days old female rabbits indicate that DA content in median eminence tended to follow a reciprocal pattern to plasma prolactin, Indeed, a negative correlation between plasma prolactin and DA content in median eminence was shown, suggesting that a decrease in median eminence DA content may be causally linked to prolactin release (Freeman et al., 2000).

Dopaminergic neurons in the arcuate nucleus receive a dense serotonergic innervation consisting of a population of brainstem neurons arising mainly from the midbrain raphe nuclei (Steinbusch, 1981). Fibers also originate from 5HT cell bodies located within the

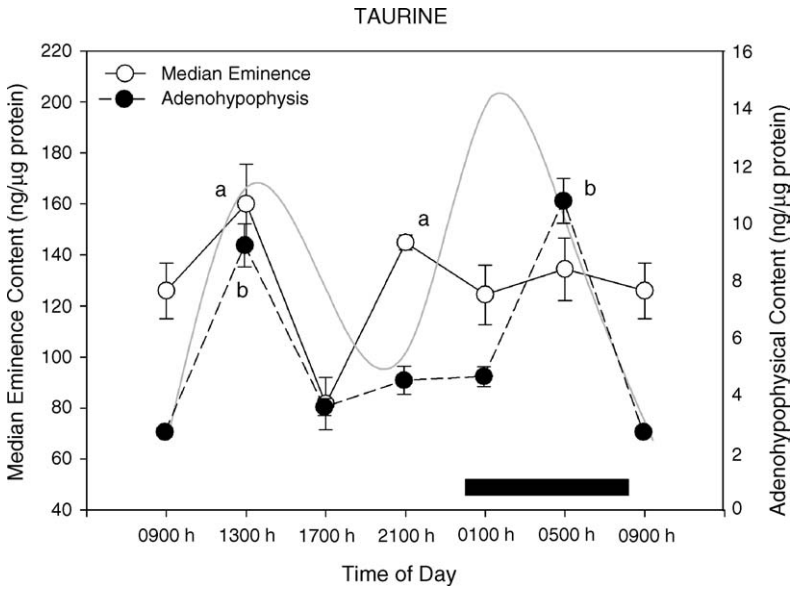

Fig. 5. Twenty-four hour changes in median eminence and adenohypophysial taurine content in 11 days old female rabbit pups. Groups of six to seven pups were killed by decapitation at six different time intervals throughout a 24-h cycle. Bar indicates scotophase duration. Results are the means ± S.E.M. Circulating prolactin levels are shown in shaded line. Letters indicate the existence of significant differences between time points within each tissue after a Tukey–Kramer's multiple comparisons test, as follows: [a] $p < 0.01$ vs. 17:00 h; [b] $p < 0.01$ vs. 09:00, 17:00, 21:00 and 01:00 h. For further statistical analysis, see text.

hypothalamus. There is a close approximation of 5HT fibers to dopaminergic cell bodies in the arcuate nucleus (Kiss and Halasz, 1986). Therefore, the effect of 5HT on prolactin release has been linked to the modulation of inhibitory dopaminergic inputs to the pituitary. The foregoing results demonstrate that 5HT content in median eminence of female rabbit pups varies throughout the day inversely to the plasma prolactin, both parameters displaying mirror patterns. In fact, a significantly negative correlation between them was found. In the adenohypophysial 5HT content positively correlated with plasma prolactin, thus pointing to a possible direct effect of 5HT on the release of prolactin.

Median eminence neurons release GABA in portal blood that reaches the anterior pituitary (Gudelsky et al., 1983) and this release can affect prolactin secretion (Casanueva et al., 1984; Selgas et al., 1997; Duvilanski et al., 1998). The present results in female rabbit pups indicate a parallel pattern of plasma prolactin and median eminence GABA during the activity phase. In fact, both parameters correlated positively in median eminence but not in the adenohypophysis.

Taurine has been also implicated in prolactin secretion control (Login, 1990; Arias et al., 1998; Duvilanski et al., 1998). Female rabbit pups showed a daily pattern of taurine content in median eminence and in the anterior pituitary, as well as a correlation with plasma prolactin levels (in the case of adenohypophysial taurine) suggesting a direct effect of taurine on prolactin release.

Summarizing, the present study in neonatal female rabbits demonstrates the existence of 24-h variations in circulating prolactin levels and in median eminence and adenohypophysial levels of some putative modulators of prolactin release. Plasma prolactin levels correlated either negatively (median eminence DA; median eminence 5HT) or positively (median eminence GABA; adenohypophysial 5HT; adenohypophysial taurine) with putative modulator content.

Acknowledgments

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