Pups removal enhances thyrotropin-releasing hormone mRNA in the hypothalamic paraventricular nucleus

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During lactation, changes in the nervous and hormonal systems of the rat alter hypothalamic neuronal functions (1-3); among these, variations in hypothalamic thyrotropin-releasing hormone (TRH) metabolism have been demonstrated. Most median eminence TRH is in nerve terminals that originate from cell bodies present in the hypothalamic paraventricular nucleus (PVN) (4). This nucleus is probably involved in the control of prolactin secretion in lactating rats because lesions of the PVN prevent the increase of prolactin in response to suckling stimulus in lactating rats (5). In spite of contradictory observations, which may be due to technical problems, portal blood TRH quantification (2) or passive immunization studies (6) indicate that TRH is released from the median eminence in response to suckling. The suckling-induced TRH release seems correlated with an increase in TRH synthesis because a brief suckling stimulus after removing the pups for 8 h induces a transient rise in the level of paraventricular TRH mRNA (7). This observation is consistent with previous studies that showed a correlation between rates of peptide synthesis and release in peptide-secreting cells (8, 9).

Suckling interruption by removal of the pups or natural weaning induces a rapid increase of tyrosine hydroxylase mRNA in the arcuate nucleus (significant at 6 h) (10) and portal blood dopamine concentration (11), which may contribute to the rapid drop of serum prolactin (12, 13). Later on (16-24 h), significant decreases of serum progesterone and corticosterone are detected (12, 14) followed by an increase of 17β-estradiol (12, 15). These events lead to the reinitiation of the estrous cycle with the first proestrous surge detected 2 or 3 days after removal of the pups (12, 15).

In contrast to the rapid changes in dopaminergic activity, there is no evidence for a rapid adaptation of TRH metabolism to suckling interruption. Paraventricular TRH mRNA or mediobasal hypothalamic (MBH) TRH levels are not modified 8 h after removal of the pups (7). One day after the end of lactation, paraventricular TRH mRNA levels are still unchanged but MBH TRH levels are decreased (16). Because TRH metabolism in hypothalamic neurons changes during the estrous cycle (16), withdrawal of the pups could also affect paraventricular TRH biosynthesis. We therefore examined the effect of long-term interruption of suckling on paraventricular TRH mRNA, an index of paraventricular TRH neuron activity, to determine if paraventricular TRHergic neurons modify their activity during the transition of lactation to the ovulatory cycle. We report here that removing the pups for 56 h at mid-lactation induced an increase in the paraventricular...
TRH mRNA and a decrease in the MBH TRH content. Because the increase of paraventricular TRH mRNA is correlated negatively with serum corticosterone levels, we determined whether this increase could be blocked by an acute increase of serum corticosterone levels produced by a 1 h suckling period 8 h after removal of the pups. This stimulus did not alter TRH and TRH mRNA levels determined 56 h after initial removal of the pups. The TRH mRNA increase was not observed in the preoptic area—anterior hypothalamus (POA-AH). In summary, interruption of lactation induced a delayed activation of paraventricular TRH biosynthesis, which may permit TRH neuron activity to be adjusted to the new physiological demands of the estrous cycle.

Material and methods

Animals

Primiparous Wistar female rats maintained in a 12-h light/dark cycle (lights on at 07.00 h) and fed ad libitum were mated, handled daily (during gestation up to the experimental day) and kept in individual cages. On the day of delivery, the litter was adjusted to eight pups until days 12–14. On the day of the experiment, rats from the same lot at around 07.00 h were divided into five groups of four to six animals, all of which were separated from their pups. Animals of one group (I) were sacrificed 8 h after removal of the pups; animals from another group (V) were maintained for 56 h without their pups before sacrifice. In the other three groups, pups were returned to their mother 8 h after removal, allowed to suckle for 60 min and killed immediately (group II), 24 h (group III) or 48 h (group IV) after the suckling stimulus. Sometimes pups did not suckle for more than 45 min; however, mothers were kept with their pups for 60 min after pups initiated suckling. All animals were sacrificed between 15.00 and 16.30 h in a room next to their housing in order to minimize stress. A schematic representation of the protocol is shown in Fig. 1. Rats were sacrificed by placing them gently on a guillotine for immediate decapitation; trunk blood was collected and the brain extracted from the skull. The MBH, containing the median eminence and ventral part of the arcuate nucleus, was excised and kept at −70°C for TRH immunoreactivity determination; the rest of the brain was frozen at −70°C. For PVN dissection, the frozen brain was kept on dry ice and a 1.5-mm thick coronal slice was cut (anterior limit: plane P 1200 µm (17)); from this slice, a square containing the PVN was taken by making three cuts: one over the fornix and two perpendicular to the first cut at the level of each fornix (16). For POA-AH dissection, a 1.5-mm coronal slice just anterior to the previous slice was cut and the POA-AH dissected as described previously (7).

For each parameter, determinations were made in either one, two or three experiments. For each experiment (except for Fig. 3A) the data were calculated as a percentage of mean group I values.

Animals were used according to the Society for Neuroscience (USA) “Guidelines for the use of animals in neuroscience research”.

Radioimmunoassays

Corticosterone was assayed with an ICN diagnostic kit; TRH was assayed as reported previously (18), with

![Fig. 1. Schematic representation of the experimental program.](image-url)
intra- and interassay coefficients of variation of 6% and 10% respectively; and prolactin was assayed with NIH reagents (intra-assay coefficient of variation = 5%).

Quantification of TRH mRNA

Paraventricular or POA-AH RNA was isolated as described previously (16). Briefly, tissue was homogenized in 308 µl of 25 mmol/l TRIS·HCl (pH 7.4), 12.5 mmol/l NaCl, 2.5 mmol/l MgCl and 0.08% Triton X-100, and the homogenate was added in 100 µl of 6% sodium dodecyl sulfate, 0.4 mol/l NaCl and 40 mmol/l ethylenediaminetetraacetic acid and extracted three times with 1:1 phenol:chloroform:isoamyl alcohol 24:1. To the aqueous phase was added 5 mol/l NaCl (1/25th vol) and two volumes of ethanol, which was kept at −70°C overnight and centrifuged at 4°C to recover total RNA.

Aliquots of 5–10 mg of RNA per slot were subjected to electrophoresis (2.2 mol/l formaldehyde, 1% agarose, 10 mmol/l sodium phosphate buffer, pH 7.0), stained with ethidium bromide (4 µg/ml), destained with water, photographed (UV transilluminator) for ribosomal RNA quantification and transferred to a nitrocellulose membrane (24–36 h, 3 mol/l NaCl and 0.3 mol/l sodium citrate); membranes were then baked for 2 h at 80°C, hybridized (19) with rat TRH cDNA (kindly donated by Dr R Goodman) and labeled by nick translation using an Amersham kit. The autoradiographic signal for TRH mRNA was quantified by laser densitometry (Biomed Instruments, Fullerton, CA) and normalized to the amount of ribosomal RNA before transfer. Ribosomal RNA levels did not vary significantly between groups (PVN: F(4,61) = 1.0758, p = 0.3763; POA-AH: F(4,22) = 1.9765, p = 0.1399).

Statistical analyses

Analysis of variance (ANOVA) was applied using the “Statistica of Stat Soft” program. When F was significant (p < 0.05), differences between means were tested using the LSD test. Differences were considered significant when p < 0.05.

Results

We previously showed that MBH TRH and paraventricular or POA-AH mRNA levels are not modified 8 h after removal of the pups (7), consequently we have compared these values (group I, taken as the control) to later time points. In order to determine whether a suckling period was able to inhibit the long-term effect of withdrawal of the pups, values after the 1-h suckling period (group II) were taken as a second control because at this time, owing to the transient nature of the effect of suckling (7), paraventricular TRH mRNA levels were equal to those in non-suckled animals. Finally, owing to the circadian rhythm of paraventricular TRH mRNA (20), later time points were from animals that were also sacrificed between 15.00 and 16.30 h.

Serum corticosterone and prolactin levels

The pattern of serum corticosterone levels (Fig. 1A) was similar to that reported previously (14), with significant differences between groups (F(4,39) = 4.5746, p < 0.01). One hour of suckling after 8 h without the pups resulted in an increase in serum corticosterone levels. The corticosterone concentrations were lower 24 or 48 h after the 1-h suckling period, resembling group 1 values (Fig. 2A). The serum prolactin concentrations varied significantly (F(4,28) = 8.7773, p < 0.0001), with a similar pattern to corticosterone (Fig. 2B).

Levels of MBH TRH and paraventricular TRH mRNA

Significant differences were observed between MBH TRH levels (F(4,11) = 4.8795, p < 0.002). As shown in Fig. 3A (open bars), there was a 46% (P < 0.005) decrease in the MBH TRH content in animals after 56 h without the pups compared to animals after 8 h without the pups. As reported previously (7), a suckling stimulus after 8 h of litter removal induced a 33% (p < 0.02) decrease in the MBH TRH levels (Fig. 3A, dashed bars). The MBH TRH content remained low 24 and 48 h after the stimulus (29%, p < 0.02 and 54%, p < 0.0005, respectively, compared to group I) (Fig. 3A, dashed bars). Fifty-six hours after the initial withdrawal of the pups there was no difference between groups with or without suckling (Fig. 3A).

The paraventricular TRH mRNA showed an opposite pattern compared to the MBH TRH content (Fig. 3B), with significant differences between groups (F(4,61) = 3.1783, p < 0.02). We observed a 151% (p < 0.02) increase in the TRH mRNA levels of the animals 56 h after removal of the pups compared with 8 h without the pups (Fig. 3B, open bars). The animals with one suckling stimulus showed a gradual increase in the TRH mRNA levels 24 (43%) and 48 h (124%, p < 0.05) after the stimulus (compared to group II) (Fig. 3B, dashed bars). In a similar way to that observed for the MBH TRH levels, paraventricular TRH mRNA levels 56 h after removal of the pups were similar to those where a 1-h suckling stimulus was introduced 8 h after withdrawal of the pups (Fig. 3B).

Levels of POA-AH TRH mRNA

In order to establish whether these TRH mRNA changes were tissue-specific, we analyzed levels in POA-AH, finding significant differences between groups (F(4,22) = 5.8034, p < 0.01) (Fig. 4). One hour of suckling induced a 51% (p < 0.01) decrease that was slowly reverted to presuckling levels. Values were similar 8 or 56 h after removal of the pups.
Discussion

Removal of the pups at mid-lactation usually leads to reinstatement of the ovulatory cycle. Our data showed an increase of paraventricular TRH mRNA 56 h after interruption of lactation. This may correlate with initiation of the estrous cycle in the animals because previous data had shown that hypothalamic TRH mRNA fluctuates during the estrous cycle (16). The increase after suckling interruption was relatively slow and may be due to some hormonal change occurring after removal of the pups.

Direct evidence for a role of 17β-estradiol on paraventricular TRH neurons was shown in vitro.
where TRH release from the median eminence is enhanced in ovariectomized rats treated with 17β-estradiol (21). On the other hand, the regulatory region of the TRH gene contains a binding site for the progesterone receptor (22). However, there is no information regarding the role of 17β-estradiol or progesterone in modulating paraventricular TRH mRNA levels. The best-characterized regulators of paraventricular TRH mRNA are serum triiodothyronine (T3) and thyroxine (T4) levels, which down-regulate it (23, 24). Because serum T3 and T4 levels increase after removal of the pups (25), the data suggest

Fig. 3. Influence of lactation interruption on the mediobasal hypothalamus (MBH) TRH (A) and paraventricular (PVN) TRH mRNA (B) levels. Histograms represent means ± SEM. Numbers within bars are the number of animals used. For panel B, the values were calculated as a percentage of the control group (I, sacrificed 8 h after removal of the pups; 100%). Dashed bar: suckling stimulus: lactating mothers separated from their pups for 8 h, with pups allowed to suckle for 60 min and killed immediately (group II), 24 (group III) or 48 h (group IV) after the stimulus. Group V: lactating rats sacrificed after 56 h without pups. Statistical significance between mean: *p < 0.05; **p < 0.02; ***p < 0.01 and ****p < 0.005; "∞" compared with group I; "£" compared with group II.
that these hormones are not a key factor in setting paraventricular TRH mRNA levels during withdrawal of the pups.

On the other hand, paraventricular TRH neurons contain glucocorticoid receptors (26), the 5'-regulatory region of the TRH gene contains a glucocorticoid response element (22) and Lechan et al. (27) showed that adrenalectomy up-regulates paraventricular TRH mRNA in male rats. Because serum glucocorticoids decrease when pups are removed (7, 14), it is possible that this decrease contributes to the up-regulation of paraventricular TRH mRNA in this condition. To test this hypothesis with a physiological paradigm, we submitted lactating rats separated from their pups for 8 h to a short-term suckling stimulus and compared the long-term response in TRH metabolism to that observed in animals without a short-term suckling. This stimulus produces a significant but transient increase in corticosterone (7, 14). Because the paraventricular TRH mRNA enhancement was not modified by the stimulus, either the hypothesis is inadequate or long-term modifications of corticosterone levels are necessary to induce an effect. Consistent with this last hypothesis, we have observed that if pups are returned with their mother 32 h after withdrawal and maintained for 24 h before sacrifice, corticosterone levels were similar to those in continuous lactation and paraventricular TRH mRNA levels were maintained at values similar to those of animals sacrificed 24 h after 1 h of suckling (Uribe RM, unpubl. results). Furthermore, we showed previously that between days 1 and 5 of lactation, paraventricular TRH mRNA levels decrease when the corticosterone levels are increasing (28). These data support the possibility that one of the negative regulators of paraventricular TRH mRNA is corticosterone.

The increased paraventricular TRH mRNA 56 h after removal of the pups suggests that paraventricular TRH neurons enter in a new functional state at this moment. This could either reflect an augmented activity of the neurons or, alternatively, that the neurons are increasing TRH biosynthesis in preparation for further demand. Changes in TRH mRNA were region-specific. Two elements distinguish POA-AH TRH mRNA levels from those in the PVN. First, as published previously, 1 h of suckling induced a drop of POA-AH TRH mRNA. Second, this change was reverted 48 h after the pups were removed but there was no increase of TRH mRNA over presuckling values. These data suggest that TRH biosynthesis in these two regions is controlled by very different events, in accordance with the distinct physiological roles of these TRH neurons.

Removal of pups for 8 h does not modify MBH TRH levels but they are decreased after 1 h of suckling, probably due to enhanced release (7). The present data show that removal of the pups for 56 h also decreases
MBH TRH levels. Because paraventricular TRH mRNA levels were increased, the long-term MBH TRH changes are probably the result of posttranscriptional regulations that may include decreased translation or precursor processing rate or increased TRH release.

In conclusion, we have shown that withdrawal of the pups induces a delayed enhancement of paraventricular TRH mRNA. This may indicate a change in the paraventricular TRH neurons in preparation for the estrous cycle. Our data are consistent with the hypothesis that corticosterone down-regulates paraventricular TRH mRNA in the rat.

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