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Comparison of the Temporal Programs Regulating Tyrosine Hydroxylase and Enkephalin Expressions in TIDA Neurons of Lactating Rats Following Pup Removal and then Pup Return

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Abstract

Dopamine (DA) and enkephalin (ENK) release from the tuberoinfundibular dopaminergic neurons (TIDA) into the hypophysial portal circulation is fundamentally different under non-lactating and lactating conditions. The aim of this experiment was to compare the effect of a brief interruption then resumption of suckling on the temporal program of tyrosine hydroxylase (TH; rate-limiting enzyme of dopamine synthesis) and ENK regulation in dams. On post partum day 10, pups were removed for a 4-h period from a group of the dams then returned for 4- and 24-h periods. It was examined whether such a brief interruption of suckling provokes full up-regulation of TH and down-regulation of ENK, and whether reinitiation of suckling limits the extent to which TH up- and ENK down-regulate. At the end of experiment, the animals were decapitated. In situ hybridization was used to examine the expression of TH and ENK mRNA in the arcuate nucleus where TIDA neurons reside. The results showed that, on one hand, the removal of pups induced TH up-regulation, on the other hand, ENK expression also increased 8 h after removal of pups and then started to slowly decline. In dams whose sucklings were reinitiated both TH and ENK mRNAs were up-regulated at least for a day. ENK expression responded more slowly to the removal of pups than expression of TH, and after reinitiation of suckling, the temporal program of regulation of both TH and ENK expressions ran parallel in the first 24 h.

Keywords

TH mRNA; ENK mRNA; Lactation; In situ hybridization

Introduction

The initiation of lactogenesis II or the onset of copious milk secretion is the result of hormonal changes, particularly the drop in placental progesterone during parturition, along with sustained high plasma concentrations of prolactin (PRL) and adequate levels of cortisol

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(Neville and Morton 2001; Pena and Rosenfeld 2001). The tuberoinfundibular dopaminergic (TIDA) neurons of the arcuate nucleus (ARC) serve as a central regulatory site of PRL secretion. Under non-lactating conditions, these neurons continuously produce dopamine (DA) and tonically release it into the hypophysial portal circulation (Ben-Jonathan et al. 1977; Freeman et al. 2000). When DA release is inhibited, PRL is rapidly emptied into the general circulation. DA acts on D2 receptors on lactotropes to inhibit PRL release (Iaccarino et al. 2002; Mansour et al. 1990). D2 receptor knockout mice develop prolactinomas (Cristina et al. 2006).

There is ample evidence that suggests that afferent activity, such as stress, sexual activity, and stimulus to the breast, is a powerful regulator of TIDA neuronal activity and thus, of PRL secretion. Mammary stimulation becomes especially critical during lactation for maintaining milk production via its ability to release PRL (Whitworth and Grosvenor 1984). Studies of electrical stimulation of the mammary nerve of lactating rats have revealed that a 3 min stimulation produces a 63% decline in hypophysial stalk and median eminence (ME) DA levels preceding the rise in plasma PRL (de Greef et al. 1981). These observations well correlate with those made in lactating animals, that is, soon after the initiation of suckling, DA release and turnover are markedly reduced (Demarest et al. 1983; Mena et al. 1976; Plotsky et al. 1982; Plotsky and Neill 1982; Selmanoff and Wise 1981) via an intensive down-regulation of the rate-limiting enzyme for DA synthesis, tyrosine hydroxylase (TH; Wang et al. 1993). In continuously lactating rats, the TH mRNA level in TIDA neurons is about ten times lower than in diestrous rats (Wang et al. 1993). Further evidence supports the importance of afferent activity to the TIDA neurons. Prevention of suckling on teats on only one side up-regulates TH expression in TIDA neurons on the contralateral side (Berghorn et al. 1995, 2001). This indicates that the sensory stimulus prompted by suckling is responsible for the TH suppression in TIDA neurons.

The expression of TH mRNA in TIDA neurons seems to be very dynamic, reflecting the changes in suckling activity. Previous studies have determined that within 1.5 h of termination of suckling, the TIDA neurons showed early signs of up-regulation of TH mRNA reflected by the appearance of 1 or 2 sites of heteronuclear RNA in the nucleus of TIDA neurons. An increase in cytoplasmic TH mRNA can be seen about 6 h after the termination of suckling and mRNA levels peak by 12–24 h. Evidence of increased protein synthesis is also noted in the terminals of ME at 6 h (Berghorn et al. 1994, 1995, 2001). From these data, it is uncertain if the early stimulus of TH neurons represents a trigger for full up-regulation of TH mRNA or whether continuous stimulation of these neurons is necessary to achieve high TH levels.

Another peptide, the expression of which varies in TIDA neurons under non-lactating and lactating conditions, is enkephalin (ENK). It is barely detectable in cycling rats, while its levels dramatically increase in ARC and ME of lactating animals (Ciofi et al. 1993; Merchenthaler 1993). Data in the literature indicate that this up-regulation of ENK is due to the hyperprolactinemia of lactation (Merchenthaler et al. 1995; Merchenthaler 1994). It is not clear what role ENK plays in TIDA neurons during lactation. Existing data show that the TH-producing activity of TIDA neurons is definitely suppressed; this does not mean that these neurons are not active in synthesizing other transmitters. Although TH expression is low during suckling, some TH is still present in TIDA neurons and in ME (Wang et al. 1993) and thus some DA release is possible (Ben-Jonathan et al. 1977). A study (Arbogast and Voogt 1998) has proposed that ENK can be co-released with DA and serves to attenuate the effect of DA on lactotropes, raising the possibility that ENK also contributes to PRL secretion.

In the present experiment, the dynamics of the changes in expression of TH and ENK mRNA in TIDA neurons were compared following a brief interruption of suckling (4 h). We have investigated whether such a brief interruption triggers full TH mRNA up- and ENK mRNA down-regulations that continue for a time after return of pups or whether reinitiation of suckling immediately stops these processes of up- and down-regulations, respectively, as a switch.

Materials and Methods

Animals

The University of Maryland's committee on Animal Care and Use approved all experimental paradigms according to NIH guidelines (after completing the experiments, the authors left the University of Maryland).

Adult timed-pregnancy Sprague–Dawley rats were housed on a 12 h light/12 h dark light schedule (lights on at 3 am and off at 3 pm) with unlimited access to food and water. The females gave birth on gestation day 21 or 22 and pups were culled to 8 on day 2 post partum (pp).

Experimental Groups

The dams were divided into three main groups with 3–11 rats investigated for each time point.

1. One group of dams continued to suckle throughout the experiment.
2. Another group had pups removed on day 10 pp and the dams were perfused at 3–4, 6, 7–8, 10–12, 16–20, or 24–28 h after removal.
3. The third group had pups removed on day 10 pp for 4 h, the time at which previous studies indicated clear heteronuclear TH RNA up-regulation (Berghorn et al. 1995; 2001), and then pups were returned. These animals were sacrificed at 3–4, 6–8, 12–16, or 20–24 h after return of pups. In the case of ENK, based on initial patterns of change (which indicated a very slow decline in ENK expression) the last two groups, in which pups were removed, were killed 48 and 72 h later.

To prepare the dams for mRNA analysis, dams were anesthetized with an overdose of sodium pentobarbital (100 mg/kg) and perfused transcardially with a solution of 0.9% sodium chloride with 2% sodium nitrite followed by 4% paraformaldehyde solution containing 2.5% acrolein (pH 6.8; Hoffman et al. 1992). Brains were removed and transferred to a 30% sucrose solution. Once the brain sunk in sucrose, they were sectioned on a freezing sliding microtome at 25 μm and collected into a cryoprotectant/anti-freeze solution (Watson et al. 1986) and stored at −20°C. This procedure enables collection of tissue over prolonged periods of time and then storage of the sections with full maintenance of mRNA levels for over 12 years with no decay (Hoffman and Le 2004).

In Situ Hybridization

Probe Preparation—The pGEM3-TH3′ construct contains a 475 base pair EcoRI/HindIII fragment corresponding to amino acids 219–377 of the rat TH enzyme. This TH fragment was derived from the RR1.2 plasmid obtained from Dr. D. M. Chikaraishi (Duke University). For antisense TH riboprobes (cRNA), the plasmid was linearized with HindIII and transcribed with T7 RNA polymerase, yielding a 509 nucleotide complementary RNA (cRNA).

A 693-bp rat ENK cDNA construct gift of Stanly Watson (University of Michigan and permission from Dr. Audrey Seasholtz) subcloned in SphI-SmaI site of pGEM3-3Z plasmid was linearized with HindIII and transcribed with T7 RNA polymerase to synthesize antisense proenkephalin cRNA probe in vitro. For sense probe, this plasmid was linearized with Ava1 and transcribed with SP6 RNA polymerase.

The in vitro transcription reaction mixture contained 1.0 mM Biotin-16-uracil triphosphate (UTP; Roche, Indianapolis, IN), 1 μg HindIII-linearized-pGEM3z-TH3′, 4 mM DTT, 40 units T7 RNA polymerase (Roche, Indianapolis, IN), 0.35 mM UTP, and 1.0 mM each of ATP, GTP, and CTP. The transcription reaction was stopped by the addition of 1 μl of ethylenediamine tetraacetic acid (EDTA). For an RNA sense probe, the pGEM3-TH3′ plasmid was linearized with EcoRI and transcribed with Sp6 RNA polymerase. The targeted sequence of nucleotides for the antisense probe are all contained within a single exon thus enabling the probe to bind to either mature or heteronuclear RNA in the tissue.

Hybridization and Visualization

Day 1 (RNAse free): Sections were removed from the cryoprotectant/anti-freeze and rinsed with a potassium phosphate buffered saline (KPBS) made with 0.1% diethyl pyrocarbonate water (KPBS+diethylpyrocarbonate (DEPC) H_2O), then incubated in 1% sodium borohydride/KPBS+DEPC H_2O to remove residual aldehydes and acrolein. Sections were then rinsed repeatedly. The tissue was rinsed with 0.1 M triethanolamine buffer (TEA, pH 8.0) followed by an incubation in 0.25% acetic anhydride in TEA at room temperature. Sections were then washed with 2× SSC (0.3 M NaCl, 0.33 M sodium citrate, pH 7.0) solution and prehybridized at 50°C for 2 h by using prehybridization buffer (50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 300 mM NaCl, 8 mM Tris pH 8.0, 0.8 mM EDTA, 15% DEPC H_2O) containing 2 mg/ml of heat denatured torula yeast RNA (TRNA; Ambion, Austin, TX). After rinsing with 2× SSC followed by hybridization with biotinylated probe (final concentration of 600 ng/kbp/ml), the probe and TRNA were heat denatured, mixed with hybridization buffer, placed on the tissue, and incubated overnight at 50°C.

Day 2: Tissue was rinsed with 4× SSC for 30 min, once with RNAse buffer (10 mM Tris pH 8.0, 500 mM NaCl, 0.75 mM EDTA pH 8.0), heated to 37°C and incubated in RNAse (20 ug/ml) in RNAse buffer at 37°C. Following rinses with RNAse buffer, the tissue was further incubated in RNAse buffer at 37°C. After an hour of 2× SSC, 1× SSCs, and 0.1× SSC rinses, the tissue was incubated in 0.1× SSC for 60 min at 55°C. The biotin was not demonstrated by ABC complex but the labeling was amplified. The amplification of the biotin labeling was described previously by Berghorn and her coworkers (2001). After incubation, the tissue was rinsed with KPBS, then incubated in goat anti-biotin (Vector Laboratories, Burlingame, CA) at a concentration of 1:100,000 in KPBS+0.4% Triton X-100 at 4°C for 48 h.

Day 4: Following incubation in primary antiserum, sections were rinsed with KPBS and then immersed into donkey anti-goat secondary antibody solution (Vector Laboratories, Burlingame, CA) at 1:600 in KPBS with 0.4% Triton X-100 at room temperature for 1 h. The tissue was then placed into avidin–biotin complex solution (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Followed rinses with KPBS and 0.175 M sodium acetate solution, the TH or ENK mRNA was visualized using a nickel sulfate 3,3-diaminobenzidine tetrahydrochloride chromogen with H_2O_2 in 0.175 M sodium acetate. The reaction was stopped by rinsing with the sodium acetate solution followed rinses with KPBS. The sections were then placed into saline and mounted onto gelatin-subbed slides and later coverslipped with Histomount (National Diagnostics, Atlanta, GA).

Image Analysis of TH and ENK mRNA

Three sections containing representative areas of ARC, between A 5.8 and 6.2 to interaural line according to the Stereotaxic Atlas by Paxinos and Watson (1986) were included in the analysis. The slides were coded so the observer was blind to the animal's treatment. The sections were placed under a Nikon Eclipse 800 microscope linked to a Cooke camera and two viewfields of each section were captured at 60× on the left side of the third ventricle. IP Spectrum Software (Vienna, VA) installed on a Macintosh G4 computer was used for capturing and analyzing the images. The entire thickness of the sections was photographed in the *Z* plane in 0.2-μm intervals. Only the 10 middle frames were then collapsed into a flattened image to visualize all mRNA clusters present within a 2 μm thickness of a section. To determine the optical density (OD) of the mRNA grains, the OD of the background was subtracted from each image. Each cell was outlined as the region of interest, segmented, and the OD for each cell containing TH or ENK mRNA was determined separately. Values were expressed as means±SEM for each experimental group. The graph charts were constructed by GraphPad Prism Program (GraphPad Software, Inc., La Jolla, CA). Differences in the mean gray levels between pup-returned, removed-but-not-returned and control (continuously suckling) groups were determined by one-way analysis of variance. Post-hoc analysis was performed using the Tukey–Kramer test. $P<0.05$ was considered statistically significant.

Results

Quantitative Analysis of TH mRNA in ARC

The analysis of TH mRNA expression by measuring OD (Fig. 1) revealed distinctive changes between rats whose pups were permanently removed and rats whose pups resumed suckling after a 4 h separation. On one hand, TH mRNA levels were significantly higher by 3–4 h in the group that did not get pups back than in continuously suckling controls ($p<0.05$). TH mRNA levels continued to rise after the complete termination of suckling and remained high even 28 h after removal of pups. On the other hand, if the pups were returned after a 4 h separation, the mean values of TH mRNA levels remained high as in dams whose pups were separated for 4 h and were significantly higher than in continuously suckling controls even 20–24 h after the resumption of suckling ($p<0.05$). By 10–12 h after initial separation, the dams whose pups were not returned had significantly higher TH mRNA levels than those whose pups were returned after 4 h. This indicates that returning the pups and thus the reinitiation of suckling started to suppress the TH mRNA production in TIDA neurons, although the already up-regulated TH mRNA did not return to continuously suckling levels during the course of experiment. It was also examined how many cells containing TH mRNA clusters could be actually detected in ARC, regardless of the intensity of the mRNA expression (Fig. 2). The number of TH mRNA expressing cells was significantly higher in all 'no return' and 'return' groups compared to continuously lactating rats ($p<0.05$); however, the standard error was much higher than in the case of TH mRNA OD.

Quantitative Analysis of ENK mRNA in ARC

The analysis of ENK mRNA expression by measuring OD (Fig. 3) showed that expression of mRNA also rose after the termination of the suckling stimulus and was significantly higher by 3–4 h after removal of pups than the OD in continuously suckling dams. After reaching peak levels around 7–8 h, the levels started to decline and approached the continuously suckling levels by 48 h. Return of the pups and thus resumption of the suckling stimulus after a 4 h pup separation still resulted in an increasing trend in the means of expressed mRNA and the levels were significantly higher than those in continuously suckling dams ($p<0.05$). The analysis of the number of ENK-mRNA-positive cells in the area of TIDA neurons in continuously suckling dams and pup-removed dams (Fig. 4) did

reveal significant increase at 7–8 h after the cessation of suckling. There was a peak at that time and the number of positive cells gradually declined thereafter and became significantly lower by 48 h than in continuously suckling dams. In dams whose pups were returned, the number of ENK expressing cells was not significantly different from that of continuously suckling ones.

Histological Appearance of TH and ENK mRNA Expressing Cells in ARC

The histology of TH and ENK mRNA expressions well supports the quantitative data (Fig. 5a). In TIDA neurons of ARC of continuously lactating dams, only a few TH expressing cells were observed. The density of the reaction product was very low. Twenty-four hours after removal of the pups the TH mRNA expression was extremely enhanced (Fig. 5b). At the same time, the ENK mRNA expression was considerably high in continuously lactating rats (Fig. 5c) and it was even stronger in the ARC of rats 7 h after removal of pups (Fig. 5d).

Discussion

The results confirm the hypothesis that the suckling stimulus is an important regulator of TH expression in TIDA neurons, at least in earlier stages of lactation. Our previous studies showed that the appearance of nuclear TH mRNA was evident as early as 1.5 h after the termination of suckling on day 10 pp (Berghorn et al. 1995) and that heteronuclear RNA levels peaked at 3 h, and then declined as cytoplasmic mRNA increased. Thus, the selection of a 4-h removal period prior to pup return in the present study represented a time after which the TIDA neurons clearly were in the process of up-regulating TH.

The TH mRNA levels, found in dams that had not received their pups back, rose gradually. It reached a peak level in 16–20 h. The results suggest that this up-regulation of TH mRNA cannot be disrupted immediately if pups are returned and the neuronal input from the nipples to the ARC is reestablished. From our data, it seems that the program of transcriptional up-regulation begins to significantly subside in 6–8 h after pups are returned compared to the groups where the pups were permanently removed ($p<0.05$). TH mRNA levels, however stayed significantly higher in both of these groups than in continuously suckling dams ($p<0.05$) for the duration of the experiment regardless of the resumption of suckling. However, mRNA levels found in dams whose pups were permanently removed did rise to higher levels than in dams that received their pups back. In theory, we expected the decline in TH mRNA in pup-returned dams to levels of continuously suckling dams with time, based on the 6-h half-life described for TH mRNA (Maurer and Wray 1997). Our observation indicates that resuckling alters the stability of the TH mRNA producing machinery after being awakened by removal of pups. One of the factors influencing the TH-producing machinery could be the adrenocorticotropic hormone (ACTH)-corticosteroid axis. It was previously described that suckling stimulus induces ACTH response (Nagy et al. 1994). In a recent publication, it was found that (Oláh et al. 2009) in lactating dams the concentration of ACTH was higher in the intermediate than in the anterior lobe, and the inhibition of DA biosynthesis by α-methyl-parathyrosine or blockade of D2 receptors by domperidone enhanced the plasma ACTH level in 1 h but did not influence the α-melanocyte-stimulating hormone (α-MSH) levels. In non-lactating (ovariectomized and ovariectomized+estradiol replaced) rats, the above-mentioned drugs enhanced the α-MSH, but did not influence the ACTH levels. It was also demonstrated in the same paper that after a short interruption of suckling (4 h) the plasma ACTH levels decreased and then after the resumption of suckling, the ACTH levels gradually increased. It is also known that during lactation a number of stressors are less effective (Kehoe et al. 1992).

To get a better understanding of how the number of cells that express TH mRNA changes with the OD of mRNA, we have to view the results together. The changes in TH mRNA OD

and the number of TH expressing cells in ARC show similar tendency upon pup removal; however, the measurement of OD seems to be more reliable and stable than the number of TH cells which express mRNA with different intensity.

Endogenous opioids have also been implicated in the regulation of suckling-induced PRL secretion during lactation (Arbogast and Voogt 1998). A possible candidate could be ENK, a δ receptor agonist. ARC contains scattered ENK immunoreactive neurons in cycling animals, but during lactation, ENK expression is strongly enhanced in TIDA neurons (Ciofi et al. 1993; Merchenthaler 1993). It was published earlier that the levels of ENK mRNA are significantly higher in continuously lactating dams than in cycling diestrous females (Merchenthaler 1994). In our experiment, the pup removal produced an increase in OD similar to the cell counts, then both declined. The return of pups did not influence the elevated ENK mRNA expression.

The up-regulation of ENK during lactation is thought to be the result of the hyperprolactinemia (Merchenthaler et al. 1995; Merchenthaler 1994). A variety of experimental paradigms show that elevated serum PRL levels are accompanied by up-regulation of ENK. The levels of PRL fall rapidly in about 2 h suckling ceases (Grosvenor et al. 1979; Lee et al. 1989; Nagy et al. 1983; Nagy and Halasz 1983). The normal peaks in PRL secretion that accompany estrous cyclicity (Lee et al. 1995) are not sufficient to prompt significant co-expression of ENK in TIDA neurons. It is unclear what kind of mechanism is responsible for maintaining ENK synthesis in TIDA neurons so long after the cessation of suckling.

On the basis of the results, it was concluded (Fig. 6) that the up-regulation of DA synthesis after termination of suckling is an active process and the return of pups 4 h later cannot interrupt this process as a simple switch. This regulatory mechanism is efficient at day 10 post partum in rats. As it was expected, lactation results in some increase in ENK mRNA expression. After removal of pups, it was transiently further increased than decreased and showed opposite changes to the TH mRNA. In the cases where pups returned after 4 h, the expression of ENK mRNA did not show any striking change and the level of both TH and ENK mRNA remained high even for a day after the resumption of suckling. The two curves indicating the mRNA levels of TH and ENK run parallel with each other.

What may be the explanation of the above-mentioned results? It is possible that ENK may have a protective role against the inhibitory effect of TH (and consequently DA) (Enjalbert et al. 1979), whose mRNA level further increases following the removal and resumption of the suckling stimulus. Arbogast and her collaborators (1998) clearly showed that naloxone (opioid receptor antagonist) infusion increased the TH mRNA levels in ARC, but not in the zona incerta, of lactating rats and prevented the suckling-induced PRL release.

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Abbreviations

ARC	Arcuate nucleus
DA	Dopamine
DEPC	Diethylpyrocarbonate
ENK	Enkephalin

KPBS	Potassium phosphate buffer-saline
ME	Median eminence
OD	Optical density
PP	Post Partum
PRL	Prolactin
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopaminergic
TRNA	Torula yeast RNA
UTP	Uracil triphosphate

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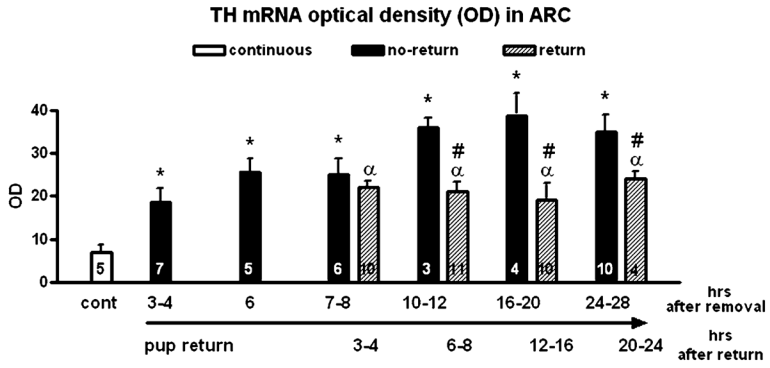

Fig. 1.

The graph chart shows optical density (OD) of TH mRNA clusters in TIDA neurons of A12 region of ARC in various experimental groups. Values are expressed as means±SE for each experimental group. The *white bar* represents the control group, in which the dams continued to lactate throughout the experiment without their pups being removed at all. The '*no-return' bars* demonstrate the levels of OD at different times after pups were removed permanently. The '*return' bars* demonstrate the OD levels at certain time points after pups were removed and then returned after a 4 h separation and continued suckling afterwards. The *numbers* in the bars indicate the number of rats used in each group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$). # indicates significant difference between 'return' group and the corresponding 'no-return' group ($p<0.05$). α indicates significant difference between continuously lactating and the given return group ($p<0.05$)

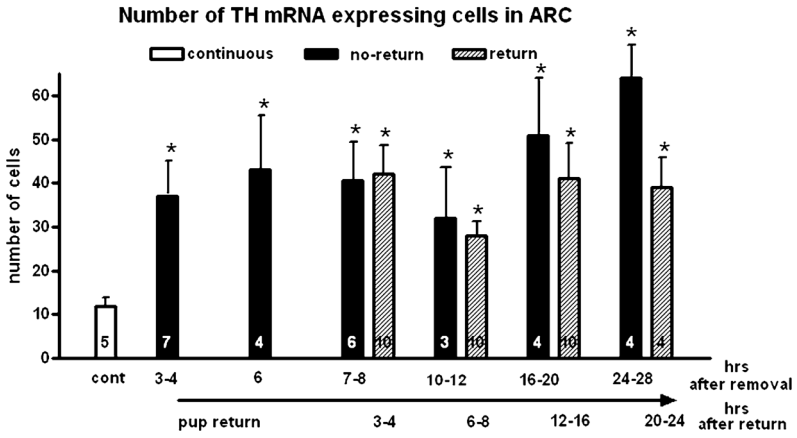

Fig. 2.

The graph chart shows the number of TH mRNA expressing cells detected in the A12 region of the ARC in the various experimental groups. Values are expressed as means±for each experimental group. The *white bar* is a control group, in which the dams continued to lactate throughout the experiment without their pups being removed at all. The '*no-return' bars* demonstrate the mean of the number of cells of each group at various times after pups were removed permanently. The '*return' bars* demonstrate the means of the number of cells at certain time points after pups were removed; however, these pups were returned after a 4 h separation and continued suckling afterwards. The *numbers in the bars* indicate the number of rats included in the group. * indicates significant difference between continuously lactating group and all the other groups ($p<0.05$)

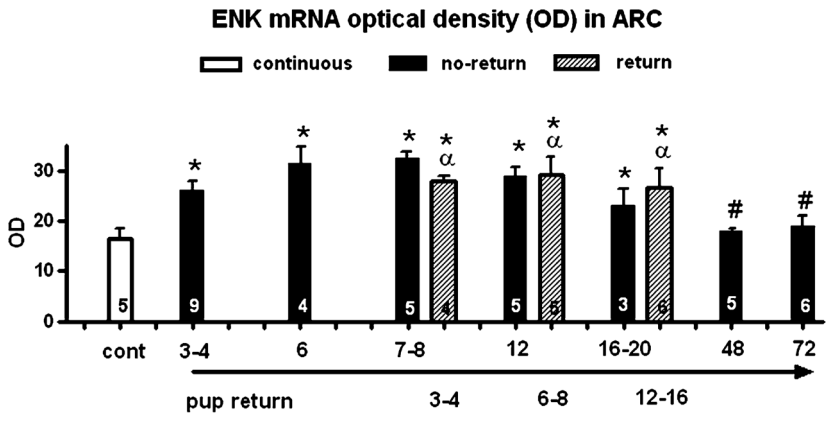

Fig. 3.

The graph chart demonstrates the optical density (OD) of ENK mRNA clusters in the A12 region of ARC in various experimental groups. Values are expressed as means±SE for each experimental group. The '*no-return' bars* demonstrate the levels of OD at the indicated times after pups were removed permanently. The '*return' bars* indicate the levels of OD at time points after pups were removed, but returned after a 4 h separation. The *white bar* indicates the OD of ENK mRNA in dams that continued to lactate and remained with their pups throughout the experiment. The *numbers in the bars* indicates the number of rats included in the group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$). # indicates significant difference between 'no-return' group (7–8 h) and 'no-return' groups (48 and 72 h; $p<0.05$). α indicates significant difference between continuously lactating and the given return group ($p<0.05$)

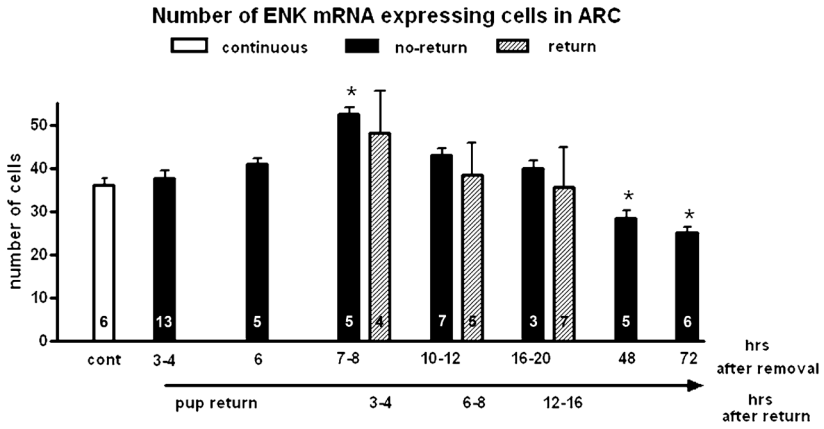

Fig. 4.

The graph chart shows the number of ENK mRNA expressing cells detected in the A12 region of the ARC in various experimental groups. Values are expressed as means±SE for each experimental group. The *white bar* is a control group, in which the dams continued to lactate throughout the experiment without their pups being removed at all. The '*no-return' bars* demonstrate the mean of the number of cells of each group at certain times after pups were removed permanently. The '*return' bars* demonstrate the means of the number of cells at certain time points after pups were removed; however, these pups were returned after a 4 h separation and continued suckling afterwards. The *numbers* in the bars indicate the number of rats included in the group. * indicates significant difference between continuously lactating group and the given 'no-return' group ($p<0.05$)

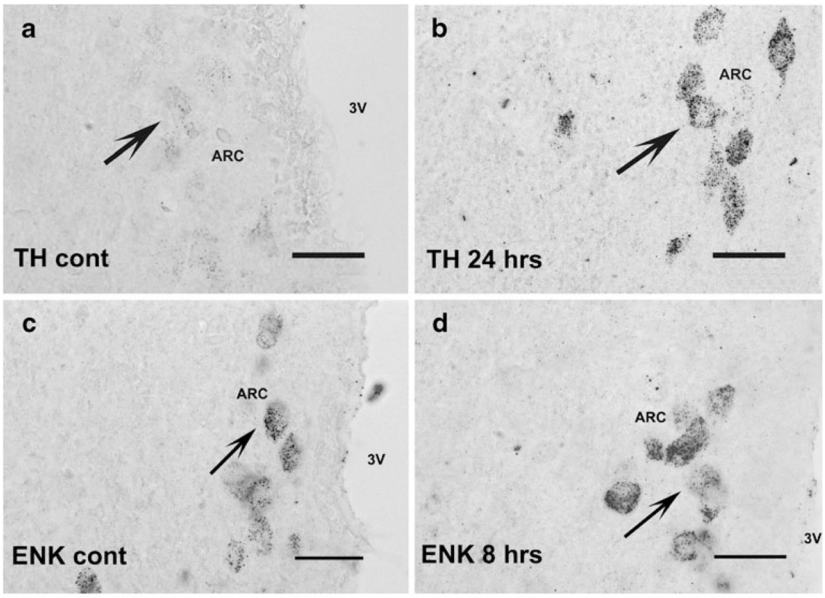

Fig. 5.

Microphotographs demonstrating TH and ENK mRNA clusters in a continuously lactating dam (**a** and **c**) and in dams whose pups were removed (**b** and **d**). *Arrows* show mRNA clusters in TH and ENK expressing cells. The *number and intensity of silver grains* indicating TH and ENK expressions are markedly higher in dams whose pups were removed than in the continuously suckling dams. *3V* third ventricle; *ARC* arcuate nucleus. *Scale* 50 μm

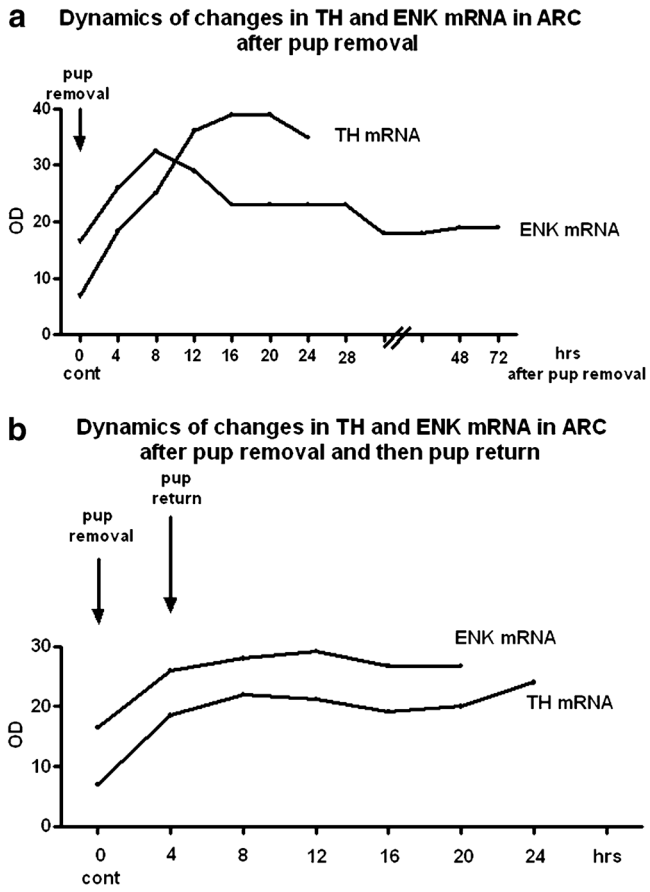

Fig. 6.

Dynamics of changes in TH and ENK mRNA optical densities (OD) in ARC during lactation upon removal of pups (**a**) and upon removal and then return of pups (**b**)