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Stimulation of Anterior Pituitary Galanin and Prolactin Gene Expression in Suckling Rats

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Recent evidence suggests that galanin may regulate prolactin (PRL) secretion during lactation. In this article, we describe the regulation of anterior pituitary galanin and PRL gene expression during pregnancy and after parturition in the rat. Expression of galanin and PRL in the anterior pituitary were significantly higher at d 20 of pregnancy compared to diestrus. One day after parturition, galanin mRNA levels increased a further 4.5-fold. This post partum increase in gene expression was not observed for PRL. The increase in galanin gene expression was maintained above the diestrous level for at least 10 d after parturition. PRL mRNA expression, on the other hand, was largely unchanged after parturition. Although the increase in galanin gene expression 1 d after parturition was independent of suckling, subsequently, galanin gene expression was significantly higher in nursing mothers. Anterior pituitary galanin gene expression was 12-fold higher in nursing mothers compared with those that were not, 3 d after parturition. Similarly, PRL gene expression was significantly lower in mothers who were not suckling their pups 3 d after parturition. Initiation of suckling alone was insufficient to stimulate galanin and PRL expression. Despite suckling for 2 d, removal of the suckling stimulus subsequently resulted in a rapid decrease in galanin gene expression. Hence, the stimulatory effect of suckling on galanin expression requires a sustained suckling stimulus. In conclusion, the data support the hypothesis that anterior pituitary galanin plays an important role during lactation, likely acting to amplify lactotroph stimulation through paracrine and autocrine mechanisms.

Key Words: Galanin; PRL; lactation; gene regulation; pituitary.

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Introduction

Galanin is a 29 amino acid neuroendocrine peptide *(1)* involved in the regulation of the hypothalamic-pituitary axis *(2)*. Although the physiological role of this peptide is not completely understood, it has been shown to modulate the release of several anterior pituitary hormones, including growth hormone *(3,4)*, prolactin (PRL) *(4,5)*, thyroid-stimulating hormone *(6)*, adrenocorticotrophic hormone *(6)*, and luteinizing hormone *(7)*. Several observations suggest an important role for this peptide in the control of reproductive function. For example, anterior pituitary galanin gene expression is dramatically activated by estrogen *(8–10)*. The physiological significance of the regulation by estrogen is currently unknown. However, anterior pituitary galanin gene expression varies more than 30-fold during the normal estrous cycle in the female rat, raising the possibility that it may be involved in the regulation of anterior pituitary hormone secretion during the estrous cycle *(10)*. Furthermore, galanin peptide and mRNA are found in anterior pituitary lactotrophs *(11)*. Recent studies by Cai et al. *(12)* show that galanin, present in the lactotrophs, plays a significant role in the paracrine regulation of PRL secretion within the pituitary gland itself. Galanin is also required for basal and vasoactive intestinal peptide-induced release of PRL from a subpopulation of lactotrophs *(13)*. Physiologically, this paracrine regulation could be important in the regulation of PRL secretion during lactation. The presence of high-affinity receptors for galanin in the pituitary further support a direct effect of galanin within the pituitary *(14)*.

To examine the potential role of galanin in the regulation of reproductive function, we studied the regulation of anterior pituitary galanin gene and PRL gene expression in pregnant and postpartum rats.

Results

Experiment 1: Anterior Pituitary Galanin and PRL Gene Expression After Parturition

Figure 1A, B shows the expression of galanin and PRL. For the first 4 d after parturition, galanin expression was

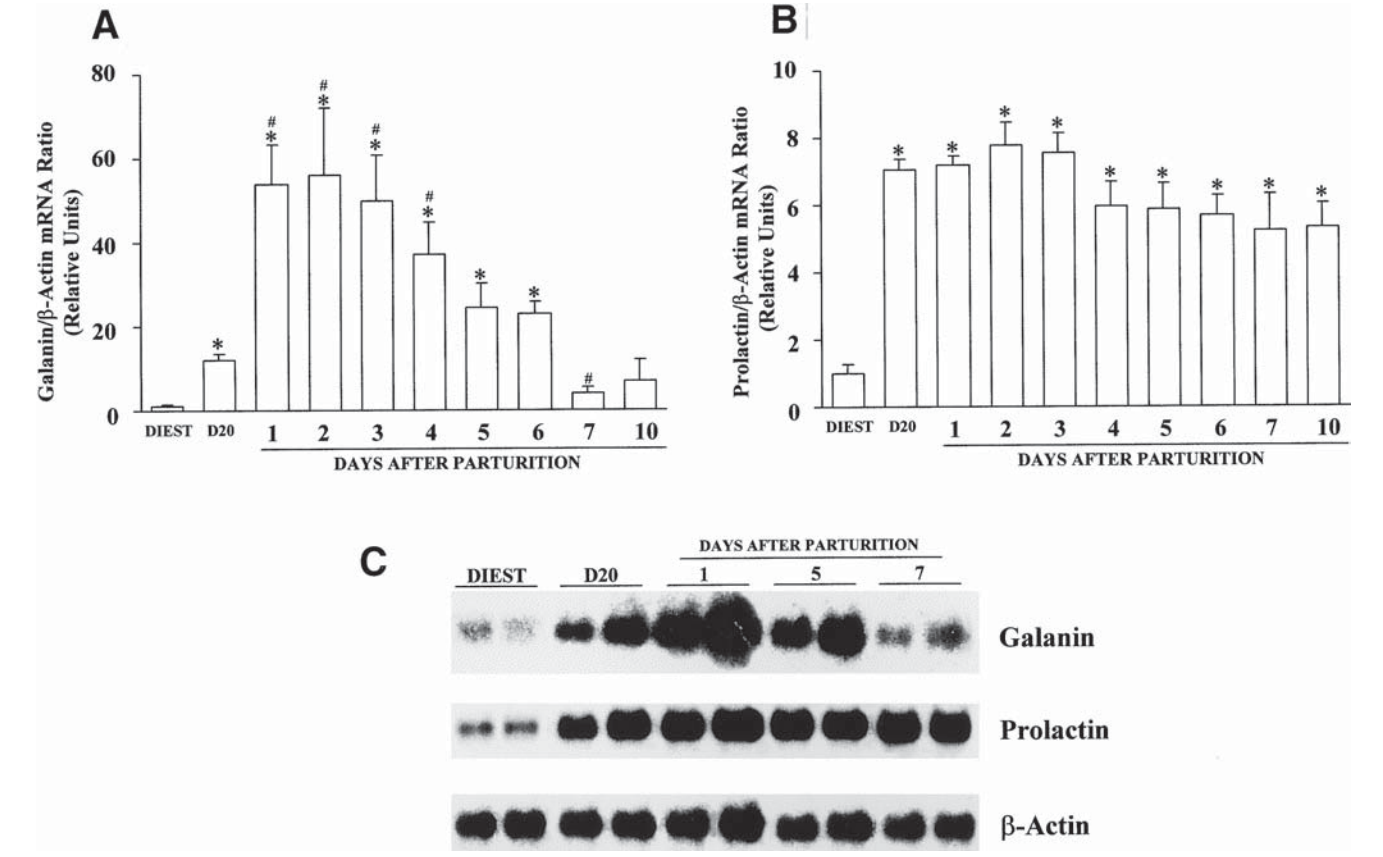

Fig. 1. Anterior pituitary galanin **(A)** and PRL **(B)** gene expression. DIEST, female rats in diestrus; D20, d 20 of pregnancy. Late pregnant mothers were closely monitored in the animal facility. On the day of parturition, the number of pups given to each mother was standardized to eight. The mothers were sacrificed after having nursed the pups for the number of days indicated. **(C)** A representative Northern blot of the results. Results shown are means ± SEM, relative to DIEST (ratio = 1). *, $p < 0.05$ relative to DIEST; #, $p < 0.05$ relative to D20.

significantly increased between 37- and 56-fold and between 3- and 5-fold compared with levels detected at diestrus and d 20 of pregnancy, respectively. Expression progressively decreased after the first 3 d, but levels were still four- and seven-fold higher at d 7 and 10, respectively, compared to diestrus, although the difference did not reach significance. PRL expression at d 20 of pregnancy was about seven-fold higher than at diestrus. However, there was no significant change in PRL gene expression after parturition compared to d 20 of pregnancy. The level of PRL expression for the first 10 d after parturition was relatively constant. Figure 1C shows a representative Northern blot of the data.

Experiment 2: Maintenance of Galanin and PRL Gene Expression by Suckling After Parturition

Figure 2A, B shows the effect of suckling on galanin and PRL expression. There was a significant increase in galanin gene expression 1 d after parturition compared to levels detected at diestrus and in pregnant animals at d 20 of gestation, regardless of whether the mothers were suckling their pups. There was no significant difference in galanin mRNA levels in suckling compared with nonsuckling control animals 1 d after parturition. However, suckling maintained a high level of expression for the next 2 d after parturition. In the absence of suckling, galanin gene expression decreased significantly over the 3 d after parturition. Galanin mRNA levels in nonsuckling mothers 2 and 3 d after parturition were 19.4 and 8.3% of the respective groups of suckling animals. Galanin levels in rats that were not suckling had returned to diestrous levels 3 d after parturition.

Figure 2B shows the regulation of PRL expression. There was no significant change in PRL gene expression 1 d after parturition compared with levels detected at d 20 of pregnancy, regardless of whether the mothers were suckling their pups. In the absence of suckling, levels were about 70% of the respective suckling animals, 2 and 3 d after parturition.

Experiment 3: Regulation of Anterior Pituitary Galanin and PRL After Initiation of Suckling

The experiment was conducted to determine whether the initiation of suckling alone could maintain high levels of galanin and PRL expression. Mother rats were allowed to suckle their pups (normalized to eight per mother) for 2 d after parturition. Subsequently, the pups either were removed from the mothers or were removed and immediately replaced. Pituitary gene expression was determined

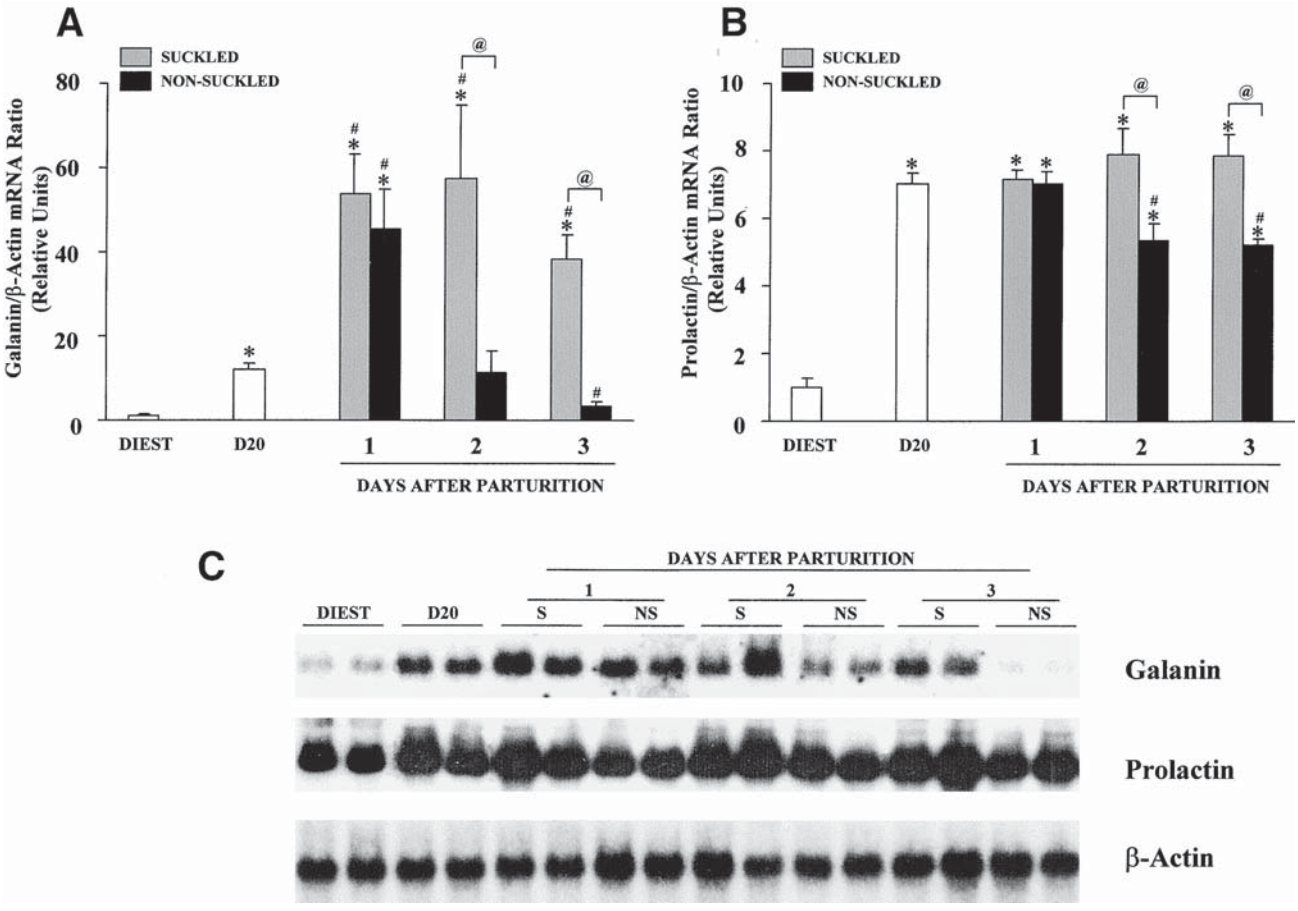

Fig. 2. Anterior pituitary galanin **(A)** and PRL **(B)** expression at diestrus (DIEST), d 20 (D20) of pregnancy and after parturition in suckling (SUCKLED) and nonsuckling (NONSUCKLED) mothers. Values shown are the means ± SEM, relative to DIEST (ratio = 1). *, $p < 0.05$ relative to DIEST; #, $p < 0.05$ relative to D20; @, $p < 0.05$. **(C)** A representative Northern blot of the results. S, suckled; NS, nonsuckled.

1 and 2 d afterward. Galanin gene expression in nonsuckling mothers was 44 and 17% of the respective groups of animals that were allowed to continue suckling at 3 and 4 d after parturition (Fig. 3A).

The pattern of PRL gene expression was similar to that of galanin. Mothers that nursed their pups had significantly higher levels of PRL mRNA compared with mothers that did not at the respective days after the start of the experiment (Fig. 3B). However, the changes were lower in magnitude compared to galanin. PRL expression in nonsuckling mothers was about 70% of that in suckling mothers.

Discussion

Anterior pituitary galanin gene expression is extremely sensitive to and potently stimulated by estrogen *(8,10)*. The expression of galanin in the anterior pituitary during pregnancy is consistent with its regulation by estrogen *(15)*. Estrogen secretion in rats is generally low throughout pregnancy except during blastocyst implantation and for several days before parturition *(16)*. The significantly higher expression of galanin and PRL at d 20 of pregnancy compared to diestrus is consistent with estrogen stimulation. In addition, there is also a further increase in estrogen levels at parturition *(17,18)*. It is likely that the increase in circulating estrogen at parturition is responsible for the subsequent increase in galanin expression 1 d after parturition, irrespective of whether the rats were suckling or not. Interestingly, although the expression of PRL was high prior to parturition, there was no significant increase in PRL expression after parturition, suggesting that PRL expression is likely less sensitive to circulating estrogen concentrations compared to galanin. The increase in galanin expression above diestrous level was maintained in nursing mothers for at least 10 d, although the difference on d 7 and 10 did not reach significance. Similarly, PRL levels were also maintained above diestrous levels during this period. The sustained increase in galanin expression was surprising in view of the decrease in estrogen levels after parturition owing to suppression of gonadotropin-releasing hormone by the suckling stimulus *(19–21)*. It has been shown that estrogen levels 1 d after parturition are undetectable *(18)*. Hence, the maintenance of a high level of galanin expression in the anterior pituitary during lactation is likely to be independent of estrogen regulation. We investigated

whether stimulation of galanin expression after parturition was dependent on the suckling stimulus.

Data in the second experiment show that in the absence of suckling, with low levels of circulating estrogen present at this time, galanin gene expression decreased significantly 2 d after parturition compared with levels on the first day of parturition. By 3 d after parturition, in the absence of suckling, the levels of galanin mRNA in the anterior pituitary had decreased to 6.5% that of d 1. On the other hand, suckling resulted in the maintenance of a high level of galanin gene expression up to 3 d after parturition. The mechanism by which suckling prevents the dramatic drop in galanin mRNA expression after parturition is unclear. One possibility is the low level of hypothalamic dopamine present at lactation *(22–24)*. Dopamine has been shown to inhibit galanin secretion from dispersed anterior pituitary cells in vitro *(25)*. It is possible that a decrease in dopamine level during suckling activates anterior pituitary galanin gene expression. Additionally, other studies have found that the termination of lactation may induce apoptosis in the hyperplastic pituitary that is not maintained by the suckling stimulus *(26)*. Apoptosis may account for the decrease in galanin and PRL gene expression in animals without a suckling stimulus. The greater effect observed for galanin expression compared to PRL may indicate that apoptosis at this time may be cell-type specific.

A major difference between suckling and nonsuckling rats, apart from whether they were suckling or not, was that the nonsuckling rats were never allowed to initiate suckling. To determine whether the initiation of suckling alone was sufficient to maintain high levels of galanin and PRL expression, mother rats were allowed to nurse their young for 2 d before the start of the experiment. Despite the initiation of suckling, there was significantly lower expression of galanin and PRL mRNA in mothers that were not allowed to suckle their pups subsequently. This showed that even after suckling had been initiated, removal of the suckling stimulus resulted in a significant and rapid decrease in galanin and PRL gene expression within 24 h, with a greater effect observed for galanin.

The significance of increased galanin expression in the anterior pituitary in nursing mothers is unclear. Although we have not determined anterior pituitary galanin peptide concentrations in this study, mRNA and peptide levels are generally regulated in parallel in studies in which both mRNA and peptide concentrations have been determined *(10,27,28)*. Similarly, the parallel regulation of mRNA and peptide has been observed for other peptides *(29–31)*. Hence, it is possible that higher galanin peptide concentrations, secreted by a subset of PRL-secreting cells, may act in a paracrine or autocrine manner to stimulate PRL secretion. Wynick et al. *(13)* showed that galanin is required for basal and vasoactive intestinal peptide-induced PRL release in a subpopulation of lactotrophs. Furthermore, it has been shown that thyrotropin-releasing hormone (TRH) is an important PRL-releasing factor during suckling *(32–34)*. Galanin potentiates the PRL-releasing effect of TRH in anterior pituitary dispersed cells *(2)* and also in normal women *(35)*. It is possible that galanin may potentiate the PRL-releasing activity of TRH during suckling. It has also been shown that galanin acts as a growth factor for lactotrophs *(13)* as well as a paracrine factor inducing PRL release *(12)*. Treatment of lactotrophs with galanin-specific antiserum prevented the mitogenic effects of estrogen on lactotrophs and also decreased PRL secretion in identified anterior pituitary cells using the hemolytic plaque assay *(12)*. The upregulation of galanin during this period would facilitate induction of lactotroph hyperplasia by estrogen, as well as PRL secretion. It is noted that the level of galanin expression progressively decreases during the first week of lactation. It is possible that galanin functions during late pregnancy and early lactation to facilitate lactotroph hyperplasia in preparation for an increase in PRL secretion. The data presented are consistent with the proposal that anterior pituitary galanin plays an important role in the induction and maintenance of lactotroph hyperplasia in lactation.

Materials and Methods

Animals and Treatments

All rats were purchased from Sembawang Animal Centre, Singapore, and housed separately in an animal room on a 12-h light, 12-h dark cycle (lights on at 8 AM) with free access to food and water. The use and care of animals were in accordance with the guidelines by the Laboratory Animal Centre of the National University of Singapore.

Experiment 1: Anterior Pituitary Galanin and PRL Gene Expression After Parturition

Female Sprague Dawley rats (150–200 g) were used for the diestrous group. The estrous cycle was determined by examination of vaginal smears under a microscope. The presence of predominantly polymorphonuclear leukocytes in the smear with little or no mucus is indicative of diestrus.

Timed-pregnant Sprague Dawley rats were killed at d 20 of pregnancy. The day when the rats were mated was designated as d 0. Mating was confirmed by the presence of a semen plug on d 1. Pregnancy was confirmed at sacrifice by the presence of fetuses in the uterus.

For groups after parturition, pregnant rats were obtained late in pregnancy and housed separately. Parturition date and time were noted, and the number of pups given to each nursing mother was standardized to eight. Mothers were sacrificed at the indicated days after parturition. There were four to five rats in each group.

Experiment 2: Maintenance of Galanin and PRL Gene Expression by Suckling After Parturition

Pregnant Sprague Dawley rats were used. Immediately after parturition, the pups were either separated (nonsuckled) or allowed to remain with their mothers (suckled). The number of pups given to each nursing mother was stan-

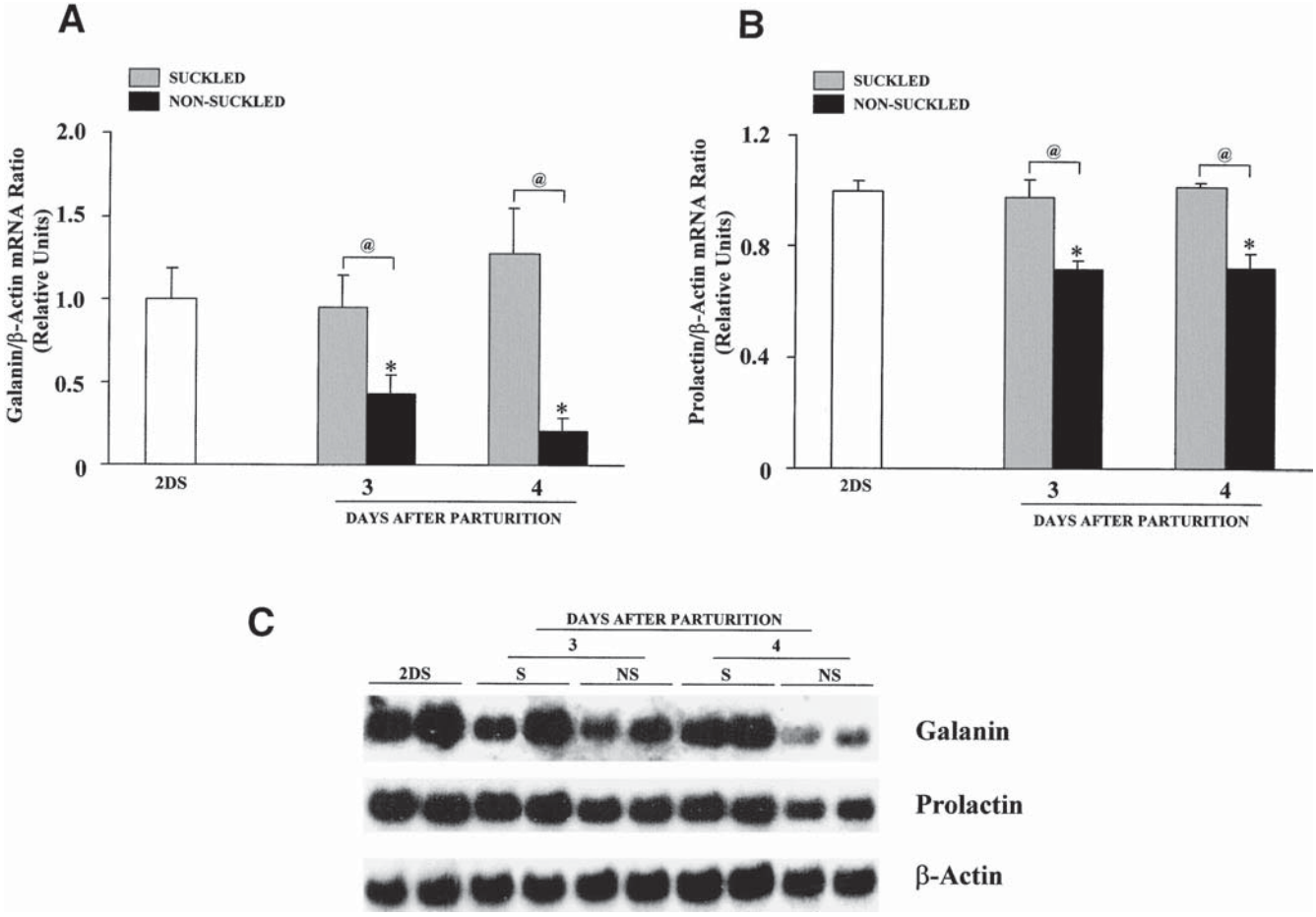

Fig. 3. Regulation of galanin **(A)** and PRL **(B)** expression after initiation of suckling. Mothers that had been suckling their pups (eight per mother) for 2 d (2DS) were used. The mothers either were allowed to continue to suckle the pups (suckled) or had their pups removed (nonsuckled) for an additional 1 and 2 d. Values shown are the means ± SEM, relative to 2DS (ratio=1). *, $p < 0.05$ relative to the 2DS; @, $p < 0.05$. (C) A representative Northern blot of the results. S, suckled; NS, nonsuckled.

dardized to eight. The rats were killed at 11:00 AM on the day of sacrifice. There were five rats in each group.

Experiment 3: Regulation of Anterior Pituitary Galanin and PRL After Initiation of Suckling

Mothers that had been suckling their young for 2 d after parturition were used. They were randomly divided into five groups (n = 5 per group). Controls were mothers that had been nursing their pups (eight per rat) for 2 d after parturition. Subsequently, a group of mothers had their pups removed and immediately replaced (suckled) whereas another group had their pups removed (nonsuckled). The rats were killed 1 and 2 d later.

Tissue Collection

All rats were killed by decapitation. The pituitaries were dissected and immediately frozen on dry ice. They were stored at –80°C until use.

Northern Blot Analysis

RNA was prepared from anterior pituitaries as previously described *(28)*. Ten micrograms of RNA from each sample was separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membrane (Qiagen, Chatsworth, CA), and hybridized to a specific rat galanin or PRL cDNA probe as previously described *(28)*. Galanin cDNA fragments were obtained by amplification of a rat galanin cDNA cloned into a pGEM vector (Promega, Madison, WI) *(9)* using T7 and M13 reverse primers. The rat galanin cDNA was a gift from Dr. Lee Kaplan, Boston, MA. The polymerase chain reaction (PCR) conditions were as follows: 30 cycles of denaturation (95°C for 45 s), annealing (50°C for 45 s), and extension (72°C for 2 min). An 8-min extension step was performed at the end of the 30 cycles to complete the extension of the amplified products.

The PRL cDNA was obtained by using the Titan™ one-tube reverse transcriptase (RT)-PCR system (Boehringer Mannheim, Germany). One microgram of rat anterior pituitary total RNA was used as template. The reaction mix was incubated at 50°C for 30 min followed by 30 cycles of denaturation (95°C for 45 s), annealing (50°C for 1 min), and extension (68°C for 2.5 min). An 8-min extension period was performed at the end of the 30 cycles

to ensure complete extension of the amplified products. The forward and reverse primers used for the amplification were 5'-GTGGTCCCAGTGGTCATCAC-3' and 5'-GGAATGAATGTAGGCTTAGC-3', respectively. The PRL cDNA fragment contained the entire coding sequence of PRL and was sequenced to confirm its identity.

The concentrations of rat galanin and PRL mRNA were determined by densitometric scanning of autoradiograms (Bio-1D, v5.08; Vilber Lourmat, France). Minor variations in loading of total RNA were corrected by normalization of data to β-actin hybridization *(36)*. The β-actin probe was obtained by using the Titan one-tube RT-PCR system (Boehringer Mannheim) as previously described. The forward and reverse primers were 5'-CGTAAAGACCTCTATGCCAA-3' and 5'-AGCCATGCCAAATGTCTCAT-3', respectively. cDNA fragments were labeled with ^{32}P dCTP using the Rediprime random prime labeling kit (Amersham, Buckinghamshire, UK).

Statistics

The means of the various treatment groups were compared using the one-way analysis of variance followed by the Student-Newman-Keuls multiple comparisons test. Differences were considered significant at $p < 0.05$.

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