Endocrine, vol. 7, no. 3, 319–323, December 1997 0969–711X/97/7:319–323/$9.25

Depriving Neonatal Rats of Milk from Early Lactation Has Long-Term Consequences on Mammotrope Development

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The sudden appearance of prolactin-releasing cells during the early postnatal period of the rat is initiated by a small milk-borne peptide. Depriving newborn rats of this early milk factor severely retards mammotrope differentiation during the neonatal period. In the present work, we extend our study of early milk deprivation to the adult. To this end, newborn litters were crossfostered onto mothers that had given birth the same day or one week earlier in order to deprive pups in the latter group of early milk. At 5, 15, and 30 d of age, rats deprived of such milk had decreased percentages of mammotropes (as measured by reverse hemolytic plaque assay, RHPA) when compared to nondeprived animals ($P < 0.05$). By 45 d, the percentage of mammotropes was similar for the two crossfostered groups ($P > 0.1$) and this persisted through d 60. Subsequently, we assessed the secretory capacity of mammotropes from 60-d old rats to secretagogues and found that early milk deprivation had no effect on basal prolactin release ($P > 0.1$), but that it augmented hormone secretion evoked by thyrotropin-releasing hormone (TRH, 100 n*M*; $P < 0.01$). The inhibitory response to dopamine (DA; 1 μ*M*) and the stimulatory response to angiotensin II (AGII; 100 n*M*) were not altered by early milk deprivation ($P > 0.1$). Taken together, these results demonstrate that factors in milk from early lactation are required for normal mammotrope differentiation, and that the delay induced by early milk deprivation leads to altered secretory function of mammotropes in adult animals.

Key Words: Prolactin; anterior pituitary; neonate; differentiation.

Introduction

In the rat, the initial appearance, and subsequent increase of prolactin-releasing cells within the anterior pituitary gland begins during the first week of neonatal life *(1)*. The sudden increase of prolactin-releasing cells between d 3 and 4 of age is delayed when pups are deprived of milk from early lactation *(2)*. A bioassay utilizing anterior pituitary cells from 1-d-old rats was developed to test whether milk itself contains the activity that stimulates mammotrope differentiation. Using this bioassay, we found that aqueous extracts of milk from early lactation directly increased the percentage of prolactin releasers *(3)*. Column chromatography of early milk, followed by pepsin digestion, demonstrated that the signal was a low-mol-wt peptide *(2,4)*. Finally, we found that this peptide was transferred rapidly from milk into neonatal circulation *(4)*. From our combined results, it is clear that milk contains signals that are obligatory requirements for normal development of mammotropes in the neonatal rat.

In this study, we attempted to assess the physiological relevance of these observations by exploring the possibility that early milk deprivation had long-term consequences on mammotrope ontogeny and function. To this end, newborn rats were deprived of early milk by placing them with mothers that had given birth 7 d earlier, a paradigm previously shown to retard mammotrope differentiation beyond the time of their normal appearance (5 d of age; *2*). We then determined whether (and if so, when) the normal abundance of mammotropes would be achieved. Finally, we assessed and compared the secretory capacities of mammotropes from adults derived from control and milk-deprived litters. Here, we report that early milk deprivation delays mammotrope ontogeny and alters the secretory capacity of mammotropes in mature animals.

Results

Initially, newborn rat litters were placed with mothers that had given birth 7 d earlier, thereby depriving these pups of signals contained within milk from early lactation. For controls, separate newborn litters were placed with mothers who had given birth the same day. When we used the reverse hemolytic plaque assay (RHPA) to measure the percentage of prolactin-releasing cells in males from these litters, we found that the delay in mammotrope differentiation induced by depriving neonates of early milk was eventu-

Received June 24, 1997; Revised August 26, 1997; Accepted August 26, 1997.

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Table 1
Consequences of Early Milk Deprivation on Body Weight and Total Anterior Pituitary Cell Number[a]

	Body weight, gms		Total number of anterior pituitary cells ($\times 10^{-4}$)	
Age	Day 0	Day 7	Day 0	Day 7
5	16.0±0.8	15.5±1.4	80.7±7.9	73.6±10.5
15	47.0±1.5	51.4±4.1	210.6±17.3	182.2±12.3
30	157.0±8.6	136.5±5.2	288.3±10.2	253.3±20.1
45	311.5±7.9	296.9 ±12.8	300.0±20.1	287.5±22.9

[a]Rats from the experiment described in Fig. 1 were weighed prior to death. In addition, the number of anterior pituitary cells was enumerated prior to the RHPA. The placing of newborn rats onto mothers that had given birth on the same day (d 0) or 7 d earlier (d 7) had no effect on litter weight or on the number of anterior pituitary cells ($P > 0.1$).

ally reversed. To be more specific, we observed, as we had previously *(2)*, that depriving newborn rats of milk from early lactation delayed mammotrope differentiation at 5 d of age (Fig. 1; $P < 0.05$). Early milk deprivation also decreased the percentage of mammotropes measured at 15 and 30 d of age ($P < 0.05$). By 45 days of age, however, the number of prolactin-releasing cells was no longer suppressed by early milk deprivation ($P > 0.1$).

The data presented in Table 1 provide evidence that the effects of early milk deprivation were specific to mammotrope differentiation. For example, depriving neonates of early milk did not alter growth rate, as demonstrated by the similarity of body weights at all age periods ($P > 0.1$). Likewise, the effects of early milk deprivation cannot be attributed to disparate growth rates of the anterior pituitary gland. Indeed, the total number of anterior pituitary cells in rats crossfostered to d 0 and d 7 mothers were similar at all ages. Thus, when viewed as a whole, the effects of early milk deprivation appear to be specific with respect to mammotrope development.

Having established that the deficit in proportional abundance of mammotropes was overcome by 45 d after birth, we next investigated whether this delay altered the secretory capacity of prolactin secretors from adult animals who were originally deprived of early milk. To this end, we used the RHPA to assess basal and regulated prolactin secretion from anterior pituitary cells of early milk-deprived and control male rats at 60 d of age. As shown in Fig. 2, the percentage of prolactin releasers was similar between the two crossfostered groups at all incubation times ($P > 0.1$), just as it was for the 45-d-old animals (Fig. 1). Furthermore, once the percentage of prolactin secretors reached a maximal plateau (usually at 6 h), the average area of prolactin plaques for 60-d-old rats was also comparable for both groups (12.5±0.8 vs 10.8±1.4 μm^2 [$\times 10^{-4}$], respectively; $P > 0.1$). Although early milk deprivation did not alter basal prolactin release or the total number of prolactin-releasing

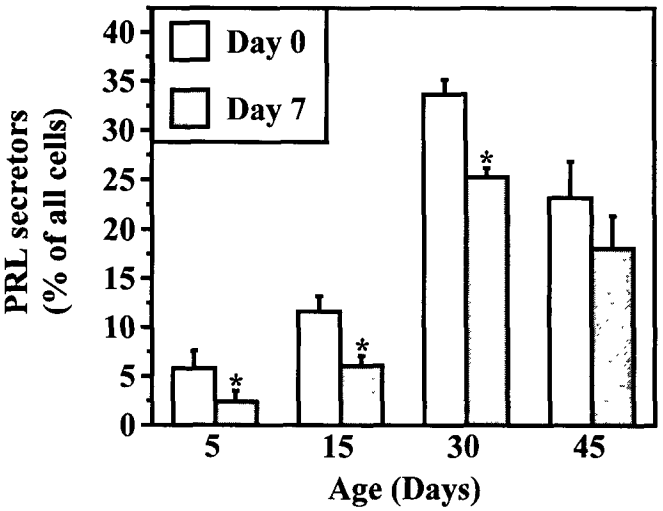

Fig. 1. Effect of early milk deprivation on ontogeny of prolactin releasors. Newborn rats (8 pups/litter) were crossfostered to mothers that had given birth on the same day (d 0) or 7 d earlier (d 7). At 5, 15, 30, and 45 d of age, crossfostered male rats (at least 2 rats from each litter, $n = 4$ separate litters) were killed, anterior pituitary glands were monodispersed, and then subjected to an RHPA to determine the percentage of prolactin secretors. At days 5, 15 and 30 of age, the percentage of prolactin-releasing cells was decreased in rats deprived of early milk (i.e., crossfostered to d 7 mothers) compared to those crossfostered to d 0 mothers (*; $p < 0.05$). By d 45, early milk deprivation no longer had a significant effect on the percentage of prolactin releasors ($P > 0.1$).

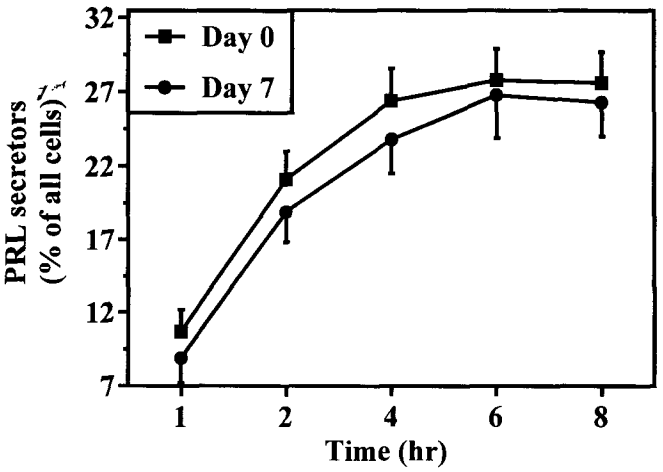

Fig. 2. Rate of prolactin plaque development in normal rats and rats deprived of early milk. Monodispersed anterior pituitary cells from 60-day-old male rats that had been crossfostered at birth to mothers that had given birth on the same day (d 0) or one week earlier (d 7) were subjected to a RHPA ($n = 4$ separate litters, 2 rats/litter). The percentage of prolactin releasors was determined after 1, 2, 4, 6, and 8 h of prolactin antibody incubation. Depriving newborn rats of early milk had no effect on the basal rate of prolactin plaque formation in the adult male rat ($P > 0.1$).

cells in mature male rats, it did alter mammotrope responsiveness. Responsiveness of mammotropes was measured by applying a short incubation period in the RHPA, which allowed the rate of plaque formation to be quantified. The increased prolactin release from stimulated cells resulted in

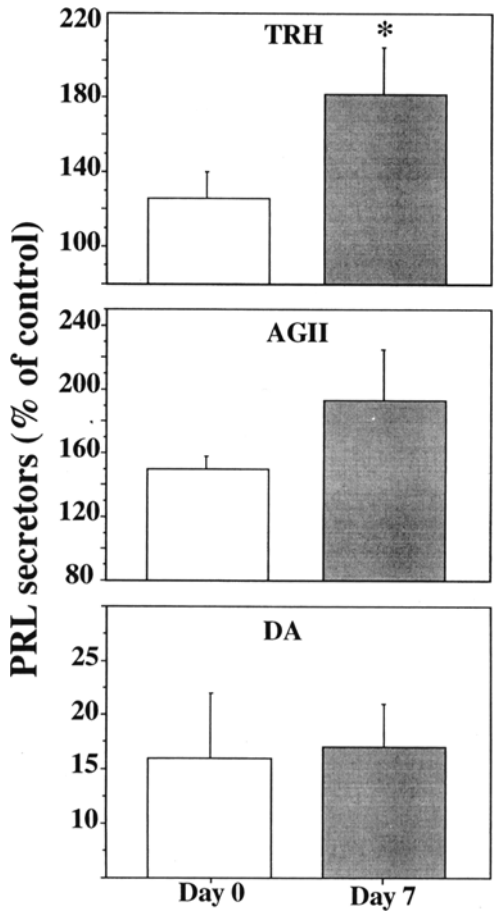

Fig. 3. Mammotrope responsiveness of early milk-deprived and nondeprived rats. At birth, newborn rats were crossfostered to mothers that had given birth on the same day (d 0) or 7 d earlier (d 7). After 60 d, crossfostered, male rats ($n = 4$ separate litters, 2 rats/litter) were killed, at which time their anterior pituitary glands were mono-dispersed and subjected to an RHPA to test their responsiveness to TRH (100 n*M*), AGII, (100 n*M*), and DA (1 μ*M*). The response of mammotropes from animals deprived of early milk to TRH was increased compared to control animals (*$P < 0.05$). Although the response to AGII was numerically higher in deprived pups, the values were not significantly different ($P > 0.1$). Inhibition by DA was similar for both groups ($P > 0.1$).

faster plaque formation than from unstimulated cells and, similarly, inhibition of prolactin release slowed plaque development. Utilizing this approach, we found that the appearance of prolactin-releasing cells from animals deprived of early milk was increased by thyrotropin-releasing hormone (TRH) compared to their crossfostered, control counterparts (Fig. 3; $P < 0.05$). However, this modulation of responsiveness was not universal. Indeed, the stimulatory response to angiotensin II (AGII) and the inhibitory response to dopamine (DA) were similar between experimental and control crossfostered groups (Fig. 3; $P > 0.1$).

Discussion

In this study, we further developed the case that factors in early milk play an obligatory role in ontogeny of the anterior pituitary gland. Specifically, we demonstrated that depriving neonates of these early milk-borne signals delayed the development of prolactin-releasing cells. Although we had previously determined this to be the case during the neonatal period *(2)*, here we extended these observations by demonstrating that the delay in mammotrope differentiation persists throughout the prepubertal period and is not overcome until maturity. There is considerable evidence that this delay is caused by lack of exposure of the developing pituitary gland to a peptide found in early milk. Indeed, we showed previously that this peptide acts directly on neonatal pituitary cells to induce differentiation of prolactin releasers *(2)*, that its concentration in milk is highest during the first days of lactation *(2)*, and that it is rapidly transferred from ingested milk to the neonatal circulation *(4)*. Inasmuch as the proportional abundance of mammotropes in early milk-deprived rats does not normalize until adulthood (long after weaning), it is obvious that the possible reappearance of the differentiative peptide in latter milk cannot explain the eventual attainment of the full mammotrope complement. Instead, a more plausible scenario is that the minority subpopulation of mammotropes that did differentiate in the absence of the milk-borne peptide actually expands after the early neonatal period. Consistent with this possibility is the report by Carbajo-Pérez and Watanabe *(5)* that the rate of mammotrope hyperplasia accelerates greatly after the second week of neonatal life in rats.

Interestingly, we also observed that delayed mammotrope differentiation altered at least one parameter of prolactin secretion in mature animals. Despite attaining equal numbers of prolactin releasers, mammotropes from early milk-deprived animals were more responsive to TRH compared to their nondeprived counterparts. Conversely, DA inhibition and AGII stimulation of prolactin release were similar between both groups, as was basal prolactin release. The mechanism by which this delay in prolactin delivery increases mammotrope responsiveness to TRH is not yet resolved. However, it should be noted that our findings of increased TRH responsiveness in animals in which mammotrope differentiation was delayed are similar to those obtained by use of another paradigm designed to deprive neonates of prolactin. Specifically, Shyr et al. *(6)* observed that rats pups that suckled from mothers injected at the time of birth with bromocryptine had dramatically reduced levels of milk prolactin available to them. Furthermore, mammotropes from these pups deprived of milk prolactin were more responsive to TRH at adulthood *(7)*. In both models (our early milk-deprived pups and their pups fed prolactin-deficient milk), the decreased delivery of prolactin resulted in later hyperresponsiveness of mammotropes to stimulatory agents.

Although we reinforce previous evidence that normal mammotrope development is dependent on exposure to early milk, a more consequential question is whether the

impact of early milk deprivation is felt beyond the secretion of prolactin *per se*. More concisely stated, we questioned whether postponing maturation of the prolactin delivery system might perturb other maturing systems that are believed to be prolactin dependent. Two strong candidates in this regard are the immune *(8,9)* and reproductive *(10)* systems. Indirect evidence suggestive of prolactin involvement in the development and functioning of these systems derives from experiments in which treatment with the hormone restored fertility *(11)* and normal immune function *(12)* to prolactin-deficient Snell and Ames dwarf mice. More direct evidence favoring a specific developmental role is that induction of luteinizing hormone receptors in the immature male rat was delayed by decreasing circulating prolactin with bromocryptine treatment *(13)*. Similarly, immunoneutralization of serum prolactin within the first few days of life altered the developmental pattern of lymphocytes within the spleen and thymus *(14)*. Collectively, these reports indicate that prolactin plays an integral role in development of the reproductive and immune systems. Therefore, perturbation of prolactin during development could have deleterious effects on these processes. Consistent with this possibility is the report by Carlos et al. *(15)*, who employed a crossfostering paradigm similar to ours to evaluate the effects of early milk deprivation on reproductive parameters in the rat. They found that normal females fertilized by early milk-deprived male rats had higher embryo implantation loss than their counterparts fertilized by nondeprived males. It was also noted that the seminal vesicles of these milk-deprived males had lower weights and fructose content than normals. Because prolactin is known to influence the male reproductive system *(10)* and growth of accessory sex glands *(16)*, it seems reasonable to conclude that a delay in onset of prolactin function contributed to this phenomenon. Resolution of the issue of whether the delay in mammotrope development also alters specific immune parameters awaits further studies.

In conclusion, we found that depriving neonates of factors in early milk had a profound effect on maturation of the prolactin delivery system. Neonates deprived of early milk exhibited not only delayed development of prolactin-releasing cells, but also altered secretory function of mammotropes once these animals reached maturity.

Materials and Methods

Animals

Timed-pregnant, Holtzman Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed under 12 h light:12-h dark conditions, fed standard rat chow, and given water ad libitum. Rats were monitored twice daily to determine the time and date of birth. Following birth, newborn rats (adjusted to 8 pups/litter, males preferred) were randomly placed with mothers whose litter had been removed. These mothers had given birth the same day (d 0) or the week before (d 7). In the first experiment, male pups within these crossfostered litters were killed at 5, 15, 30, and 45 d of age. Their anterior pituitary glands were removed, monodispersed and subjected to an RHPA for prolactin. In the second experiment, anterior pituitary cells from crossfostered, male rats at 60 d of age were also subjected to prolactin RHPA.

RHPA

The RHPA for prolactin has been described previously in detail *(17)* and therefore will only be outlined here. Monodispersed, anterior pituitary cells derived from crossfostered animals were combined with protein A-conjugated ovine erythrocytes (Colorado Serum Co., Denver, CO) and infused into Cunningham chambers. After 1 h, nonattached cells were removed, and prolactin antibody (1:40) was infused into the chamber. Cells were incubated for 20 h at 37°C before plaques were developed by the infusion of guinea pig complement (Gibco BRL, Grand Island, NY; 1:60, 45 min). The reaction was stopped by the infusion of glutaraldehyde (Sigma, St. Louis, MO; 2% in 0.9% NaCl) into the chamber. Prior to enumeration, anterior pituitary cells were stained with toluidine blue (Sigma; 1% in 2% glutaraldehyde) to demarcate cells containing a nucleus. The relative abundance of plaque-forming and nonplaque-forming pituitary cells was then quantified (3 slides/litter), and mammotrope percentages were determined by dividing the number of plaque formers by the total number of epithelial cells evaluated.

In the second experiment, anterior pituitary cells from crossfostered, 60-d-old, male rats were subjected to RHPA with a paradigm similar to that described above with the following exceptions. First, the time of incubation with the prolactin antibody was varied (1, 2, 4, 6, and 8 h) before addition of complement for development of plaques. Second, prolactin plaque formers were also evaluated after just 1 h of antibody incubation in the presence of DA (Astra USA, Inc., Westborough, MA; 1 μM), TRH (Peninsula Laboratories, Belmont, CA; 100 nM) and AGII (Sigma; 100 nM). These doses were chosen from preliminary experiments as being maximally effective doses (data not shown). The short, 1-h incubation period allows the monitoring of those cells that secrete the most prolactin per unit time. Formation of plaques from stimulated cells occurs before the formation from unstimulated cells, and similarly, formation of plaques from inhibited cells slows the appearance of plaques. Finally, basal release of prolactin was quantified by measuring the plaque areas with the aid of an ocular reticle once the maximum percentage of plaque-forming cells reached a plateau (usually at 6 h). The diameters of at least 50 plaques/slide were measured.

Statistics

Data were analyzed using predetermined paired *t*-tests. To analyze mammotrope responsiveness of crossfostered

animals, data were first normalized to a percentage of basal values. Data are expressed as means (SEM).

Acknowledgment

This work was supported by National Institutes of Health Grant HD-31487 to L. S. F.

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