

Contribution of Suckling Stimulation to the Daily Periodic Display of Nursing Behavior in Non-Lactating Virgin Rabbits

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Abstract

A female rabbit (does) nurse once a day for three min with circadian periodicity. This timing is controlled by the amount of suckling stimulation received as small litters (2-4 kits) disrupt nursing periodicity and increase the duration of nursing bouts. Yet, as suckling induces: a) the secretion of oxytocin and prolactin and b) milk ejection, the relative contribution of those two types of factors to the daily timing of nursing needs to be independently explored. Therefore, the objective of the present work was to determine the relevance of milk output for the circadian display of rabbit nursing behavior. Methods: We used the model of anosmic virgins, as they can be induced to behave maternally (by lesioning the olfactory epithelium), they allow suckling from (foster) kits, but they are unable to produce milk. Does were given five foster kits, aged 1-5 days, placed inside a transparent nest box within the maternal cage, and replaced daily. The does' behavior was recorded continuously for 15 days through a video system allowing us full view of the animals. Results: By day 6 post-lesion 70% of virgins (i.e., 14/20) were entering the nest box, adopting a crouching posture over the litter, allowing suckling, and leaving ca. 3 min. later. 55% (i.e., 11/20) of lesioned virgins displayed nursing behavior with a ca. 24 hr periodicity, as determined by a Rayleigh analysis (3 does) or a linear regression (8 does). Conclusions: a) daily periodicity of nursing behavior can occur regardless of milk output or a preceding pregnancy; b) such factors, however, contribute to the display of a single daily episode of circadian nursing behavior in most does.

Keywords: Circadian rhythms; Nursing; Maternal behavior; Rabbits; Lactation; Suckling; Oxytocin; Olfactory epithelium

Introduction

Lactating rabbits (does) nurse the young inside the maternal nest only once a day, for around three minutes [1-3] with circadian periodicity, under continuous light [4] or light: dark conditions [5,6]. These characteristics are modulated by suckling stimulation as rabbit does nursing four kits or less enter the maternal nest several times a day [6] and they stay inside it for more than three min [7-9]. Accordingly, a maximal expression of the PER1 protein (product of the clock gene *per1*) is observed in specific regions of the rabbit forebrain at eight hrs post-suckling, independently of the time of day at which mothers nursed [10]. Taken together, the above findings suggest that the timing of circadian nursing behavior and the time inside the nest box rely on suckling stimulation. Indeed, pre-emptying the mammary glands of milk (by suckling a foster litter under anesthesia) does not antagonize the doe's entrance into the nest box to nurse her own kits [11]. Moreover, virgin rabbits can be induced to behave maternally by lesioning the olfactory epithelium with ZnSO₄ [12] despite their incapacity to produce milk. When foster kits are introduced into the nest box at a specific time of day such anosmic virgins will almost immediately jump inside and adopt a crouching posture over the litter. The kits will then start searching for the doe's nipples and initiate suckling guided by the so-called mammary pheromone [13], which is produced by lactating mothers and also by virgins [14,15]. After ca. 3 min of receiving suckling stimulation from the foster litter, and despite the lack of milk output, anosmic virgins will jump out of the box, as do regular lactating mothers. This evidence shows that milk output is irrelevant for determining the female's motivation to enter the nest box and the time she spends inside it. Yet, such information fails to reveal if milk output contributes to the spontaneous circadian display of nursing because the cited studies only measured the doe's willingness to nurse and the time she spent inside the nest box [8,11,12]. Thus,

in the present work we hypothesized that, if milk output is irrelevant for determining the circadian display of nursing in rabbits, anosmic virgins provided with a (foster) suckling litter (larger than four pups) would show a daily pattern of circadian nursing behavior.

Materials and Methods

Animals and housing conditions: Virgin New Zealand white adult female domestic rabbits (3.5-4.5 Kg body weight) bred in our colony was used. They were housed in individual wire mesh maternal cages (90 cm long x 60 cm wide x 40 cm high) that contained a transparent acrylic nest box (50 cm long x 30 cm wide x 32 cm high). Conditions inside the vivarium were: controlled light (14:10 L:D; lights-off at 21:00 h) and natural temperature (13-25 °C). Water and Purina rabbit pellets were provided ad libitum every day between 07:00 and 08:00 h. Throughout this work animal care complied with the Law for the Protection of Animals (Mexico) and with international guidelines regarding animal research [16].

Lesion to the olfactory epithelium: Females were made anosmic as described earlier [10], following the procedure of Mulvaney and

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Heist [17]. Briefly, females were lightly anesthetized with ketamine (1 mg/kg; Imalgem; Rhone-Merieux, Mexico) and xylazine hydrochloride (rompun; 25 mg/Kg; Beyer, Mexico). 1.0-1.5 ml of 5% ZnSO₄ (n=20) or saline (n=8) was then infused into each nostril (using polyethylene tubing, Clay Adams PE 60; O.D=1.22 mm; I.D=0.76 mm) while rabbits were gently held in the supine position. Two minutes later does were held with their head down to allow the solution to flow out of the nostrils and thus minimize spread to the oral cavity. Rabbits were then returned to their home cages.

Olfactory perception and ambulation tests: To verify that the infusion of ZnSO₄ induced anosmia, while the infusion of saline did not, we used the olfactory discrimination test reported earlier [12]. Females were placed inside a square wire mesh arena (1.2 m/side x 60 cm high, with the floor raised 10 cm above the ground) which had a painted grid dividing the floor into nine squares. Two glass jars containing water or freshly collected male urine were placed under two opposing corners of the arena. We counted, for the next 10 min, the number of times the rabbit sniffed each of the jars. To determine non-specific effects provoked by the chemical lesion to the olfactory epithelium, we simultaneously counted the number of times the rabbit crossed any of the lines painted on the floor (ambulation in an open field). These tests were made on two consecutive days before the infusion of ZnSO₄ (or saline; baseline) and again on post-infusion days 1 and 2. To confirm the recovery of olfactory function at the end of the experiment these tests were performed again on day 18.

Nursing behavior measurement: Starting on post-infusion day 3 video cameras were installed inside the vivarium and connected, via underground cables, to a computer placed in a different room. The transmitted images were recorded directly onto the hard drive, a procedure used in our previous study [6] that allowed us to review the videos off line at any time of day. Six kits from a lactating mother of our colony were placed inside the nest box of each experimental virgin at 10:00 h and remained there for the next 24 h. The kits were then returned to their mother for nursing and replaced with new litters of the same age. These had been fed their own mothers a few hours earlier. This procedure was repeated daily for five days after which new litters of newborn kits were used. Thus, the experimental virgins were continuously exposed to foster kits, aged 1-5 days. Because the nest box used was transparent (see above) we were able to have a full view of the behavioral interactions between virgins and foster litters at any time, particularly if the kits suckled, presumably guided by the “nipple pheromone”, as described in our earlier study [12]. The following parameters were recorded without interruption for the following 15 days: a) hour of day when the doe entered the nest box and adopted a crouching posture (indicative of nursing behavior) over the litter; b) duration of time spent inside the nest box at each visit (determined by subtracting the time of exit from the time of entrance).

Statistical analysis: To compare the number of sniffs and crossings (ambulation in an open field) shown before vs after the infusion of ZnSO₄ (or saline) a Wilcoxon test was performed [18]. To determine the vector that best described a female's individual time for displaying nursing behavior across days 1-15 a Rayleigh test for randomness [19] was performed. From this analysis we derived the phase angle and rho of every individual vector. Only females that showed nursing behavior on at least five days were included in the population vector, which was obtained from the Rayleigh analysis of the mean phase angle vector of each individual. When this criterion was not met, and visual inspection of the actograms revealed an apparently drifting (as free running) daily pattern of nursing behavior, a linear regression analysis was performed on the time of display of this activity. This allowed us to determine,

for each individual, the period and the significance of r from the lines adjusted to such daily patterns.

Results

Figure 1 shows that in females infused with saline the number of sniffs directed towards urine was significantly higher than those directed to water. These differences were evident on the days pre-infusion and persisted until the last day of testing, indicating that the infusion of saline did not induce anosmia. In contrast, does infused with ZnSO₄ directed more sniffs towards urine before the infusion, were unable to discriminate between these stimuli on post-infusion days 1 and 2, and recovered this capacity by day 18 (Figure 2). These findings confirm that, as reported previously [12,17] the infusion of ZnSO₄ provokes a degeneration of the olfactory epithelium, which leads to anosmia, followed by a regeneration of such tissue which, in turn, allows the recovery of function. Ambulation in an open field (Figure 3) significantly declined with respect to baseline on post-infusion days 1 and 2 in both experimental groups but had fully recovered by day 18.

Infusion of ZnSO₄ promoted the expression of nursing behavior as up to 70% of does in such group (i.e., 14/20) showed this response by day 6 (Figure 4). In contrast, no saline-infused rabbit entered the nest box and crouched over the litter on any day of testing, confirming that virgin does are not ‘spontaneously maternal’ unless they become anosmic. Nursing behavior in anosmic virgins, however, was expressed in an irregular fashion (Table 1). For instance, the latency for the onset of this activity (relative to the days of exposure to kits) ranged from 1-6 days and the maximal number of consecutive days on which an anosmic rabbit showed nursing behavior ranged from 2-13. That is, in contrast to ‘regular’ nursing mothers, which nurse every day from parturition onwards, anosmic virgins performed nursing behavior on some days and not on others.

A Rayleigh analysis revealed that the values of rho (indicating the direction of the vector describing the most likely time of nursing behavior) were statistically significant for three females. Indeed, the actograms of such does show that their nursing behavior occurred usually once a day, during the dark phase (Figure 5 A, B, C). Other rabbits, by contrast, entered the nest box several times across the day (Figure 6 A, B, C). When double-plotted their actograms revealed that entrances into the nest box on any given day did not occur randomly but in a timed manner, during a restricted portion of the

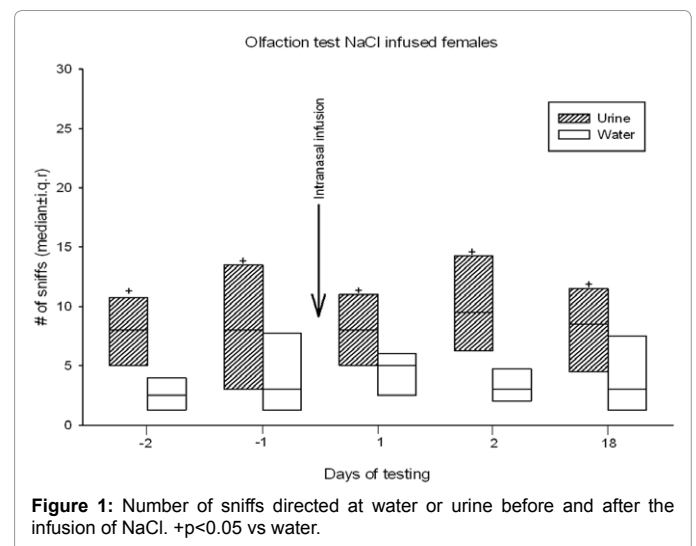
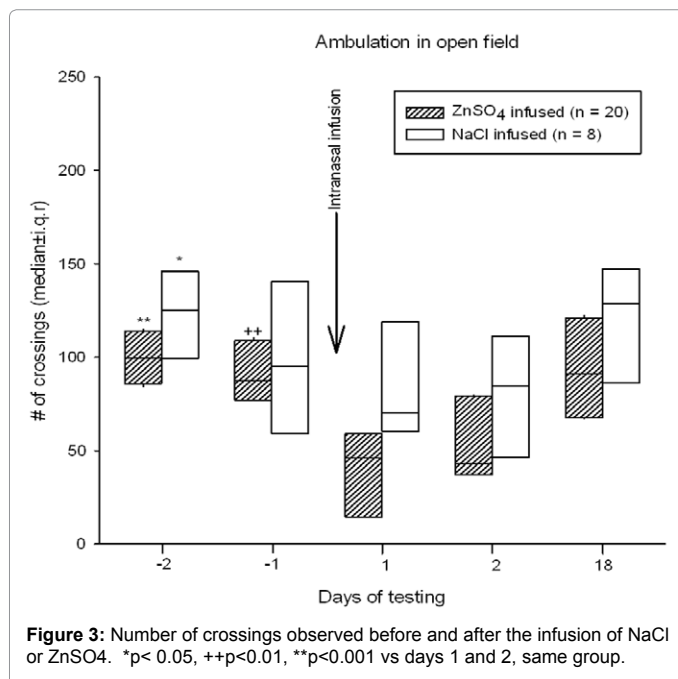
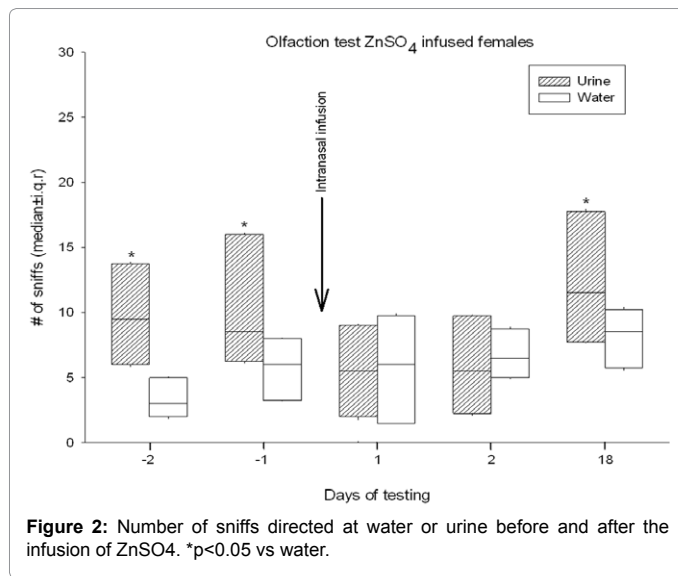


Figure 1: Number of sniffs directed at water or urine before and after the infusion of NaCl. *p<0.05 vs water.



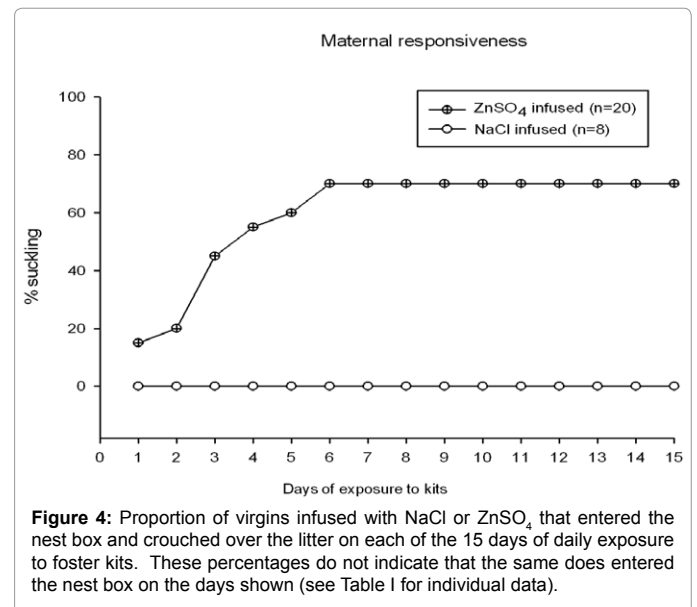
day, resembling a free-running period shorter than 24 h. Indeed, linear regression analysis of the lines adjusted to these patterns revealed that the values of *r* were statistically significant for 8 out of 12 does (*p*<0.05, Table 2). It is interesting that in one doe (#1985), in which the Rayleigh analysis indicated a significant circadian vector, the linear regression analysis did not reach statistical significance. This seems to be due to the successive back and forward shifts in the timing of nursing in this animal, which did not affect the *rho* value from the Rayleigh analysis but had a great influence on the *r* value of the linear regression. Other anosmic virgins entered the nest box only on a few days (Table 1; Figure 7 A,B) and, consequently, no statistical analysis could be performed on them. In contrast to the above-described differences in the frequency and time of day at which virgins displayed nursing behavior, whenever they entered the nest box and allowed suckling from the kits, they stayed inside for ca. 3-5 min, as occurs with lactating rabbits (Table 1). In summary, a total of 11 out of 20 virgin does sprayed with ZnSO₄

(i.e., 55%) showed nursing behavior in a fashion consistent with a periodicity of ca. 24 hrs, as determined through a Rayleigh analysis (three females) or a linear regression (eight rabbits).

The relationship between anosmia and the expression of nursing behavior on a given day was not a direct one. Table 3 shows that at the beginning of the experiment (early period) all females were anosmic on the day following the infusion of ZnSO₄ (day 1), but only 21% of them (i.e., 4/20) showed nursing behavior two days later (day 3). By contrast, at the end of the experiment (late period) 60% of ZnSO₄-infused virgins (i.e., 12/20) showed nursing behavior (day 17), despite the fact that, on day 18, only 1/20 individuals (5%) was anosmic.

Discussion

In agreement with our earlier report [8] the suckling stimulation provided by six kits allowed a normal time inside the nest box (i.e., ca.



# ♀	First day showing nursing behavior*	Maximal number of consecutive days showing nursing behavior	Rho	p	phase angle (α)	Geographic hour	% sucklings in dark phase	
1951	1	3	3.58	0.522	<0.114	28°	1.87	80
2130	1	3	4.15	0.600	0.165	318°	21.2	29
2045	1	13	5.93	0.337/0.575	<0.201/ <0.014	14°/ 318°	21.2	54
1940	2	0	5.05	0.12	<0.87	139°	9.27	40
1944	3	8	6.78	0.220	<0.659	87°	5.8	33
1947	3	6	3.8	0.368	<0.245	103°	6.87	44
1996	3	3	5.45	0.725	<0.02	45°	3.00	100
1986	3	2	3.85	0.985	nd	nd	nd	100
1989	3	0	3.37	0.767	nd	nd	nd	33
1990	4	9	3.05	0.856	<0.001	354°	23.6	100
1985	4	7	2.67	0.661	<0.009	105°	7.00	50
1993	5	5	4.88	0.528	<0.085	9°	0.60	82
1959	6	2	7.05	0.545	nd	nd	nd	67
1939	6	0	3.32	0.66	nd	nd	nd	0

Table 1: Characteristics of nursing behavior and parameters determined through a Rayleigh analysis.

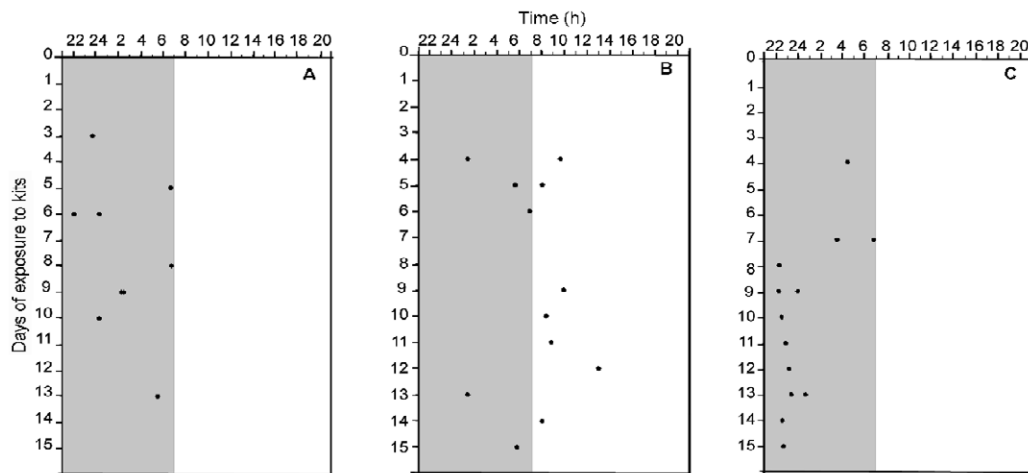


Figure 5: Actograms of the three virgins (a, b, c, corresponding to #1996, 1985, and 1990 in Table I) from which we confirmed a circadian display of nursing behavior following a Rayleigh analysis.

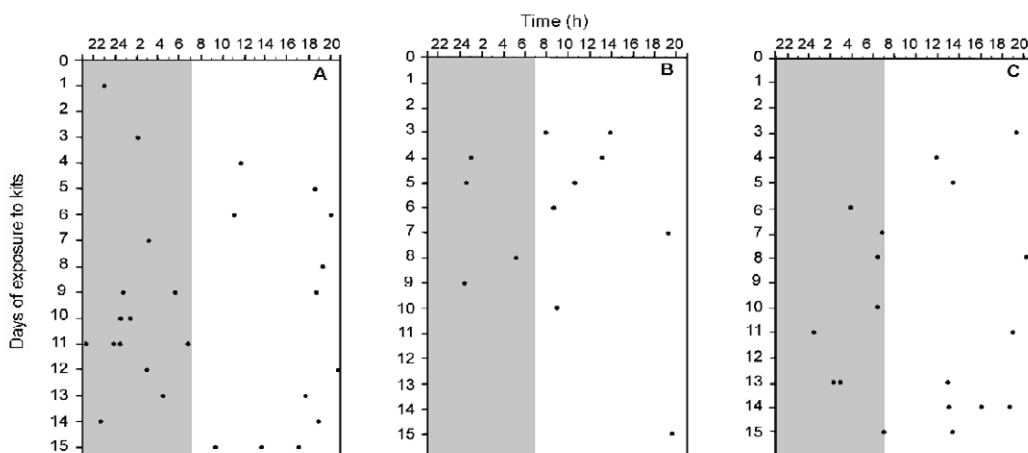


Figure 6: Actograms of three virgins (a, b, c, corresponding to #2045, 1944, and 1947 in Table I) that entered the nest box several times a day, from which we could not determine a significant vector that confirmed circadian periodicity of nursing behavior.

# ♀	slope	period	r	p
1951	-19.0	23.02	0.9698	0.018
2130	32.0	24.75	0.9867	0.004
2045	-11.2	22.85	0.9491	0.03
1940	-59.0	23-57	0.9669	0.018
1944	-10.9	22.84	0.9752	0.011
1947	-17.0	23.00	0.9071	0.064
1996	-6.0	22.65	0.9981	0.004
1986	-36.0	23.34	0.3873	0.34
1989	-22.0	23.05	0.5329	0.27
1990	92.0	24.26	0.9690	0.018
1985	-50.46	23.52	0.7948	0.14
1993	-10.89	22.84	0.9897	0.004

*does # 1939 and 1959 were excluded from this analysis due to the few days on which they entered the nest box (see Table I)

Table 2: Linear regression analysis to determine circadian periodicity of nursing*

3 min) despite the virgins' incapacity to produce milk. Moreover, the consistency in the time spent inside the nest box is an indirect indicator that the kits provided did engage in suckling: we have shown that a

threshold of suckling stimulation is essential to induce the crouching posture over the litter and to allow a normal time inside the nest box [8]. In contrast, the other characteristic of nursing behavior, i.e.,

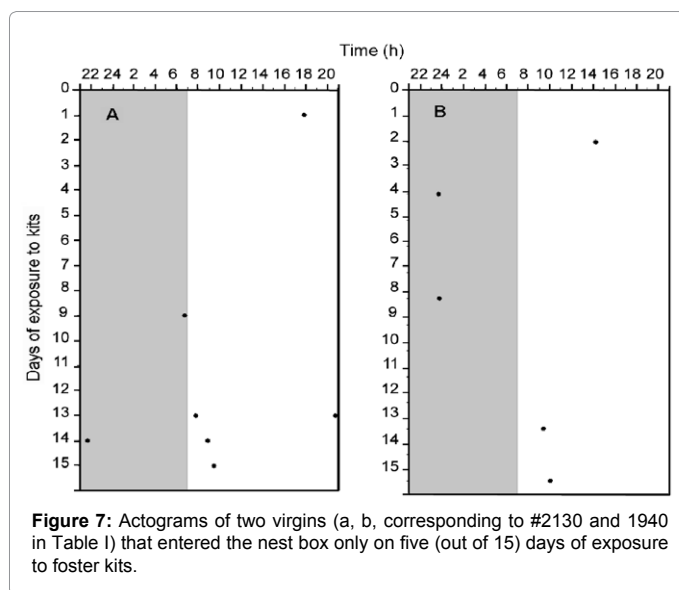


Figure 7: Actograms of two virgins (a, b, corresponding to #2130 and 1940 in Table I) that entered the nest box only on five (out of 15) days of exposure to foster kits.

Early period	Percent anosmic on post-infusion day 1			Late period	Percent anosmic on post-infusion day 18		
	Yes	No	Total		Yes	No	Total
Percent nursing on post-infusion day 3	21	0	21	Percent nursing on post-infusion day 17	7	53	60
	79	0	79		0	40	40
	100	0	100		7	93	100

Table 3: Relationship between anosmia and display of nursing behavior in ZnSO₄-infused virgins.

its occurrence once a day with a period close to 24 h [4-6], differed from that of lactating mothers in the following aspects: a) the onset of nursing behavior was not immediate: most virgins started displaying it on the third to fourth day of pup exposure, i.e., on post-lesion days 5-6; b) none of the females entered the nest box every single day to crouch over the litter: the maximal number of consecutive days on which they showed nursing behavior ranged between 2 and 13; c) the proportion of virgin does sprayed with ZnSO₄ that showed nursing behavior with a periodicity of ca. 24 hrs was 55% (11/20). Most of the nursing behavior episodes of these virgins occurred during the dark phase, as reported for mothers given free access to nurse their litters [5,6], despite the fact that we introduced the foster kits to virgins at 1000 hrs. That is, even though the virgins had access to the litters for 11 hrs of the light period (i.e., from 1000 to 2100 hrs), they entered the nest box during the dark phase, as occurs in lactating rabbits [5,6].

Although the present results confirm that anosmia promotes nursing behavior in virgin rabbits the mechanisms underlying this effect are unclear. Although ZnSO₄ intranasal infusions reduced ambulation on the following two days this effect cannot explain the latency of 3-4 days to initiate nursing behavior because virgins infused with NaCl also showed such reduced ambulation but did not become maternal. Moreover, the fact that anosmia was present immediately after ZnSO₄ infusions but nursing behavior was not and, vice versa, that at the end of the experiment up to 60% of lesioned virgins were maternal but only one was still anosmic, does not support a direct relationship between the perception of signals from the young and the likelihood of maternal responsiveness in virgin rabbits. Rather, we believe that the lesion to the olfactory mucosa (provoked by ZnSO₄ infusions) may lead to the degeneration of fibers that interfere with the function of brain

circuits that tonically inhibit the expression of maternal responsiveness in virgins. Indeed, there is evidence that the olfactory system inhibits 'abnormal' behaviors, specifically: lesions to the olfactory system promote the display of the lordosis reflex (characteristic of sexually receptive females) in castrated steroid-treated male rats [20,21].

Although the present results do not rule out a participation of milk output in timing the circadian periodicity of actual nursing they do show that such process is not essential. Perhaps milk output per se and/or the secretion of hormones like oxytocin and prolactin act together with nipple stimulation to activate a neural network that times the unfailing display of nursing with a periodicity of ca. 24 hrs. Indeed, the representation of the receptive field of the ventrum in cortical area S1 is larger in lactating rats than in virgins [22]. If something similar occurs in rabbits it may account for the larger variability in the display of nursing behavior of virgins vs lactating does.

A limitation of the present study is, of course, that a major difference between lactating mothers and 'maternal virgins' is that the latter have not been exposed to the hormones of pregnancy [2]. These agents 'prepare' the maternal brain to respond to the litter ab initio in a species-specific way. Thus, even primiparous rabbits upon first exposure to the young crouch over them inside the nest, nurse them for ca. 3 min [8] and repeat this activity with circadian periodicity [6]. Moreover, the activation of several forebrain nuclei following nursing (as determined by the number of c-FOS protein-immunoreactive cells) is the same on the first as on the seventh day of lactation [23]. Consequently, the lack of pregnancy hormones may partly account for the 'less than perfect' circadian timing of suckling behavior in maternal virgins. Another caveat of the present work is that, as the kits did not obtain any milk despite their suckling, we cannot determine how their tactile stimulation of the doe's ventrum or the intensity of the nipple stimulation provided compares to that of kits who do obtain milk. Moreover, we can anticipate that the release of PRL and OT by suckling was probably lower in these virgins than in truly lactating does. Clearly, more work is warranted to determine the relative contribution of milk output, hormones, and peripheral stimulation to the circadian display of rabbit nursing.

Conclusion

Our results show that suckling stimulation per se has a major role in timing the periodicity of nursing, even in the absence of a preceding pregnancy, and is sufficient to determine a normal duration of suckling episodes. That is, a neural network intrinsic to doe rabbits is activated by suckling stimulation, regardless of milk output, to allow the periodic expression of nursing behavior once a day. However, other factors, like milk output and the hormones of pregnancy and lactation, are necessary in most females to allow a 'flawless', unfailing display of nursing behavior like that observed in truly lactating rabbits.

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