Suckling induces a daily rhythm in the preoptic area and lateral septum but not in the bed nucleus of the stria terminalis in lactating rabbit does

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Abstract

Maternal behavior in the rabbit is restricted to a brief nursing period every day. Previously we demonstrated that this event induces **daily** rhythms of PER1 protein, the product of the clock gene *Per1*, in oxytocinergic and dopaminergic populations in the hypothalamus of lactating rabbit does. This is significant for the **periodic** production and ejection of milk, but the activation of other areas of the brain has not been explored. Here we hypothesized that **daily** suckling will induce a rhythm in the preoptic area, lateral septum and bed nucleus of the stria terminalis, which are important **areas** for the expression of maternal behavior in mammals including the rabbit. To this **end,** we analyzed PER1 expression in those areas through a complete 24-h cycle at lactation day 7. Does were scheduled to nurse during either the day at 10:00 (ZT03) or the night at 02:00 (ZT19) h. Non-pregnant, non-lactating females were used as controls. In contrast to control **females,** lactating does show a clear, significant rhythm of PER1 that shifts in parallel to timing of nursing in the preoptic area and lateral septum. We determined that the maximal expression of PER1 at 8 h after scheduled nursing decreased significantly at 24 and 48 h after the absence of suckling. This effect was more **pronounced** in the lateral septum than in the preoptic area. We conclude that daily suckling is a powerful stimulus that induces rhythmic activity in brain structures in the rabbit that **appear** to be part of a maternal entrainable circuit.

Keywords

Nursing; maternal behavior; Per1 protein; temperature rhythm

Introduction

In mammals, maternal behavior (MB) consists of a series of behaviors that contributes to the survival of the young by providing food, warmth, shelter, protection from predators and conspecifics and appropriate stimulation to the young (Olazábal et al., 2013). Most of **our** knowledge about the neural basis of MB comes from studies in rodents and sheep, and research in other species reveal that this behavior is diverse and expressed in different forms

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and modalities (González-Mariscal & Poindron, 2002). In this regard MB in the rabbit represents an exceptional example as **mothers** after parturition visit daily their pups only for nursing without providing any other care to their progeny (Zarrow, *et al*, 1965; Caba & González-Mariscal, 2009). More remarkably, this daily visit occurs at **about the same hour every day** (Jilge, 1995; González-Mariscal *et al.,* 2013), which is unique among mammals. Previously we demonstrated daily rhythms of PER1 protein, product of the *Per1* clock gene, that shift in parallel to the timing of **daily** nursing in hypothalamic oxytocinergic and dopaminergic cells of the lactating doe (Meza *et al.,* 2008; 2011). This **was** not a general effect **on neuronal activity** in the doe's brain, as a dopaminergic population in the diencephalon was not **affected** by this stimulus (Meza *et al.,* 2011). These results were important because they laid groundwork to explore the neuroendocrine mechanisms that control the **periodic** production and ejection of milk. However, the neural basis of the periodic **maternal** behavior remains poorly explored. Specifically, it is unknown whether other areas in the brain, such as the preoptic area (POA), lateral septum (LS), and the bed nuclei of the stria terminalis (BNST), important for the expression of MB in several species, including the rabbit (Numan & Insel, 2003; Olazábal *et al.,* 2013) **display rhythmic activity** related to **timed** nursing. We considered it important to explore these regions to **gain further** insight into the possible neural **substrates mediating** control of MB in the rabbit, taking into account **the fact** that the suprachiasmatic nucleus (SCN), the master circadian clock, is not entrained by timing of nursing (Meza *et al*., 2008). Here, we hypothesized that the POA, LS and BNST will show rhythmic activity associated **with** timing of nursing (Meza et al., 2008). To this aim we explored the expression of PER1 in those prosencephalic areas in lactating does scheduled to nurse either during the day or the night.

Materials and Methods

Animals and housing

New Zealand white female rabbits bred in the colony in Xalapa, México, were housed under a LD cycle (12:12 h; lights on at 07:00= ZT0). Rabbits were provided with food pellets (Purina, México D.F., México) and water *ad libitum*. Females were mated and housed individually in stainless steel cages at room temperature of $23 \pm 1°C$ and were monitored daily from day 28 of pregnancy until delivery. Each cage had two compartments, one for the mother and one for the nest (0.60 m wide × 0.50 m long × 0.40 m high) linked by a tunnel (0.25 m wide × 0.50 m long × 0.40 m high) between them. The tunnel had a sliding door on each end, permitting the experimenter to control the mother's access to the tunnel and nest. Before parturition, does had free access to the nest compartment and they were provided with approximately 100 g of straw to build a nest. Pups were born in the nest. On the day of parturition, litters were adjusted to four to five pups. Experiments and animal handling were conducted according to the national guide for care and use of animal experimentation approved by the Ethics Committee of Universidad Veracruzana, in accordance with the procedures of the National Guide for the Production, Care and Use of Laboratory Animals (Norma Oficial Mexicana NOM-062-ZOO-1999), which complies with international guidelines laid down by the Society for Neuroscience and were approved and conducted according to the Statement of Assurance with Standards for Humane Care and Use of

Laboratory Animals approved by the National Institutes of Health (NIH) to the Universidad Veracruzana.

Experimental design

On the day of parturition (PP0), the door between mothers' compartments and the tunnel was closed. Starting the next day (PP1), the door was opened for nursing at either ZT03 (Nursing ZT03 group) or ZT19 (Nursing ZT19 group). The doe entered the nest immediately after the door was opened to nurse. The mean daily duration of nursing was 235 ± 8 s (Mean±S.E.) and 224 ± 3 s for pups nursed at ZT03 and ZT19, respectively. Does were sacrificed at PP7 starting at their scheduled time of nursing (ZT03 or ZT19) and then at 4 h intervals at ZT03, ZT07, ZT11, ZT15, ZT19 and ZT23 (n=4 at each time point). We also determined the maintenance of the PER1-ir cells in nursing deprived does. Following the nursing schedules above, does were deprived of one (Nursing-Dep 24 h) or two (Nursing-Dep 48 h) cycles in both nursing groups. They were sacrificed 8 h after their previous scheduled time of nursing (n=4 at time point) at the time of expected maximum expression of PER1-ir cells in the POA, LS and BNST of nursing does. Non-pregnant, non-lactating adult females were used as controls (Control group, n=4 at each time point). Control females were handled as the lactating ones and sacrificed at 4 h intervals. For the present experiment, we used the same **animals in which** we **previously** demonstrated synchronization of locomotor behavior **and rhythms in** oxytocin **cells**, but not suprachiasmatic nucleus **cells**, in the hypothalami of lactating female rabbits (Meza et al., 2008).

Immunohistochemistry

Rabbits were anesthetized with an overdose of sodium pentobarbital (60 mg/kg, i.v.) and were perfused transcardially with saline solution (0.9%), followed by 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). Brains were removed immediately after perfusion, cryoprotected successively in 10, 20 and 30% sucrose in PB and then sectioned coronally at 50 μm with a cryostat (Microm, Walldorf, Germany). Serial sections were collected in PB from the level of the organum vasculosum of lamina terminalis to the mammillary bodies. The fourth section of each set was used for PER1 labeling as described below, following similar protocols previously established for nuclear proteins (Caba *et al.,* 2008) in rabbit brain. Tissue was washed in PB four times, 5 min each, to remove excess aldehydes and then exposed for 10 min in 0.5% hydrogen peroxide solution to eliminate endogenous peroxidase activity. Nonspecific antibody reactions were blocked by placing the sections in 3% normal horse serum (Vector Labs; Burlingame, CA) for 1 h at room temperature. Sections were then incubated for 48 h at 4 °C in polyclonal PER1 antibody (sc-7724) diluted at 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA) in 3% normal horse serum with 0.3% Triton X-100 (Sigma). Tissue was then placed in biotinylated horse anti-goat serum (1:200, Vector Labs) for 1 h. After 3 washes in PB, sections were incubated in avidin-biotin complex (1:250, Vector Labs) for 1 h. PER1 antibody-peroxidase complex was stained with a solution of 0.05% diaminobenzidine (DAB; Polysciences) in the presence of nickel sulfate (10 mg/ml, Fisher Scientific, Fair Lawn, NJ), cobalt chloride (10 mg/ml, Fisher scientific) and 0.01% hydrogen peroxide, which produced a black-purple precipitate. After 10 minutes, tissue was transferred to PB to stop the reaction. Sections were mounted onto gelatin-subbed slides, dehydrated, and cleared in Hemo-De (Fisher Scientific), then coverslipped with

Permount. In all cases, tissue sections from subjects **at** each time point were processed together. The sc7724 PER1 antibody has been **previously** characterized and **validated** in rabbit **brain tissue** (Caba *et al.,* 2008).

Quantification of Immunostaining

PER1-ir was identified as a black-purple precipitate from the DAB-nickel/cobalt reaction in the cell nucleus. To quantify immunoreactive nuclei staining, we determined the background optical density in a nearby region lacking immunoreactivity with the Image-Pro Plus program, v. 5 (Media cybernetics, Silver spring, MD). Immunoreactive cells that reached five times the optical density background level were considered positive. Cells below this staining level were considered negative. The numbers of PER1-ir positive cells were counted unilaterally by two observers blind to the experimental conditions with an Olympus BX41 (Tokyo, Japan) microscope using a grid according to a protocol previously published (Caba *et al.,* 2008; Meza *et al.*, 2008). The localization of brain structures was determined using terminology according to Girgis and Shi-Chang (1981) and our previous experience in studying the forebrain of this species (Caba *et al.,* 2003). At the time of maximum expression, PER1-ir in the preoptic region was observed in the periventricular as well as in the medial preoptic area, being more abundant in the proximity to the third ventricle. Then we selected an area of 591,839 μm^2 (Fig. 1) that included both PER1-ir cells in the periventricular and medial preoptic area (**termed POA**), similar to a previous study in the rabbit (Gonzalez-Mariscal *et al.,* 2009). For the LS we **analyzed** the dorsal (LSD), intermediate (LSI) and ventral (LSV) subdivisions where we selected an area of 271 518, 227 320 and 220 440 μm^2 respectively (Fig. 1). For the BNST, we **analyzed** its ventral subdivision (vBST; Numan & Insel, 2003), which lies dorsolateral to the POA, and selected an area of 370,416 μm^2 (Fig. 1). In all cases cell counting was done in one representative sectioncorresponding to levels AP1 for the LS and BNST and AP2 for the POA from Bregma of the stereotaxic atlas of the rabbit (Girgis & Shi-Chang, 1981).

Statistical analysis

Analysis of the number of PER1-ir cells was performed by one-way ANOVA to determine whether there were differences across different time points and between each group; this was followed by a post hoc analysis using the Tukey test (SigmaStat 3.5). Comparisons between groups at specific time points in deprived nursing does were performed using Student's t-test. Probability levels of $P<0.05$ were considered significant. Values are given as means ± S.E. Additionally, acrophases of the number of PER1-ir were evaluated by Cosinor analysis using Acro.exe software (version 3.5) described by Refinetti (2006), where a rhythm was considered to be significant when $P<0.05$.

Results

In control animals all studied structures expressed PER1 without a clear rhythm, however, nursing induced a robust **and** significant PER1 rhythm in the POA, LSD and LSV, which shifted in parallel to timing of nursing. PER1 expression **reached** a peak 8 h after scheduled nursing in these structures, regardless of whether nursing **occurred** during day or the night. In the LSI and in BNST there was a completely different pattern. In the LSI, there was a

significant rhythm in the Nursing ZT03 group where the maximum level was observed 16 h after nursing, **whereas** in the Nursing ZT19 **group**, no significant rhythm was expressed. In the BNST, no significant rhythm was observed in any group. **The absence** of suckling significantly decreased PER1 expression at peak time in both POA and LSD and LSV but the effect was more **pronounced** in **the** LS than in **the** POA.

PER1-ir in the POA

The POA showed a rhythm of PER1-ir in the nursing groups, with a peak at 8 h after nursing. No rhythm was detected in the Control group. The photomicrographs in Figure 2A show the POA of a representative section of a doe at the time of nursing and 8 h later. ANOVA revealed a significant difference across time in the Nursing ZT03 ($F_{5,18} = 56.848$, $P < 0.001$) and the Nursing ZT19 ($F_{5,18} = 131.537$, $P < 0.001$) groups. No significant differences across time were seen in the control group ($F_{5,18} = 1.051$, $P = 0.419$). For the Nursing ZT03 group, the highest value at ZT11 was significantly different from the remaining time point values ($P < 0.001$ in all cases). In the Nursing ZT19 group, the maximal value at ZT03 was significantly different from the remaining time point values ($P < 0.001$ in all cases; Fig. 2B). The cosinor analysis revealed significant rhythmicity of PER1-ir in both Nursing ZT03 (amplitude 101.0; acrophase 17.0h; $P < 0.001$) and Nursing ZT19 (amplitude 137.0; acrophase 9.0h; $P < 0.001$), but not in the Control group (amplitude 17.5 acrophase 17.0h; $P > 0.05$; Fig. 2C).

PER1-ir in the LSD

The LSD showed a rhythm of PER1-ir in the nursing groups, with peak values at 8 h after nursing. No rhythm was detected in the Control group. The photomicrographs in Figure 3A show the LSD in a representative section from a doe at the time of nursing and 8 h later. The ANOVA analysis revealed a significant difference across time in Control ($F_{5,18} = 8.816$, $P < 0.001$), Nursing ZT03 ($F_{5,18} = 39.085$, $P < 0.001$) and Nursing ZT19 ($F_{5,18} = 11.303$, P < 0.001) groups. In the Control group the peak value at ZT03 was significantly different from the ZT15 and ZT19 ($P < 0.05$ in both cases) values. In the Nursing ZT03 group, the higher values at ZT11 and ZT15 were significantly different from values at ZT03, ZT07, ZT19 and ZT23 ($P < 0.001$ in all cases). In the Nursing ZT19 group, the higher values at ZT03 and ZT07 were significantly different from remaining time point values ($P < 0.001$ for ZT15, ZT23 and $P < 0.05$ for ZT11 and ZT19; Fig. 3B). Cosinor analysis revealed significant rhythmicity of PER1-ir in both Nursing ZT03 (amplitude 80.0, acrophase 21.0h; $P < 0.001$) and Nursing ZT19 (amplitude 71.5; acrophase 9.0h; $P < 0.001$) groups, but not in the Control group (amplitude 23.0; acrophase 13h; $P > 0.05$; Fig. 3C).

PER1-ir in the LSI

The LSI showed variations of PER1-ir in the nursing and control groups. The Nursing ZT03 group expressed a maximal value of PER1 at 16 h after nursing and was the only group with a significant rhythm. ANOVA revealed a significant difference across time in PER1-ir in the Control ($F_{5,18} = 4.178$, $P < 0.05$), Nursing ZT03 ($F_{5,18} = 6.657$, $P < 0.001$) and Nursing ZT19 ($F_{5,18} = 7.870$, $P < 0.001$) groups. For the Control group, the maximum value at ZT11 was only different from ZT03 ($P < 0.05$). For the Nursing ZT03 group, the maximal value at

ZT19 was significantly different from the remaining time points ($P < 0.001$ in all cases). In the Nursing ZT19 group, maximal values at ZT11 and ZT19 were significantly different from ZT03, ZT07 and ZT23 (P < 0.001 for ZT03 and ZT07, $P < 0.05$ for ZT23). Cosinor analysis revealed significant rhythmicity of PER1-ir in Nursing ZT03 (amplitude 43.5; acrophase 1.0h; $P < 0.05$), but not in Nursing ZT19 (amplitude 35.0; acrophase 1.0h; $P > 0.05$) and control groups (amplitude 29.5; acrophase 21.0h; $P > 0.05$).

PER1-ir in the LSV

The LSV showed a rhythm of PER1-ir in the nursing groups, with maximal values at 8 h after of nursing. No rhythm was detected in the Control group. The photomicrographs in Figure 4A show the LSV in a representative section from a doe at the time of nursing and at 8 h later. ANOVA revealed a significant difference in PER-ir **across time** in the Control ($F_{5,18} = 142.439$, $P < 0.001$), Nursing ZT03 ($F_{5,18} = 20.452$, $P < 0.001$) and Nursing ZT19 ($F_{5,18} = 22.918$, $P < 0.001$) groups. For the Control group, the maximal value at ZT07 was significantly different from the remaining time point values ($P < 0.001$ in all cases). For the Nursing ZT03 group, the maximal values at ZT11 and ZT15 were significantly different from values at ZT03, ZT07, ZT19 and ZT23 ($P < 0.001$ in all cases). For the Nursing ZT19 group, the highest value at ZT03 was significantly different from the remaining time point values ($P < 0.001$ in all cases; Fig. 4B). Cosinor analysis revealed significant rhythmicity of PER1-ir in Nursing ZT03 (amplitude 85.0; acrophase 17.0h; $P < 0.05$) and Nursing ZT19 (amplitude 69.0; acrophase 9.0h; $P < 0.01$) groups, but not in Control group (amplitude 40.0; acrophase 9.0h; $P > 0.05$; Fig. 4C).

PER1-ir in the BNST

The BNST showed no variation in the number of PER1-ir cells in any group. ANOVA revealed no significant difference in PER1-ir **across time** in the Control ($F_{5,18} = 1.051$, $P = 0.419$), Nursing ZT03 ($F_{5,18} = 1.966$, $P = 0.133$) **or** Nursing ZT19 ($F_{5,18} = 0.628$, $P = 0.680$) groups. Cosinor analysis indicated no significant rhythmicity of PER1-ir in Control (amplitude 1.6; acrophase 21.40; $P > 0.05$), Nursing ZT03 (amplitude -6.5; acrophase 26.4; $P > 0.05$) and Nursing ZT19 (amplitude -8.5; acrophase 26.04; $P > 0.05$) groups.

PER1-ir in nursing deprived does

Figure 5 shows the number of PER1-ir cells 8 h after nursing compared to 8 h after the time of expected nursing in nursing-deprived does for 24 h and 48 h in POA, LSD and LSV. In the POA, quantitative analysis revealed a decrease of PER1-ir in Nursing-Dep 48h group in comparison to corresponding nursing group ($P < 0.01$ in both cases; Fig. 5A). In LSD (Fig. 5B) and LSV (Fig. 5C) a significant decrease was identified in Nursing-Dep 24h ($P < 0.01$, in both cases) and Nursing-Dep 48 h ($P < 0.01$; in both cases) groups in comparison to corresponding nursed does.

Discussion

The results of this study confirm our hypothesis that timed nursing is associated with **the** rhythmic activity of areas in the forebrain critical for the expression of maternal behavior. Specifically we found that the PER1 protein rhythm shifts in parallel to **the** timing of

nursing in the POA, and in the dorsal and ventral portion of LS, but not **in** the intermediate portion of this structure and the BNST.

The medial preoptic area in the rostral hypothalamus is critical for MB regulation in the rat and other species. In rodents, implants of estradiol, prolactin and placental lactogens in this area stimulate the onset of MB. Conversely, electrical lesions in the POA disrupt established MB or its onset at parturition but stimulation in this area induces the display of MB in sensitized virgin females (**reviewed** in Numan & Insel, 2003). In female rabbits treated with progesterone, estradiol implants in the POA elicited digging, and following progesterone withdrawal, straw carrying, two of the three components of maternal nest-building in this species (González-Mariscal *et al.,* 2005). Moreover, after parturition, there is a significant three- to four-fold increase in the number of FOS expressing cells in the POA, including the periventricular preoptic region, in lactating rabbit does that express MB in comparison to virgins (González-Mariscal *et al.,* 2009). Together, this evidence confirms the importance of POA for the establishment and expression of MB in the rabbit. In agreement, present results provide evidence that the POA can play an important role in the regulation of **periodic rhythm of** MB in this species as the rhythm of PER1 shifts in parallel to the timing of nursing and no rhythmic expression was observed in control animals. In this regard, the rhythmic activity of the POA is particularly interesting in light of the rhythm in body temperature that **occurs** during nursing. In pregnant rabbits, **body temperature displays** a rhythm that **free runs** with a period length greater than 24 h. But after parturition this rhythm is entrained by timing of scheduled nursing and the temperature peak coincides with the phase of nursing (Jilge *et al.,* 2001). **The** POA contains temperature sensitive neurons, **specifically located** in the periventricular and medial POA, and together with other regions of the forebrain is considered the thermoregulatory center **of** the brain (Nakamura, 2011). We consider that this is an important point as perhaps temperature sensitive neurons in POA are important for the control of the **daily** rhythm of nursing in this species, and **this possibility could be** explored in future experiments.

Furthermore, our results in the POA are important as this area, **together with mesolimbic circuitry** that involves the ventral tegmental area and nucleus accumbens, promotes the activation or inhibition of brain pathways involved in maternal responses (Numan & Woodside, 2010). **Thus the** present results open the opportunity to explore the possible synchronization of this proposed pathway, as **the** POA **may participate** together with other structures as part of a multioscillatory mechanism to control the **regulation of daily** nursing. In this regard, it is interesting that the rhythm of locomotor behavior of lactating does shifts in parallel to timing of nursing, **regardless of** whether this is scheduled during the day or the night (Meza *et al.,* 2008). Furthermore, females show an increase in locomotor behavior in the hours preceding nursing time (Meza *et al.,* 2008). This behavioural pattern is similar to **that** observed in rodents under a food restriction schedule and **which is** known as food anticipatory activity (FAA; Mistlberger, 1994). **FAA is** present in rabbit pups (Caba *et al*., 2008) **and it has** been suggested that FAA is elicited by a multi-oscillatory system in which several central and peripheral structures interact (Mistlberger, 2011). **In considering the** present results **with respect to the** POA and LS (see below), we propose that **these structures** are part of an "entrainable maternal circuit" that supports regulation of daily

maternal behavior in this species. Future studies are warranted to determine **the identity and timing** of hormonal factors during pregnancy or lactation **that may be** responsible for the establishment of this PER1 rhythm. Also, it will be important to determine the neurochemical phenotype of the PER1 expressing cells in order to get a better idea about neurotransmitters and/or peptides involved in the control of **daily** maternal behavior in this species.

The lateral septum is a complex structure in the telencephalon involved in the integration of several physiological and behavioral process related to higher cognitive functions, such as learning and memory, emotion, fear, aggression, and stress, among others (Jakab & Leranth, 1995). Additionally, this structure **has** been **linked** to the control of complex behaviors **such as** maternal behavior in rodents (Numan & Insel, 2003). Lesions of the septum disrupt MB, including deficiency of lactation and cannibalism, in mice (Carlson *et al.,* 1968) and rats (Slotnick & Nigrosh, 1975). In female rabbits with lesions of the septum, pregnancy and parturition is normal, but females show marked disturbances in nest building and after parturition, lack of care of the young, unwillingness to nurse the litters and cannibalism (Cruz & Beyer, 1972). Together this evidence suggests that lesions of the septum affect both the neural control of this complex behavior and perhaps also the neuroendocrine system responsible for lactation. However, in a careful study of postpartum females with lesions in the septum, Fleischer and Slotnick (1978) demonstrated in the rat that failure to nurse their pups is not due to an interruption of neuroendocrine pathways necessary for lactation or milk ejection reflex. Nevertheless the septum massively projects to the limbic system, particularly the hippocampal formation, as well as to the diencephalon where several hypothalamic regions and nuclei such as the preoptic area are their main targets. **In addition**, many regions of the brain send projections to the LS, one of the heaviest **being** from the lateral preoptic area (Jakab & Leranth, 1995). Then, although is possible that the deficits in MB are due to the interruption of neural pathways to other areas related to the control of MB, **such** as those with the POA, it is possible that cells in the **LS** are indeed involved in the regulation of MB. The reason for this assumption comes from two sources. First, in lactating female rats expressing MB, there is a significant increase of FOS expressing cells in the LS, even when they are exposed to pups without suckling (Lonstein *et al.,* 1998; Stack & Numan, 2000). In rabbits, the brief interaction with the pups for **only one** daily nursing episode on the day after parturition induces a two-fold increase in the number of FOS expressing cells in LS compared to lactating females **that are** not given pups for suckling on the corresponding day, or to virgin females (González-Mariscal *et al.*, 2009). The second source of evidence comes from the present results. Nursing induces a clear rhythm of PER1 in the LSD and LSV that shifts in parallel to the timing of nursing either during the day or the night in comparison to non-lactating, non-pregnant females. Future experiments using excitotoxic axon-sparing lesions are warranted to investigate the specific importance of LS cells in MB (Numan *et al.,* 1988). It is very important to take into account the massive afferent and efferent projections of the septum, which make it difficult to interpret the complex MB disturbances **by lesions**.

The area of the BNST analyzed, the vBST, lies dorsolateral to the mPOA, and shows activation, as indicated by FOS, during the expression of MB in the rat (Numan & Numan,

1994). The vBST, together with mPOA, are considered to form a functional system important for maternal behavior in rats (Numan & Insel, 2003). However, vBST lesions made by excitotoxic amino acids disrupt mainly retrieval behavior, and, in general, the deficit **in** MB is not as severe as that **seen after lesions of the** POA (Numan & Numan, 1996). Perhaps due to **its** importance in retrieval behavior, this region seems to be not activated in lactating rabbits (Gonzalez-Mariscal *et al.,* 2009) **and does** not show a rhythm related to timing of nursing (present results) as the rabbit doe does not retrieve their pups during their only visit for nursing.

It is important to emphasize that the **rhythms observed** in the LS and POA **are driven rhythms as they depend** on suckling stimuli. **As shown,** skipping just one suckling bout is sufficient to significantly decrease the number of PER1 expressing cells in the LS. Missing two suckling bouts induces a further 3-4 fold decrease in comparison to animals that nursed. In contrast, the decrease of PER1 by nursing deprivation in the POA is less severe but **is** already significant after the missing of two suckling bouts. This PER1 decrease was also observed in neuroendocrine cells in the lactating doe (Meza et al., 2008; 2011) and confirms that suckling is a powerful stimuli that induce rhythmic activity in the female rabbit brain. Additionally, our results confirm that specific stimuli, other than light, are capable of inducing rhythms of clock genes and their protein products in brain regions outside the suprachiasmatic nucleus (Guilding & Piggins, 2007; Kriegsfeld & Silver, 2006).

We conclude that **daily** suckling of pups is a powerful stimulus that induces **driven rhythms** of PER1 in brain areas **relevant for** expression of **daily** maternal behavior in the rabbit, which perhaps are part of an entrainable maternal circuit.

Acknowledgements

Abbreviations

BNST	bed nuclei of stria terminalis
DAB	3,3 diaminobenzidine
FAA	food anticipatory activity
ir	immunoreactive or immunoreactivity
LS	lateral septum
LSD	lateral septum dorsal
LSI	lateral septum intermediate
LSV	lateral septum ventral
LD	light–dark

MB	maternal behavior
OT	oxytocin
PB	phosphate buffer
PER1	Period1 (protein)
PP	postpartum
POA	preoptic area
ZT	zeitgeber time

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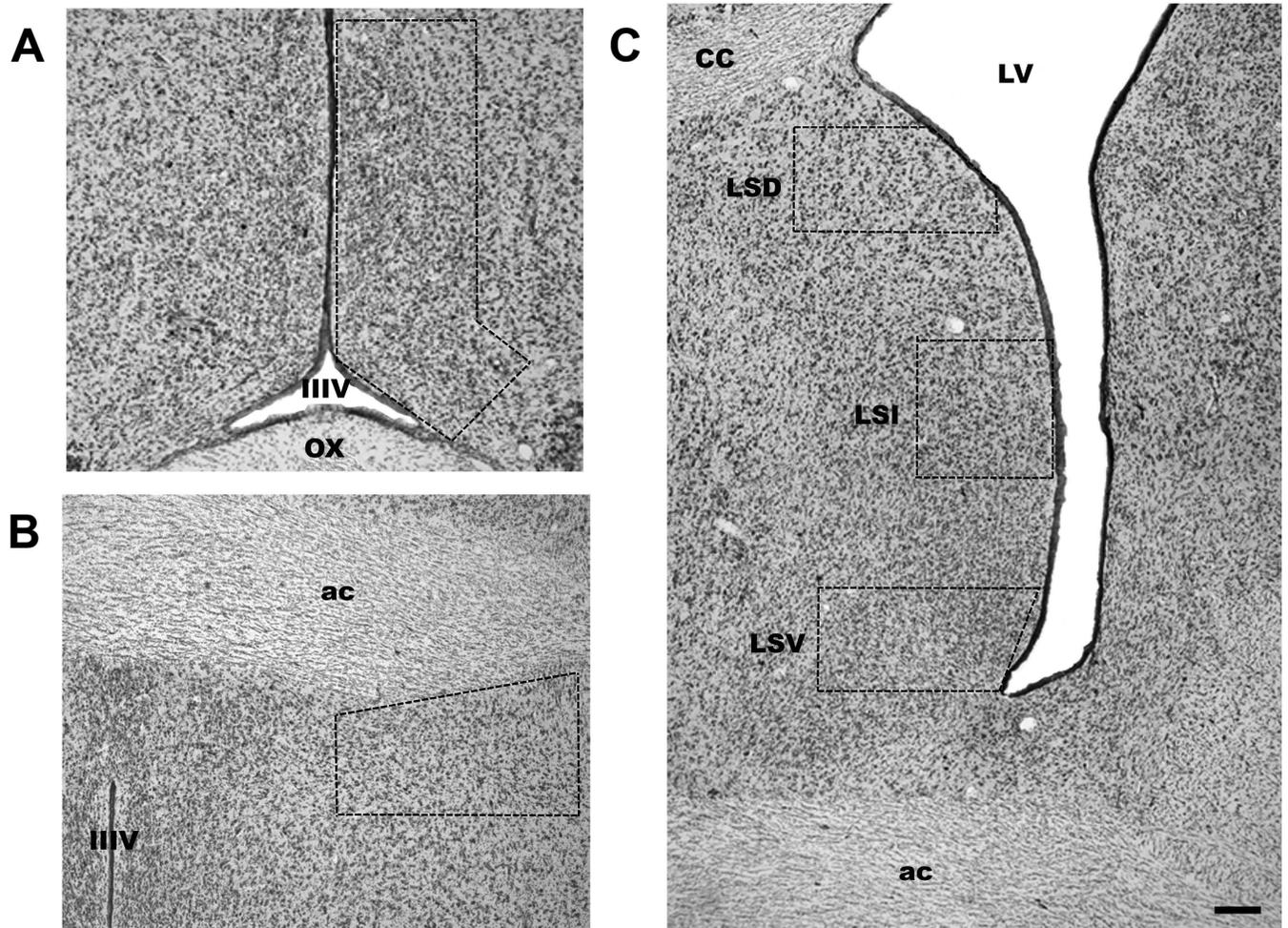
Figure 1.
Photomicrographs (Thionine stain) showing **the location of** the POA (A), **BNST (B)** and the LS **(C).** Dotted lines delimit the counted area. LSD = lateral septum dorsal; LSI = lateral septum intermediate; LSV = lateral septum ventral. IIIV = third ventricle; OX = optic chiasma; LV = lateral ventricle; CC = corpus callosum; ac = anterior commissure. Scale bar 50 μm.
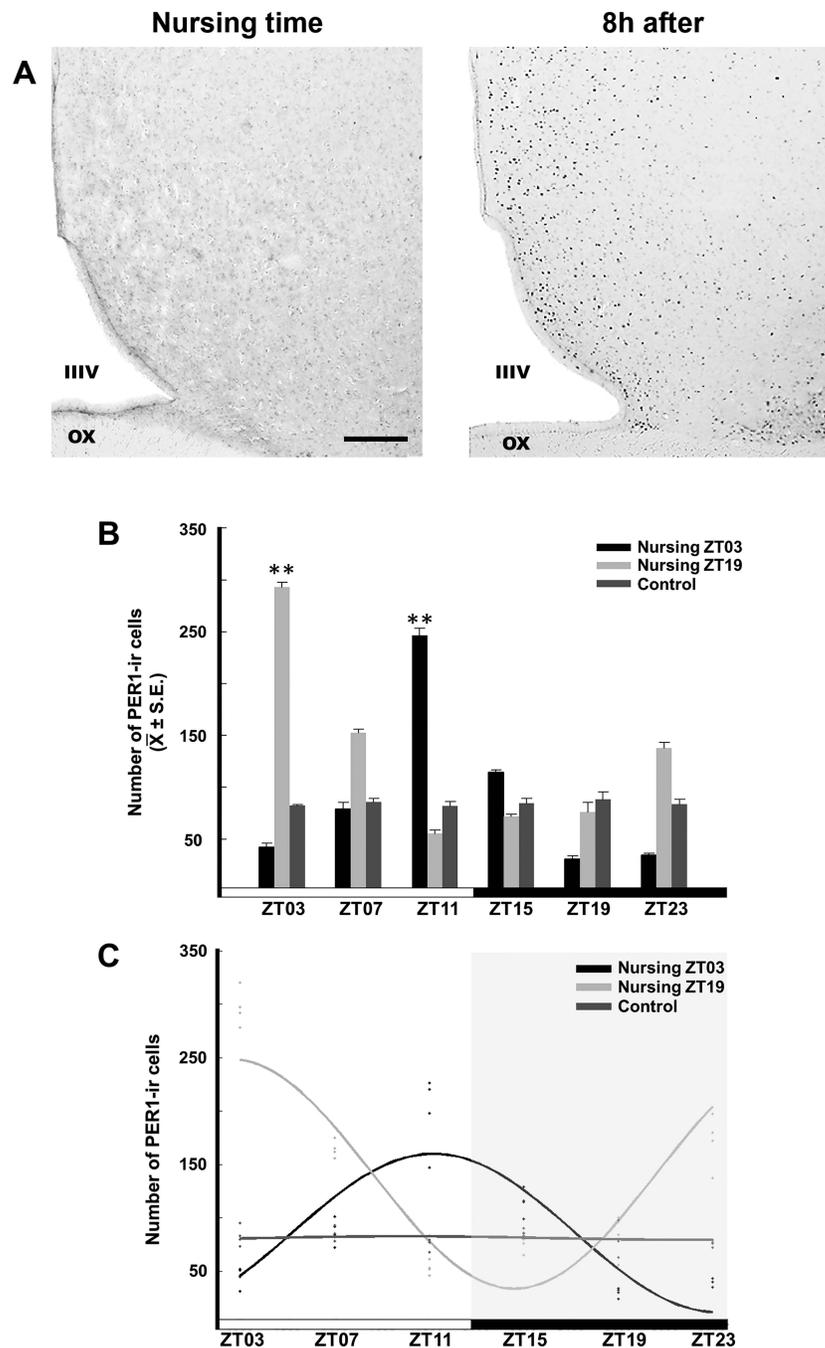
Figure 2.
Expression of PER1 in the POA. (A) Photomicrographs of representative sections at nursing time and at 8h after nursing in the Nursing ZT03 group. Note the density of PER1-ir cells 8h after nursing. IIIV, third ventricle, OX, optic chiasma. Scale bar 100 μm. (B) PER1-ir cells in Control, Nursing ZT03 and Nursing ZT19 groups; ** Denotes significant difference between higher and lower values within the same group. ** $P < 0.001$. Values are mean ± SEM. The black and white bar at bottom represents the LD condition. (C) Cosinor analysis

indicates a significant rhythmicity of PER1-ir cells in Nursing ZT03 and Nursing ZT19, but not in Control group. See text for details.
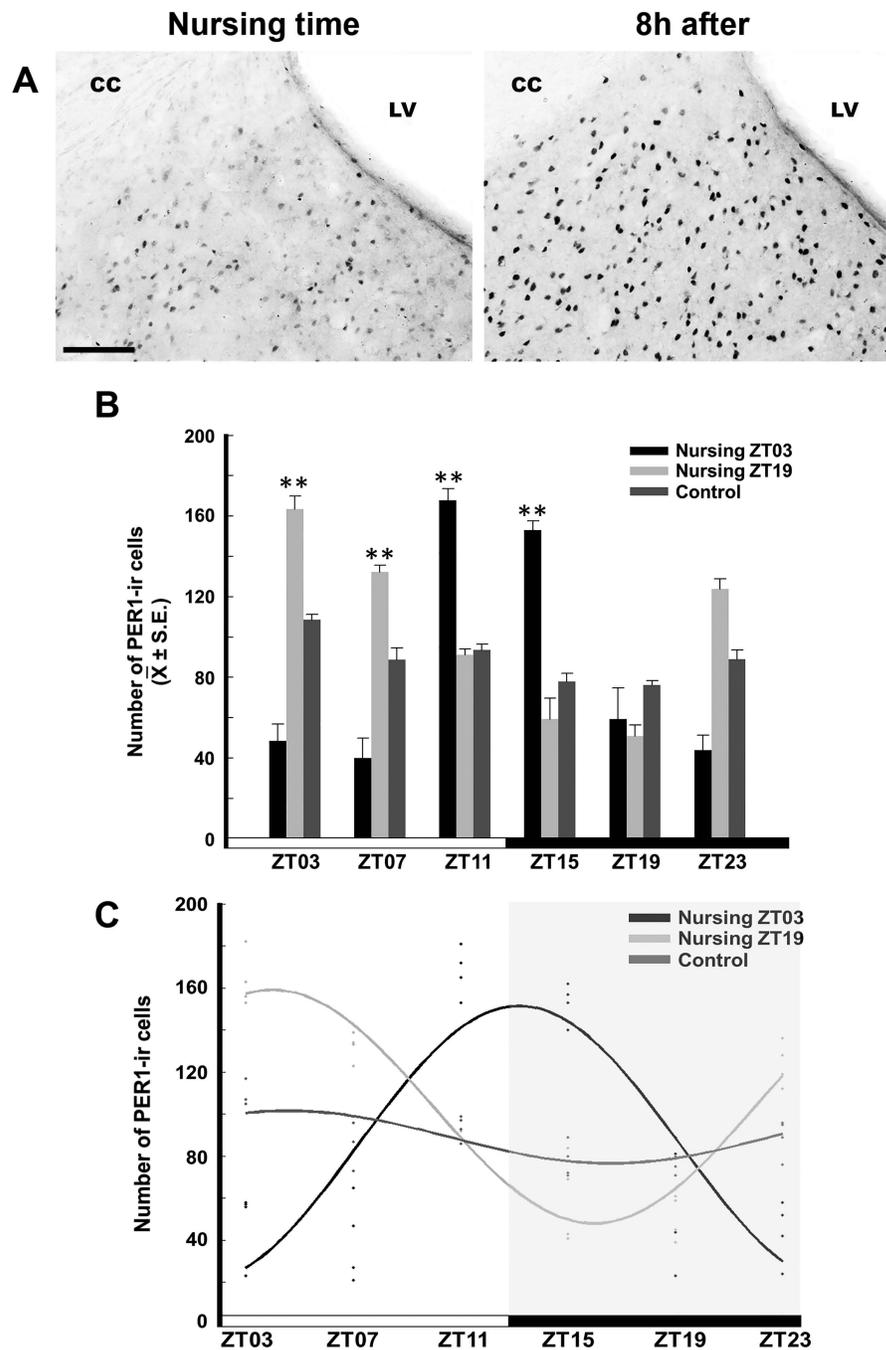
Figure 3.
Expression of PER1 in the LSD. (A) Photomicrographs of representative sections at nursing time and at 8h after nursing in the Nursing ZT03 group. Note the density of PER1-ir cells 8h after nursing. LV, lateral ventricle, CC, corpus callosum. Scale bar 100 μm. (B) PER1-ir cells in Control, Nursing ZT03 and Nursing ZT19 groups; ** Denotes significant difference between higher and lower values within the same group.. ** $P < 0.001$. Values are mean ± SEM. The black and white bar at bottom represents the LD condition. (C) Cosinor analysis

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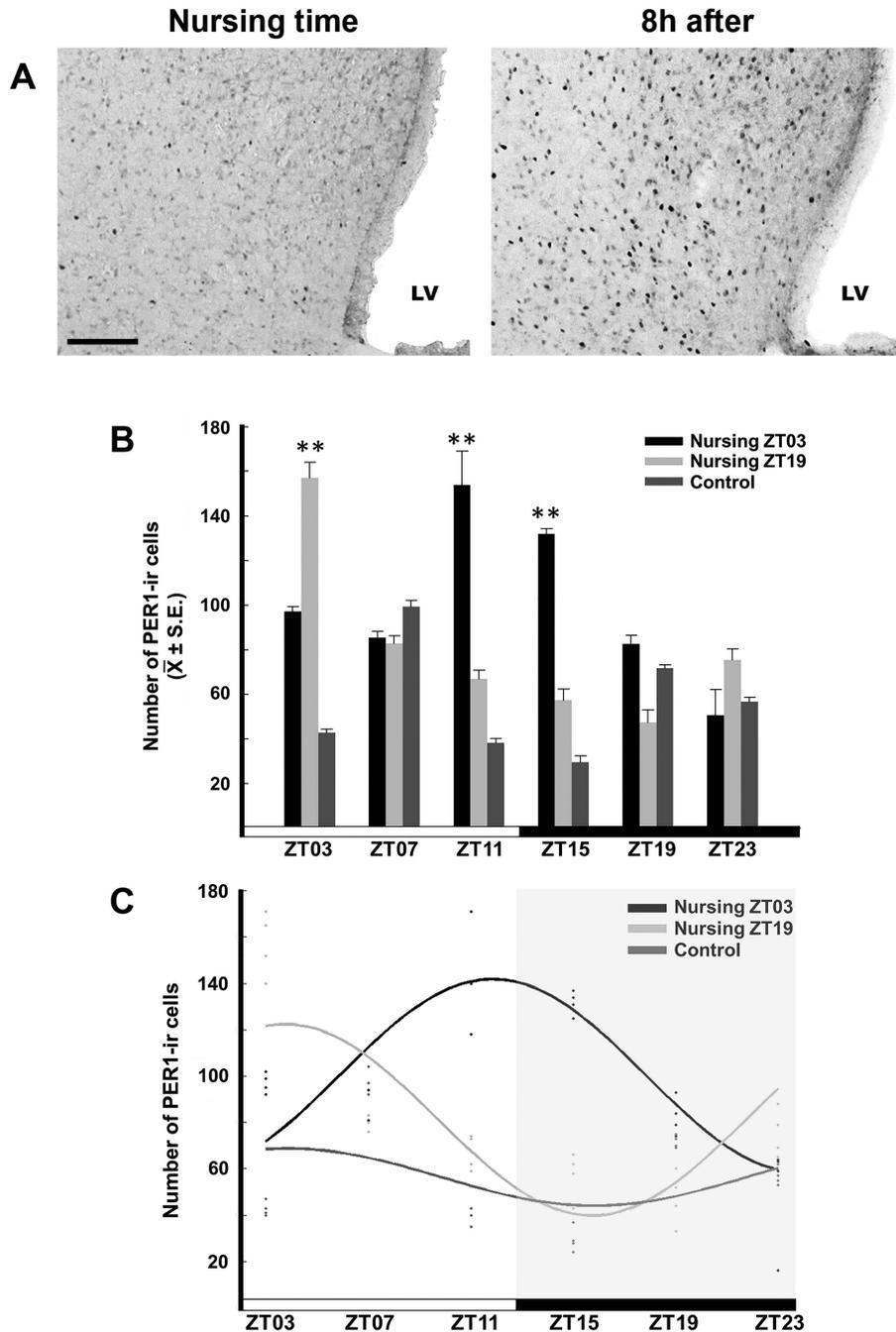
Figure 4.
Expression of PER1 in the LSV. (A) Photomicrographs of representative sections at nursing time and at 8h after nursing in the Nursing ZT03 group. Note the density of PER1-ir cells 8h after nursing. LV, lateral ventricle. Scale bar 100 μm. (B) PER1-ir cells in Control, Nursing ZT03 and Nursing ZT19 groups; ** $P < 0.001$ Denotes significant difference between higher and lower values within the same group. ** $P < 0.001$. Values are mean ± SEM. The black and white bar at botton represents the LD condition. (C) Cosinor analysis indicates a

significant rhythmicity of PER1-ir cells in Nursing ZT03 and Nursing ZT19, but not in Control group. See text for details.
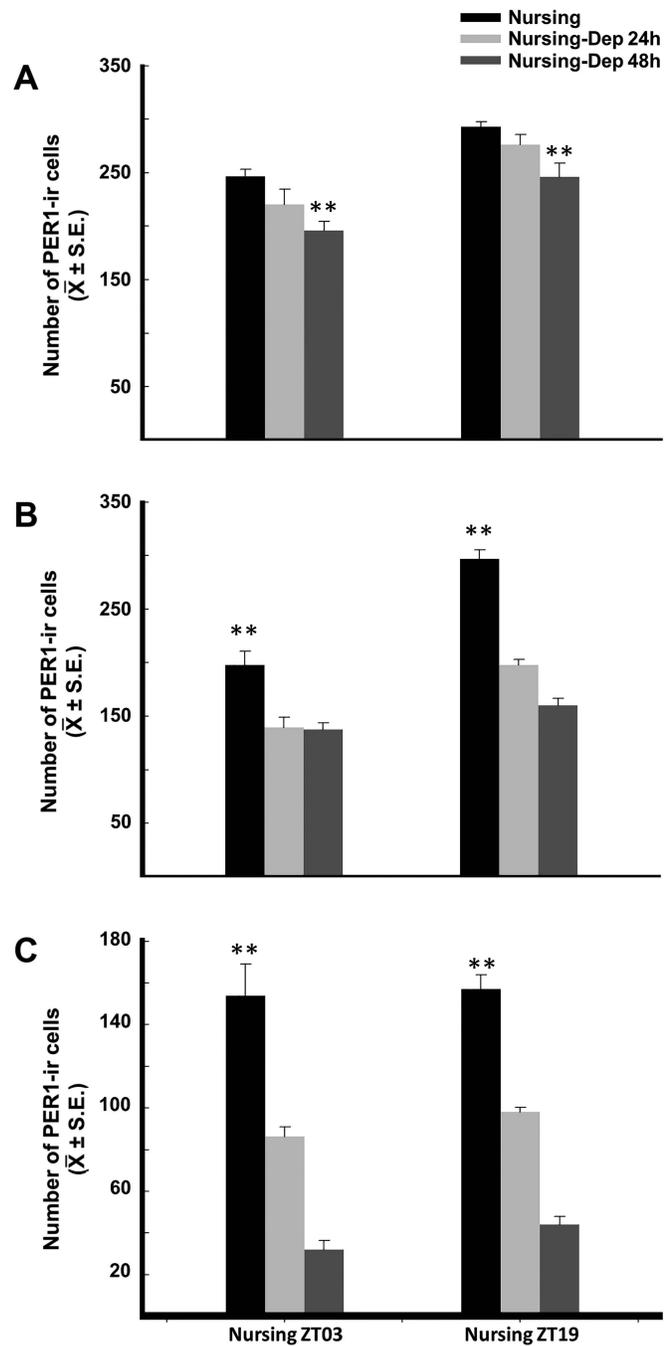
Figure 5.
Expression of PER1-ir cells in nursed does 8 h after nursing in comparison to nursed deprived females for 24 and 48 h, 8 h after expected nursing time in POA (A), LSD (B) and LSV (C). **Denotes significant differences between higher and lower values. See text for details. ** $P < 0.001$. Values are mean ± SEM.