Central Clock Regulates the Cervically Stimulated Prolactin Surges by Modulation of Dopamine and Vasoactive Intestinal Polypeptide Release in Ovariectomized Rats

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Key Words

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Abstract

Background/Aims: Cervical stimulation induces a circadian rhythm of prolactin secretion and antiphase dopamine release. The suprachiasmatic nucleus (SCN) controls this rhythm, and we propose that it does so through clock gene expression within the SCN. **Methods:** To test this hypothesis, serial blood samples were taken from animals injected with an antisense deoxyoligonucleotide cocktail for clock genes (generated against the 5' transcription start site and 3' cap site of *per1*, *per2*, and *clock* mRNA) or with a random-sequence deoxyoligonucleotide in the SCN. To determine whether disruption of clock genes in the SCN compromises the neural mechanism controlling prolactin secretion, we sacrificed another group of rats (under the same treatments) at 12.00 or 17.00 h. Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using HPLC/electrochemical detection in the median eminence as well as the intermediate and the neural lobe of the pituitary gland, and the DOPAC:dopamine ratio was used as an index of dopamine activity. Vasoactive intestinal polypeptide (VIP) content was determined in tissue punches of the SCN and paraventricu-

lar nucleus (PVN), an SCN efferent. **Results:** Treatment with clock gene antisense deoxyoligonucleotide cocktail abolished both the diurnal and nocturnal prolactin surges induced by cervical stimulation. This treatment abolished the antiphase relationship established by cervical stimulation between dopamine neuronal activity and prolactin secretion. Also, VIP content increased in the SCN and decreased in the PVN. **Conclusion:** These results suggest that the SCN clock determines the circadian rhythm of prolactin secretion in cervically stimulated rats by regulating dopamine neuronal activity and VIP inputs to the PVN.

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Introduction

In rodents, cervical stimulation induces twice-daily surges of prolactin (PRL) which can be reproduced, in the presence or absence of ovaries, by infertile mating or artificial stimulation of the uterine cervix. This characteristic cervical stimulation-induced PRL secretion is observed for several days without reapplication of the stimulus and independent of the time of day at which the stimulus is applied [for references, see 1]. When animals are placed in a 12-hour light and 12-hour dark environment with lights on at 06.00 h, the nocturnal PRL surge

occurs at approximately 03.00 h and the diurnal surge at 17.00 h [2]. Alternating photoperiods entrain these surges and they free-run in either constant light or constant darkness [3], showing that this secretory PRL pattern is an endogenous circadian rhythm.

In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus is the central clock controlling circadian rhythms. Lesions of the SCN cause a loss of the circadian rhythms of both the central nervous system and peripheral tissues, and transplants of the SCN into SCN-lesioned animals restore some of these rhythms [4–6]. A transcriptional-translational network driven by interactions between positive and negative feedback loops of the activators CLOCK and BMAL and the repressors PERIOD (PER1-2), CRYPTOCHROME (CRY 1-2), and REV-erba have been proposed to generate circadian rhythmicity in the SCN. This loop is considered the core molecular mechanism by which the SCN synchronizes (or determines) circadian rhythm [7]. Disruption of the molecular clock mechanism leads to impairment of several circadian rhythms, such as locomotor behavior and the secretion of corticosterone [8–12]. We have shown that an injection of deoxyoligonucleotide antisense sequence of *rPer 1*, *rPer 2*, and *rclock* (which promotes an acute and transitory knockdown of these genes and their respective protein expression within the SCN) disrupts the preovulatory surge of PRL as well as the PRL surge induced by estradiol in ovariectomized (OVX) rats [13]. This treatment also disrupts the circadian rhythm of drinking behavior, the circadian release of corticosterone, and CLOCK expression [13, 14]. A great deal of evidence indicates that the SCN controls PRL secretion during mating. SCN lesions abolish both the mating- and cervical stimulation-induced PRL surges [15–18], but how the core molecular mechanism within the SCN is involved with the circadian rhythm of cervical stimulation-induced PRL secretion is not known.

A definite increase in PRL secretion occurs following its release from inhibitory hypothalamic dopaminergic tonus. Once PRL has been secreted, it feeds back on the hypothalamus to stimulate synthesis [19] and turnover [20, 21] of dopamine (DA), as well as to promote the release of DA into pituitary portal blood [22, 23]. This short-loop feedback has been proposed to be the gear for the PRL surges observed several days after cervical stimulation in OVX rats. In fact, cervical stimulation induces an antiphase relationship between PRL surges and DA tonus [24]. DA reaches the lactotrophs in the anterior lobe of the pituitary gland from each of 3 hypothalamic dopaminergic systems: the tuberoinfundibular (TIDA), tuber-

ohypophyseal dopaminergic (THDA), and periventricular hypothalamic dopaminergic (PHDA) systems [25]. TIDA neurons arise from the dorsomedial arcuate nucleus, and their axons terminate in the capillary bed of the external zone of the median eminence (ME), where DA diffuses and is transported to the anterior pituitary via the long portal vessels. THDA neurons arise from the rostral arcuate nucleus, and their axons terminate on short portal vessels in the neural lobe (NL) and intermediate lobe (IL) of the pituitary. PHDA neurons arise from the periventricular nucleus, and their axons terminate exclusively on short portal vessels in the IL [26].

In general, the SCN is divided into at least 2 subdivisions, a ventrolateral (core) and dorsomedial (shell) region. The ventrolateral portion of the SCN harbors the majority of vasoactive intestinal polypeptide (VIP) neurons, whereas the dorsomedial region contains vasopressin-producing neurons [27, 28]. Because antagonism of VIP expression within the SCN abolishes the cervical stimulation-induced PRL surges in OVX rats [29], we propose that the VIP projections from the SCN are responsible for setting the time (or phase) of the cervical stimulation-induced surges of PRL. DA-producing cells that project to the ME receive VIP inputs from the SCN [30, 31], and antagonism of VIP disrupts the DA circadian rhythm activity observed in OVX rats [32]. Another target of VIP input from the SCN is the paraventricular nucleus (PVN) of the hypothalamus [33], which is also involved in the cervical stimulation-induced secretion of PRL; this nucleus has VIP receptors [34], and the phase of VIP activity in the PVN throughout the day mirrors the secretory pattern of PRL secretion in cervically stimulated OVX rats [35].

We therefore hypothesize that clock genes are essential for VIP expression within the SCN. Disruption of VIP in SCN neurons would disrupt the ability of the SCN to communicate photoperiodic cues to target regions such as DA-producing neurons and the PVN. In the present study, we tested these hypotheses by transient interruption of the expression of *per1*, *per2*, and *clock* in the SCN and then evaluated the effects on: (1) the PRL secretory profile induced by cervical stimulation, (2) DA neuronal activity, and (3) VIP concentration in the SCN and in the PVN.

Materials and Methods

Animals

Adult female Sprague-Dawley rats weighing 250–300 g (Charles River Labs, Wilmington, Mass., USA) were housed under a standard 12:12 light:dark cycle with lights on at 06.00 h

and constant temperature (25°C) and humidity. Standard rat chow and water were available ad libitum. All experimental protocols were approved by the Florida State University Animal Care and Use Committee. Animals were bilaterally ovariectomized under isoflurane vapor and submitted to the experimental design described below.

Experiment 1: Effect of Acute Knockdown of Clock Genes on Cervical Stimulation-Induced PRL Surges

Seven to 10 days after ovariectomy, guide cannulae were implanted bilaterally within the dorsal border of the SCN. After 5 days, each animal was cervically stimulated at 17.00 h.

On the following day, animals were cervically stimulated again at 09.00 h. A cannula was then implanted in the jugular vein from 17.00 to 18.00 h (1 h before lights off). At 18.00 h (Zeitgeber, ZT, 12) animals received bilateral injections into the SCN of an antisense deoxyoligonucleotide cocktail (AS-ODN) of *clock*, *per1* and *per2* genes or of random-sequence deoxyoligonucleotides (RS-ODN). We have previously shown that this treatment disrupts circadian drinking behavior for 72 h [13, 14], suggesting that it compromises the feedback loop of clock gene expression, and consequently SCN rhythmic function for this period of time. On the following day, blood samples were taken at 13.00 h and every 2 h afterward until 05.00 h for measurement of plasma PRL by radioimmunoassay (RIA). At the end of the experiment, the animals were sacrificed, and their brains were frozen on dry ice for sectioning in a cryostat for verification of the SCN cannula placement. Rats in which the guide cannulae were positioned directly above the SCN were considered to have received effective AS-ODN or RS-ODN treatments.

Experiment 2: Effect of Acute Knockdown of Clock Genes in the SCN on the Neuronal DA Activity Induced by Cervical Stimulation in OVX Rats

Animals were treated as for experiment 1, but did not receive jugular cannulae. On the day after injections of AS-ODN or RS-ODN, they were sacrificed at 12.00 and 17.00 h, and the ME, IL, and NL were rapidly dissected for DOPAC and DA measurements by HPLC/electrochemical detection. These times were selected because we have previously shown that DAergic neurons are more active at 12.00 h and relatively inactive at 03.00 and 17.00 h [24, 36]. Trunk blood was collected in these animals for PRL measurements by RIA.

Experiment 3: Effect of Acute Knockdown of Clock Genes on VIP Content of Punches of Both SCN and PVN

Brains from each animal used in experiment 2 were frozen on dry ice for VIP measurements in punches of both the SCN and the PVN.

General Methods

Cervical Stimulation. The uterine cervix was stimulated with an electrode constructed from a Teflon rod (diameter 5 mm) with 2 platinum wires protruding from the tip. The times chosen for stimulation mimicked normal mating on proestrus evening and the morning of estrus. Stimulations were applied as 2 consecutive trains of electric current of 10-second durations (rectangular pulses 1 ms of 25 V at 200 Hz). This procedure has been shown to yield the highest success rate in initiating the twice-daily PRL surges that are characteristic of mated rats [37].

Bilateral Cannula Implantation into the SCN. The animals were anesthetized (100 μ l/100 g weight) with a ketamine (49 mg/ml)/xylazine (1.8 mg/ml) cocktail, and implanted stereotactically with bilateral stainless steel guide tubes (1.5 mm apart, 9.5 mm in length, 27 gauge) whose tips were placed at the dorsal border of the SCN (0.8 mm posterior to bregma, 7.9 mm ventral to the dorsal surface of the dura mater). Bilateral 33-gauge solid steel mandrills were placed inside the guide tubes, and dust caps were used to secure the apparatus.

Jugular Cannulation and Blood Samples. Animals were anesthetized with isoflurane (Butler, Dublin, Ohio, USA), and sterile Micro-Renathane® tubing (OD = 0.040, ID = 0.025, MRE-025; Braintree Scientific, Braintree, Mass., USA) was inserted into the right jugular vein. The tubing, filled with heparinized saline (30 U/ml), was fitted subcutaneously and exteriorized at the back of the animal's neck. A daily flush with sterile-heparinized saline kept the line patent until the beginning of blood collection. Blood samples of 200 μ l were drawn into plastic heparinized syringes. After removal of each blood sample, the same volume of sterile saline was injected through the catheter to replace the volume of blood removed. Plasma was separated by centrifugation at 1,200 g for 15 min at 4°C and was stored at -20°C until assayed for PRL.

SCN Injection of Deoxyoligonucleotides. Animals were anesthetized with isoflurane, and the 33-gauge bilateral mandrills were removed. Bilateral internal cannulae (33 gauge, 10.5 mm total length, 1 mm extension beyond the guide tube) were inserted into the guide tubes, and 800 nl of AS-ODN or RS-ODN (2.5 mg/ml) was injected at 200 nl/min with two 10- μ l Hamilton syringes attached to an automated microinfusion pump (KD Scientific, Fisher Scientific, Fair Lawn, N.J., USA). As noted previously [14], AS-ODNs were generated against the 5' transcription start site (5'INI) and 3' cap site of *per1*, *per2*, and *clock* mRNA. Control animals were injected with RS-ODNs, which had the same nucleotide content (% AGCT) as the AS-ODN but were not complementary to clock gene mRNA sequences. As demonstrated previously in our laboratory, this treatment leads to a knockdown of PER1, PER2, and CLOCK expression in the SCN by more than 60% within 6 h of injection and to a decrease in the number of CLOCK-positive neurons in the SCN at 08.00 and 16.00 h [13, 14]. We have also previously reported that this treatment disrupts the circadian drinking behavior of OVX rats [14].

Tissue Preparation. Rats were sacrificed at 12.00 h and 17.00 h. The ME, IL, and NL were separated under a stereomicroscope and stored in vials with 200 μ l of homogenization buffer (0.15 N perchloric acid, 50 μ M EGTA, 13.6 nM dihydroxybenzylamine) at -80°C until the day of assay. The brains of these animals were also removed after decapitation for micropunches of the SCN and PVN regions. Seven consecutive slices of 300- μ m thickness were obtained with a cryostat and were placed on Superfrost/Plus pre-cleaned microscope slides (Fisherbrand, Fisher Scientific, Waltham, Mass., USA) on dry ice. From the first 3 slices, bilateral punches of the SCN were taken under a stereomicroscope, with a micropunch needle (Electron Microscopy Sciences, Hatfield, Pa., USA) attached to a tip with inside diameter of 500 μ m. In the third slice and in the 3 following ones, bilateral punches of the PVN were taken, also under a stereomicroscope, with a micropunch needle of the same size. Prior to and following the micropunch procedure, slices were evaluated under a 5 $\times$ microscope objective for verification of guide-cannula placement and that only SCN

Fig. 1. Effects of PER1, PER2, and CLOCK expression knockdown in the SCN on the profile of plasma PRL secretion induced by cervical stimulation in OVX rats. A cocktail of either AS-ODN or RS-ODN for clock genes was injected into the SCN, and blood samples were taken the following day. ^a $p < 0.05$, RS-ODN (17.00 and 03.00 h) vs. RS-ODN (other times) (one-way ANOVA followed by Bonferroni's test); ^b $p < 0.001$, AS-ODN vs. RS-ODN (2-way ANOVA followed by Bonferroni's test). There were no differences among times within AS-ODN.

and PVN tissues were punched. The 3 punches obtained from the SCN and the 4 from the PVN were separately pooled in 500- μ l tubes and stored at -80°C until the day of the assay.

Radioimmunoassay. Plasma concentrations of PRL were determined by RIA with specific kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases. The antiserum for PRL was anti-rat PRL-S9, and the reference preparation was PRL-RP3. To prevent interassay variation, we assayed all samples in the same RIA. The lower limit of detection for PRL was 0.10 ng/ml. The intraassay coefficient of variation was 5%. VIP contents in the SCN and PVN were determined with a kit (Phoenix Pharmaceuticals, Burlingame, Calif., USA). Protein was extracted from the punches by the addition of 0.1 N HCl followed by placement in a boiling water bath for 10 min. After 45 min on ice, samples were homogenized, and 25 μ l of each sample was taken for protein-content determination with the DC Protein Assay Kit (Bio-Rad, Hercules, Calif., USA). The remaining homogenates were centrifuged at 5,200 g. Supernatants were removed and lyophilized in a Modulyo D lyophilizer (Thermo Scientific, Waltham, Mass., USA) and reconstituted in RIA buffer. The RIA kit was validated before use with tissue extracts. Data were expressed as picograms of VIP per milligram of protein.

DA and DOPAC Measurement by HPLC Coupled to Electrochemical Detection. Samples containing ME, IL, or NL were thawed, homogenized, and sonicated. The homogenate was filtered through a 0.2-mm nylon microfiltration unit (Osmonics, Livermore, Calif., USA) and then placed in autosampler vials. The concentration of DA and 3,4-dihydroxyphenylacetic acid (DOPAC) was measured by HPLC coupled to electrochemical detection. Twenty microliters of each sample were injected by an autosampler (Model 542 Autosampler; ESA, Chelmsford, Mass., USA). The mobile phase, consisting of 75 mM sodium dihydrogen

phosphate monohydrate (EM Science, Gibbstown, N.J., USA), 1.7 mM 1-octane sulfonic acid (Fisher Scientific), 100 ml/l triethylamine (Aldrich, Milwaukee, Wisc., USA), 25 μ M EDTA (Fisher Scientific), 6% acetonitrile (EM Science), titrated to pH 3.0 with phosphoric acid (Fisher Scientific), was delivered by a dual piston pump (LC-20AD; Shimadzu Co., Analytical & Measuring Instruments Division, Kyoto, Japan) at 600 μ l/min. Water was purified on a Milli-Q system (Millipore, Bedford, Mass., USA) to 18 M Ω resistance and polished with a Sep-Pak minicolumn (Millipore). Catecholamines were separated on a reverse phase C18 column (MD-150; Dimensions 150 $\times$ 3 mm, particle size 3 μ m, ESA), oxidized on a conditioning cell (E: +300 mV, ESA 5010 Conditioning Cell, ESA), and then reduced on a dual-channel analytical cell (E1: -65 mV; E2: -225 mV, ESA 5011 High Sensitivity Analytical Cell, ESA). The change in current on the second analytical electrode was measured by a coulometric detector (ESA Coulochem II) and recorded by EZStart 7.3 SP1 (Shimadzu, Kyoto, Japan). The amount of catecholamine or internal standard, dihydroxybenzylamine, in all sample peaks was estimated by comparison with the area under each peak for known amounts of each. Recovery of dihydroxybenzylamine was used as the internal standard corrected for any loss of sample. The sensitivity of the assay was 6 pg of DA and 19 pg of DOPAC.

Statistical Analysis

Data from experiment 1 are presented as mean $\pm$ SEM plasma PRL concentrations, and statistical differences between groups were determined by 2-way ANOVA followed by Bonferroni's test. Data from experiments 2 and 3 are presented as means $\pm$ SEM of the DOPAC:DA ratio, VIP content, and plasma PRL levels of animals sacrificed at 12.00 h or 17.00 h from AS-ODN-injected and RS-ODN-injected groups. Statistical differences in experiments 2

Fig. 2. Effects of knockdown of PER1, PER2, and CLOCK expression in the SCN on the DOPAC:DA ratio levels of the ME (a), IL of the pituitary (b), and NL of the pituitary (c), as well as on plasma PRL levels (d). ^a $p < 0.05$ AS-ODN vs. RS-ODN group (1-way ANOVA followed by Newman-Keuls' test); ^b $p < 0.05$, 12.00 vs. 17.00 h (1-way ANOVA followed by Newman-Keuls' test).

and 3 were determined by 1-way ANOVA followed by Newman-Keuls' test. All analyses were performed with GraphPad Prism (GraphPad Software; San Diego, Calif., USA), and $p < 0.05$ was considered statistically significant.

Results

Experiment 1: Knockdown of Clock Genes in Neurons of the SCN Abolishes Both the Diurnal and Nocturnal Surges of PRL in Cervically Stimulated OVX Rats (fig. 1)

Based on the histological analyses of the guide-canula placement, out of 10 animals, 7 animals were considered successfully AS-ODN treated; and out of 10 animals, 8 animals were considered successfully RS-ODN

treated. The injection of AS-ODN cocktail for clock genes into the SCN abolished both the diurnal ($p < 0.001$) and nocturnal ($p < 0.001$) surges of PRL, as revealed by 2-way ANOVA followed by Bonferroni's test (RS-ODN vs. AS-ODN). The AS-ODN did not affect basal PRL levels. In animals in which injections missed the SCN on one or both sides, the PRL levels were similar to those injected with RS-ODN (data not shown).

Experiment 2: Knockdown of Clock Genes in Neurons of the SCN Alters the DA Activity Profile Induced by Cervical Stimulation in OVX Rats (fig. 2)

Based on the histological analyses of the guide-canula placement, out of 10 animals, 7 animals were considered successfully AS-ODN treated at both 12.00 and

Fig. 3. Effects of knockdown of *PER1*, *PER2*, and *CLOCK* expression in the SCN on VIP content in punches of both the SCN (a) and the PVN (b). * $p < 0.05$ AS-ODN vs. RS-ODN group (1-way ANOVA followed by Newman-Keuls' test).

17.00 h; and out of 10 animals, 7 animals were considered successfully RS-ODN treated at 12.00 h, and 8 animals at 17.00 h. The DOPAC:DA ratios of animals injected with RS-ODN were higher at 12.00 h in both the NL and ME (fig. 2a, c), whereas the PRL concentration was low (fig. 2d). The injection of AS-ODN cocktail for clock genes into the SCN disrupted this relationship. DOPAC:DA ratio in the ME of animals injected with AS-ODN did not change between 12.00 and 17.00 h, nor did PRL concentration in this group (fig. 2a, c, d). In the NL of AS-ODN-treated animals, the DOPAC:DA ratio is higher at 17.00 h. Comparisons between treatment groups (RS-ODN and AS-ODN) showed that AS-ODN lowered this ratio at 12.00, but not at 17.00 h in both ME and NL. PRL levels of AS-ODN-treated animals were lower than those of RS-ODN-treated animals (fig. 2d). No differences in the DOPAC:DA ratio of the IL were found between ani-

mals injected with RS-ODN and those injected with AS-ODN (fig. 2b).

Experiment 3: After Knockdown of Clock Genes in Neurons of the SCN, VIP Accumulated in the SCN and Decreased in the PVN of Cervically Stimulated OVX Rats (fig. 3)

Based on the histological analyses of the guide-cannula placement, out of 10 animals, 7 animals were considered successfully AS-ODN treated at both 12.00 and 17.00 h; and out of 10 animals, 7 animals were considered successfully RS-ODN treated at 12.00 h, and 8 animals at 17.00 h. The injection of AS-ODN cocktail for clock genes into the SCN increased the VIP content in this nucleus (fig. 3a), whereas a decrease in the PVN (fig. 3b) was observed at both 12.00 and 17.00 h. No differences between these 2 times were found within either the RS-ODN group or the AS-ODN group.

Discussion

After mating-like cervical stimulation, 2 surges of PRL secretion are observed in OVX rats [38]. In the present study, an AS-ODN injection that decreased the expression of *per1*, *per2*, and *clock* [14] within neurons of the SCN abolished both PRL surges in cervically stimulated OVX rats. We have previously demonstrated this same effect on the PRL secretory surge induced by estradiol in OVX and proestrus rats [13]. Taken together, these data link PRL secretion observed in different stages of the reproductive cycle to the molecular clock mechanism in the SCN. The knockdown of *per1*, *per2*, and *clock* in the SCN presumably has profound effects on regions downstream from the SCN (arcuate nucleus and PVN) that control PRL secretion induced by cervical stimulation. No changes in the DOPAC:DA ratio in the ME were observed in the AS-ODN-treated rats when compared between times (12.00 and 17.00 h). In addition, this treatment promoted an accumulation of VIP in the SCN and a decrease in VIP content of the PVN.

Considering that the treatment was performed at ZT12, after the peak of *per1* and *per2* gene expression (ZT 6), and that *clock* expression is constitutive [39], the effect of the AS-ODN cocktail for clock genes on PRL secretion may be only attributed to a downregulation of the *clock* gene. On the other hand, we have previously shown that this treatment disrupts the circadian drinking behavior for 72 h [13, 14], suggesting that the feedback loop of clock gene expression in the SCN is disrupted during this pe-

riod of time. A typical cycle of clock gene expression begins in the first hours of the day after the activation of *per* and *cry* transcription by heterodimer CLOCK/BMAL1, the accumulation of mRNA occurs maximally around noon (ZT 6), and 2 h later the cytoplasm protein levels are maximum [40–42]. A decrease in *clock* gene expression may lead to a disturbance in the positive loop of the cycle, and consequently to less *per* gene expression around the time of the beginning of blood sampling (ZT 13) in the present study.

We have previously observed that cervical stimulation establishes a short-loop feedback between PRL and TIDA and THDA neurons, but not PHDA neurons [24]. In the present study, the DOPAC:DA ratio was higher in cervically stimulated OVX rats at 12.00 h (when PRL levels were low) and lower at 17.00 h (when PRL levels were high) in the ME and NL, whereas no changes were observed in the IL. These results reinforce the participation of TIDA and THDA neurons in PRL secretion observed in cervically stimulated OVX rats. The decrease in TIDA neuronal activity at the time of PRL surges confirms previous observations in pseudopregnant rats [43, 44]. However, pseudopregnant rats show a biphasic decrease in DA neuronal activity not only in the TIDA, but also in PHDA neurons [36]. The presence of ovarian steroids in pseudopregnant rats may account for the discrepancy of these results, as the lack of ovarian steroids contributes to gradual waning of PRL surges in OVX rats not seen in intact rats [45, 46].

AS-ODN against *per1*, *per2*, and *clock* mRNA injected into the SCN prevented the decrease in DA activity induced by cervical stimulation at 17.00 h in the TIDA and THDA neurons (fig. 2a, c). This treatment also decreases TIDA and THDA activity at 12.00 h; however, PRL levels are still low at this time, reinforcing the necessity of PRL stimulatory factors in order to impact PRL secretion. Surges of PRL were not observed in these animals (fig. 2d) or in the animals in which serial blood samples were taken (fig. 1). Intriguingly, this same treatment affects the TIDA and THDA neuronal activity, but not the PHDA in OVX rats [14]. Cervical stimulation therefore decreases TIDA and THDA neuronal activity at the time of cervical-stimulation-induced PRL surges, and affects the entrainment by photoperiod cues transduced by the SCN on specific populations of DA neurons.

Cervical stimulation appears to link PRL secretion to a circadian oscillatory mechanism. TH-containing neurons of both the arcuate and periventricular nucleus express clock genes [14, 47], but only the TIDA has been characterized as under SCN control [48], presumably by

the central clock, as AS-ODN against *per1*, *per2*, and *clock* mRNA injected into the SCN disrupts the circadian rhythm of TIDA activity observed in OVX rats [14] and in cervically stimulated OVX rats (present study). THDA, however, does not show a circadian rhythm [49] and is not controlled by the SCN in OVX rats [14]. On the other hand, the DOPAC:DA ratio observed in the NL (where the axons of the THDA neurons terminate) of cervically stimulated OVX rats treated with clock gene AS-ODN were similar at 12.00 and 17.00 h. This result demonstrates a strong effect of the central clock on this DA neuronal population. We conclude from these results, taken together, that TIDA and THDA have a stronger SCN input in cervically stimulated OVX rats, being activated directly or indirectly by cervical stimulation.

Cervical stimulation may not directly affect DA activity, because DA levels decreased several hours after the stimulus [50], although the physiological decrease in DA neuronal activity is only seen after cervical stimulation (as demonstrated in the present study and by McKee et al. [24]). Administration of a DA antagonist to OVX rats induces an increase in PRL secretion, although of lower magnitude than the cervical stimulation-induced PRL surges [51], suggesting that, besides lowering DA activity, cervical stimulation activates a PRL-stimulatory factor. Oxytocin (OT) has been described as a strong PRL-stimulatory factor candidate under physiological conditions [29, 52, 53]. We have shown that the administration of an OT antagonist abolishes the cervical-stimulation-induced PRL surges as well as the relationship between DA activity and PRL secretion established by cervical stimulation. This treatment does not, however, interfere with the low level of DA activity observed in the afternoon [24], probably because the DA tonus depends strongly on a circadian signal. Indeed, in the present study, the DA activity in the ME is higher at 17.00 h in the AS-ODN when compared to the RS-ODN group. In the NL, although no difference was found between groups, when compared between time, DA activity is higher at 17.00 h in the AS-ODN group. This result suggests that the central clock is (and/or drives) the circadian signal required for the regulation of DA tonus responsible for PRL secretion in cervically stimulated OVX rats. Because TIDA and THDA neurons have receptors for VIP, and VIP fibers synapse with these neurons [30], VIP may be the SCN signal affected by the disruption of clock genes. In agreement, VIP antagonism within the SCN prevents the decrease in DA activity at the time of the diurnal PRL surge [18], and as a consequence disrupts the occurrence of this surge [29]. In addition, VIP seems to be responsible

for the nocturnal surge of PRL. A VIP antagonist injection decreases domperidone (DA antagonist)-induced PRL secretion at 03.00 h, but not at 17.00 h [52]. We further illustrated the role of VIP on PRL secretion in a mathematical model, which ascribes VIP as a DA inhibitory factor responsible for the generation of the nocturnal PRL surge [24, 29]. Thus, VIP inputs to DAergic neurons may be responsible for their lowered inhibitory activity at the time of the PRL surges. Without this effect, higher DA tonus is observed, and no surges of PRL occur in cervically stimulated OVX rats treated with AS-ODN for clock genes.

VIP inputs to the PVN may also involve OT release that ultimately induces the secretory PRL surges in cervically stimulated OVX rats. Like the DAergic neurons, the OTergic neurons of the PVN exhibit a circadian rhythm of activity [54], and importantly, the OTergic neurons of the PVN express VIP receptors [29], specifically in the anterior zone of this nucleus [55]. In addition, VIP AS-ODN injected within the SCN of cervically stimulated OVX rats abolishes the diurnal surge of PRL [29]. In the present study, we showed that treatment with clock gene AS-ODN decreased VIP content in the PVN, but increased it in the SCN. These results probably reflect the blocking of VIP transport from the cell body in the SCN and release from the axon terminal in the PVN. In fact, the majority of SCN synaptic connection to the PVN is composed of VIP input [33]. We are not aware of studies directly describing VIP as clock controlled; thus, alternatively, the effect of clock genes on PRL secretion may be through arginine-vasopressin peptide (AVP) that has been described as clock controlled [56]. This peptide is synthesized in the dorso-medial portion of the SCN [28], and an inhibitory role on PRL secretion induced by estradiol of female rats has been described [57]. However, no study has been done, so far, to demonstrate its role on PRL secretion induced by cervical stimulation. Because AVP acts as an intrinsic-excitatory electrical signal with-

in SCN cells through its type 1a receptors that are detected in the majority of SCN neurons [58], it is possible that AS-ODN for clock genes generally decreases the SCN electrical activity by affecting the expression of AVP. This, in turn, decreases VIP inputs from the SCN. The central clock therefore may be involved in the regulating process of VIP delivery to the PVN, and may ultimately regulate PRL secretion by affecting OT release from the PVN.

Clock mutations interrupt estrous cyclicity and interfere with pregnancy [59]. Perinatal and postpartum problems result in a premature decline in progesterone levels at midpregnancy [13] and a shortened duration of pseudopregnancy and disruption of daily maternal behavior [27] occur, suggesting that PRL secretion is compromised. In addition, PRL gene expression in anterior pituitary cells (lactotrophs) of lactating rats depends on an E-BOX that binds CLOCK-BMAL1, suggesting that this heterodimer drives PRL gene transcription through DNA recognition at E-BOX motifs at the level of the pituitary [60]. In the study reported here, however, we showed that PRL secretion induced by cervical stimulation depends specifically on the central clock (the expression of *per1*, *per2*, and *clock* within the SCN), which may communicate with the pituitary through VIP connections to the PVN and to TIDA/THDA neurons. The results of the present study, together with our previous data, show that the secretion of PRL depends on the central clock [13], and the expression of clock genes within SCN neurons generates the circadian signal for the circadian secretory PRL surges in female rats.

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