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Vasoactive intestinal polypeptide modulates the estradiol-induced prolactin surge by entraining oxytocin neuronal activity

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Abstract

In female rats, estradiol is responsible for a circadian secretory PRL pattern which requires an intact suprachiasmatic nucleus (SCN). SCN outputs involved in this secretory profile remain elusive. Because oxytocin has been proposed to stimulate PRL secretion, we investigated whether the projections of vasoactive intestinal polypeptide (VIP) from the SCN to neurons producing oxytocin in the paraventricular and periventricular nuclei (PVN and PeVN, respectively) are responsible for timing PRL surges induced by estradiol (E₂). E₂-treated ovariectomized (OVX) rats received an injection of antisense or random-sequence oligodeoxynucleotide for VIP in the SCN and blood samples were taken for prolactin measurements by radioimmunoassay. Additionally, the percentage of oxytocin-positive neurons immunoreactive to FOS-related antigens (FRA) was determined in the PVN and PeVN, as an index of neuronal activity. In the PVN, oxytocinergic neuronal activity increased in the early evening regardless of E₂ treatment, whereas E₂ induced an increase of activity in the PeVN. VIP antisense attenuated this increase observed in both neuronal populations. Additionally, in the PeVN, VIP antisense advanced this increase by 2 hours (from 19:00h to 17:00h). This same effect was observed in the PRL surge that occurred at 17:00h in the VIP antisense injected animals. Thus, the SCN influences the precise timing of the E₂-induced PRL surge via VIP projections to oxytocinergic neurons of the PVN and PeVN.

Keywords

circadian; paraventricular; periventricular; suprachiasmatic

1. Introduction

Cycling rats display a surge of prolactin (PRL) every 4–5 days on the afternoon of proestrus (Freeman et al., 2000). Estradiol (E₂) treatment in ovariectomized (OVX) rats changes this secretory PRL profile in that it induces a daily surge of PRL at approximately the same time, but of a lesser magnitude, than that observed on proestrus in cycling rats (Neill, 1972). Both surges, the proestrous surge and the E₂-induced surge, shift in response to a shift in the photoperiod (Blake, 1976; Pieper and Gala, 1979). Therefore, PRL secretion is not only dependent on an increase in E₂, but also depends on a source of entrainment.

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Estradiol sensitizes the lactotroph to secrete PRL, and lowers the PRL-inhibiting hypothalamic dopaminergic tone. However, inhibition of dopaminergic tone alone does not induce a full proestrous surge suggesting that an additional factor, a prolactin releasing hormone (PRH), is also necessary to induce PRL secretion (Freeman et al., 2000). Many PRH candidates have been suggested, but overwhelming evidence suggests oxytocin (OT) as the main PRH. The cell membrane of lactotrophs bear OT receptors (Chadio and Antoni, 1989), administration of OT stimulates PRL secretion (Chadio and Antoni, 1993; Lumpkin et al., 1983; Salisbury et al., 1980; Samson et al., 1989) and antagonism of OT prevents PRL release (Arey and Freeman, 1989; Arey and Freeman, 1990; Johnston and Negro-Vilar, 1988; McKee et al., 2007; Sarkar, 1988). Estradiol also influences OT synthesis (Breton et al., 1995) and secretion (Yamaguchi et al., 1979), suggesting an additional permissive role of E₂ on PRL secretion via control of OT.

A circadian component has been proposed to coordinate both the PRL inhibiting factors (dopamine) and the stimulating factors (potentially OT). The suprachiasmatic nucleus (SCN) is well established as the primary circadian pacemaker in mammals responsible for entraining circadian rhythms throughout the central nervous system and periphery (Stephan and Zucker, 1972). Lesions of the SCN abolish the proestrous surge of PRL, as well as the E₂-induced surges (Brown-Grant and Raisman, 1977; Pan and Gala, 1985a). However, because the surge of PRL is abolished, the SCN lesion experiments do not reveal the mechanisms involved in the entrainment of PRL secretion to photic cue.

The majority of light entrained SCN projections are composed of vasoactive intestinal polypeptide (VIP), a 28 amino acid peptide, which was originally isolated from porcine duodenum (Said and Mutt, 1970) and was later localized to the central nervous system (Said and Rosenberg, 1976). VIP is found in the ventrolateral SCN and projects to a variety of regions throughout the brain (Watts and Swanson, 1987). It is found in high concentrations in the hypothalamus, hypophyseal portal blood (Shimatsu et al., 1981) and is also synthesized in the pituitary (Arnaout et al., 1986). The role of VIP in PRL secretion has remained elusive. Disruption of VIP in the SCN has been shown to abolish the diurnal PRL surge induced by cervical-stimulation and decrease the estradiol-induced PRL surge in OVX rats (Egli et al., 2004; Harney et al., 1996). Disruption of VIP in the SCN has also caused a delay and/or decrease in the magnitude of the estrogen-induced PRL surge (van der Beek et al., 1999). Therefore, the function of VIP from the SCN remains unclear.

Previously, our lab has demonstrated that the population of dopaminergic neurons in the arcuate and periventricular nuclei (PeVN) responsible for the tonic inhibition of PRL secretion express VIP₂ receptors (Gerhold et al., 2001). The endogenous rhythms of these dopaminergic neurons are disrupted in response to VIP antisense oligodeoxynucleotides (VIP AS-ODN) injections in the SCN (Gerhold et al., 2002). This treatment decreases VIP protein content (Harney et al., 1996) and expression of VIP neurons of the SCN (Egli et al., 2004; Gerhold et al., 2002). VIP₂ receptors have also been localized to OT neurons within the PeVN and paraventricular nucleus (PVN) of the hypothalamus (Egli et al., 2004). Our current hypothesis is that VIP input to OT neurons in the hypothalamus are the time-component in the mechanism controlling PRL surges. The current study evaluated the effect of VIP on E₂-induced PRL surges, and also investigated its influence on the activity of OT neurons in the PVN and PeVN, using VIP AS-ODN to disrupt VIP protein synthesis.

2. Results

2.1 Experiment 1

Following E₂ treatment, OVX rats exhibited a surge of PRL which peaked at 19:00h on day 2. Administration of either VIP RS-ODN or VIP AS-ODN into the SCN did not alter this

secretory PRL surge (Fig. 1A). However, on day 3, although no change in the magnitude of the PRL surge was observed, VIP AS-ODN treatment caused a two-hour phase advance of the PRL surge, which peaked at 17:00h compared to VIP RS-ODN (Fig. 1B) which peaked at 19:00h.

2.2 Experiment 2

In the PVN, there was no difference in the percent of OT neurons expressing FRA between OVX and OVX-E₂ treated groups (Fig. 2 A-B). However, in both groups, a gradual increase across time was observed with the greatest percent of OT neurons expressing FRA starting at 19:00h (Fig. 2 A-B) until 21:00h. The increase at 19:00 h ($p < 0.001$) and 21:00 h ($p < 0.01$) was prevented in the OVX-E₂ treated group by VIP AS-ODN infusion in the SCN (Fig. 2 C and Table 2).

In the PeVN, E₂ induced a gradual increase in the percent of OT neurons expressing FRA across time (Fig. 2E). Comparisons between OVX and OVX-E₂ treated groups clearly showed that E₂ affects OT neuronal activity. This activity differed significantly between OVX and OVX-E₂ treated groups at 13:00h ($p < 0.05$), 15:00h ($p < 0.05$) and 17:00h ($p < 0.01$) (Fig. 2 D-E). Administration of VIP AS-ODN in the SCN caused a two-hour phase advance in activity of OT neurons within the PeVN with the highest expression at 17:00h (Fig. 2F and Table 3) compared with VIP RS-ODN.

In the PeVN of OVX-E₂ treated animals, the percent of OT neurons expressing FRA increased at the same time as the E₂-induced PRL surge. Additionally, following VIP AS-ODN administration in the SCN, both the surge of PRL (Fig. 1 B) and the increase in the percent of OT neurons expressing FRA were advanced to 17:00h (Fig. 2 F). Fig.3 shows photomicrographs of representative sections of the PVN and PeVN of OVX and OVX-E₂ at 19:00h, in which staining for FRA and OT can be observed. At this time point steroid treatment does not significantly change the percentage of OT neurons expressing FRA in the PVN or in the PeVN. However, an increase in FRA expression in non-OT neurons in the PVN of OVX animals can be observed at 19:00h.

3. Discussion

These data suggest that endogenous VIP influences the timing of PRL secretion. The disruption of VIP synthesis influenced the temporal pattern of E₂-induced PRL secretion as well as the activity of OT neurons believed to be involved in E₂-induced PRL secretion. Based on previous research which found that disrupting VIP protein synthesis prevents the decrease in dopamine activity (Gerhold et al., 2002) which is normally responsible for stimulating PRL secretion (Ben-Jonathan and Hnasko, 2001), we hypothesized that the administration of VIP AS-ODN in the SCN would prevent the circadian profile of PRL secretion observed in OVX-E₂ treated rats. Instead, the E₂-induced PRL surge phase advanced 2 hours on day 3 post-infusion. This unexpected outcome suggested that VIP was influencing an additional factor, possibly a PRH. OT neurons in the PVN and PeVN were the first candidates considered, as they are one of the strongest PRH candidates, and also because these regions also receive projections of VIP from the SCN (Watts and Swanson, 1987) and have VIP₂ receptors (Egli et al., 2004).

In order to test the hypothesis that VIP influences OT neurons to modulate the circadian profile of PRL secretion, we first described the rhythm of OT neurons expressing FRA in the PVN and the PeVN in OVX and OVX-E₂ treated animals. Interestingly, the rhythm of OT neurons expressing FRA in the PVN was not dependent on E₂, whereas the rhythm in the PeVN was. OT neurons expressing FRA in both the PVN and PeVN of OVX-E₂ treated animals, as well as the PVN of OVX animals all rose gradually across the day peaking at approximately the same time as the E₂-induced surge of PRL. In the PVN, VIP AS-ODN attenuated the increase

in OT neurons expressing FRA. Interestingly, disruption of VIP synthesis phase advanced the activity of OT neurons expressing FRA in the PeVN of OVX-E₂ treated rats by 2 hours. This phase advance was also observed in the E₂-induced PRL surge of animals treated with VIP AS-ODN.

The effects of OT at the anterior pituitary are necessary for PRL secretion (Arey and Freeman, 1990; Egli et al., 2004; McKee et al., 2007). We hypothesize that the sequence of events may be: 1) an increase of OT synthesis, 2) an increase of OT release in the median eminence (eventually transported to the anterior pituitary gland) and 3) OT acting in the pituitary gland to increase PRL secretion. Therefore, changes in FRA expression could reflect one or all of these events. In any case, changes in OT neuronal activity should happen before a PRL surge to guarantee that OT will stimulate PRL secretion at the time of the surge. Because it has been described that FRA expression is delayed by approximately 1 hour (Kovacs, 1998), the increase observed in the present study should reflect changes in neuronal activity at least 1 hour prior to the time of interest. Additionally, it is important to note that FRA is constitutively expressed (Hoffman et al., 1993). Not surprisingly, FRA expression which did not colocalize with OT was present at all time points examined. However, the phenotype of other neurons with which FRA colocalized is unknown but will be explored in future work.

Previous studies have suggested that the SCN is involved in PRL secretion. However, the mechanism through which it is involved has remained unclear. Although the SCN is usually involved in entrainment, lesions of this structure prevented the proestrous (Brown-Grant and Raisman, 1977) and E₂-induced surges of PRL (Pan and Gala, 1985a). However, lesions of other structures such as the medial basal part of the suprachiasmatic area (Kawakami and Arita, 1981), medial preoptic area and the ventromedial hypothalamus (Pan and Gala, 1985a) also prevent the PRL surge. Because these areas all receive SCN projections, there is a possibility that SCN lesions indirectly influence PRL secretion by disrupting projections to areas more critically involved in the magnitude of the PRL surge. Furthermore, because the PRL surge is abolished in SCN lesion studies, the component involved in entraining PRL secretion has not previously been described.

The majority of light entrained SCN projections are composed of VIP and a stimulatory role in PRL secretion has been suggested (Akema et al., 1988; Bogacka et al., 2002; Huang and Pan, 1996; Samson et al., 1980). The mechanism by which it does so has remained unclear. In agreement with Harney et. al. (1996), our current data have shown that VIP AS-ODN had no effect on PRL secretion 2 days after E₂ and VIP AS-ODN infusion. However, the present experiment observed the phase advance 3 days after E₂ and VIP AS-ODN infusion. This time point was not taken into consideration in previous papers. This suggests that the surges persist because of possible residual VIP in axon terminals. Possibly, by day 3 the residual VIP has cleared and the VIP AS-ODN has prevented further synthesis. Additionally, a delay and decrease in the magnitude of the PRL surge has also been observed (van der Beek et al., 1999). The current data also show an effect on the time of the surge, while a decrease in magnitude was not observed. These differences may be due to differing methodologies. VIP AS-ODN administration has also been shown to be effective in blocking the diurnal, but not nocturnal, surge of PRL induced by artificial cervical stimulation in OVX rats (Egli et al., 2004). Therefore, a different mechanism appears to be involved in these surges. Because the cervically stimulated animals are OVX and do not receive E₂ treatment, the E₂-dependent activity of OT neurons expressing FRA in the PeVN may be inconsequential. Furthermore, because VIP AS-ODN prevented the increase in OT neurons expressing FRA in the PVN, this may be the reason no PRL surge was observed.

Because the current study examined the E₂-induced surge of PRL, an additional aim was to determine how E₂ acts centrally to influence circadian PRL secretion. Neurons in the SCN

do not concentrate E₂ (Pfaff and Keiner, 1973) and implants of estradiol in the SCN have no effect on PRL secretion (Pan and Gala, 1985b), suggesting that any effects of E₂ would be at a downstream site from the SCN. E₂ has previously been shown to increase the presence of VIP₂ receptors on hypothalamic dopamine neurons (Gerhold et al., 2001), suggesting a modulatory role for ovarian steroids in VIPergic transmission of photic cues from the SCN to these neurons.

An additional permissive role of E₂ on PRL secretion may be its ability to influence OT activity. OT receptor mRNA on lactotrophs dramatically increases in response to exogenous E₂ (Breton et al., 1995). Furthermore, plasma OT concentrations increase in response to E₂ administration (Yamaguchi et al., 1979), and are highest in hypophyseal portal blood on the afternoon of proestrus (Sarkar and Gibbs, 1984). The high levels of OT in hypophyseal blood presumably reflect the secretory activity of OT neurons in the median eminence. While the majority of OT neurons in the paraventricular nucleus project to the posterior pituitary, they also send collaterals to the median eminence (Zimmerman et al., 1984). OT may also reach the anterior pituitary from the posterior pituitary via short portal vessels (Murai and Ben-Jonathan, 1987; Murai et al., 1989). Based on this, one would hypothesize that the activity of the OT neurons in the PVN would reflect the levels of OT observed in the hypophyseal portal blood and within the anterior pituitary in response to changing levels of E₂. However, the current data show that OT neurons in the PVN do not differentially respond to E₂ treatment, suggesting that E₂ may not play a modulatory role in OT neuronal activity in the PVN. Indeed, only a small population of neurons in the PVN concentrates E₂ (Rhodes et al., 1981; Rhodes et al., 1982). However, a high level of E₂ concentration was found along the third ventricle (Pfaff and Keiner, 1973) where the PeVN OT neuronal population is found. As observed in our data, E₂ treatment induces an increase in the activity of OT neurons expressing FRA in this population. Therefore, E₂ may induce PRL secretion by affecting the activity of OT neurons in the PeVN.

Of particular interest was the consistency in profiles associated with the effect of VIP AS-ODN on E₂-induced PRL surge and on the activity of OT neurons in the PeVN. In this neuronal population VIP AS-ODN promoted a 2-hour advance in the increase of OT neurons expressing FRA. This phase advance was also observed in the E₂-induced PRL surge. This suggests that this population of OT neurons may be directly involved in the secretory profile of PRL induced by E₂. Not only does this offer further evidence of OT as a PRH, it also suggests VIP as a timing mechanism influencing hypothalamic factors involved in the control of PRL secretion.

The current data suggest an additional role of VIP in the coordination of PRL secretion. Not only does it influence the pituitary (Bogacka et al., 2002; Samson et al., 1980), it also acts centrally to entrain the activity of neurons responsible for inhibiting (dopamine) (Gerhold et al., 2002) or stimulating (oxytocin) E₂-induced PRL secretion. Because the current study did not discriminate between magnocellular and parvocellular neurons, future studies will address this.

4. Experimental procedures

4.1 Animals

Adult female Sprague-Dawley rats (> 60 days of age) weighing 250–300g (Charles River Laboratories, Raleigh, N.C.) were kept in standard rat cages under a 12h light : dark cycle (lights on at 06:00h) with water and rat chow available ad libitum. All animals were anesthetized with Halothane and OVX bilaterally and then allowed to recover for 7–10 days. At 10:00h on the day of ODN infusion (day 0), all animals were injected subcutaneously with 20 µg of estradiol-17β (Sigma, St. Louis, MO) in corn oil. This paradigm results in E₂ levels similar to those found on the afternoon of proestrus (Neill, 1972). Control animals received

vehicle injections of corn oil. All animal procedures were approved by the Florida State University Animal Care and Use Committee.

4.2 General procedures

4.2.1 SCN guide cannula implantation—Animals were anesthetized with Ketamine (0.7mL; 49 mg/mL) and Xylazine (0.3mL; 1.8 mg/mL) and placed in a stereotaxic device (David Kopf, Tujunga, CA). A midline incision exposed the skull and the location of the SCN was determined using stereotaxic coordinates (Paxinos and Watson, 1998). A small opening in the cranium was made with a dental drill and the surface of the brain exposed. Guide cannulae (1.5 mm apart; 8.5 mm in length; 26 gauge) were lowered to the dorsal border of the SCN (0.8 mm anterior to bregma; 7.9 mm ventral to the dural surface) and fixed to the skull. Subsequent procedures began a minimum of 5 days post-implantation to allow for complete recovery.

4.2.2 Jugular vein catheter implantation—Animals were anesthetized with Halothane and Polyurethane tubing (Micro-Renathane, Braintree Scientific Inc., Braintree, MA) was inserted into the 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 heparinized saline kept the line open until the start of the blood collection.

4.2.3 VIP antisense oligodeoxynucleotide injections in the SCN—For ODN injections, animals were anesthetized with Halothane and placed under an injection mini-pump attached to two 1 μ L syringes (Hamilton Inc.), which were connected to a bilateral delivery cannula (Plastics One Inc.) through Silastic (PE-10) tubing. VIP AS-ODN and RS-ODN directed against VIP mRNA (B.A.S.S. Laboratory, Florida State University) were diluted in sterile water (0.5 μ g in 0.5 μ L). On the day of E₂ administration (day 0), ODN were infused for 4 minutes through a bilateral cannula (1.5 mm apart 9.5 mm in length; 33 gauge) which was placed in the guide cannula directed at the SCN. This dose has previously been reported not to result in side effects or toxicity when infused into the SCN (Gerhold et al., 2002). Treatment with the AS-ODN and RS-ODN were given at least 36h prior to the first blood collection point or sacrifice. This time allows for maximum protein synthesis disruption. Previous work from our lab has shown that treatment with VIP AS-ODN decreases VIP-immunoreactivity in the ventrolateral SCN by 75% when the cannula is successfully placed in the medial to caudal regions of the SCN (Gerhold et al., 2002). To verify bilateral cannula placement, one series of brain sections were processed for immunohistochemistry for FRA and brains with cannulae placed in the medial to caudal regions of the SCN were considered successful. Animals with misplaced cannulae were discarded.

4.2.4 Blood Collection—Two and three days after E₂ treatment (day 2 and day 3), 400 μ L of blood were collected in heparinized syringes at a 2 hour interval beginning at 13:00h and ending at 21:00h. Blood lost during sampling was replaced with sterile saline. Samples were stored at 4 C until centrifuged (4000 g). Serum was collected and stored at -20 C until analysis for PRL concentration by radioimmunoassay.

4.3 Radioimmunoassay

The concentration of rat PRL in serum was determined in duplicate by radioimmunoassay (RIA) using NIDDK materials supplied by Dr. Albert Parlow through the National Hormone and Pituitary Program (Torrance, CA). Serum concentrations of PRL are expressed as ng/ml in terms of the rat PRL RP-3 standard. Assay sensitivity is 1 ng/ml and the intra-assay coefficient of variation was 5%. To prevent inter-assay variation, all samples were assayed in the same RIA.

4.4 Immunohistochemistry (IHC)

Animals were deeply anesthetized with 83 mg/Kg body weight of pentobarbital solution (NembutalR, Abbott Laboratories, Chicago, IL) and perfused intracardially with 50 mL of 0.1 M phosphate buffered saline (PBS) at pH 7.35, followed by 200 mL of cold 0.4% paraformaldehyde in 0.1 M PBS at pH 7.35. Whole brains were post-fixed, and then placed in 20% sucrose until they sank. Each brain was blocked using a coronal brain matrix and collected in three parallel series of 30 μ m sections. The brains were sectioned coronally from 600 to 2600 μ m post bregma with a cryostat (Microm, Zeiss, Germany) and stored in cryoprotectant at -40 C. To process for IHC, one series of free floating sections was placed in Falcon 6 or 12 well plates, covered with 1.5 to 3 mL of 0.1 M PBS with 0.1% Triton X-100 (PBS-X) and placed on an orbital shaker. All IHC was initiated with 3 $\times$ 10 min rinses in PBS-X followed by background blocking with 10% normal donkey serum (Chemicon, Temecula, CA) in PBS-X for 1 h at room temperature. Primary antibody incubations of OT (rabbit anti-OT, IHC8152, Peninsula Laboratories, Inc., Belmont, CA; dilution 1:10000) and Fos related antigen (FRA) (goat anti-cFOS, K-25, Santa Cruz Biotechnologies, Santa Cruz, CA; dilution 1:5000) were performed in PBS-X with 0.01% Na-Azide at 4 C overnight. Secondary antibody incubations of Cy2 (donkey anti-rabbit, Jackson ImmunoResearch, West Grove, PA; dilution 1:600) and Cy3 (donkey anti-goat, Jackson ImmunoResearch, West Grove, PA; dilution 1:600) were also performed in PBS-X with 0.01% Na-Azide at 4 C overnight. Sections were mounted on clean slides and allowed to dry overnight. Coverslips were secured with Aqua Polymount (Polysciences, Inc., Warrington, PA) and were sealed with nail polish. All sections with neurons expressing OT and FRA were acquired using a Leica DMR stereoscope attached to a Spot-RT Digital camera (Diagnostic Instr. Inc.). All gray scale images were pseudocolored and then blindly analyzed using MetaMorph analysis software (Universal Imaging, Inc. Molecular Devices). OT neurons in the PVN and PeVN expressing FRA were considered active neurons. In 6 sections containing the PVN and/or the PeVN, the number of OT neurons and those which express FRA were counted. The results are presented as the percentage of OT neurons expressing FRA.

4.5 Experiment #1: Effect of VIP-AS on E₂-induced PRL secretion

At 10:00h on day 0, groups of OVX rats received a subcutaneous bolus injection (20 μ g/rat) of estradiol-17 β . Animals were then bilaterally injected with VIP AS-ODN or RS-ODN into the SCN. On day 2 and day 3, blood samples were collected every 2 hours from 13:00h until 21:00h from the jugular vein. To verify bilateral cannula placement, animals were then perfused and one series of brain sections were processed for IHC for FRA. Brains with cannulae placed in the medial to caudal regions of the SCN were considered successful. Animals with misplaced cannulae were discarded.

4.6 Experiment #2: Effects of VIP AS-ODN on OT activity in the PVN and PeVN

At 10:00h on day 0, groups of OVX rats received a subcutaneous bolus injection (20 μ g/rat) of estradiol-17 β or oil. On day 3, groups of animals were perfused with fixative solution every two hours beginning at 13:00h and ending at 21:00h. Three rats were perfused per time point per treatment group. Brains were collected and processed for IHC. Six sections containing the PeVN or the PVN were incubated with antibodies against OT and FRA (a well established marker of neuronal activity (Hoffman et al., 1993)). OT neurons in the PVN and PeVN expressing FRA were considered double labeled. In a separate group, OVX-E₂ treated animals were bilaterally injected with VIP AS-ODN or VIP RS-ODN into the SCN on day 0. Following this treatment, the animals underwent the same perfusion schedule and IHC treatment as described above.

4.7 Statistical Analysis

Data are presented as the mean $\pm$ SEM. Data analysis was conducted using a two-way ANOVA for the comparison of differences between treatment groups, followed by Bonferroni post-hoc comparison. A one-way ANOVA was used for comparison of time differences within treatment groups, followed by Bonferroni *post-hoc* comparison. Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA) was used for statistical analyses and graph preparation. Differences were considered significant when $P < 0.05$.

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A. 2 days after E₂ treatment B. 3 days after E₂ treatment **Figure 1.**

Mean ($\pm$ SEM) concentration of serum PRL obtained from animals ($n=6$ /group) either 2 days (Panel A) or 3 days (Panel B) after estradiol treatment given prior to VIP AS-ODN or VIP RS-ODN injections. Letter *a* indicates the significantly greater concentration of PRL versus other times within groups (one-way ANOVA, followed by Bonferroni's post-hoc test $p < 0.01$). The surge of PRL secretion is observed 2 h earlier in the VIP AS-ODN treated group than in the VIP RS-ODN treated group (two-way ANOVA, followed by Bonferroni's post-hoc test $**p < 0.01$).

Figure 2.

Percentage of OT neurons expressing FRA in the PVN and PeVN of OVX (Panel A, D), OVX-E₂ treated rats submitted to RS-ODN injection (Panel B, E) or to AS-ODN (Panel C, F) in the SCN. One-way ANOVA, followed by Bonferroni's post-hoc test, indicates difference in the percentage of OT neurons expressing FRA at 17:00h or 19:00h versus 13:00h. Asterisks indicate difference between AS-ODN and RS-ODN treated groups (two-way ANOVA, followed by Bonferroni's post-hoc test * $p < 0.05$, *** $p < 0.001$).

Figure 3.

Photomicrographs of representative sections containing OT neurons (green) and neurons expressing FRA (red) in the PVN of OVX-E₂ treated (A) or OVX (B) animals and in the PeVN of OVX-E₂ treated (C) or OVX (D) animals. Solid arrows point to representative OT neurons expressing FRA. Steroid treatment does not significantly change the percentage of OT neurons expressing FRA at 19:00 h in the PVN or in the PeVN. In the PVN of OVX animals, an increase in FRA expression is observed in non-OT neurons at 19:00 h. Scale bar = 100 μ m.

Table 1

	Antisense sequence oligodeoxynucleotide (AS-ODN)	Random sequence oligodeoxynucleotide (RS-ODN)
Cap site	5'-GCTCTGCACTACAACCTGAC-3'	5'-CCGATCGACACCTAGCTATC-3'
Translation start site	5'-TTGCTTCTGGATTCCATCTC-3'	5'-GTTATCCGTCTCATTGCTCT-3'

Table 2

	Treatment & Time of day (h)	OT neurons (+) (Number $\pm$ SE)	OT and FRA (+) neurons (Number $\pm$ SE)
OVX	1300	155.5 $\pm$ 23.40	81.05 $\pm$ 8.7
	1500	201.39 $\pm$ 74.22	112.89 $\pm$ 39.66
	1700	119.22 $\pm$ 37.42	72.05 $\pm$ 25.45
	1900	129.7 $\pm$ 16.02	85.78 $\pm$ 9.48
	2100	125.8 $\pm$ 9.9	78.16 $\pm$ 4.67
OVX-E₂ (RS-ODN)	1300	183.83 $\pm$ 9.33	91.33 $\pm$ 2.5
	1500	105.61 $\pm$ 11.54	58.72 $\pm$ 8.31
	1700	168.67 $\pm$ 15.42	95.28 $\pm$ 11.24
	1900	109.1 $\pm$ 45.21	74.5 $\pm$ 24.61
	2100	157.1 $\pm$ 40.52	90.89 $\pm$ 22.89
OVX-E₂ (AS-ODN)	1300	41.89 $\pm$ 21.19	18.33 $\pm$ 9.34
	1500	59.39 $\pm$ 5.42	28.06 $\pm$ 2.55
	1700	41.89 $\pm$ 13.24	25.56 $\pm$ 9.56
	1900	107.67 $\pm$ 10.78	47.22 $\pm$ 7.17
	2100	44.61 $\pm$ 8.64	20.89 $\pm$ 3.5

Table 3

	Treatment & Time of day (h)	OT neurons (Numbers $\pm$ SE)	OT and FRA (+) neurons (Numbers $\pm$ SE)
OVX	1300	16.56 $\pm$ 0.34	10.11 $\pm$ 0.66
	1500	49.78 $\pm$ 16.77	36.5 $\pm$ 11.73
	1700	24.03 $\pm$ 5.52	11.56 $\pm$ 2.31
	1900	25.89 $\pm$ 4.77	17.72 $\pm$ 2.28
	2100	31.67 $\pm$ 6.8	23.39 $\pm$ 4.75
OVX-E₂ (RS-ODN)	1300	33.44 $\pm$ 11.16	15.61 $\pm$ 5.86
	1500	23.89 $\pm$ 5.44	11.83 $\pm$ 1.29
	1700	27.95 $\pm$ 10.42	19.67 $\pm$ 7.56
	1900	12.67 $\pm$ 5.78	9.56 $\pm$ 3.74
	2100	22.39 $\pm$ 4.39	14.44 $\pm$ 4.06
OVX-E₂ (AS-ODN)	1300	13.06 $\pm$ 6.23	6.72 $\pm$ 4.04
	1500	25.61 $\pm$ 10.06	14.94 $\pm$ 5.62
	1700	24.89 $\pm$ 10.56	20.61 $\pm$ 9.7
	1900	15.55 $\pm$ 1.78	8.28 $\pm$ 1.81
	2100	10.72 $\pm$ 3.37	5.83 $\pm$ 2.49