Title: Systemic oxytocin induces a prolactin secretory rhythm via the pelvic nerve in ovariectomized rats.

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Abstract

We have shown previously that an intravenous (iv) injection of oxytocin (OT) in ovariectomized (OVX) rats initiates a circadian rhythm of prolactin (PRL) secretion similar to that observed after cervical stimulation (CS). In this study, we investigated the pathway through which OT triggers the PRL rhythm. We first tested whether an intracerebroventricular injection of OT could trigger the PRL secretory rhythm. As it did not, we injected OT iv while an OT receptor antagonist was infused intravenously. This antagonist completely abolished the PRL surges, suggesting that a peripheral target of OT is necessary for triggering the PRL rhythm. We hypothesized that OT may induce PRL release, which would be transported into the brain and trigger the rhythm. In agreement with this, OT injection increased circulating PRL by 5 min. To test whether this acute increase in PRL release would induce the PRL rhythm, we compared the effect of intravenously-administered thyrotropin-releasing hormone (TRH) and OT. Although TRH injection also increased PRL to a comparable level after 5 min, only OT-injected animals expressed the PRL secretory rhythm. Motivated by prior findings that bilateral resection of the pelvic nerve blocks CS-induced pseudopregnancy and OT-induced facilitation of lordosis, we then hypothesized that the OT signal may be transmitted through the pelvic nerve. In fact, OT injection failed to induce a PRL secretory rhythm in pelvic-neurectomized animals, suggesting that the integrity of the pelvic nerve is necessary for the systemic OT induction of the PRL secretory rhythm in OVX rats.

Key words: oxytocin receptor, pseudopregnancy, lactotrophs, pelvic nerve
Introduction

Mating or cervical stimulation (CS) induces a circadian rhythm of prolactin (PRL) secretion in female rats, consisting of nocturnal (0300 h) and diurnal (1700 h) surges. The rhythm persists for 10-12 d, which is about half the duration of pregnancy (19). As the rhythm in secretion continues for several days without additional stimuli, it has been suggested that a hypothalamic ‘memory’ is activated by CS and acts to sustain the daily PRL surges (17). This rhythm can be produced in ovariectomized (OVX) rats, demonstrating that the memory does not require ovarian steroids (43). Despite these findings, the mechanism by which the memory is triggered is not understood.

PRL secretion by lactotrophs is tonically inhibited by hypothalamic dopamine (DA) (3; 11). While the full generation of PRL surges requires a decrease in DA inhibition, the actions of one or more stimulating factors are also needed (16; 18). Several lines of evidence suggest that oxytocin (OT) may act as a PRL-releasing factor to induce PRL surges in several physiological paradigms (23; 39; 40), including mating. Since it has been shown that there is an immediate release of OT (34) after CS in rats, we hypothesized that this initial burst of OT could be responsible for triggering the PRL surges induced by CS. In agreement with this, we observed that a single intravenous (iv) injection of OT in OVX rats was indeed able to induce a PRL secretory rhythm and a DA release pattern similar to that initiated by CS (13). Early in vivo work demonstrated that the concentration of PRL in the blood is increased within 15-20 min after mating or artificial CS (7; 44). Complementing these in vivo studies, we and others have demonstrated that OT stimulates PRL secretion in vitro when administered to anterior pituitary cells in culture (28) through a calcium-dependent mechanism (12; 45). This led to the hypothesis that the OT-induced PRL rhythm could be triggered by the PRL released by lactotrophs following OT injection.
To evaluate the role of PRL in mediating the OT- or CS-induced circadian PRL rhythm, we recently tested whether a bolus of PRL was sufficient to trigger the rhythm by injecting ovine PRL (oPRL) into OVX rats. We found that either peripheral or central oPRL injections were able to trigger a PRL rhythm that is similar to that induced by OT or CS (21). The concentration of oPRL required to trigger the rhythm was much larger when injected peripherally than when injected centrally, consistent with a mechanism that involves a central action of PRL. Peripheral PRL presumably enters the central nervous system through a carrier-mediated transport system in the choroid plexus (47).

The objective of this study was to clarify the mechanism through which OT triggers the PRL rhythm when injected into OVX rats. We hypothesized the existence of a neurogenic component to mediate the effects of systemic OT on the initiation of the PRL secretory rhythm. The integrity of the pelvic nerves has been previously inferred to be necessary for the initiation of the mating-induced surges of PRL, since pelvic neurectomy prior to mating blocks pregnancy (44) and pseudopregnancy (10). As pelvic nerve integrity is also required for OT-induced lordosis facilitation (33), our last experiment tested the effect of pelvic neurectomy on the OT-induced PRL secretory rhythm in OVX rats.

Methods

Animals

Adult female Sprague-Dawley rats weighing 250-300 g (Charles River, Raleigh, NC) were kept in a Laboratory Animal Resources care facility, housed in groups of three in plastic cages under a 12:12 light:dark cycle (lights on at 0600 h) and controlled temperature (25 C). Food and water were provided ad libitum. All rats were OVX bilaterally through a single ventral midline incision under isoflurane anesthesia (Aerrane; Baxter, Deerfield, IL) and allowed to
recover for at least 1 week. At the end of this and other surgical procedures (see below), rats were treated with a single ip injection of an anti-inflammatory analgesic (Metacam, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO; 1 mg/kg). Animal procedures were approved by the Florida State University Animal Care and Use committee.

**Experimental design**

*Experiment 1: Central vs peripheral OT action to trigger rhythmic PRL secretion*

We first injected saline or OT icv in order to determine if central OT could trigger the PRL secretory rhythm in OVX rats. In a second set of OVX animals, we performed an iv OT injection in the presence of an OT antagonist that had been infused peripherally for approximately 24 h to prevent OT binding to its receptors in the anterior pituitary. The control group also received an iv OT injection, but no OT antagonist was infused. Both central and peripheral OT injections were performed at 1600 h, the same time used in our previous work in which a single OT injection in OVX animals induced the PRL secretory rhythm (13). Blood samples were drawn every 2-4 h on days 2 and 3 to determine the dynamics of PRL secretion.

*Experiment 2: Correlation between immediate PRL release and the PRL secretory rhythm*

Animals were injected with OT or TRH vs saline iv at 1600 h and blood samples were taken 5 and 10 min later, to evaluate the acute changes in PRL levels in plasma after the injections. Blood samples were additionally drawn every 2-4h, on days 2 and 3 after the injections to assess the occurrence of the PRL secretory rhythm.

*Experiment 3: Effect of pelvic neurectomy on the OT-induced rhythmic secretion of PRL*

OVX animals had their pelvic nerve sectioned bilaterally (see below) and after 1 week of recovery were subjected to OT injection iv at 1600 h. Sham animals were dissected down to the
level of the pelvic nerve, but no sectioning was performed. Blood samples were taken 5 and 10 min immediately after the injection and every 2-4 h on days 2 and 3 for the measurement of PRL levels.

The icv cannulation

Under ketamine (Ketaset; Fort Dodge, IA; 49 mg/mL) and xylazine (Anased; Lloyd Laboratories, Shenandoah, IO; 1.8 mg/mL) anesthesia (100 µL/100 g body weight), animals were positioned in a stereotaxic apparatus with the incisor bar at –3.3 mm. For the central injection of OT, a 22-gauge guide cannula (C313G, Plastics One Inc., Roanoke, VA) was implanted in the right cerebral lateral ventricle (coordinates: 1.0 mm posterior to bregma, 1.6 mm lateral to the midline, and 3.2-3.7 mm below the outer surface of the skull). The correct vertical positioning of the cannula in the lateral ventricle was determined by displacement of the meniscus in a water manometer that detects pressure differences among compartments. The cannula, protected with a plastic-capped mandril (C313DC, Plastics One), was attached to the bone with stainless-steel screws and acrylic cement. After surgery all rats were allowed to recover for 1 week in individual cages.

Jugular vein catheter implantation and blood samples

One week after icv cannulation, rats were anesthetized with isoflurane and a catheter (Micro-Renathane; MRE-040, 0.040" O.D. x 0.025" I.D.; Braintree Scientific, Braintree, MA) filled with sterile saline (0.9% NaCl; Teknova, Hollister, CA) was inserted through the external jugular vein into the right atrium, fitted subcutaneously and exteriorized at the back of the animal, as previously described (20). All stainless-steel surgical instruments were immersed in chlorhexidine disinfectant (Novalsan; Fort Dodge, IA) until surgery. After the surgery, the catheter tube was filled with gentamicine sulfate (Alexis, San Diego, CA) to prevent bacterial growth and to maintain catheter patency (46). On the morning of the first day of the experiment,
an extension of the catheter tubing filled with saline was connected to the jugular catheter and the
rats were left undisturbed in their cages. Blood samples of 300 μL were withdrawn into plastic
heparinized syringes and the same volume of sterile 0.9% NaCl was injected through the catheter
immediately after removal of each blood sample.

**Injections and infusions**

All drugs were dissolved in 0.9% NaCl. Oxytocin (H-2510, Bachem Americas, Torrance,
CA; 0.3 μg/μL) or saline were injected icv under light isoflurane anesthesia via a stainless steel
needle (0.2 mm diameter) connected by PE-10 polyethylene tubing to a Hamilton syringe
(Hamilton, Reno, NV) controlled by an injection pump (KDS100, KD Scientific, Holliston, MA)
calibrated to dispense 5 μL solution/min. After each injection the needle was left inside the
cannula for an additional 60 sec to avoid solution reflux. This dose of OT has been shown to
elicit known central effects of OT, such as induction of maternal behavior in virgin rats (36).
Peripheral OT (5μg /200 μL), TRH (1μg /200 μL, P1319, Sigma, St Louis, MO) or saline
administration was performed iv using the jugular vein catheter implantation described above.

For the peripheral infusions, 200 μL of the OT antagonist solution (225 μg /200 μL,
DesGly-NH$_2$-d((CH$_2$)$_5$[D-Tyr$^2$,Thr$^4$]) OVT, GenScript Corporation, Scotch Plains, NJ) (30) was
inserted in each osmotic pump (AP-2001D; Alzet, Durect Corp., Cupertino, CA) and infused at a
rate of 9 μg/ h for 1 d. This peptide OT antagonist, unlike non-peptide OT antagonists, has
limited penetration through the blood brain barrier (8). Infusion of the OT antagonist alone in
OVX rats did not modify PRL levels over the next 1-3 days (data not shown).

**Pelvic Neurectomy**

Immediately after ovariectomy, the mid-ventral incision was extended from the pubis
toward the xyphoid process. The bifurcation of the vena cava into the common iliac veins was
first located and then followed 1 cm caudally to the site of origin of the internal iliac veins. These veins run perpendicular to the long axis of the body, toward the rat’s dorsum, and were visualized by gentle retraction of the surrounding muscle with sterilized cotton swabs. The pelvic nerve was located approximately 5 mm from the origin of the internal iliac vein, running rostrocaudally across its axis, perpendicular to the vein, deep in the space between the internal iliac vein and the adjacent muscles (for detailed explanation, see (10)). The nerve was transected bilaterally with fine scissors under a Zeiss surgical microscope. Sham surgery consisted of dissection down to the level of the pelvic nerve, but no sectioning was performed. As bladder evacuation is compromised by pelvic neurectomy, bladders were emptied manually twice daily until the end of the experiment. The same procedure was conducted in sham animals to ensure identical handling procedures, but little urine was expelled in these animals. Bladder distention due to urine retention was used as the indicator of successful neurectomy.

**Radioimmunoassay**

Blood samples were centrifuged at 1200 g for 15 min at 4°C; the plasma was separated and frozen at –20°C until assayed. Plasma PRL was determined by radioimmunoassay (RIA) using a kit provided by Dr. Albert F. Parlow through the National Hormone and Pituitary Program (Torrance, CA). \(^{125}\)I was purchased from PerkinElmer Life Sciences (Shelton, CT) and PRL-I-6 was radiiodinates the Chloramine-T method. The antiserum for PRL was anti-rat PRL-S9 and the reference preparation was PRL-RP3. The lower limit of detection was 0.10 ng/mL and the intra- and inter-assay coefficients were less than 4 and 12%, respectively.

**Statistical analysis**

Data are expressed as mean ± S.E.M. Statistical differences were determined by two-way ANOVA followed by the Bonferroni post-hoc test. Comparisons among times within the same
experimental group were analyzed by one-way ANOVA followed by the Newman-Keuls post hoc test. $P < 0.05$ was considered statistically significant.

**Results**

*OT acts peripherally to induce the PRL secretory rhythm in OVX rats*

The icv injection of OT (0.3µg) had no effect on PRL levels in OVX rats (Fig. 1A). However, peripheral injection of OT (5µg) triggered a two-pulse per day PRL rhythm (Fig. 1B), as demonstrated previously (12). This suggests that OT acts peripherally, not centrally, in initiating this PRL rhythm. In another set of experiments an OT receptor antagonist (OTa) was infused peripherally for 24 h during the time of OT injection. In these animals the surges were completely blocked (Fig. 1B, $P < 0.001$ for the nocturnal surges and $P < 0.05$ for the diurnal surges), even after clearance of the antagonist. This suggests that a peripheral target of OT, possibly anterior pituitary lactotrophs, is necessary for triggering the OT-induced PRL rhythm.

*An acute release of PRL is not sufficient to trigger the PRL secretory rhythm*

The immediate blood sampling after the peripheral OT injection revealed an acute increase in the circulating concentration of PRL after 5min (Fig. 2A, $P < 0.05$). As expected, the PRL secretory rhythm was induced in these animals, with elevated PRL at 1700 h and 0300 h ($P < 0.05$). We repeated the same experimental design, but injecting TRH instead of OT, into another group of animals. TRH is known to evoke PRL release from lactotrophs (25; 26). The results are shown in Fig. 2B. Although TRH induced an acute release of PRL comparable to that observed after OT injection ($P < 0.05$), no increase of PRL concentration was observed in the subsequent blood samples. This suggests that the acute release of PRL does not mediate the OT-induced triggering of the PRL secretory rhythm.
Pelvic neurectomy blocks the OT-induced PRL rhythm

In a final series of experiments, resection of the pelvic nerve (shown in Fig. 3A) was performed. Successful pelvic neurectomy was assessed by comparing bladder size (Fig. 3B). Sham animals exhibited normal size bladders (left) while pelvic neurectomized animals presented distension of the bladder (right), due to interference with the micturition reflex. The results of peripheral OT injection to animals subjected to pelvic neurectomy are shown in Fig. 3C. OT administration at 1600 h in sham-operated animals increased the concentration of PRL at the expected times in subsequent days (Fig. 3C): 0300 h (P < 0.05) and 1700 h (P < 0.01) on day 2, and 0300 h on day 3 (P < 0.001). Bilateral transection of the pelvic nerves completely abolished the OT-induced increases of PRL occurring on subsequent days (Fig. 3C, P < 0.05 (day 2), and P < 0.001 (day 3)).

Discussion

We intended to clarify the mechanism by which a bolus injection of OT triggers a PRL secretory rhythm that mimics the rhythm observed after CS in OVX rats. Our first hypothesis was that OT could act centrally in order to induce this rhythm. This proposition was ruled out, since an icv injection of OT known to induce maternal behavior in virgin rats was ineffective in starting the rhythmic secretion of PRL. Therefore, it is very unlikely that the peripheral injection of OT acts at the CNS to trigger the PRL rhythm. Our results corroborate the fact that OT has poor blood-brain barrier permeability (24). In contrast, central OT release is known to be involved in several reproductive behaviors, including facilitation of the onset of sexual and maternal behavior (22). CS has been shown to alter OT receptor affinity and density in the medial preoptic area (9) and to increase the expression of FOS in OT neurons of the parvicellular PVN. These neurons release OT centrally and into the median eminence (38) and hence to the anterior pituitary through the long portal vessels, which very likely influences circadian PRL secretion. Thus, although our
results show that an icv injection of OT does not initiate a PRL rhythm, we must point out that a central role for OT on CS or mating-induced PRL surges is possible. In addition, our mathematical model also suggests that the CS-induced PRL rhythm requires activation of hypothalamic OT neurons (5).

Our next experiments confirmed that OT must be acting on a peripheral target to trigger the PRL rhythm, as peripheral infusion of an OT receptor antagonist completely blocked the occurrence of the OT-induced PRL surges. The surges were abolished even after the clearance of the antagonist, suggesting that the antagonist effectively prevented OT from triggering the memory of this rhythm. These results differ from our results in OVX rats subjected to CS, in which peripheral infusion of an OT antagonist initially abolished the CS-induced PRL surges that nonetheless returned after clearance of the drug (31). Thus the current evidence indicates that peripheral OT can trigger the rhythmic PRL surges, but is not necessary to trigger the CS-induced PRL rhythm.

Convincing evidence has been published for a stimulatory role of OT on PRL secretion in rats, and several reports have shown that PRL may gain access to the brain through a receptor-mediated mechanism (27; 29; 47). Even though evidence has been presented to support an immediate release of PRL after CS (7; 42) or mating (44), it is not yet clear that this PRL release is related to the incidence of pseudopregnancy or pregnancy. More recent studies show that the PRL levels are higher 1h after mating among females that become pseudopregnant than among those in which mating did not induce pseudopregnancy (15). Conversely, the diurnal surges and pseudopregnancy smears are observed in CS rats treated with ergocornine (a blocker of PRL release) (48), consistent with the early assumptions that activation of the corpora lutea of pregnancy or pseudopregnancy does not require a PRL surge within the first 12 h after mating, that occurs in delayed pseudopregnancy (2).

Given the requirement for a peripheral action of OT and its stimulatory effect on PRL, our next proposition was that OT might act directly on the lactotrophs, inducing PRL secretion
which in turn would act in the CNS to trigger its own rhythm. This hypothesis was based on our prior observations that central PRL injection initiated the circadian PRL rhythm (6; 21). If this hypothesis is true, 1) OT injection must result in acute PRL secretion, and 2) other factors that stimulate PRL secretion should also induce the PRL secretory rhythm. We tested this possibility in experiment 2. OT increased peripheral plasma PRL 5 min after its injection, and induced the PRL secretory rhythm on subsequent days, supporting preceding in vivo findings that peripheral administration of OT results in a rapid release of PRL (28). Injection of TRH in another group of animals also induced acute PRL secretion, similar to that induced by the OT injection. However, TRH did not elicit the PRL secretory rhythm on the following days, suggesting that the PRL increase observed after OT peripheral injection is not sufficient to trigger the PRL secretory rhythm. Alternatively, the acute PRL increase may act in concert with other peripheral effects of OT to trigger the memory and initiate the rhythm.

These results led us to the hypothesis that other peripheral targets of OT injection might be more relevant than the anterior pituitary lactotrophs to induce the PRL secretory rhythm. Systemic or peripheral administration of OT has been demonstrated to facilitate lordosis in steroid-treated OVX rats (1) and this facilitation is blocked after pelvic neurectomy (33). In female rats, the internal genitalia are innervated primarily by the hypogastric nerves (sympathetic) and pelvic nerves (sympathetic and parasympathetic) that terminate peripherally in the pelvic or uterine cervical ganglia (37). Visceral afferent fibers of the pelvic nerve are more sensitive than hypogastric nerve fibers to uterine and cervical mechanostimulation (4). Electrical stimulation of the pelvic nerve elicited contractions in both cervix and uterus (41) and increased intravaginal pressure (35). Accordingly, recordings from the pelvic nerve activity showed that it responds to mechanical stimulation of the vagina and to stretching of the cervix during parturition (37). Pelvic neurectomy blocks induction of pseudopregnancy (10), presumably by blocking the PRL increase after mating (44). The integrity of the pelvic nerve is also necessary for the pacing behavior during mating (14). Since the cervix and uterus are targets of peripheral OT, it is
reasonable to hypothesize that intravenous OT administration could result in increased pelvic nerve activity and neurotransmission to the CNS. Interestingly, the OT-induced facilitation of lordosis is blocked after removal of either the cervix or uterus, demonstrating that both are important components in the peripheral mechanism transmitting the signal to the CNS (32). Consistently with this, our results demonstrated that pelvic neurectomy also blocked the OT-induced PRL rhythmic secretion, suggesting that the pelvic nerves may relay the signals triggered by the OT injection to the CNS to elicit lordosis and rhythmic PRL secretion.

**Perspectives and Significance**

The mating stimulus induces a circadian PRL rhythm that rescues the corpora lutea of the rodent estrous cycle and prolongs their ability to secrete progesterone (19). This is critical for successful pregnancy and implantation. This rhythm of two surges per day can be induced by artificial cervical stimulation in the absence of gonadal steroids, which indicates that this reflex operates through a memory triggered independently of gonadal steroids. In this study we show that an OT injection can also initiate a circadian PRL rhythm through peripheral, not central, actions of OT. The peripheral signal is transmitted to the CNS through the pelvic nerve. In this regard, peripheral OT can cause contractions of the cervix and uterus which in turn may signal the CNS through the pelvic nerve, thus initiating the circadian PRL rhythm. This possibility would be consistent with our results and needs to be tested. An interesting question is to what extent the pathway for the response initiated by cervical stimulation and that initiated by OT injection overlap. Oxytocin is released into the plasma after CS, a response known as the Ferguson reflex in humans (34), so it is plausible that OT acts as the trigger for the CS-induced rhythm. However, the blockade of OT receptors abrogated the OT-induced surges, but not those induced by CS, indicating that the latter stimulus might activate additional receptor systems and sensory pathways to those targeted by OT. Thus, although the mechanisms for triggering the OT-
induced and the CS-induced PRL secretory rhythm may both involve the pelvic nerve, additional sensory afferents are likely utilized in the CS pathway. These additional elements, as well as the brain areas involved in both the CS and OT induction of the PRL rhythm, will be the focus of future experiments.

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**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the authors.

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Legends

Figure 1. Central (icv) vs peripheral (iv) effect of OT to induce the PRL secretory rhythm. (A) OVX rats were injected with OT (0.3 μg, n=12) or vehicle (n=7) icv at 1600 h of Day 0 (arrow). (B) OVX rats were injected iv with OT (5 μg) at 1600 h of Day 0 (arrow). In 13 rats the OT antagonist (OTa, 9 μg/h) was infused iv beginning around 1200 h of Day 0 for 24 h. Blood samples were withdrawn during the next 2 days to observe the PRL diurnal and nocturnal surges. Data are presented as mean ± S.E.M. #, P < 0.05 and ###, P < 0.001 vs. control group at the same time. *, P < 0.05, ***, P < 0.001 vs. 1:00 h of Day 2 PRL in the same experimental group.

Figure 2. Correlation between immediate PRL release and the PRL secretory rhythm. (A) OVX rats were injected with saline (n=8) or OT (n=9) iv at 1600h of Day 0 (arrow). Blood samples were taken immediately before and 5, and 10 min following peripheral injection and thereafter during the next 2 days to observe the PRL diurnal and nocturnal surges. (B) The same experimental design was repeated injecting rats with saline (n=8) or TRH (n=9). Data are presented as mean ± S.E.M. #, P < 0.05 and ##, P < 0.01 vs. control group at the same time. *, P < 0.05 vs. 1600 h PRL in the same experimental group.

Figure 3: The effect of pelvic neurectomy on the OT-induced PRL secretory rhythm. (A) Photograph showing the location of the pelvic nerve in a perfused rat. (B) Comparison between bladders from two sham-operated animals (left) and one from a pelvic neurectomized animal (right). (C) OVX rats had their pelvic nerve cut bilaterally (PEX, n=9) or were submitted to sham surgery (SHAM, n=12). After recovery, they were injected with OT iv at 16 00h of Day 0 (arrow). Blood samples were withdrawn immediately after the injection and during the next 2 days to observe the PRL diurnal and nocturnal surges. Data are presented as mean ± S.E.M. #, P
< 0.05 ##, P < 0.01 and ###, P < 0.001 vs. sham group at the same time. *, P < 0.05 and ***, P < 0.001 vs. 1600 h PRL in the same experimental group.
Figure 1

A

- Saline (7)
- OT icv (12)

B

- OT iv (11)
- OT iv + OTa iv (13)
Figure 3

A

B

C

1 cm

Day 0

OT

Day 2

Day 3

PEX (9)

Sham (12)

Prolactin (ng/mL)

Time (h x 10^{-2})

0

25

50

75

100