## REVIEW ARTICLE

# A Tale of Two Rhythms: The Emerging Roles of Oxytocin in Rhythmic Prolactin Release

R. Bertram,* C. V. Helena,† A. E. Gonzalez–Iglesias,† J. Tabak† and M. E. Freeman†

*Department of Mathematics and Programs in Neuroscience and Molecular Biophysics, Florida State University, Tallahassee, FL, USA.

†Department of Biological Science, Florida State University, Tallahassee, FL, USA.

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Hormone secretion often occurs in a pulsatile manner. In this review, we discuss two rhythms of *in vivo* prolactin release in female rats and the ongoing research that we and others have performed aiming to understand the mechanisms underlying them. The peptide hormone oxytocin appears to play an important role in both rhythms. One rhythm occurs during the first half of pregnancy, but can also be induced in ovariectomised rats. This is characterised by a circadian pattern with two prolactin surges per day. Two methods for triggering this rhythm are discussed, each utilising a unique physiological pathway that includes oxytocin action, presumably on pituitary lactotrophs. The second rhythm occurs during the oestrous cycle and is characterised by a surge of prolactin on the afternoon of pro-oestrus. We discuss recent findings that oxytocin is more effective at stimulating prolactin release from lactotrophs taken from animals on the afternoon of pro-oestrus than from those of animals on the morning of dioestrus 1, raising the possibility that this hormone plays a physiological role in the regulation of prolactin secretion during the oestrous cycle.

**Key words:** dopamine, oxytocin, prolactin.

Correspondence to:
Richard Bertram, Department of Mathematics, Florida State University, Tallahassee, FL, USA (e-mail: bertram@neuro.fsu.edu).

Prolactin is one of the most versatile hormones and its release from pituitary lactotrophs in female rats is stimulated by suckling and mating, and also occurs on the afternoon of pro-oestrus (1). The wide array of factors that contribute to the control of prolactin release have been reviewed (2). Suckling evokes a classic neuroendocrine response, in which prolactin release starts when the suckling begins and ends when the suckling stops. By contrast, mating evokes a prolactin response that lasts for 10 days, indicating that some type of 'memory' is activated by the stimulus. During pregnancy, this response is rhythmic, consisting of two prolactin surges per day, one in the morning (the nocturnal surge) and one in the afternoon (the diurnal surge). Likewise, prolactin released during the oestrous cycle is rhythmic, with a surge occurring every 4–5 days, on the afternoon of pro-oestrus. There is now evidence that the peptide hormone oxytocin is involved in both of these rhythmic behaviours. In this review, we provide an overview of recent work performed in our laboratory aiming to determine the role that oxytocin plays in rhythmic prolactin secretion.

### Rhythm 1: circadian prolactin rhythm induced by cervical stimulation

The circadian prolactin rhythm induced by cervical stimulation received during mating occurs during the first half of pregnancy in the female rat and is characterised by two surges per day (3, 4). The released prolactin is necessary to rescue the corpus luteum and maintain its ability to secrete progesterone for 10 days (1, 2). Subsequently, progesterone secretion is sustained for the remainder of the 20–22 days pregnancy by placental lactogens (5, 6). A similar prolactin rhythm, lasting up to 12 days, can be induced by artificial cervical stimultion in both intact and ovariectomised animals, demonstrating that ovarian steroids are not necessary for triggering or maintaining the prolactin rhythm (7). However, ovarian steroids do play a role in the termination of these surges in intact animals (1). Although it has been known for many years that the cervical stimulation-induced prolactin rhythm involves interactions between the hypothalamus and pituitary lactotrophs (8), questions regarding the mechanism for the initiation and maintenance of this rhythm have been hard to answer, and are largely unanswered even today. Three questions immediately come to mind: (i) How does cervical stimulation trigger the memory in ovariectomised rats? (ii) What is the memory? (iii) What are the elements required for the production of the prolactin rhythm that is maintained by the memory?

We have found that peripheral injection of oxytocin or central injection of ovine prolactin into ovariectomised rats can start the circadian prolactin rhythm (9, 10). Motivated by these findings, we investigated whether cervical stimulation was capable of producing a prolactin rhythm when either an oxytocin receptor antagonist or a

prolactin receptor antagonist was applied centrally (via i.c.v. infusion) during and/or after the cervical stimulation. Central infusion of the oxytocin receptor antagonist desGly-NH$_2$-d(CH$_2$)$_5$[D-Tyr$^2$,Thr$^4$]OVT into the lateral cerebral ventricle had little or no effect on the cervical stimulation-induced rhythm (C. Helena, unpublished observation), suggesting that central actions of oxytocin are not involved in the triggering of the memory and are not part of the rhythm mechanism. [In a different strain of rats, however, a direct injection of the oxytocin receptor antagonist into the ventrolateral region of the ventromedial hypothalamus inhibited the prolactin rhythm induced by mating, rather than cervical stimulation (11).] Central infusion of the prolactin receptor antagonist S179D inhibited the rhythm when the antagonist was present but, if the prolactin receptor antagonist was present only on the day of cervical stimulation, the prolactin rhythm was still produced (10). This suggests that the central action of prolactin is necessary for the production of the rhythm (i.e. the rhythm does not occur when a prolactin receptor antagonist is present), but is not required for triggering the memory (i.e. cervical stimulation induced a delayed prolactin rhythm even though central prolactin receptors were blocked at the time of stimulation). Thus, although it remains unclear how cervical stimulation triggers the memory that maintains the prolactin rhythm, these data argue against a role for central oxytocin or prolactin in triggering the memory for the rhythm.

The identity of the memory also remains elusive, although there has been recent progress. Some recent work suggests that the ventrolateral region of the ventromedial hypothalamic nucleus may be a component of the memory (11) and, because parvocellular neurones send projections to the ventromedial hypothalamus (12), the paraventricular nucleus may also be involved. Whatever the identity and location of the memory, one may ask whether it acts directly on anterior pituitary lactotrophs or whether it mediates its effect on prolactin secretion through modulation of dopaminergic neurone activities in the arcuate and periventricular nuclei of the hypothalamus. Dopamine released from these neurones is known to have a strong inhibitory action on lactotrophs (13).

The elements involved in the production of the prolactin rhythm have largely been identified. Hypothalamic dopamine is known to inhibit lactotroph activity via binding to D$_2$-type receptors (14). By contrast, prolactin from lactotrophs stimulates hypothalamic dopamine neurones (15) through specific receptors (16). Thus, prolactin indirectly inhibits its own release through activation of the dopamine neurones. *In vitro* studies in rat hypothalamic slices (17) and cell cultures from the mediobasal hypothalamus of the embryonic rat pup (18) have shown that prolactin application increases dopamine synthesis through phosphorylation-mediated up-regulation of tyrosine hydroxylase activity. This enzyme, which converts tyrosine into L-DOPA, is the rate-limiting enzyme in dopamine production, and these studies showed that the increase in catecholamine levels occurs within 1–2 h of prolactin application. Prolactin also increases the mRNA level for tyrosine hydroxylase, but this occurs on a slower time scale of 4 h (18). This slower time scale is similar to that observed in an *in vivo* study (19). Another *in vivo* study showed that dopamine neurone activity was elevated and serum prolactin levels were inhibited within 1 h of peripheral injection of ovine prolactin (15). These findings suggest an increase in

dopamine secretion in response to prolactin. (There was an additional effect, with increased dopamine neurone activity and decreased prolactin levels, 3 h after ovine prolactin injection.) Using mathematical modelling, we have demonstrated that the mutual feedback loop between lactotrophs and dopamine neurones, with a time delay, is capable of generating a two-pulse per day prolactin rhythm (20). A key prediction of the model is that dopamine neurone activity should peak at times between the prolactin surges. Because the ratio of DOPAC (3,4-dihydroxyphenylacetic acid, a metabolite of dopamine) to dopamine is an index of dopamine neuronal activity at the synaptic terminals (21, 22), we measured this ratio in the median eminence, the intermediate lobe, and the neural lobe of cervically-stimulated and ovariectomised rats to investigate this prediction. We found that the ratio peaked at noon in both the median eminence and the neural lobe, which is between the two prolactin surges and is consistent with the model prediction (23).

Although interactions between lactotrophs and dopamine neurones appear to be crucial for the prolactin rhythm, additional factors must be involved. Modelling suggests that vasoactive intestinal polypeptide-ergic neurones in the suprachiasmatic nucleus, which innervate (24) and inhibit dopamine neurones of the arcuate nucleus early in the morning in a circadian fashion (25, 26), are responsible for setting the time of the nocturnal surge and for making the nocturnal prolactin surge larger than the diurnal surge (20). Moreover, the fact that administration of vasoactive intestinal polypeptide antisense oligonucleotides in the suprachiasmatic nucleus altered the prolactin (and oxytocin) rhythms in cervically stimulated rats (27) demonstrates an important role for this input from the suprachiasmatic nucleus. Earlier work from our laboratory suggested a role for oxytocin in the regulation of prolactin surges in cervically stimulated rats (28). More recently, we demonstrated that peripheral infusion of an oxytocin receptor antagonist blocked the cervical stimulation-induced prolactin rhythm as long as this antagonist was present. However, once the oxytocin antagonist had cleared from the system 2 days after the end of the infusion, the prolactin rhythm appeared (23). Because the oxytocin antagonist does not cross the blood–brain barrier, this suggests that the action of oxytocin on the lactotrophs is a necessary element of the prolactin rhythm. These findings also indicate that peripheral oxytocin actions are not essential for the triggering of the memory because cervical stimulation was able to trigger the memory even in the presence of a peripheral oxytocin receptor antagonist (although the prolactin rhythm was not expressed until after the oxytocin antagonist was cleared from the system).

Figure 1 illustrates our proposed model for the cervical stimulation-induced prolactin rhythm. The prolactin rhythm is mediated primarily by the mutual interactions between lactotrophs and hypothalamic dopamine neurones (solid arrowhead indicates a stimulatory action, in this case with a delay of $\tau = 2$ h, and an open arrowhead indicates an inhibitory action). Lactotroph and dopamine neurone activity oscillate out of phase, so that the prolactin level is high when dopamine neurone activity is low and vice versa. The rhythm is facilitated by the stimulatory actions of oxytocin released from neurones of the paraventricular nucleus into the neural lobe of the pituitary (29) and into the median eminence (30). This
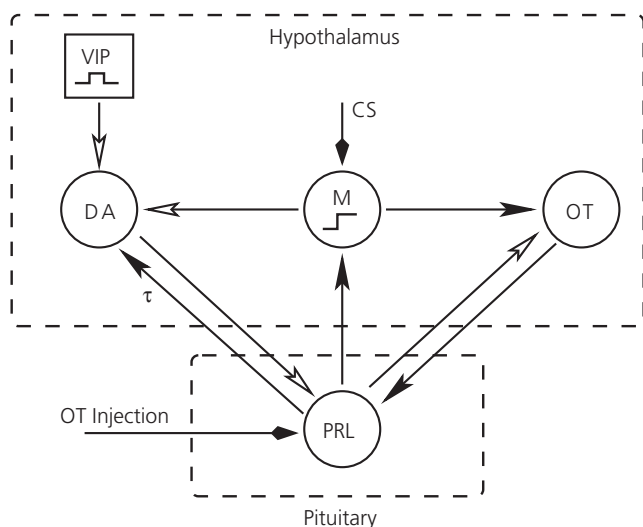
**Fig. 1.** Proposed model for the prolactin rhythm initiated by cervical stimulation (CS) or by oxytocin (OT) injection. Closed arrowheads indicate stimulatory actions, open arrowheads indicate inhibitory actions. The stimulation of dopamine (DA) neurones by prolactin (PRL) has a delay of $\tau$ hours. A pulse of vasoactive intestinal polypeptide (VIP) from suprachiasmatic neurones occurs every morning, setting the phase of the prolactin oscillation. The memory, M, is switched on by CS or by PRL.

**Fig. 2.** Numerical simulation of the mathematical model; for equations, see (39). (A) A prolactin (PRL) rhythm (solid) is initiated by cervical stimulation, as described in Fig. 1. The dopamine (DA) neurone activity (dashed) oscillates out of phase with prolactin levels. (B) The cervical stimulation (CS) activates oxytocin (OT) neurones of the paraventricular nucleus, which oscillate out of phase with prolactin levels as a result of the inhibitory influence of prolactin on the neurones. (C) The memory (M) is switched on by the cervical stimulation and stays on throughout the simulation. The variables PRL, DA and OT have all been normalised to facilitate comparison.

oxytocin has been shown to increase $Ca^{2+}$ levels (27) in lactotrophs and to stimulate prolactin secretion both *in vivo* (9, 31, 32) and *in vitro* (27, 33–35). The prolactin, in turn, has a rapid inhibitory action on oxytocin neurones of the supraoptic nucleus (36, 37) and the paraventricular nucleus (Sirzen-Zelenskaya A, unpublished data). Both of these actions are included in the model. The circadian inhibitory action of vasoactive intestinal polypeptide neurones of the suprachiasmatic nucleus onto dopamine neurones is also included. Finally, cervical stimulation activates a memory that maintains the prolactin rhythm. It does this by partially inhibiting the dopamine neurones and by stimulating oxytocin neurones. Evidence for the latter comes from experimental data, showing that peripheral oxytocin levels are elevated subsequent to cervical stimulation (9, 38). Evidence for an inhibitory effect of the memory on secretion from dopamine neurones comes from a mathematical modelling study (20). Here, we demonstrated that a direct stimulatory input to lactotrophs would lead to an elevated prolactin level, but would not initiate a rhythm. By contrast, partial inhibition of dopamine neurones both increased the mean prolactin level and, most importantly, induced a circadian prolactin rhythm. Thus, although we are still far from identifying the central location of 'the memory', we consider that progress has been made in understanding the mechanism through which it works.

When the descriptive model shown in Fig. 1 is translated into mathematical equations (20, 39), a simulated prolactin rhythm is produced (Fig. 2A, solid). In this simulation, the prolactin level is low prior to cervical stimulation. A rhythm is started by the stimulus, with two prolactin surges per day. The second, (diurnal) surge is smaller than the first, as is typically observed experimentally (23, 27). Along with the prolactin rhythm, there are rhythms in the
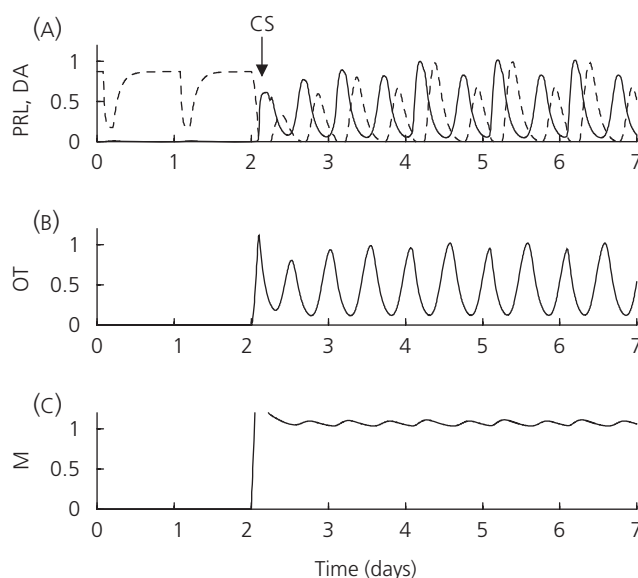
activities of dopamine neurones (Fig. 2A, dashed) and oxytocin neurones (Fig. 2B). These variables oscillate out of phase with the prolactin rhythm, peaking when the prolactin level is declining (in the case of dopamine) or at its nadir (in the case of oxytocin). This rhythm persists as long as the memory is on (Fig. 2C).

## Rhythm 1 (continued): circadian prolactin rhythm induced by peripheral oxytocin injection

It has been shown that oxytocin is released into the circulation following cervical stimulation in sheep (40), rats (38), and humans (41, 42). This motivated us to inject oxytocin peripherally into female ovariectomised rats to attempt to start the circadian prolactin rhythm. We found that oxytocin injection starts a prolactin rhythm that is very similar to that induced by cervical stimulation (9). That is, there are two prolactin surges per day, one in the morning and another in the afternoon, which continue for at least 7 days. Do cervical stimulation and peripheral oxytocin injection trigger the prolactin rhythm in the same way? To investigate this, we performed a series of experiments with oxytocin and prolactin receptor antagonists. The results of these experiments are shown in Fig. 3, with the mathematical model (for equations, see Helena C. V., unpublished data).

When the oxytocin receptor antagonist is administered peripherally during the cervical stimulation, the prolactin rhythm is initially inhibited. However, once the oxytocin receptor antagonist clears the
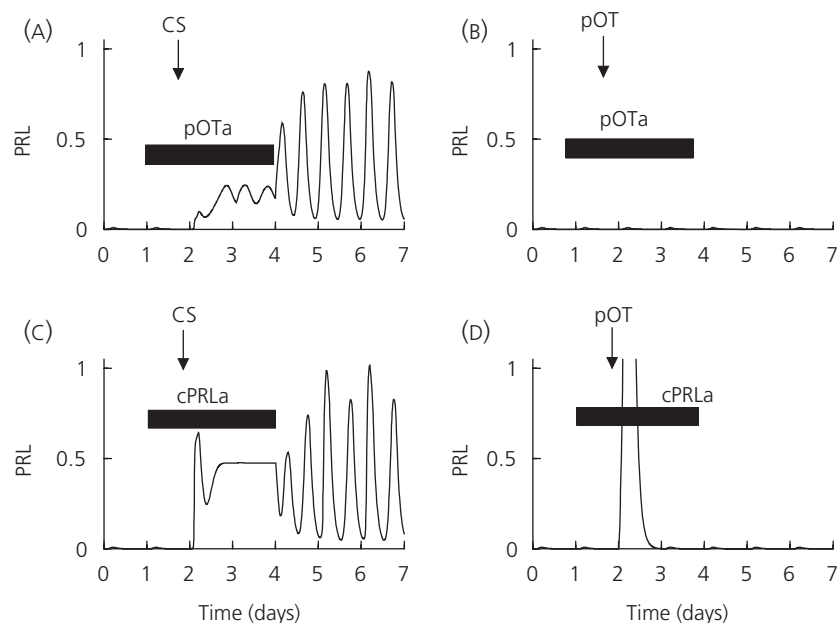
**Fig. 3.** Numerical simulation of the mathematical model illustrating the effects of two antagonists on the triggering of the memory for the biphasic circadian prolactin rhythm. (A), (C) Both peripheral oxytocin receptor antagonist (pOTa) and central prolactin antagonist (cPRLa) prevent the rhythm induced by cervical stimulation (CS) when the antagonist is present. However, the rhythm emerges once the antagonist has cleared from the system. (B), (D) The prolactin rhythm induced by peripheral oxytocin injection (pOT) is not triggered when the same antagonists are present during the injection.

system, a prolactin rhythm emerges (Fig. 3A). This was also demonstrated previously (23); the prolactin rhythm emerged 2 days after the cessation of the oxytocin receptor antagonist infusion. The explanation for this is that oxytocin stimulatory action on the lactotrophs is necessary for the rhythm to occur, but the trigger for the memory does not require peripheral actions of oxytocin. By contrast, when oxytocin is injected peripherally in the presence of a peripheral oxytocin receptor antagonist, there is little or no effect on prolactin levels (Fig. 3B), even after the oxytocin receptor antagonist has cleared the system (Helena C. V., unpublished data). This shows that the peripheral injection of oxytocin triggers the prolactin rhythm by acting peripherally, most likely on lactotrophs. This is in contrast to the way that cervical stimulation triggers the rhythm, which does not require peripheral actions of oxytocin.

How can peripheral oxytocin actions trigger the memory? One possibility is that the peripheral oxytocin injection stimulates lactotrophs sufficiently so that the dopamine inhibition is overcome and prolactin is released into the blood. The prolactin then crosses the blood–brain barrier, possibly through receptors in the choroid plexus (43, 44), and triggers the memory. [Indeed, peripheral injection of ovine prolactin at high concentration initiates the prolactin rhythm, bypassing oxytocin altogether (10).] A model simulation illustrating the critical role played by central prolactin is shown in Fig. 3(D), where a central prolactin receptor antagonist (cPRLa) is simulated. When peripheral oxytocin injection is simulated in the model, there is a single large surge of prolactin, due to the direct effect of oxytocin on lactotrophs. However, when a central prolactin receptor antagonist is present at the time of the oxytocin injection, no rhythm is initiated, even after the prolactin receptor antagonist is removed. This reflects the model hypothesis,

supported by experimental data (10), that central prolactin can trigger the memory (Fig. 1). When this link is impaired, by the central administration of a prolactin receptor antagonist, the prolactin released in response to the peripheral oxytocin injection cannot trigger the memory so, even after the prolactin receptor antagonist is cleared, there will be no prolactin rhythm. This model prediction (i.e. that peripheral oxytocin injection fails to initiate the prolactin rhythm when the prolactin receptor antagonist is infused centrally on the day of the oxytocin injection) has been experimentally confirmed in our laboratory (Helena C. V., unpublished data).

This result differs significantly from a similar experiment conducted on cervically stimulated rats (without oxytocin injection). Here, the cervical stimulation-induced prolactin rhythm was prevented when the central prolactin receptor antagonist infusion was maintained continuously. However, when it was infused only during the day of cervical stimulation, the prolactin rhythm started 2 days later (10). This suggests that cervical stimulation triggers the memory through a mechanism that does not utilise central actions of prolactin. This is illustrated by the model shown in Fig. 3(C). The cervical stimulation triggers the memory, thereby partially inhibiting dopamine neurones and stimulating oxytocin neurones. This results in an elevation of peripheral prolactin. However, while the central prolactin receptor antagonist is present, the positive feedback of prolactin onto the dopamine neurones is blocked, so the rhythm mechanism is dysfunctional. Once the prolactin receptor antagonist is cleared and the prolactin feedback onto dopamine neurones is restored, a prolactin rhythm is produced because all the required elements (triggered memory and a functional rhythm generating mechanism) have now been re-established.

From these experiments, it appears that, although both cervical stimulation and oxytocin injection can induce a biphasic circadian prolactin rhythm, the pathways through which they operate are different. The model illustrated in Fig. 1 is our current view of the interaction pathways whereby cervical stimulation and peripheral oxytocin (or prolactin) injection produce the prolactin rhythm. Although our understanding of the 'memory' and its location in the brain is very limited, the structure of the model is consistent with all of our recent data on cervically stimulated, ovariectomised rats (10, 23) and ovariectomised rats in which either oxytocin or prolactin is injected peripherally (9, 10) (see also Helena C. V., unpublished data). Furthermore, it clearly predicts that feedback interactions between pituitary lactotrophs and dopamine neurones are insufficient to account for mating-induced rhythmic prolactin release and establishes that additional interactions are required.

## Rhythm 2: prolactin rhythm induced by the ovarian cycle

While the first rhythm involving oxytocin does not require hormone steroids, the pro-oestrous afternoon surge is likely a result of the rhythmic variations in oestradiol levels during the oestrous cycle. Indeed, in response to a brief exposure to oestradiol, ovariectomised rats secrete daily surges of prolactin at times corresponding to that on pro-oestrus (45). This is largly a result of the decrease in the inhibitory influence of dopamine on lactotrophs mediated by the rise in oestradiol during the cycle (1, 2). Besides this reduction in inhibition, the pro-oestrous prolactin surge may be facilitated by a stage-specific increase in either the plasma level of a stimulating hormone, or in the responsiveness of lactotrophs to such a hormone, or both. It is well established that plasma oxytocin concentrations increase in response to oestradiol administration (46) and it has been demonstrated previously that the concentration of oxytocin in pituitary portal blood reaches a peak on the afternoon of pro-oestrus (47), following the peak oestradiol concentrations observed in the morning. Moreover, passive immunoneutralisation of endogenous oxytocin or peripheral administration of an oxytocin receptor

antagonist has been both shown to inhibit the prolactin surge in the afternoon of pro-oestrus (32, 48) as well as suckling-induced prolactin secretion (33, 49). Complementary to this, we have recently found that pro-oestrous lactotrophs are significantly more responsive to oxytocin than those obtained from dioestrus 1 (50). This constitutes a second rhythm, linked to the 4–5 day long oestrous cycle of female rats.

In our experiments, anterior pituitaries were obtained from cycling rats on the morning of dioestrus 1 or the afternoon of pro-oestrus. Oxytocin was then applied to the anterior pituitary cells (100 nM for 10 min) and the prolactin response measured using radioimmunoassay. As shown in Fig. 4(A), the prolactin response to oxytocin was much greater in cells harvested during pro-oestrus than in cells harvested during dioestrus 1. This effect was shown to be statistically significant when data were analysed from seven experiments from dioestrous cells, and ten experiments from pro-oestrous cells (50). The enhanced prolactin secretion on pro-oestrus corresponded to an enhanced intracellular $Ca^{2+}$ response from lactotrophs (Fig. 4B). Indeed, both the number of lactotroph cells showing a $Ca^{2+}$ response to oxytocin and the single-cell response were greater on pro-oestrus than on dioestrus 1 (50). This agrees with the observation that oxytocin receptors are up-regulated by oestradiol in the anterior pituitary as shown by our laboratory (49) and others (51). In addition to receptor up-regulation, it is likely that ovarian steroids have additional effects because the dose-response curve of the prolactin-releasing effect of oxytocin was left-shifted in lactotrophs obtained from pro-oestrous versus dioestrous rats. Receptor up-regulation alone would have no effect on the half-maximal location of the curve. Thus, it is possible that the enhanced oxytocin response on pro-oestrus is the result of an increase of anterior pituitary oxytocin receptor expression and signalling produced by the elevated oestradiol levels present in the preovulatory phase of the oestrous cycle.

These data show a rhythmic variation in the responsiveness of lactotrophs to oxytocin during the oestrous cycle. The increased responsiveness on pro-oestrus follows a common theme in oxytocin signalling, where the actions of oxytocin are typically preceded by an increase in responsiveness of the target tissue. For example,
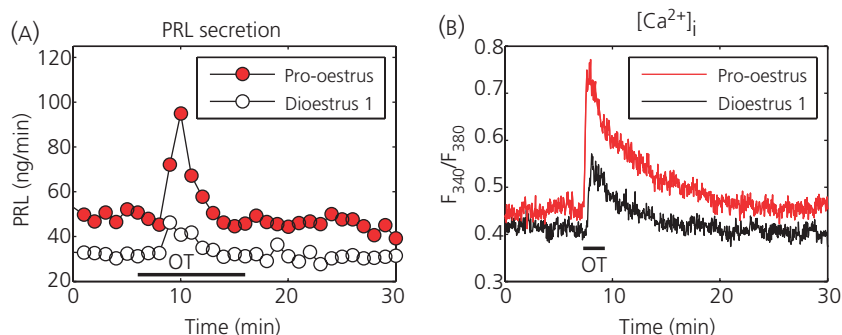


**Fig. 4.** Comparison of the oxytocin (OT)-induced responses in perfused lactotrophs obtained from female rats on the morning of dioestrus 1 (open symbols) and the afternoon of pro-oestrus (closed red symbols). (A) Oxytocin application (100 nM for 10 min) evoked a larger prolactin (PRL)-releasing effect from pro-oestrus cells. Samples were collected every minute. (B) Oxytocin application (100 nM for 2 min) elicited a larger increase in the free intracellular $Ca^{2+}$ concentration, as measured by the fura-2 fluorescence ratio. Details and a full description of experimental procedures are provided elsewhere (50).

myometrial cells of the uterus are sensitised to the contractile effect of oxytocin immediately prior to parturition (52, 53). In the mammary gland, there is an increase in the number of oxytocin binding sites throughout gestation that remains during the period of lactation (54). Thus, the finding that the oxytocin action on lactotrophs is magnified at the time point when such enhancement is needed is consistent with a physiological role for oxytocin in the regulation of prolactin secretion.

## Summary

As is the case with many hormones, prolactin secretion is often rhythmic. The particular rhythm depends on the physiological state of the animal, and the factors mediating one rhythm could be different from those mediating others. However, oxytocin and dopamine appear to be factors involved in most (if not all) of the rhythms. Much work remains to be carried out with respect to the identification of the 'memory' involved in the circadian prolactin rhythms initiated by mating, cervical stimulation or peripheral oxytocin/prolactin injection. This memory is also likely responsible for the phenomenon of 'delayed pseudopregnancy' in the rat, in which mating (55), electrical stimulation of the cervix (56), or electrical stimulation of the hypothalamus (57) early in the oestrous cycle leads to a circadian prolactin rhythm only after ovulation occurs at the end of the oestrous cycle. In addition to the systems-level question of the memory, important cellular questions remain. For example, the intracellular signalling pathway involved in the oxytocin stimulation of prolactin release is largely unknown, as is the mechanism through which this is magnified on pro-oestrus. We hope that the many questions that remain will be answered in the near future.

## Acknowledgements

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