Ovulation triggers oxytocin gene expression in the bovine ovary

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The gene for the nonapeptide neurohormone oxytocin is highly expressed in the bovine corpus luteum. Measurements of oxytocin-specific mRNA through the oestrous cycle of non-pregnant cows show that transcription is maximal accompanying ovulation and decreases rapidly thereafter. In contrast, immunohistochemistry shows neurophysin peptide levels to be greatest at mid-cycle. Low levels of oxytocin mRNA are detected in follicles and in the luteolytic half of the cycle. This mRNA is virtually absent in the corpus luteum of pregnant cattle. No cyclicity is evident in hypothalamic oxytocin mRNA levels.

Oxytocin *Neurophysin* *Corpus luteum* *Estrous cycle* *Gene expression*

1. INTRODUCTION

The biosynthetic pathway for the hormone oxytocin has been clarified in the mammalian hypothalamus. A single gene comprising 3 exons encodes a precursor polyprotein of approx. 12800 Da; oxytocin immediately follows the N-terminal signal sequence, the remainder of the molecule being occupied by neurophysin I, a polypeptide thought to act as a carrier for oxytocin during its axonal transport from the hypothalamus to the nerve endings of the posterior pituitary [1]. Hypothalamic oxytocin appears to be involved in the milk ejection reflex and in uterine contraction accompanying parturition. Recently, reports have suggested the presence of oxytocin in other peripheral tissues, where it may subserve quite different functions [2–4]. So far confirmation by sequence analysis of extra-hypothalamic oxytocin synthesis has only been made for the bovine corpus luteum [5]. cDNA and primer extension sequencing indicate that the hypothalamic and luteal oxytocin mRNAs are identical, except for a shorter poly(A) tail in the latter molecule [5,6]. Here, we show that the expression of the oxytocin gene in the bovine corpus luteum occurs as a single large pulse correlating with ovulation and the initial luteinizing stimuli. In contrast, oxytocin gene expression in the hypothalamus exhibits no variation with the oestrous cycle.

2. EXPERIMENTAL

2.1. *Preparation of RNA*

Corpora lutea were dissected from the ovaries of slaughtered cattle within 20 min of exsanguination and divided into portions. One portion was immediately frozen in liquid nitrogen, to be later crushed and directly homogenized in 5–10 vols of 4 M guanidinium thiocyanate/1 M β-mercaptoethanol. Total RNA was isolated from the homogenate by centrifugation through a 5.7 M CsCl cushion [7], followed by 2 ethanol precipitations. Controls indicated >70% recovery of RNA using this procedure, and comparison with an alternative technique using phenol/chloroform extraction [5] showed no difference either in total RNA yield or in proportion of oxytocin message. The remaining portions of corpora lutea were processed for immunohistochemical analysis as in the legend to fig.2.

Follicles were punctured to remove follicular

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fluid before weighing and freezing in liquid nitrogen prior to extraction. Hypothalami were dissected to include ~3 g tissue containing the paraventricular, supraoptic and suprachiasmatic nuclei and extending anteriorly into the preoptic area and posteriorly as far as the mammillary bodies. Corpora lutea from pregnant cows were obtained from a commercial abattoir; measurements of foetal crown-rump length indicated pregnancy stages from 50 to 240 days.

2.2. *Dot blot procedure*

10 μg of the extracted total RNA was then spotted onto nitrocellulose filters in formaldehyde solution [5] and hybridized at 60°C in 0.72 M NaCl, 40 mM Na-phosphate, pH 7.0, 4 mM EDTA, 0.1% (w/v) SDS, 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinylpyrrolidone-40, 0.02% bovine serum albumin, 25 μg/ml denatured herring sperm DNA, with 10^6 cpm/ml ($>10^8$ cpm/μg) of a ^{32}P nick-translated DNA fragment (*Pst*I-*Pst*I) comprising ~190 bp from the 3′-end of the hypothalamic oxytocin cDNA [8]. This probe included no plasmid and only 12 adenosines of the poly(A) tail, and was highly specific for the bovine oxytocin gene. Autoradiograms of dot blots were densitometrically quantitated and compared with a serial dilution of oxytocin cDNA blotted with the RNA as internal standard. Negative controls were provided by bovine muscle and cerebellum RNA preparations. As a further control for the non-selectiveness of the extraction procedure, the filters were rehybridized with a chick β-actin probe which cross-reacted with the bovine gene product. The resulting autoradiograms showed that this structural gene was present in an approximately constant proportion throughout the oestrous cycle (not shown).

3. RESULTS AND DISCUSSION

Unlike the hypothalamus, the corpus luteum is an ephemeral, steroid-producing tissue whose hormonal expression is entirely dependent on the endocrine status of the animal. It therefore appeared likely that the corpus luteum may express the oxytocin gene in a manner also reflecting the influence of the hormonal environment, i.e. the oestrous cycle.

Although preliminary data indicated that the highest level of oxytocin mRNA is present in the first half of the oestrous cycle ([5]; unpublished), it was not possible to correlate precisely the onset of oxytocin gene expression with the stage of the oestrous cycle. This can be explained in part by the subjective assessment of the oestrous status of commercial abattoir animals and the irregularity of the cycle within individual animals, some entering the luteotrophic or luteolytic phases at markedly different rates.

To alleviate some of these difficulties, a laboratory herd of Angus and Angus cross-bred cows were slaughtered at precise dates within their oestrous cycles. Oestrous (day 0) was determined by mounting behaviour and later confirmed by the state of vaginal mucus, subjective appearance of the ovary and plasma progesterone levels at time of exsanguination. Corpora lutea, corpora albicanta and follicles from all stages of the oestrous cycle, as well as hypothalami of the same animals, were extracted for RNA using the guanidinium thiocyanate procedure (see legend to table 1). Total RNA from individual corpora lutea of various stages in the oestrous cycle, as well as from animals through pregnancy was then spotted onto nitrocellulose and hybridized with a ^{32}P-labelled DNA probe derived from the 3′-end of the cDNA and specific for the oxytocin mRNA. Densitometric analysis of these dot blots yielded the data in table 1 and fig.1, presented as oxytocin cDNA equivalents. In a parallel set of experiments the tissue was immunohistochemically analyzed using neurophysin I antibodies.

The results show that oxytocin mRNA is already detectable at a low level in the mid-cycle follicles. On ovulation, however, there is not only a burst of total RNA production concomitant with the growth of the luteinizing tissue, but also a dramatic rise in the specific proportion of oxytocin mRNA. Maximum proportions are reached around day 3 and then decline swiftly, so that from day 11 onwards only very low levels are detectable. In contrast, all hypothalamic measurements remained around a constant value of ~1 ng oxytocin cDNA equivalent per hypothalamus and showed no variation dependent on oestrous status.

Fig.1 expresses the levels of oxytocin mRNA in relation to total RNA thereby serving as an index of transcription and does not take into account growth, proliferation and demise of the corpus

Table 1

Oxytocin mRNA levels (means ± SD) in follicles, corpora lutea and hypothalami of Angus and Angus cross-bred cattle

Stage of cycle	*n*	Follicular or luteal size (g)	Total RNA (μg/g tissue)	Oxytocin mRNA		
				(pg/10 μg RNA)	(ng/g tissue)	(ng/organ)
Follicles						
Mid-cycle	2	0.62 ± 0.13	104 ± 9	10.0 ± 4.7	0.10 ± 0.04	0.06 ± 0.03
Day 1	5	0.25 ± 0.18	278 ± 179	9.4 ± 2.3	0.26 ± 0.17	0.05 ± 0.02
Day 0	4	0.52 ± 0.46	107 ± 54	16.1 ± 10.7	0.12 ± 0.06	0.06 ± 0.04
Corpora lutea						
Day 1	3	0.39 ± 0.02	954 ± 294	131.3 ± 3.5	12.58 ± 4.18	4.92 ± 1.42
Day 3	4	0.68 ± 0.34	2204 ± 1039	154.8 ± 42.7	36.91 ± 27.67	19.55 ± 8.10
Day 7	4	3.54 ± 0.95	1397 ± 714	50.0 ± 48.1	6.65 ± 6.33	21.37 ± 18.52
Day 11	3	3.77 ± 0.76	1989 ± 411	9.6 ± 8.5	1.90 ± 1.82	7.83 ± 7.63
Day 14	4	4.30 ± 1.42	2296 ± 306	10.4 ± 3.3	1.90 ± 1.58	6.67 ± 5.37
Day 17	2	3.33 ± 1.89	1118 ± 627	10.3 ± 3.3	1.27 ± 1.03	4.46 ± 5.38
Day 19	4	3.07 ± 0.87	1348 ± 1027	11.5 ± 5.2	1.51 ± 1.56	3.54 ± 3.08
Day 21	2	2.05 ± 0.01	127 ± 139	20.7 ± 7.6	0.24 ± 0.23	0.50 ± 0.48
Day 22	5	0.91 ± 0.37	185 ± 87	7.1 ± 2.7	0.09 ± 0.02	0.10 ± 0.05
Day 24	3	0.83 ± 0.31	955 ± 628	< 1.0	<0.05	<0.03
Pregnancy	9	5.80 ± 1.80	636 ± 467	1.3 ± 0.9	0.05 ± 0.03	0.28 ± 0.09
Hypothalamus	21		280 ± 60	12.4 ± 2.9	0.35 ± 0.13	1.10 ± 0.39

The value for one corpus albicans (see legend to fig.1) was extraordinarily high and excluded from this analysis. No significant trend was evident in luteal oxytocin mRNA levels from pregnant cattle

luteum through the cycle. The endocrinological state of the organ is expressed more effectively as total oxytocin mRNA per whole organ (table 1). Maxima of total oxytocin mRNA are reached between days 3 and 7 and decline more gradually, such that oxytocin message is still detectable, albeit at a low level, also in the luteolytic half of the cycle.

In general, corpora albicanta as well as corpora lutea from pregnant animals have very low or undetectable amounts of oxytocin mRNA. An exception is suggested by one corpus albicans from day 24 of its cycle, i.e. day 3 of the new cycle, with a very high level of oxytocin message (fig.1). This may illustrate the ephemeral recovery of a corpus luteum under the influence of ovulatory stimuli.

Significantly, levels of oxytocin peptide within the corpus luteum remain low, similar to those in follicles, until about day 3, after which there is an increase to a peak on days 8–13 [9], several days after the maximum in oxytocin mRNA level. Similarly, immunohistochemical analysis using neurophysin antibodies showed that, although luteal cell bodies were very weakly stained on day 3 (not shown), optimal staining was not obtained until day 11 (fig.2). These findings suggest that translation may not correlate with the highest levels of oxytocin mRNA and/or that processing of the common precursor may be delayed. This would reflect the physiological requirement for the hormone, which appears to be released in vivo towards the luteolytic half of the cycle.

Although clearly connected with ovulation the factor(s) triggering transcription of the oxytocin gene are at present unknown. The marked oxytocin mRNA production of day 1 is anticipated by a small but clear increase of oxytocin mRNA in 2 out of 4 preovulatory follicles from the day of oestrous (day 0), suggesting that follicle rupture alone may not be the signal. Release of the oxytocin peptide takes place mainly from day 7 onwards until oestrous, resulting in a cyclic pattern of

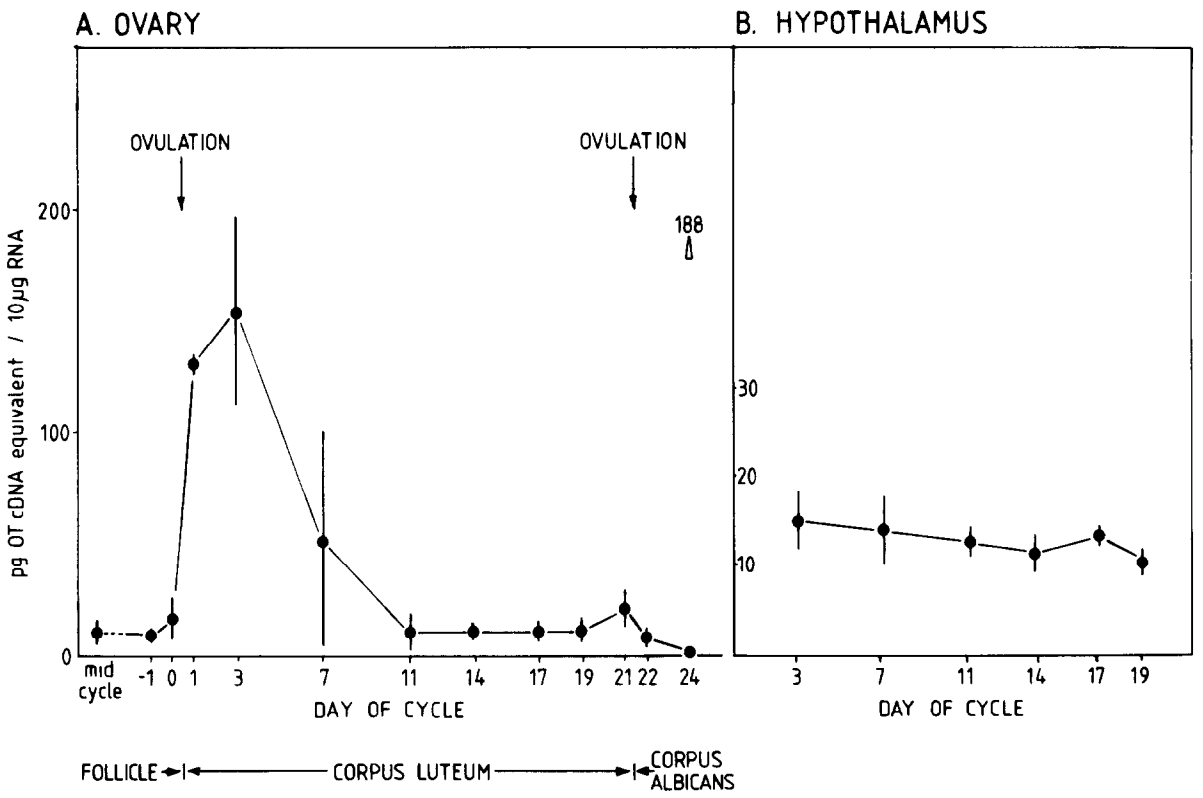

Fig.1. Follicular/luteal (A) and hypothalamic (B) levels of oxytocin mRNA through the oestrous cycle of the non-pregnant cow. Data are derived from table 1. One value appeared not to fit the general trend (small arrow): a corpus albicans from day 24 had a very high oxytocin mRNA proportion (188 pg cDNA equiv./10 μg RNA).

oxytocin and neurophysin in the peripheral serum [9]. This released oxytocin has been functionally linked to the luteolytic role of prostaglandin $F_{2\alpha}$ in determining the lifespan of the corpus luteum [10]. However, since oxytocin mRNA is produced maximally at ovulation, the factors influencing transcription must be removed from those involved in luteolysis per se. Furthermore, the low level of oxytocin mRNA in the luteolytic phase also implies that stores of the nonapeptide once depleted probably cannot be regenerated.

Taken together, these results show that specific transcription of the oxytocin gene occurs at the time of ovulation and is shut off again shortly thereafter. The absence of oestrous variation in hypothalamic oxytocin gene expression emphasizes the independent nature of the 2 expression systems in terms of their regulatory control. Such control must therefore reside within the immediate cellular environment, probably involving differential cell-specific receptor systems.

The pattern of oxytocin gene expression in the bovine ovary parallels close the fate of the granulosa-derived large luteal cells, which appear to predominate in the early luteotrophic half of the oestrous cycle [11]. It is primarily large luteal cells that contain membrane-bound secretory granules [12], presumably the mechanism by which endocrine tissues secrete peptidyl hormones. In addition, only the large luteal cell produced oxytocin in vitro [13,14]. The immunohistochemical analysis also clearly shows neurophysin immunoreactivity only in the large luteal cells of presumed granulosa origin, the small cells remaining unstained (fig.2).

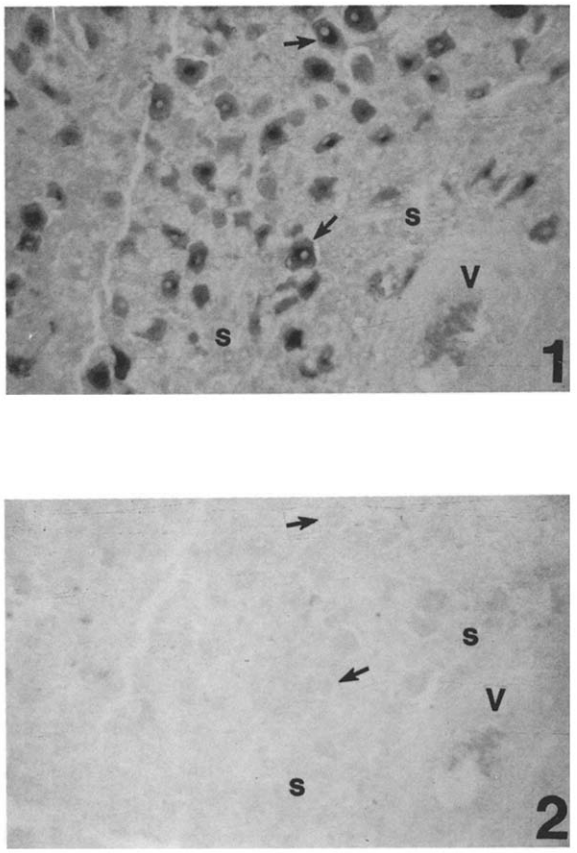

Fig.2. Neurophysin immunoreactivity in day 11 corpora lutea. (1) Non-pregnant cow corpora lutea (day 11) incubated with rabbit anti-porcine oxytocin-neurophysin serum (1:5000) and stained with the Vectastain ABC reagents (avidin-biotin-horseradish peroxidase; Vector Laboratories, Burlinghame, CA). Parallel sections were stained with haematoxylin and eosin to determine cell types. The small luteal cells (s) do not stain. Blood vessel (v). 353 ×. (2) Serial section of (1) incubated with rabbit anti-oxytocin-neurophysin serum that was absorbed with bovine oxytocin-neurophysin (50 μg/ml) prior to staining. Large luteal cells (arrows) do not stain. Blood vessel (v). 353 ×. A further control using rabbit pre-immune serum in place of anti-neurophysin, gave similar results (not shown).

Within the follicle it is the granulosa cells, rather than the theca, which are capable of synthesizing oxytocin and its mRNA ([9]; unpublished). It is therefore tempting to postulate a regulatory link between this cell type and expression of the nonapeptide gene.

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