THE EFFECT OF PROGESTERONE ON OXYTOCIN MESSENGER RNA IN HYPOTHALAMIC NEURONS OF ESTROGEN-TREATED FEMALE RATS STUDIED WITH QUANTITATIVE *IN SITU* HYBRIDIZATION HISTOCHEMISTRY

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ABSTRACT

Using *in situ* hybridization histochemistry with tritiated synthetic oligodeoxynucleotide probes and a computer-assisted image analysis system, we examined possible progesterone effects on oxytocin messenger RNA (mRNA) in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the female estrogen-primed rat. Conditions of combined estrogen and progesterone exposures were used to address questions of relevance to the combined effect of these two hormones on female reproductive behavior. Hybridization signals for oxytocin mRNA were shown by control maneuvers to be quite specific, and were observed in neurons of the dorsolateral part of the SON and in the peripheral part of the PVN. Statistic analysis of the Student *t*-test and Kolmogorov-Smirnov test showed that progesterone treatment of estrogen-primed ovariectomized female rats did not affect hybridization for oxytocin mRNA in neurons of the SON, whereas prolonged progesterone exposure did significantly increase the number of silver grains in neurons of the PVN. Among the subdivisions of the PVN, the posterior ventromedial part showed a particularly strong increase in oxytocin mRNA levels. These results suggest that progesterone, working in concert with estrogen priming, has an effect on oxytocin mRNA levels in that portion of the PVN, which might be relevant for the control of female reproductive behavior.

Oxytocin is synthesized in neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus, whose most predominant projections are to the neurohypophysis, where it is released from nerve terminals into the blood stream as a hormone which induces uterine contractions during labor and milk ejection (3, 17, 31). In addition to this hypothalamo-neurohypophyseal system, oxytocin-containing neurons in particular subnuclei of the PVN and accessory magnocellular nuclei in the hypothalamus send their axons to other brain regions, eliciting central neural effects important, at least, for female repro-

ductive and maternal behaviors (7, 8, 10, 14-16, 33, 46).

It has been demonstrated that the ovarian steroid, estrogen, can have multiple effects on oxytocin-producing neurons: estrogen enhances the electrical activity of the oxytocinergic neurons (1, 30), stimulates the release of oxytocin into the blood stream and also into the cerebrospinal fluid (50), changes the amount of oxytocin-immunoreactivity in the neuronal cell bodies and in terminal areas in both hypothalamic and extrahypothalamic sites (8, 36). Some effects may be due to hormonal action at the mRNA level, since in neurons

Fig. 1 Protocol of estrogen and progesterone treatments. At 12:00 AM of day 1, estradiol benzoate was subcutaneously (s.c.) injected. At 10:00 AM of day 2, before estrogen treatment alone would be adequate for reproductive behavior or LH surge, control rats were sacrificed and tissue processed for *in situ* hybridization. At 12:00 AM of day 2, remaining rat groups had estradiol benzoate injected. At 10:00 AM of day 3, progesterone was injected and after that rats were sacrificed for hybridization histochemistry at 2:00 PM and 7:00 PM.

as in peripheral tissues, steroid hormones bind receptor molecules which interact specifically with DNA sequences upstream of regulated genes to influence the expression of those genes (28). Ultrastructural studies and experiments using *in situ* hybridization in the hypothalamic ventromedial nucleus, where estrogen and progesterone receptors are profuse, show that gene expression and protein synthesis are regulated in a biologically significant way (13, 20). Indeed, estrogen induces the appearance of progesterone receptors in neurons of the hypothalamus, increasing the level of receptors such that estrogen and progesterone can act synergistically in hypothalamic neurons (4, 21, 26, 29, 41); and this effect on progesterone binding is due to a recruitment of neurons which express the gene for the progesterone receptor (40).

Since female reproductive behavior in rats depends on the synergistic actions of estrogens followed by progesterone, the question arises as to whether progesterone can influence oxytocin mRNA levels in estrogen-primed rats. In the present study we examined changes of oxytocin mRNA in neurons of the SON and PVN of the hypothalamus by the effect of estrogen and/or progesterone treatment with the use of *in situ* hybridization histochemistry in conjunction with cell-by-cell resolution analysis.

MATERIALS AND METHODS

Animals and Treatments

Adult female Sprague-Dawley rats (200–250 g)

were kept under controlled lighting conditions, and food and water were available *ad libitum*. Ten to 14 days after ovariectomy, the animals received subcutaneous injections of estradiol benzoate followed by progesterone in sesame oil. The injection protocol is summarized in Fig. 1. The first group ($n=3$) was only administered estrogen, whereas the second ($n=3$) and third ($n=3$) groups were treated with both estrogen and progesterone. At designated time points (Fig. 1), rats from each experimental group were decapitated and the brains were rapidly removed from the skull onto a chilled glass plate. The brain blocks were frozen by immersion in chlorodifluoromethane, chilled by liquid nitrogen. Cryostat sections were cut at 6 μ m-thickness in the coronal plane, thaw mounted on to gelatin chrome alum-subbed slides, and dried at 37°C for at least 1 h before fixation.

In Situ Hybridization

The hybridization procedure is described in previous papers (22–24, 27). In brief, the sections were fixed by immersion in ethanol-acetic acid (3:1, v/v) for 15 min, followed by placing the slides (30 min, 70°C), in a solution of $2\times$ SSC (0.3 M sodium chloride/0.03 M sodium citrate, titrated to pH 3.5 with acetic acid) containing diethylpyrocarbonate (20 μ l/100 ml), and in a solution of $2\times$ SSC containing pepsin (1 mg/ml) for 15 min at 37°C. The slides were then washed in solution of $0.2\times$ SSC for 1 min and in a solution of 5 mM dithiothreitol in autoclaved water. Following a 2-h incubation with a prehybridization-formamide solution, heat-denatured hybridization buffer containing tritiated oxytocin oligodeoxynucleotide probe (20,000–50,000 cpm/20 μ l) was applied to the sections for 24 h at room temperature. After hybridization, the sections were rinsed in $2\times$ SSC for 10 min, and washed in $0.5\times$ SSC for 48 h at room temperature. The slides were dehydrated in successive solutions of ammonium acetate/ethanol, and dried at room temperature.

Autoradiography

The sections which were to be processed for emulsion autoradiography were dipped in Kodak NTB-3 emulsion and allowed to expose in light-tight boxes for 4 weeks. At the appropriate exposure time, as determined by test slides, all slides were photodeveloped with Kodak D19 developer and fixed by Fuji

Fixer. Sections were stained with cresyl violet, dehydrated, cleared and coverslipped.

Probe

The oligodeoxynucleotide probe, 5' CTCGGAGA-AGGCAGACTCAGGGTCG 3', generated on a DNA synthesizer (Applied Biosystems Incorporations, Type 381A), was complementary to bases 912 to 936 of the oxytocin gene (23, 47). The three-prime (3') end of the probe was labeled with [³H]deoxycytidine triphosphate (1.48–2.22 TBq/mmol, Amersham, Buckinghamshire, U.K.) by incubation with terminal deoxynucleotidyl transferase. The labeled probe was separated from free unlabeled mononucleotide by Nensorb column (Du Pont, Boston, U.S.A.). See Kawata *et al.* (22, 24) for details.

Specificity Controls

To test for potential positive chemographic artifacts, some sections were hybridized without probe. Several sections received pretreatment with RNase (Sigma, 20 µg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) for 30 min at 37°C before hybridization was carried out. To test the specificity of the hybridization reaction, some sections were hybridized with 25-fold excess of unlabeled probe before the application of labeled probe. Finally, some adjacent sections were used for immunohistochemical staining with oxytocin-antiserum (Incstar, Sillwater, U.S.A.).

Quantification of Silver Grains

Approximately twenty glass slides were analyzed in each group. Each glass slide contained two sections, therefore almost forty sections were measured in each experimental group. Neurons which were analyzed by the Bioquant system were selected to meet the criteria for having nucleoli in their perikaryon. The analysis of the changes between each group was performed blind.

The number of silver grains per neuron was quantitatively analyzed with the aid of a computerized image analysis system (Bioquant system). Individual neurons were drawn using a camera lucida, and pixel number as a reflection of grain quantity over individual labeled neurons was recorded on the drawings. To achieve the criterion for a neuron to be classified as labeled, a cell had to

have at least five times as many silver grains over the cytoplasm as were found over a similar size region of surrounding neuropil (24, 41). Statistical analysis of the data was accomplished using Student *t*-test and Kolmogorov-Smirnov test.

RESULTS

Clusters of silver grains were seen in the cytoplasm of magnocellular neurons, predominantly in the dorsolateral part of the SON and near the periphery of the PVN, when using the labeled oxytocin oligodeoxynucleotide probe (Fig. 2). Some neurons adjacent to blood vessels, located between the SON and the PVN, also showed positive hybridization signals. Incubation of sections in hybridization buffer without the tritiated probe did not yield any clusters of silver grains over neurons. Similarly, pretreatment of sections with the enzyme RNase completely eliminated silver grain clusters in the autoradiograms, indicating that the positive hybridization signal was due to binding of the oligodeoxynucleotide probe to tissue RNAs. Prior incubation of the sections with a 25-fold excess of unlabeled oligodeoxynucleotide probe greatly reduced hybridization signal, thus showing the expected limited-capacity binding of the oligodeoxynucleotide probe to oxytocin mRNA. Finally, immunohistochemical studies have been performed to confirm that oxytocin-expressing neurons under our conditions are seen predominantly in the dorsolateral part of the SON and near the periphery of the PVN, in addition to their occurrence around blood vessels between these two magnocellular nuclear groups. All of these controls indicate the validity of the oxytocin hybridization results in these experiments.

Quantitative analysis revealed that in the dorsolateral part of the SON, the average number of pixels per labeled neuron showed no significant differences between groups sacrificed at 10:00 AM (estrogen only), 2:00 PM (4 h exposure to progesterone) and 7:00 PM (9 h exposure to progesterone) (by *t*-test) (Fig. 3). There was no intra-group variability. Likewise, in the SON, the distribution of labeled neurons within the nucleus and their proportion in the cellular population were not different between treatment groups: neurons with positive hybridization signal were always observed in the dorsolateral part of this cell group. The frequency distribution histogram from the dorsolateral part of the SON was plotted according to treat-

Fig. 2 Dark-field photomicrographs of the PVN (a) and SON (b) showing positive hybridization results as indicated by clusters of grains. Oxytocin-expressing cells were easily detected. $\times 130$

ment group and the Kolmogorov-Smirnov statistical analysis of this distribution indicated no significant differences in the SON (Fig. 4).

In the PVN, the distribution of neurons with a positive hybridization signal for oxytocin expression was similar among the experimental groups sacrificed at 10:00 AM (estrogen only), 2:00 PM (4 h exposure to progesterone) and 7:00 PM (9 h exposure to progesterone) (Fig. 5). However, in the

PVN, the group sacrificed at 7:00 PM (9 h exposure to progesterone) had a significant increase in the average number of pixels per neuron ($P < 0.001$, by *t*-test), compared to the 10:00 AM and 2:00 PM groups (Fig. 3). Analyzing the frequency distribution histogram of pixels per neuron (Fig. 4), the Kolmogorov-Smirnov analysis of the distribution revealed a significant shift towards higher values in the 7:00 PM group (9 h exposure to progester-

one), compared to the animals sacrificed at 10:00 AM or 2:00 PM ($P < 0.001$).

Within the PVN, by visual inspection it appeared that the increases in the numbers of pixels per neuron were particularly strong in the posterior ventromedial part of this cell group, among the neurons classified as a subnucleus by Sawchenko and Swanson (45) (Fig. 6). Special attention was paid to divide the subnuclei of the PVN by watching alternative sections of hybridized and non-hybridized ones stained by cresyl violet. In order to document a possible progesterone effect in this region of PVN quantitatively, sections were selected which, for each rat, optimized the sampling of this subnucleus, for subsequent Bioquant analysis of pixels per neuron (Fig. 7). When the frequency distributions of the absolute numbers of pixels in neurons expressing different amounts of oxytocin mRNA were compared across groups, there was an evident shift to the right in the 7:00 PM group (exposed to 9 h of progesterone) compared to the other two

Fig. 3 Histogram showing the average number of pixels in neurons of SON and PVN measured on the Bioquant computer program. Comparisons and statistics are in the text.

Fig. 4 Frequency histogram showing the grains per neuron, actually measured on the Bioquant computer program as pixels per neuron, from each experimental group in SON and PVN. Comparisons and statistics are in the text.

Fig. 5 Camera lucida drawings of representative sections through the PVN of each experimental group. Filled neurons are hybridization-positive cells, whereas open neurons are hybridization-negative cells. Placements of positive oxytocin-expressing cells were similar in all three experimental groups.

groups, by statistical analysis on the Kolmogorov-Smirnov test ($P < 0.001$). Since the strongest increases in the 7:00 PM group were seen with the absolute numbers of pixels in neurons, the cytoplasmic area changes of labeled neurons must be a parameter which is significantly affected by prolonged progesterone exposure, inducing hypertrophic effects on neurons. Then, in order to correct for possible changes in neuronal size with hormonal treatment, as well as possible inequalities of sampling through cytoplasmic area, the numbers of pixels per unit area of cytoplasm in oxytocin-expressing cells were also calculated (Fig. 8). Again, the 7:00 PM group showed a shift to the right, towards greater oxytocin mRNA expression per unit area, using the Kolmogorov-Smirnov test ($P < 0.001$).

DISCUSSION

Locations and proportions of oxytocin-expressing neurons in the SON and PVN as shown by hybridization with our tritiated labeled oligodeoxy-nucleotide probe agree well with those of oxytocin-containing neurons identified immunohistochemically in the present investigation and previous studies and also with those of labeled oxytocin neurons by previous *in situ* hybridization histochemistry (18, 25, 36, 38, 48, 49, 51, 57). Absence of labeling in sections treated with RNase or processed without

the probe indicates that the oligomer we used hybridized in a valid fashion with oxytocin mRNA. The value of quantitative analysis using *in situ* hybridization for cell-by-cell resolution was shown, taking into account the fact that the PVN of the rat has been divided into several subnuclei with different projections (2, 36, 45).

Following systemic injections of [^3H]estradiol, estrogen-concentrating neurons have been demonstrated in the rat brain using autoradiography, and combining immunohistochemistry with tritiated estrogen autoradiography in the same tissue allowed the argument that some neurons in posterior subnuclei of the PVN concentrate estrogen in the cell nucleus and express immunoreactive oxytocin in the cytoplasm (37). In addition, estrogen receptor-immunoreactive cells tend to confirm the distribution discovered with steroid autoradiography (12). Indeed, estrogens have already been shown to affect the distribution of oxytocinergic cells and processes as well as oxytocin immunoreactivity with the use of immunohistochemistry (9, 19) and radioimmunoassay (11, 52). Immunoreactivity of oxytocin-containing neurons can be decreased by estradiol treatment in the anterior commissural nucleus (37). Jirikowski *et al.* (19) reported that the estradiol treatment influences the topographical distribution of immunostained oxytocinergic neurons and processes: short-term estradiol treatment increased the number of immunoreactive cells in

Fig. 6 Autoradiographs of neurons from the ventromedial part of the PVN after estrogen only (control rats) (a) and 7:00 PM, after 9 h of progesterone (b). Note the increased number of silver grains overlying the cell bodies in (b) compared to (a). $\times 400$

the septohypothalamic nucleus, the lateral sub-commissural area, the medial preoptic area, the perifornical region, the zona incerta, and the ansa lenticularis, but the distribution of oxytocin-immunoreactive neurons in the magnocellular SON and PVN were not altered with changing estradiol level. In agreement with this, the current progester-

one experimental treatment did not alter the anatomical distribution of oxytocin-expressing cells but, instead, did change their quantitative properties of expression. Following estrogen treatment, van Tol *et al.* (52) and recently Chung *et al.* (11) have reported quantitative increases in oxytocin mRNA expression, a result predicted by the large

Fig. 7 Frequency distribution histograms for oxytocin hybridization results in the posterior ventromedial portion of PVN analyzed by Bioquant computer according to pixels per neuron. Diagonal stripe, 10:00 AM; horizontal stripe, 2:00 PM; solid black, 7:00 PM. Statistics in the text

literature showing estrogen stimulation of oxytocin release (34).

Progesterone greatly facilitates female reproductive behavior in estrogen-primed ovariectomized female rats (34), and the main purpose of our experiment was to evaluate oxytocin gene expression under the influence of combined estrogen and progesterone treatments, emphasizing oxytocinergic cells which project to the other part of the brain. Steroid autoradiography has indicated the presence of progesterone-concentrating neurons within areas already shown to contain estrogen receptor-containing cells in both rat and guinea-pig brains (44, 53, 54, 56). Likewise, progesterone receptor-immunoreactivity has been found primarily in estrogen receptor-immunoreactive cells in the guinea-pig brains, including the arcuate nucleus, the periventricular preoptic nucleus, medial preoptic nucleus, hypothalamic ventrolateral nucleus, as well as, notably, the PVN (5, 6, 55). Since

gene expression for the progesterone receptor increases after estradiol priming (40), we were able to form the hypothesis that estrogen priming would allow an effect of progesterone on oxytocin gene expression. In fact, Caldwell *et al.* (9) have measured oxytocin-immunoreactivity using the tissue-punch method, and found that estrogen and progesterone treatment causes a significant increase in oxytocin-immunoreactivity in the PVN, compared to estrogen treatment alone. The present findings are consistent with this, showing an increase in oxytocin mRNA in the posterior ventromedial portions of the PVN following combined estrogen-progesterone treatment, and the projections of some of the cells in this portion of the PVN suggests relevance for behavioral involvement (45). Eventually, combining retrograde neuroanatomic methods with *in situ* hybridization in the same tissue will allow us to discern whether individual neurons which project to the brain stem

Fig. 8 Frequency distribution histogram for oxytocin hybridization results in the posterior ventromedial portion of PVN analyzed by Bioquant computer program according to pixels per unit cytoplasmic area. Diagonal stripe, 10:00 AM; horizontal stripe, 2:00 PM; solid black, 7:00 PM. Statistics in the text

are influenced in their production of oxytocin mRNA following hormone treatment (11).

What are the mechanisms through which oxytocin gene expression is altered following hormone treatment? As has been discussed (12), estrogen and progesterone-stimulated increases in oxytocin in the plasma (39) could be due to indirect mechanisms rather than by direct action of the hormones on magnocellular neurons which project to the neurohypophysis. Indeed, even for direct steroid action on PVN, a rapid action in the cell nucleus has not been proven. For example, in the present

result, if a rapid elevation of oxytocin mRNA was required following progesterone treatment and before reproductive behavior could occur, then we should have seen marked increases 4 h after progesterone (i.e. at 2:00 PM). This did not occur. Instead, the present data suggest that under the circumstances of these experiments, increased oxytocin gene expression has more of a restorative role (i.e. at 7:00 PM, 9 h exposure to progesterone), after reproductive behavior would have occurred (lordosis increases between 1 and 8 h following progesterone treatment) and may, in fact, be a consequence

of elevated oxytocin peptide secretion. In this respect, oxytocin mRNA responses to hormones may be directly analogous to the situation with LHRH gene expression: elevated LHRH mRNA levels (32, 42, 43) can be tied to circumstances correlated with elevated LHRH peptide secretion. This is particularly interesting for oxytocin neurons, in which estrogen treatment can lead to increased occurrence of action potentials (1, 30) which, through stimulus-secretion coupling (35), would lead to increased peptide release which, in turn, may drive increased oxytocin gene expression.

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