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# Changes in Oxytocin Receptor mRNA in Rat Brain During Pregnancy and the Effects of Estrogen and Interleukin-6

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## Abstract

Changes in brain oxytocin receptor (OTR) binding sites during the course of pregnancy may influence the sudden onset of maternal behavior in female rats at parturition. *In situ* hybridization was used to identify changes in OTR messenger ribonucleic acid (mRNA) expression during pregnancy and parturition. Two patterns of mRNA regulation were observed. Relative to diestrus virgin control females, OTR mRNA was elevated in the lateral septum and medial preoptic area at days 13–15 of pregnancy but not on the morning of parturition. In the central nucleus of the amygdala and ventromedial nucleus of the hypothalamus (VMH), OTR mRNA was most abundant on the morning of parturition. Strong signals for OTR mRNA were detected in the bed nucleus of the stria terminalis, hypothalamic paraventricular nucleus, supraoptic nucleus and suprachiasmatic nucleus. However no group differences were detected in these areas. As estrogen and interleukin-6 have been suggested to modulate OTR gene expression and both are elevated at the time of parturition, their effects on OTR mRNA in the brain were examined. Estrogen and interleukin-6, given simultaneously, significantly elevated the concentration of OTR mRNA in the VMH, but not in the amygdala. The increase in the VMH was significantly greater than that produced by estrogen alone, and interleukin-6 alone had no effects. These results demonstrate that transcriptional regulation of OTR gene expression mediates changes in receptor density in the brain in a region specific manner during pregnancy and suggests a potential mechanism for some of these changes.

Oxytocin (OT) plays a crucial role in several functions necessary for reproduction in the female rat, including sexual behavior (1, 2), uterine contractions during parturition (3), lactation (4) and maternal behavior (5, 6). Both peripheral and central actions of OT are mediated by a member of a G-protein coupled, seven transmembrane domain family of receptors (7, 8). Oxytocin receptor (OTR) mRNA is increased several-fold at parturition in rat and human uterus (9, 10). Oxytocin receptor binding and mRNA expression in the uterus and in select brain regions are regulated by estrogen (9, 11–13). Quantitative analysis of OTR by receptor autoradiography has demonstrated increases in receptor binding in the hypothalamus, amygdala and bed nucleus of the stria terminalis at parturition (14, 15). In the rat, the onset of maternal behavior coincides with these increases in OTR binding. Central infusions of OT antiserum (16) or a selective antagonist (5) delays the onset of maternal behavior.

The present study was designed to characterize the changes in OTR mRNA expression during pregnancy and at parturition as well as to investigate a possible mechanism of these changes. *In situ* hybridization was used to quantify changes in mRNA expression during pregnancy in specific brain regions. The 5′ flanking region of the OTR gene contains several sequence elements known to mediate genomic responses to estrogen as well as interleukin 6 (IL-6) (17, 18). As circulating concentrations of both estrogen and IL-6 increase at the onset of spontaneous labor in humans (19), these substances could mediate the changes in OTR gene expression in the uterus and brain associated with parturition. Therefore, we examined the influences of estrogen and IL-6 on the expression of brain OTR in ovariectomized rats.

## Results

*In situ* hybridization with the antisense probe resulted in specific labeling in each of the areas which exhibit strong binding with receptor autoradiography, including the lateral septum (LS), bed nucleus of the stria terminalis (BnST), central amygdala (AmC), and the ventromedial nucleus of the hypothalamus (VMH) (Fig. 1). In addition, a strong signal was detected in several other regions previously reported to express OTR mRNA, including the paraventricular nucleus of the hypothalamus (PVN), medial preoptic area (MPOA), suprachiasmatic nucleus (SCN) and the supraoptic nucleus (SON), although strong binding was not detected with receptor autoradiography in these areas. Sense strand control hybridization gave a uniform background over all of these regions.

The results of the quantitative analysis of OTR mRNA expression in the brains of diestrus virgins, pregnant (days 13–15), and parturient females are presented in Fig. 2. Significant group

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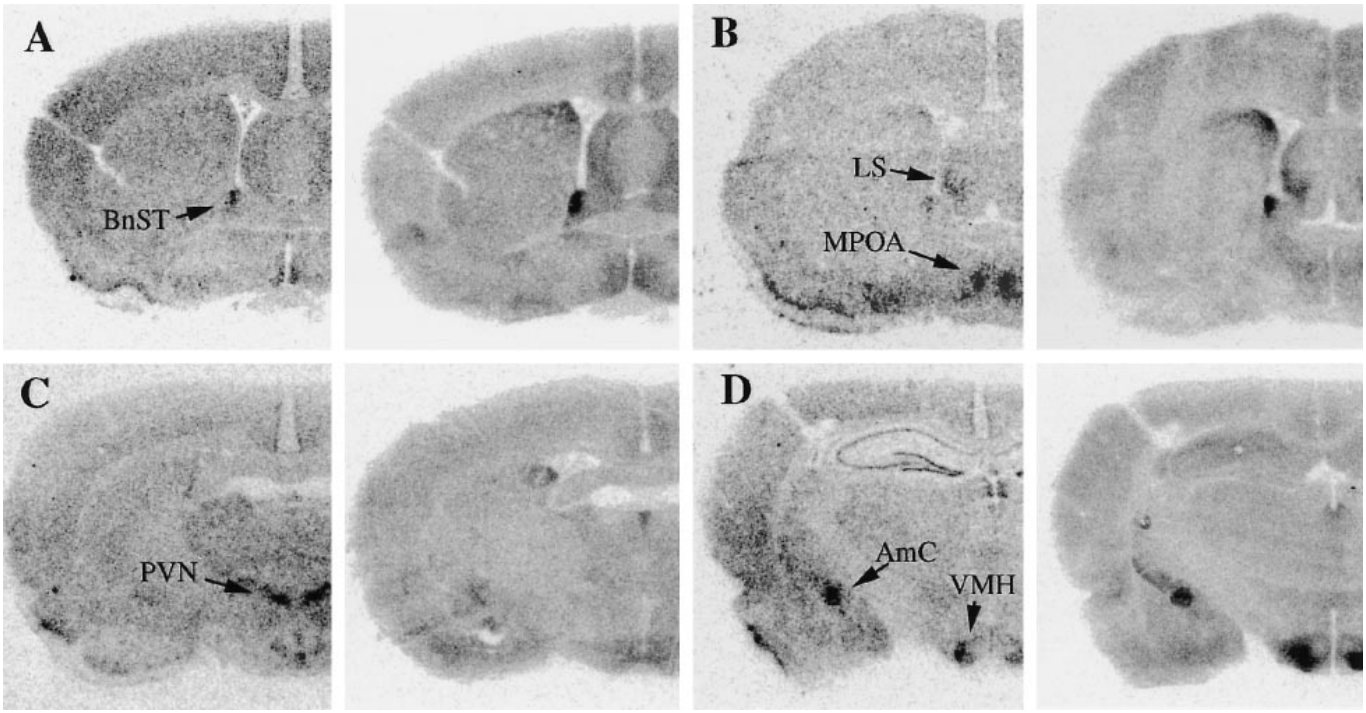


FIG. 1. Photomicrographs comparing OTR mRNA distribution (left panel) and $^{125}$OTA binding pattern (right panel) of adjacent sections of a pregnant rat brain. *In situ* hybridization and receptor autoradiography give similar results in the bed nucleus of the stria terminalis (BnST) (A); lateral septum (LS) (B); central nucleus of the amygdala (AmC) (D); and the ventromedial nucleus of the hypothalamus (VMH) (D). However, note the high levels of OTR mRNA in the medial preoptic area (MPOA) (B) and paraventricular nucleus of the hypothalamus (PVN) (C) in contrast to the low levels of binding in these areas.

differences were found for the VMH ($F=9.0$, $P<0.005$), AmC ($F=16.3$, $P<0.001$), LS ($F=6.2$, $P<0.01$), MPOA ($F=14.2$, $P<0.001$). Two patterns of regulation were apparent. OTR mRNA increased in the MPOA ($P<0.001$; Fig. 3) and LS ($P<0.01$) during pregnancy compared to diestrus controls but dropped to diestrus levels by the morning of parturition. In contrast, OTR mRNA in the VMH ($P<0.002$; Fig. 3) and the AmC ($P<0.001$) were significantly elevated on the morning of parturition. In the AmC, signal was also elevated during pregnancy compared to diestrus controls ($P<0.05$). OTR mRNA concentrations were similar in the BnST, PVN, SON and SCN of all groups (data for SON and SCN not shown).

In the ovariectomized females, significant treatment effects of estradiol benzoate (EB) and IL-6 were found only in the VMH (Figs 4 and 5). EB treatment resulted in a 2-fold increase in OTR mRNA in the VMH of ovariectomized females. This increase in OTR mRNA in the VMH is statistically significant when analysed separately ($P<0.01$, *t*-test), however, fails to reach the criteria for statistical significance in the post-hoc analysis ($P>0.05$). IL-6 treatment alone did not result in an increase in OTR mRNA in any brain region analysed. However, the combination of EB and IL-6 greatly increased OTR mRNA in the VMH ($P<0.001$). Significantly higher levels of OTR mRNA were found in the VMH of the EB/IL-6 treated animals compared to EB treated animals ($P<0.01$), suggesting a synergistic effect.

## Discussion

Virgin female rats display little interest in infants and when presented with foster young will either avoid or cannibalize them. Prior to parturition, there is a rapid, dramatic shift in motivation from a lack of interest to a driven, relentless pursuit of nest-building, retrieval, licking, grouping and protection of pups. Oxytocin plays a significant role in the induction (5, 6, 20), but not the maintenance of maternal behavior in the rat (21, 22). The induction of OTR in the brain at the time of parturition may facilitate the behavioral actions of OT at this time. Receptor autoradiographic studies have reported increases in receptor binding in the VMH, AmC and BnST at the time of parturition (14, 15). The present study indicates that, at least in the VMH and AmC, this increase in binding is the result of increased gene expression at parturition. The lack of an increase in OTR mRNA in the BnST at parturition may reflect temporal differences in the regulation of gene expression and the appearance of functional protein. Increases in OTR mRNA in the VMH are also associated with estrus in the rat and, therefore, the increase in OTR in that region could be related to post-partum estrus rather than the induction of maternal behavior. However, we have recently found a similar post-partum increase in OTR binding in the VMH of voles (Wang Z, Young L and Insel T, unpublished data), a species which does not show an increase in OTR binding associated with estrus (23). Therefore, the post-partum increase of OTR in the VMH may influence behaviors other than receptivity and may be regulated by hormonal events specifically associated with parturition.

Earlier studies of OTR mRNA in the brain have noted inconsistencies between OTR binding sites and mRNA (24, 25). Our comparison of OTR binding sites and mRNA expression in adjacent sections clearly demonstrates OTR synthesis in each brain region in which OTR binding sites are detected with

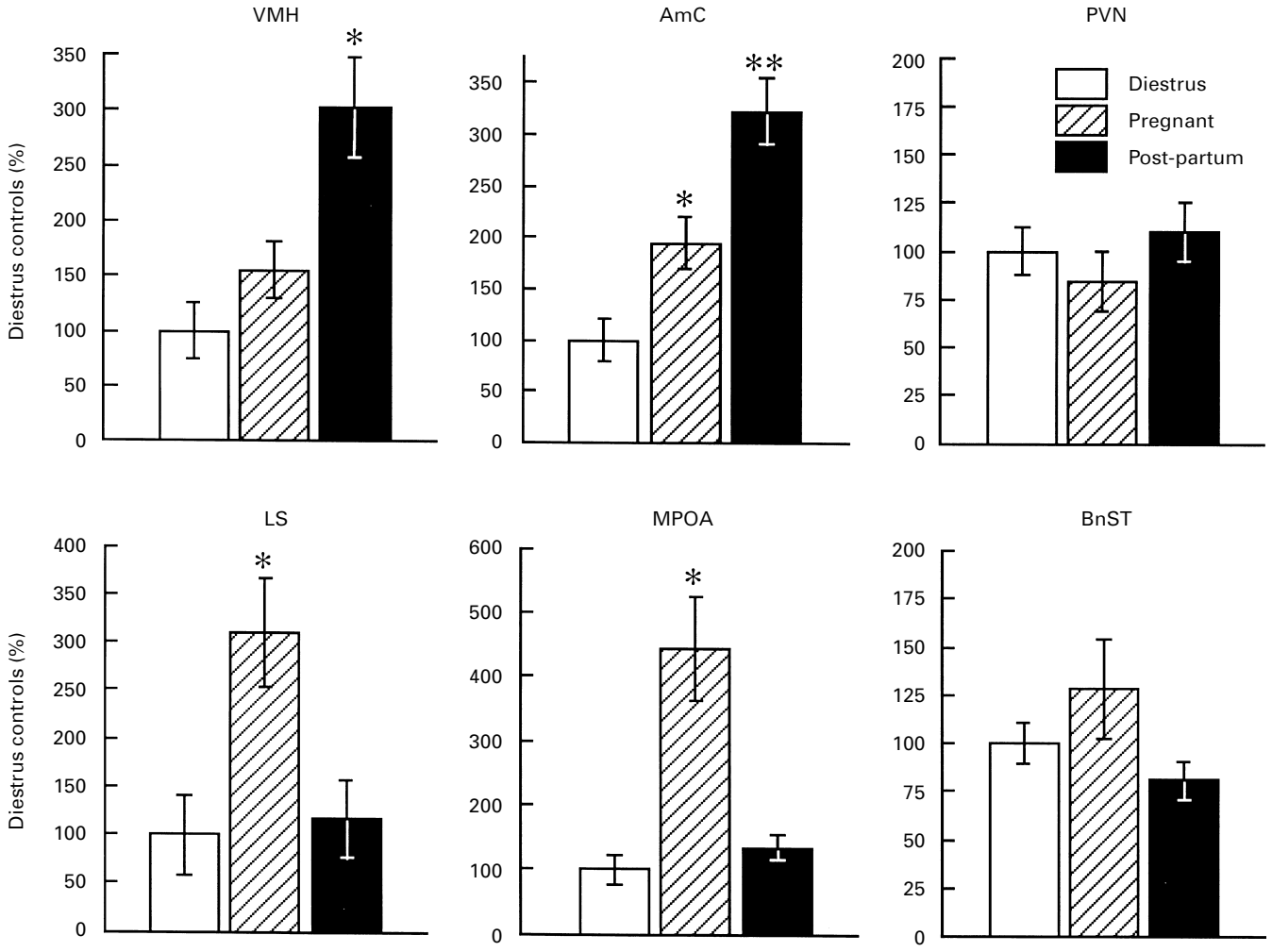


FIG. 2. Quantitative analysis of OTR mRNA concentrations in the brains of diestrus virgin, pregnant (days 13–15), and parturient rat brains. Data are presented as percent of the mean of the diestrus controls ±SE. *$P<0.05$ compared to controls; **$P<0.05$ compared to pregnant females. VMH, ventromedial nucleus of the hypothalamus; AmC, central nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus; MPOA, medial preoptic area; LS, lateral septum; BnST, bed nucleus of the stria terminalis.

autoradiography. Inconsistencies still exist, as previously noted, in several regions in which OTR mRNA is detected while binding is not. A strong signal for OTR mRNA (25) as well as OTR immunoreactivity (26) has been reported for the PVN and SON, while binding above background is rarely detected. One study reported OTR binding in the PVN and SON of lactating rats if the animals were given an icv injection of OT antagonist (27). Several studies have suggested that OTR located within these regions may be involved in positive feedback mechanisms during parturition and lactation (28–30). A recent report in sheep demonstrated that oxytocin release in the PVN at parturition plays an important role in the induction of maternal behavior (31). These studies suggest the presence of functional OTR in these regions even though they are not detected with autoradiography. Because the PVN and SON are the major sites of synthesis of OT in the brain (32), it is possible that the binding sites in these regions are not typically detected by receptor autoradiography, as they are masked by endogenous ligand. Whatever the mechanism, it is clear that limitations exist for quantitative analysis of OTR using receptor autoradiography and these limitations may extend to other brain regions as well. Our data indicate that the synthesis of OTR in the SON and PVN is relatively constant throughout pregnancy and is apparently not modulated by gonadal steroids.

Inconsistent results between immunocytochemistry, *in situ* hybridization, and receptor binding for OTR in the AmC have led to the suggestion that either multiple subtypes of OT binding sites may exist in the brain (26) or OTR localized in this region, is synthesized elsewhere and transported to this area (24). The present data demonstrate that OTR mRNA is found in the AmC and that changes in OTR mRNA synthesis at parturition parallel the increase in OTR binding found at parturition (15).

The increase in OTR mRNA in the MPOA during pregnancy deserves attention. OTR binding sites in this region, as visualized by receptor autoradiography, are diffuse and difficult to distinguish from the background (Fig. 1B). Strong signals for OTR mRNA were detected in the MPOA of the pregnant females, and were often barely detectable in other animals. A similar pattern

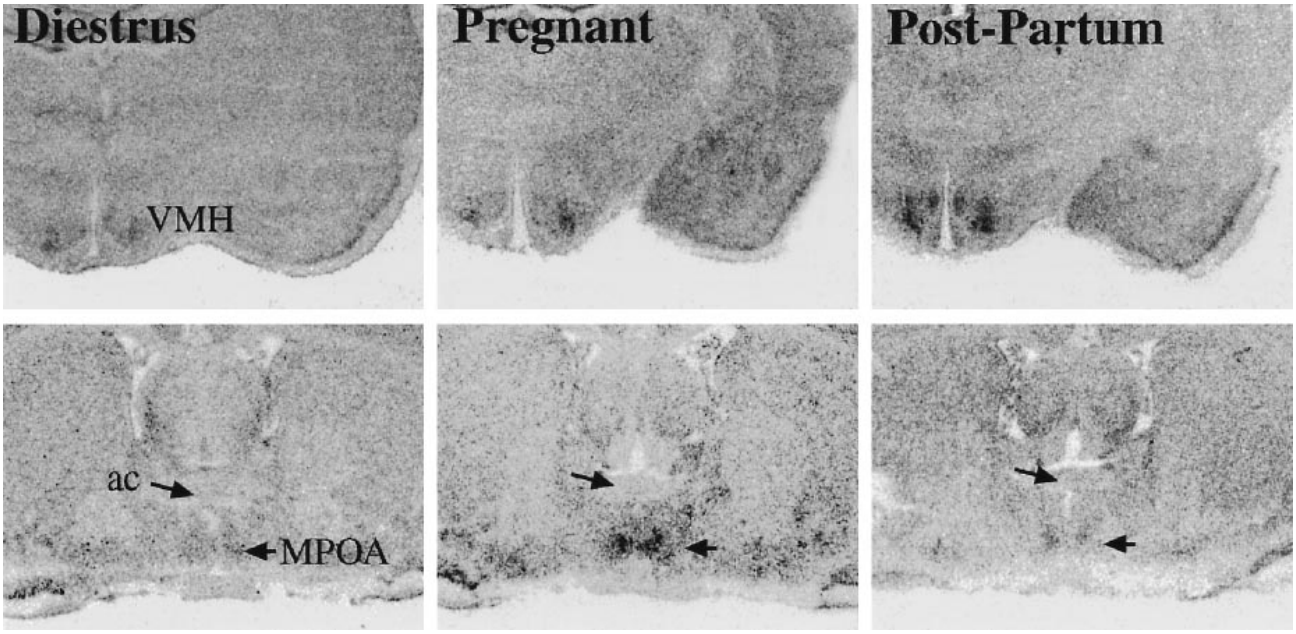


FIG. 3. Photomicrographs illustrating the levels of OTR mRNA in the ventromedial nucleus of the hypothalamus (VMH) and the medial preoptic area (MPOA) of diestrus (left column), pregnant (middle column) and post-partum (right column) females. ac, Anterior commissure.

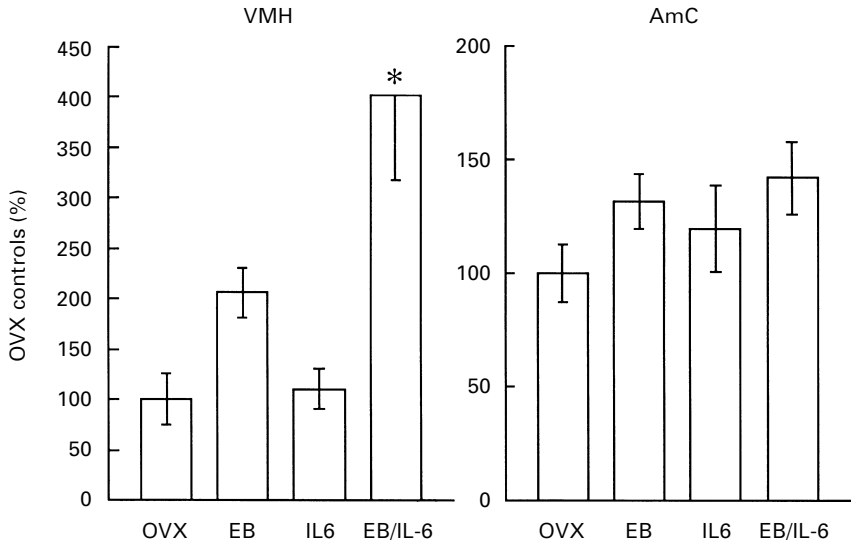


FIG. 4. The effects of estradiol benzoate (EB) and interleukin-6 (IL-6) on OTR mRNA concentrations in the ventromedial nucleus of the hypothalamus (VMH) and the central nucleus of the amygdala (AmC) of ovariectomized females. EB and IL-6, given simultaneously, significantly increased OTR mRNA over all other treatments in the VMH but not the AmC. *$P < 0.001$ compared to ovariectomized (OVX) controls.

of regulation was found in the LS. The MPOA and the LS are thought to be critical for the expression of normal maternal behavior (33–35). Infusion of OT in the MPOA has powerful effects on maternal behavior, suggesting a role of this region in the OT-mediated induction of maternal behavior (20). A previous homogenate receptor binding study suggested that the concentration of OTR protein is elevated in the MPOA at parturition relative to mid pregnancy (20). It is possible that the increase in OTR mRNA in this region during pregnancy results in an accumulation of OTR at the end of pregnancy, which in turn facilitates the behavioral response of OT released during parturition. The present study suggests that OTR gene expression in the LS and MPOA is elevated during pregnancy and synthesis decreases dramatically post-partum. As physiological changes in progesterone during pregnancy parallel the changes in MPOA and LS OTR mRNA (36), progesterone is a candidate for mediating these transcriptional events. Because OT is required for the induction, and not the maintenance of maternal behavior, the sustained synthesis of receptors in this region after parturition may not be necessary.

The discovery of several IL-6 responsive elements on the 5′ flanking region of the rat OTR gene led to the prediction that

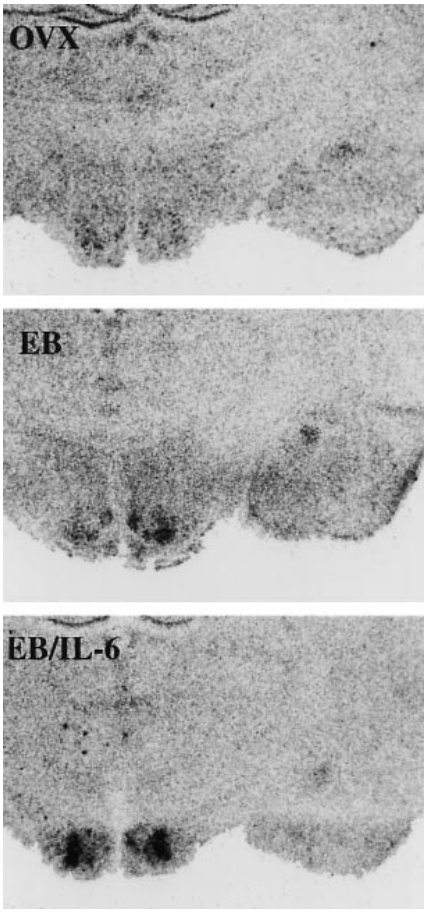


FIG. 5. Photomicrographs illustrating the relative effects of estradiol benzoate (EB) and the combination of EB and interleukin-6 (IL-6) on OTR mRNA concentrations in the ventromedial nucleus of the hypothalamus (VMH) of ovariectomized (OVX) females.

cytokines may influence the induction of OTR gene expression in the uterus at the onset of spontaneous labor (17). IL-6 is synthesized by the placenta and maternal serum levels increase dramatically at the onset of labor (19). Estradiol levels also increase at the end of pregnancy (37). Because the induction of OTR binding sites in the brain coincides with the increase of OTR bind in the uterus, we hypothesized that IL-6 may also influence OTR gene expression in the brain. The results from this study support this hypothesis. Although IL-6 alone did not increase OTR gene expression, the combination treatment of EB plus IL-6 resulted in an increase in OTR mRNA in the VMH. EB and IL-6, together, had a greater effect on OTR synthesis than EB treatment alone, suggesting a synergistic interaction between these substances, which may be physiologically relevant to parturition.

In addition to its role in defense to infection and injury (38), IL-6 appears to have a number of actions in the central nervous system, including reduction in food intake (39), induction of fever (40) and activation of the hypothalamic-pituitary-adrenal axis (41). IL-6 has also been reported to induce the release of OT from hypothalamic explants (42). However, the present data are the first indication of a potential link between IL-6 released during labor and the post-partum induction of maternal behavior. We are not aware of any studies that have investigated the influence of IL-6 on maternal behavior.

Because IL-6 was administered peripherally in this study, in order to mimic the systemic release during parturition, it was not possible to determine whether IL-6 is acting directly on the OTR gene as predicted. Although we did not measure the circulating concentration, the dosage of IL-6 used should not produce unusually high levels of IL-6 for an extended length of time. For example, IL-6 concentrations at the time of parturition in humans reach 0.5 ng/ml during contractions (43); the rats in this study received 50 ng IL-6 (ip) once per day. Clearly the IL-6 in this study could be acting either peripherally or centrally. For example, IL-6 is known to increase permeability of the blood-brain barrier (44, 45), which could affect the diffusion of estrogen or other factors into the brain. Some effects of IL-6, such as the stimulation of neurohypophyseal OT and vasopressin release, require prostaglandin synthesis (42). However, IL-6 may be acting directly on OTR gene expression. IL-6 is capable of passing through the blood-brain barrier, and evidence for a specific IL-6 transport system on the blood-brain barrier has been reported (46). Furthermore, IL-6 receptor mRNA has been detected in neurons in the VMH of the rat (47). Further studies comparing the relative efficacies of peripheral and central IL-6 administration, along with studies using specific receptor antagonists, will be necessary to identify the mechanism by which IL-6 and estrogen synergize to affect OTR gene expression. Regardless of the mechanism of action, this study suggests that cytokines may influence neural responsiveness to neuropeptides, and potentially influence the expression of social behavior.

## Materials and methods

### *Animals*

Thirty-six female Sprague Dawley rats were obtained from Harlan (Prattville, AL, USA) and housed at 21 °C, provided free access to food and water, and exposed to a 12 h light, 12 h dark cycle. Two groups of rats were used; intact and ovariectomized. Timed pregnant rats were purchased for the pregnancy and parturition groups. Animals were killed by rapid decapitation and brains were frozen on dry ice and stored at −80 °C until sectioning. Brains were harvested from pregnant rats at days 13–15 of pregnancy (n = 5) and from parturient rats on the morning of parturition after all pups were delivered (n = 5). Five virgin females were monitored for the estrus cycle using vaginal smears for at least 8 days and were killed on the morning of diestrus. A second group of rats were virgin females (250–260 g, n = 21), which were ovariectomized under anesthesia (sodium pentobarbital) 1 week prior to treatment for the regulation studies.

### *Hormone treatment*

Four treatment groups were used in this study: (1) vehicle-vehicle (OVX), n = 5; (2) estradiol benzoate-vehicle (EB, n = 6 ), (3) vehicle-IL-6 (IL-6, n = 5) and (4) EB-IL-6 (n = 5). One week after ovariectomy, each animal received an injection of IL-6 (50 ng/100 µl saline, Boehringer Mannheim, Indianapolis, IN, USA) or saline alone and estradiol benzoate (EB) (10 µg/100 µl sesame oil, Sigma, St. Louis, MO, USA) or oil alone. All injections were given ip on two consecutive days. Each animal received a total of four injections. Twenty-four hours after the last injection, brains were harvested as described above.

### In situ *hybridization*

*In situ* hybridization was performed on 20 µm thick cryostat sections as previously described (48). Sense and antisense $^{35}S$ labeled riboprobes were generated using SP6 or T7 RNA polymerase from a PCR fragment of the rat uterine OTR cDNA spanning amino acids 198–342 (7). This probe contained portions of both the second and third exons of the gene.

Adjacent sections were used for receptor autoradiography in order to correlate the distribution of OTR mRNA and receptor binding. Hybridized slides were exposed to Kodak BioMax MR film for 6 weeks before developing.

*Receptor autoradiography*

Receptor autoradiography was performed using $^{125}$I-D$(CH_2)_5$ [Tyr$(Me)_2$,Tyr-$NH_2^9$]OVT (NEN, Boston, MA, USA; OTA) as previously described (14). After a pre-wash in 50 mM Tris-HCl (pH 7.4) containing 50 μM GTP, slides were exposed to a 60 min incubation (at room temperature) with 60 pM $^{125}$I-OTA in Tris with $MgCl_2$ (10 mM), bovine serum albumin (0.1%; RIA grade, fraction V, Sigma), and bacitracin (0.05%). Non-specific binding was defined in adjacent sections by adding 50 nM Thr$^4$Gly$^7$-OT (Peninsula, Belmont, CA, USA) to the incubation buffer. The slides were then rinsed in 50 mM Tris pH 7.4, 10 mM $MgCl_2$. After air drying, the slides were exposed to BioMax MR film (Kodak, Rochester, NY, USA) for 48 h.

*Statistical analysis*

Film autoradiograms were analysed using a computer aided image analysis system utilizing the Image computer program (version 1.55, NIH) as described previously (48). Optical density measurements of mRNA signals on the X-ray film were converted to cpm using $^{35}$S standards prepared in brain paste. Specific labeling for each region (in cpm) was calculated by subtracting background from an adjacent area of the same section. Means of bilateral measurements in 1–3 sections, depending on the brain region, were analysed. Data are presented as percent of control levels. One-way ANOVA and Fisher's least significant difference post hoc tests ($P<0.05$) were used to evaluate differences in OTR mRNA expression.

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