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Oxytocin receptor binding in the hypothalamus during gestation in rats

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Oxytocin receptor binding in the hypothalamus during gestation in rats. Am J Physiol Regul Integr Comp Physiol 291: R53–R58, 2006; doi:10.1152/ajpregu.00766.2005.—Central oxytocin receptors (OTR) may be involved in adaptations of the brain oxytocin (OT) system during gestation, which are critical for systemic release of OT during parturition and lactation. We used quantitative autoradiography to determine changes in OTR binding in numerous brain sites during the course of gestation in the rat. Furthermore, to evaluate the importance of ovarian steroids in mediating pregnancy-related changes in OTR binding, we measured binding in ovariectomized animals treated with progesterone and/or estrogen, and in pregnant animals treated with exogenous progesterone during late gestation. We found that OTR binding was significantly increased in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) by midgestation (day 15) compared with control. In addition, there was a further significant increase in OTR binding in these nuclei by late gestation (day 20). The bed nucleus of the stria terminalis (BNST) and the medial preoptic area (MPOA) also showed significant gestation-associated increases in OTR binding, which were similar during mid- and late pregnancy. Treatment with exogenous progesterone throughout pregnancy did not alter the increase in OTR binding characteristic of late gestation in any of these brain sites. Finally, estrogen treatment in ovariectomized animals resulted in increased OTR binding in the SON, BNST, and MPOA, but not the PVN. These data demonstrate that OTR binding in the hypothalamus is increased during mid- and late-gestation, compared with ovariectomized control animals, which may be mediated by increased estradiol.

OXYTOCIN (OT) IS SYNTHESIZED in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. Primary functions of OT released into the systemic circulation from magnocellular neuron terminals in the neural lobe include regulation of uterine contractions during parturition and milk ejection during lactation (17, 40). In addition, OT is released at several brain sites, including the medial preoptic area (MPOA), where it has been implicated in maternal behavior (41), and locally within the PVN and SON (intranuclear release), where it appears to exert autocrine and paracrine actions to enhance and coordinate systemic release of the hormone during parturition and lactation (34, 35, 39).

The central OT system undergoes a number of biochemical, anatomical, and electrophysiological adaptations during gestation in anticipation of parturition and lactation. For example, OT gene expression is increased (20, 33), and the OT magnocellular neurons undergo anatomical reorganization (37, 46) during late gestation. Furthermore, electrophysiological response characteristics (24) and membrane properties (44) of OT magnocellular neurons are altered at this stage of pregnancy. We have recently found that central infusion of a selective OT receptor (OTR) antagonist during the final 2 wk of gestation delays the pulsatile release of OT during suckling and impairs pup development during lactation, without altering maternal behavior (31). These data suggest that during gestation, OT exerts central actions that are critical for the normal function and sensitivity of this neuroendocrine system subsequently during lactation.

It is possible that these effects are the result of increased release of OT locally within the magnocellular nuclei during gestation, but such increased intranuclear release has not been observed (11, 32). Alternatively, there may be changes in OTR expression and/or activation during this time. Previous studies have demonstrated that OTR mRNA (49) and immunoreactivity (1) are present in magnocellular neurons, although OT ligand binding appears low in these nuclei (12, 28). Furthermore, one study reported increased OTR binding in magnocellular nuclei during lactation, but only after central administration of an OTR antagonist (14). In addition, OTR mRNA expression in the PVN during pregnancy is not increased during midgestation (days 13–15) compared with diestrus female rats (50). However, measurement of OTR mRNA expression and OTR binding at other times during gestation have not been reported. Consequently, changes in OTR dynamics throughout gestation and lactation have not been completely defined.

Consequently, to more fully characterize the potential importance of changes in OTR in magnocellular nuclei during gestation, we evaluated OTR binding during both mid- and late gestation. Furthermore, we measured OTR binding in other brain regions previously demonstrated to show an increase in either binding (23) or OTR mRNA (5, 48, 50) during gestation or immediately postpartum. These included the central nucleus of the amygdala (CeA), the lateral septum (LS), MPOA, and the bed nucleus of the stria terminalis (BNST). Finally, because estrogen and progesterone regulate OTR binding and/or OTR mRNA expression in brain (5, 11, 26, 47), we examined OTR binding in these brain sites in pregnant animals given exogenous progesterone until late gestation, and in ovariectomized...
(OVX) rats subjected to an ovarian steroid regimen, which mimics changes observed during gestation and which induces the increased OT mRNA expression characteristic of late gestation (4, 6).

MATERIALS AND METHODS

Animals

Timed-pregnant or virgin female Sprague-Dawley rats were obtained from a commercial supplier (Charles River Laboratories, Wilmington, MA). The animals were maintained on a 12:12-h light-dark cycle in single cages with ad libitum access to food and water. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Ovariectomy

Virgin female rats were anesthetized with 2,2,2-tribromoethanol (Avertin, 300 mg/kg). After a midlateral incision into the body cavity, the ovaries were removed. The body wall was sutured shut, and the external incision was closed with wound clips.

Protocols

Experiment 1. OTR binding during gestation. Gonadally intact females on gestation day 15 (Mid-Preg) or 20 (Late Preg), and nulliporous females at least 1 wk after ovariectomy, were deeply anesthetized with 2,2,2-tribromoethanol (Avertin, 300 mg/kg) and decapitated. Brains were removed, quick-frozen in dry ice, and stored at −80°C until processed.

Experiment 2. Effects of progesterone treatment on OTR binding in late-gestation rats. These timed-pregnant females were administered exogenous progesterone through late gestation to determine the effects of the progesterone withdrawal, which occurs during late gestation on OTR binding. On gestation day 12, females were anesthetized with Avertin and implanted subcutaneously with three Silastic capsules (L: 40 mm, ID: 1.98 mm, OD: 3.175 mm) containing progesterone crystals (powder). A control group was implanted with blank capsules in a similar manner. On gestation day 20, females were killed and their brains were removed, quick-frozen and stored in a −80°C freezer until processed.

Experiment 3. Effects of estradiol and progesterone treatment on OTR binding in OVX female rats. Ovariectomized animals were implanted subcutaneously between the scapulae with a single Silastic capsule (L: 30 mm, ID: 1.98 mm, OD: 3.175 mm) filled with estradiol (0.25 mg/ml sesame oil) or with an empty capsule on treatment day 1. On treatment day 3, the animals with estradiol-containing capsules were divided into three groups, two of which were implanted with capsules containing progesterone (see above), and the third group was implanted with empty capsules. Animals receiving empty capsules on treatment day 1 were implanted with three additional empty capsules on day 3 (Cont). On treatment day 14, progesterone capsules were removed from one group of animals with estrogen capsules (E+P−), whereas the second group with both estrogen and progesterone underwent control surgery, but the progesterone capsules were not removed (E+P+). The animals treated with estrogen on treatment day 1 and an empty capsule on treatment day 3 had the empty capsule removed on treatment day 14 (E−). Similarly, animals implanted with empty capsules on treatment days 1 and 3 had one empty capsule removed on treatment day 14. On treatment day 16, all animals were killed. The brains were removed, quick-frozen, and stored in a −80°C freezer until processed.

Tissue Processing

Sectioning. Twelve-micrometer-thick frozen sections were cut in a cryostat, thaw-mounted onto RNase-free coated slides, air-dried, and then stored at −80°C until processed for OTR autoradiography.

Immediately adjacent sections were also collected and stained with cresyl violet. The sections were selected to contain the SON and PVN, MPOA, CeA, BNST, and LS.

Autoradiography. We carried out OT receptor autoradiography using the selective iodinated OT antagonist d(CH2)5[Tyr(Me)2,Tyr-NH2]2-ornithine vasotocin ([125I]OTA, New England Nuclear, Boston, MA), as described previously (8, 30). Briefly, after thawing, sections were washed in Tris·HCl (pH 7.4) and then exposed to 60 PM [125I]OTV in Tris with MgCl2 (10 mM), BSA (0.1%) and bacitracin (0.05%) for 75 min. Nonspecific binding was determined in adjacent sections by adding 50 nM [d(CH2)5,Tyr(Me)2,Omethyl]-vasotocin to the incubation buffer. A final 35-min wash was performed in 50 mM Tris (pH 7.4), 100 mM MgCl2 to reduce background. The dry slides were then exposed to X-ray film (Kodak Biomax, MR, Eastman Kodak, Rochester, NY) for 1–2 days.

Densitometry

 Autoradiograms were analyzed using National Institutes of Health (NIH) Image software (Bethesda, MD). Briefly, digital images were taken from the film autoradiograms, the image colorized, and the light intensity was adjusted so that the intensity of the densitometric signal fell within the linear range of the system response. For analysis, the brain area to be analyzed on the autoradiogram was identified on the cresyl violet stained section, and the intensity of the signal measured. All densitometric measures were corrected for nonspecific binding and background staining.

Statistical Analysis

Optical density data (arbitrary units) are presented as means ± SE. Means were analyzed with one-way ANOVA to compare optical densities in each brain area. Differences between individual means were determined using Neuman-Keuls a posteriori comparisons. A P < 0.05 was considered significant.

RESULTS

Experiment 1. OTR Binding During Gestation

Mean optical density measures for the PVN and SON from all groups are shown in Fig. 1A. Optical density (arbitrary units) in both magnocellular nuclei were significantly elevated by gestation day 15, compared with OVX control animals. Furthermore, there was an additional significant increase in OTR binding between gestation days 15 and 20, as optical density on both SON and PVN were significantly greater in late-gestation animals compared with both midgestation and OVX control rats. Figure 2 is a representative photomicrograph of coronal sections taken through the level of the PVN and SON (A, schematic) from an OVX control (B), midgestation (C), and late gestation (D). As demonstrated in Fig. 1, density of binding progressively increases throughout gestation compared with the OVX control rat.

Figure 1B presents mean optical density measures in the BNST and MPOA from OVX, midgestation, and late-gestation animals. Both brain areas showed a significant increase in OTR binding, compared with OVX, by midgestation. There was no further increase in OTR binding by late gestation. OTR binding in the LS and CeA in both groups of pregnant animals was not significantly different from OVX control rats (data not shown).

Experiment 2. Effects of Progesterone Treatment on OTR Binding in Late-gestation Rats

OTR binding in PVN, SON, BNST, and MPOA from late-gestation rats and from late-gestation rats implanted with
capsules containing progesterone is shown in Fig. 3, A and B. There were no differences in OTR binding between untreated, late-gestation rats and late-gestation rats treated with progesterone for the duration of gestation.

**Experiment 3. Effects of Estradiol and Progesterone Treatment on OTR Binding in OVX Female Rats**

Figure 4 shows the effects of estradiol alone for 16 days (E+), estradiol and progesterone for 16 days (E+P+), and estradiol with simultaneous progesterone for 14 days followed by progesterone withdrawal (E+P−), compared with OVX animals that received no steroid replacement (Cont). Although OTR binding in the PVN showed no apparent effect of steroid treatment in OVX animals, binding in the SON was significantly elevated in E+ and E+P+, compared with Cont (Fig. 4A, left). Furthermore, although not statistically significant, there was a tendency for OTR binding to be increased in E+P− animals in the SON.

A similar relationship exists between groups for OTR binding in the BNST (Fig. 4B, left), E+ significantly increased OTR binding, whereas E+P+ and E+P− groups tended to be higher, but these differences were not significant. Similar to pregnant animals, OTR binding in the LS and CeA was not increased in OVX rats receiving steroid replacement (data not shown).

**DISCUSSION**

The central OT system in the magnocellular nuclei undergoes a number of adaptations during gestation to prepare for the hormone demands of parturition and lactation. These include increased OT mRNA expression (20, 33), anatomical modifications of OT neurons in magnocellular nuclei (37, 46), development of a bursting pattern of OT neuron response (24),...
and altered membrane properties of OT neurons (44). These developmental responses associated with gestation are likely to be necessary to establish the response characteristics and sensitivity of the system to ensure adequate secretion of systemic OT in response to suckling.

Results from the present experiments demonstrate for the first time that OTR binding in several sites of the hypothalamus is significantly increased in rats during mid- and late gestation, compared with OVX control animals. Furthermore, OTR binding in magnocellular nuclei of animals during late gestation is higher than both mid-gestation and OVX control rats. Although OTR binding was not measured during early pregnancy, these results suggest that OTR binding undergoes an adaptive upregulation during gestation, not only in sites associated with maternal behavior, such as the BNST and MPOA, but also in magnocellular nuclei that control systemic OT secretion during parturition and lactation. These changes in receptor binding may be important for central actions of OT that are important in organizing this neuroendocrine system for heightened activity during parturition and lactation.

The functional significance of increased OTR binding in PVN and SON during gestation has not been defined. It is well documented that intranuclear release of OT within the magnocellular nuclei acts in an autocrine and paracrine manner to produce a positive feedback, stimulatory effect on systemic OT secretion (15, 39), as well as coordinating and facilitating excitation of the entire population of OT neurosecretory neurons to induce a synchronous discharge (29, 38) during parturition and lactation. It is possible that OTR are upregulated during gestation to increase the sensitivity of magnocellular neurons to intranuclear release of the peptide during parturition and lactation.

In addition, there is evidence that OTR stimulation during gestation may mediate the biochemical, anatomical, and/or electrophysiological adaptations of the magnocellular OT system during gestation. For example, increased OT mRNA expression in magnocellular nuclei induced by steroid treatment in OVX animals is attenuated by OTR blockade (42). Furthermore, many of the morphological changes in the OT neurons of the PVN and SON associated with gestation and lactation are mediated by activation of central OTR during pregnancy (36, 45). Consequently, upregulation of OTR binding and subsequent OTR stimulation during gestation may be necessary for initiating and/or maintaining adaptations essential for normal systemic OT release, as well as increasing magnocellular neuron sensitivity to intranuclear OT during parturition and lactation.

It is possible that the enhanced OTR binding present in hypothalamic nuclei of pregnant animals results from increased neuron size, with no change in receptor density or affinity. Neuronal hypertrophy during late gestation has been demonstrated in the medial preoptic area of the hypothalamus, which is associated with maternal behavior (27). Furthermore, magnocellular OT neurons show hypertrophy during periods of intense stimulation, such as lactation (16, 18). However, although some structural adaptations in neurosecretory OT neurons present during lactation appear by late gestation (19, 46), we were unable to find any demonstration of cellular hypertrophy in the PVN or SON before parturition. A systematic evaluation of neuronal hypertrophy throughout the hypothalamus during gestation has not been completed. Consequently, it

Fig. 3. Oxytocin receptor binding indicated by optical density (arbitrary units) in the PVN, SON, BNST, and MPOA of late-gestation control rats (LATE PREG) and late-gestation animals treated with progesterone (LATE PREG + P). n = 6–8 animals/group.

Fig. 4. Oxytocin receptor binding indicated by optical density (arbitrary units) in the PVN, SON, BNST, and MPOA in ovariectomized (Cont) rats and ovariectomized animals treated with estrogen alone (E+), estrogen and progesterone (E+P+), and estrogen and progesterone with progesterone withdrawal (E+P−). *P < 0.05 compared with Cont. n = 5–7/group.
is not possible to determine the relative contributions of increased receptor density, receptor affinity, and/or neuronal hypertrophy to enhanced OTR binding observed in specific areas of the hypothalamus in the present experiments.

Because estrogen increases OTR mRNA (5) and OTR binding (7, 9, 11, 25) in many brain sites, we investigated the role of ovarian steroids in increased OTR binding, using a steroid replacement regimen designed to mimic changes in estradiol and progesterone observed during normal gestation in the rat (7). These steroid hormone replacement procedures are routinely used by us (32) and others (2–4) as a model for the changing hormonal milieu present during gestation. In these studies, treatment with estrogen alone, or combined estrogen and progesterone both produced significant increases in OTR binding in BNST, MPOA, and the SON. These brain areas also exhibited increased OTR binding during mid- and late gestation, thus, suggesting that the increased estrogen associated with pregnancy stimulates the upregulation of OTR.

Even though OTR in PVN and SON changed in parallel during mid- and late gestation, ovarian hormone treatment increased OTR binding in the SON, but not PVN; the reason for a lack of effect in PVN is unclear at present. It is possible that inasmuch as the PVN is composed of a wide variety of cell types, compared with the SON, highly discrete changes in a subpopulation of PVN neurons may not have been detectable. Alternatively, the contribution of estrogen to upregulation of OTR in the PVN may differ from the SON and other brain sites. It is possible that other factors associated with pregnancy, not present in OVX animals, are involved in the upregulation of OTR in the PVN. It is not unprecedented that PVN and SON might be subject to separate regulatory mechanisms. For example, earlier work from this laboratory has shown that OT mRNA expression during lactation is regulated by different stimuli, catecholamines, and central OT in SON, but not PVN (43). Furthermore, expression of GABA-A receptor subunit mRNA is upregulated by estrogen and progesterone in the SON, but not the PVN (13).

Previous studies have demonstrated that progesterone withdrawal following a period of high concurrent estradiol and progesterone is essential for the increase in OT mRNA expression observed during gestation (10), and during steroid hormone replacement treatment in OVX rats (2, 4). In contrast, our results suggest that increased OTR binding during gestation does not appear to require progesterone withdrawal, as late-gestation rats administered exogenous progesterone until gestation day 20 demonstrated increased OTR binding equivalent to untreated late-gestation animals, and E+P+ rats had similar levels of OTR binding as animals treated with estrogen alone (E+).

In the present experiments, OTR binding in response to ovarian steroids, and during gestation, was compared with binding observed in OVX female animals. This preparation has been frequently used as a control group for evaluating the potential role of ovarian steroids in adaptations of the central OT system during gestation (2, 10), including studies on changes in OTR binding (22) and expression of OTR mRNA (21).

In summary, these experiments have demonstrated that OTR binding in several hypothalamic structures is significantly increased in mid- and late gestation, compared with OVX control animals. Furthermore, OTR binding in magnocellular nuclei of late-gestation pregnant animals was greater than either midgestation or OVX rats. Although OTR binding during early gestation was not measured in these studies, the findings suggest a progressive increase in OTR binding in the magnocellular nuclei over the course of gestation. This increase in magnocellular OTR function may be in preparation for mediating autocrine and paracrine responses to intranuclearily released OT during parturition and lactation, or may contribute to ongoing adaptations of the OT magnocellular system characteristic of gestation. Furthermore, it appears that upregulation of OTR in most hypothalamic sites is primarily due to estradiol.

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