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Gonadal Steroid Modulation of Stress-Induced Hypothalamo-Pituitary-Adrenal Activity and Anxiety Behavior: Role of Central Oxytocin

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Intracerebroventricular administration of oxytocin reduces anxiety behavior and hypothalamo-pituitary-adrenal (HPA) responses to stress in female rats. Similar changes are seen in late-pregnant rats, and oxytocin-sensitive pathways may mediate these effects. This study investigated anxiety behavior and stress responses using a gonadal steroid model of late pregnancy, which is known to increase endogenous oxytocin expression. Compared with continuous progesterone treatment, 3-d withdrawal of progesterone after 11-d treatment of ovariectomized rats with estradiol and progesterone resulted in increased binding of the oxytocin receptor ligand [^{125}I]*d*(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]ornithine vasotocin in selective forebrain regions, including the ventrolateral septum and ventromedial hypothalamus. Behavior in the elevated plus-maze indicated that progesterone withdrawal had an anxiolytic effect, and this was associated with lower levels of c-*fos* mRNA expression in the ventral hippocampus, an area previously shown to be sensitive to oxytocin. In other groups of animals, the plasma corticosterone response to a psychological stress (10 min of 114 dB white noise) was significantly attenuated by this steroid manipulation. Furthermore, simultaneous infusion of the selective oxytocin receptor antagonist desGlyNH$_2$, *d*(CH$_2$)$_5$[Tyr(Me)^2,Thr^4]OVT during the period of progesterone withdrawal reversed this attenuation of noise-induced HPA activation, indicating a role for endogenous oxytocin in this effect. Thus, mimicking the steroid profile of late pregnancy leads to a reduction in anxiety behavior and attenuates HPA activity induced by mild stress. These effects appear to be mediated through the involvement of central oxytocin neurotransmission. (*Endocrinology* 147: 2423–2431, 2006)

A GROWING BODY of evidence indicates that oxytocin possesses potent anxiolytic properties in the central nervous system (1–5). Furthermore, intracerebroventricular (icv) infusion of low doses of oxytocin attenuate hypothalamo-pituitary-adrenal (HPA) activity induced by either white noise stress (3) or restraint stress (6) and attenuates restraint-induced expression of the immediate-early gene c-*fos* in the hypothalamic paraventricular nucleus (PVN) (6). Importantly, the potentiation of stress-induced HPA activity and anxiety behavior in the elevated plus-maze by central administration of an oxytocin antagonist indicates that endogenous oxytocin may participate in these effects (7–9). The role of endogenous oxytocin is substantiated by evidence that transgenic mice that lack the oxytocin gene show higher levels of anxiety in the elevated plus-maze (10, 11) and display altered emotional reactivity (12).

In the rat, an attenuation of stress responses and anxiety behavior occurs toward the end of pregnancy (13, 14) and into early lactation (15). This coincides with an increase in both the levels of oxytocin mRNA in the hypothalamus (16, 17) and the release of oxytocin into the limbic system (18). Oxytocin receptor binding (19) and mRNA levels (20) also increase in selected regions of the forebrain, and this correlates with an increased sensitivity of neurons to the excitatory effects of the peptide (21). However, whether these changes in oxytocin and its receptor mediate the pregnancy-related change in anxiety levels has been questioned (22).

A gonadal steroid paradigm involving treatment of ovariectomized rats with progesterone and estradiol, followed by progesterone withdrawal, has been used as a model of late pregnancy and has been shown to increase the expression of oxytocin mRNA in the PVN and supraoptic nuclei (23–25). In the current study we have employed this model to examine its effect on the density of oxytocin-binding sites, which are known to be sensitive to gonadal steroids (26–32), anxiety behavior in the elevated plus-maze, and the regional neuronal activation after exposure to the elevated plus-maze (measured by the expression of mRNA for the immediate-early gene c-*fos*), and the HPA response to noise stress.

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Abbreviations: BSTo, Bed nuclei of the stria terminalis; CeA, central nucleus of the amygdala; desGlyOTA, desGlyNH$_2$,D-(CH$_2$)$_5$[Tyr(Me)^2, Thr^4]OVT; HPA, hypothalamo-pituitary-adrenal; icv, intracerebroventricular; IAL, interaural line; OTA, [D-(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT; PVN, paraventricular nucleus.

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Materials and Methods

Animals and steroid treatment

Female Sprague Dawley rats were obtained from Bantin and Kingman (Hull, UK; body weight, 225–250 g) at least 7 d before surgery.

Animals had free access to food and water throughout the study and were maintained on a 14-h light, 10-h dark illumination schedule (lights on at 0500 h). For the steroid paradigm, animals were anesthetized using a combination of Hypnorm (0.32 mg/kg fentanyl citrate and 10 mg/kg fluanisone, im; Janssen Pharmaceuticals Ltd., Oxford, UK) and diazepam (2.6 mg/kg, ip; Phoenix Pharmaceuticals Ltd., Gloucester, UK), and ovaries were removed by bilateral incisions. Before suturing the skin, a single estradiol implant was positioned beneath the skin on the flank. This implant comprised a piece of SILASTIC brand tubing (Dow Corning, Midland, MI; inside diameter, 1.6 mm) filled with an oleaginous solution of estradiol (150 μg 17β-estradiol benzoate [Sigma-Aldrich Corp., St. Louis, MO)/ml vegetable oil] and sealed with SILASTIC brand rubber compound. The length of tubing was adjusted for body weight (10 mm/100 g body weight). At the time of surgery (d 0) and on the afternoons of the following 11 d (at ~1700 h), animals received an sc injection of progesterone [5 mg progesterone (Sigma-Aldrich Corp.) in 0.1 ml vegetable oil]. On d 12–14, half the animals had the progesterone injection replaced by an injection of the oil vehicle (progesterone-withdrawn group); the others continued to receive progesterone (progesterone-maintained group). Studies were performed on the 15th d of the protocol, 3 d after withdrawal of progesterone. Animal treatments were coded and were not broken until after analysis had been performed.

Study 1: anxiety-related behavior

On d 15 of the steroid protocol, progesterone-maintained and progesterone-withdrawn animals (n = 8/group) were tested for anxiety behavior on an elevated plus-maze (55-cm-long arms; 12-cm-high walls on closed arms; elevated 80 cm from ground; painted black). Animals were placed in the central zone with their heads facing a closed arm and were allowed to explore for a 30-min period, during which behavior was video taped. This length of exposure to the elevated plus-maze was used to provide a sufficient stimulus to evoke immediate-early gene expression. Behavior was analyzed in 3-min segments, during which the number of full entries into one of the four arms was recorded; an entry was defined as when all four paws had crossed the threshold of the arm. Entries into the open arm, expressed as both absolute number and proportion of the total entries, and the time spent on the open arms were also recorded. After 30-min exposure to the maze, the animal was killed by decapitation, and trunk blood collected into heparinized tubes. After centrifugation, the plasma fraction was frozen and stored at −20 C for later determination of the levels of progesterone and estrogen. The brain was also removed and frozen on dry ice for analysis of the regional expression of oxytocin-binding sites and c-*fos* mRNA.

Study 2: HPA response to noise stress

On d 10 of the steroid regimen, animals from the progesterone-maintained and progesterone-withdrawn groups were reanesthesized using Hypnorm and diazepam, and the right jugular vein was cannulated with a SILASTIC brand-tipped polythene cannula (OD, 0.96; inside diameter, 0.58 mm; Portex, Hythe, UK) filled with 10 U/ml heparinized isotonic saline. The free end of the cannula was exteriorized through a scalp incision and passed through a protective steel spring anchored to the skull using two screws and self-curing dental acrylic. Once recovered, the end of the spring was attached to a mechanical swivel, which allowed complete freedom of movement in the cage. On d 15, automated blood collection was conducted. For collection of blood samples, each animal was connected to an automated blood-sampling system, as previously described (33, 34). The collection of 20-μl blood samples for the measurement of corticosterone concentrations commenced at 0600 h on the 15th d and continued every 10 min for 4 h. At 0800 h, a noise generator was activated, and rats were exposed to 114 dB white noise (12,000–60,000 Hz) for 10 min. Sampling continued for an additional 120 min, after which each animal was given an overdose of pentobarbitone, and the brain was quickly removed onto dry ice for determination of oxytocin binding.

Study 3: effect of oxytocin receptor antagonists

To determine whether the effect of progesterone withdrawal on noise-induced HPA activity with either sterile 0.9% saline or one of two selective oxytocin antagonists [D-$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr-NH_2^{9}]OVT (OTA) (35) or desGlyNH_2,D-$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT (desGly-OTA) (7, 9)], gifts from Dr. Maurice Manning (Medical College of Ohio, Toledo, OH). *In vivo* bioassays show that these two antagonists show selectivity for action at the oxytocin receptor over the vasopressin V_{1a} receptor (36). At the same time that the jugular cannulation was performed, an osmotic minipump (model 1007D, Durect Corp., Cupertino, CA) was implanted. Pumps, rated to deliver 0.52 μl/h over a 7-d period, were filled with either saline or the appropriate peptide (600 ng/μl), connected to a brain infusion device (Durect Corp.), and allowed to equilibrate overnight in isotonic saline. During surgery, an area of parietal bone was trephined, and the brain infusion device was stereotaxically positioned in the lateral ventricle. The minipump was positioned sc between the scapulae and the infusion device and was secured by means of dental acrylic holding the iv cannula and protective spring. The remaining protocol for sampling and noise-induced HPA activity was the same as that for study 2.

Hormone measurements

Total plasma corticosterone concentrations were measured directly in diluted whole blood using an RIA that employed citrate buffer, pH 3.0, to denature the binding globulin, antiserum (supplied by Prof. G. Makara, Institute of Experimental Medicine, Budapest, Hungary), and [^{125}I]corticosterone (ICN Biomedicals, Irvine, CA; specific activity, 2–3 mCi/μg). The assay had a limit of detection of 5 ng/ml. Plasma estradiol and progesterone levels were measured in trunk blood obtained from progesterone-maintained and progesterone-withdrawn animals as well as from additional groups of pregnant rats on d 16 of pregnancy before luteolysis and on d 19 after luteolysis, for comparison. Estradiol and progesterone were measured using commercially available kits (ICN; catalog no. 07-238102 and 07-270102, respectively).

Oxytocin autoradiography and mRNA expression

Brains from the animals in study 1 were analyzed for expression of c-*fos* mRNA, and brains from these animals plus some additional, similarly treated animals were analyzed for oxytocin-binding sites by autoradiography. Coronal sections (15 μm thick) were cut with a cryostat and mounted on gelatin-coated slides from four levels of the forebrain: the level of the ventrolateral septum (LSV; 8.7 mm rostral to the interaural line (IAL)), the level of the PVN (7.2 mm rostral to the IAL), the level of the ventromedial hypothalamic nucleus (VMH; 6.2 mm rostral to IAL), and through the ventral hippocampus, approximately 5.7 mm rostral to IAL [stereotaxic coordinates according to the atlas of Paxinos and Watson (37)]. This cutting protocol encompassed both areas of known oxytocin binding (4, 19, 26–32) and the PVN, which displays stress-induced c-*fos* mRNA (6, 38).

Autoradiographic detection of oxytocin-binding sites. The density of oxytocin-binding sites was determined using the selective ligand OTA, employing iodination and autoradiographic procedures previously described (39). Briefly, slides were allowed to dry at room temperature and then were preincubated for 15 min in 50 mM Tris-HCl (pH 7.4). Sections were incubated in [^{125}I]OTA (50–67 nM) diluted in Tris-HCl containing 0.1% BSA and 5 mM $MgCl_2$ for 2 h before washing (twice, 10 min in Tris-HCl), rinsed with cold deionized water, and air dried. Specificity was determined by displacement of OTA binding using 10 μM oxytocin in the incubation buffer. When dry, sections were exposed to [^{3}H]Hyperfilm (Amersham Biosciences, Little Chalfont, UK) for 4–5 d at room temperature before developing. All sections were processed simultaneously and exposed on a single film. Autoradiograms were quantified by computer-assisted densitometric analysis (Kontron Zeiss Electronics Ltd., Watford, UK). The areas chosen for analysis were the LSV, VMH, oval nucleus of the bed nuclei of the stria terminalis (BSTo; also known as the lateral dorsal BST), and central nucleus of the amygdala (CeA).

In situ hybridization histochemistry of c-fos mRNA. In situ hybridization was performed as previously described (6) using a 680-bp riboprobe complementary to the coding sequence rat c-*fos*. All sections were hybridized in the same incubation and exposed to photographic film (Hyperfilm, Amersham Biosciences) together with a series of ^{35}S-labeled standards. Developed films were analyzed using public domain image analysis software (MD Image program; National Institutes of Health, Bethesda, MD), and the integrated optical densities were calculated.

Statistical analysis

Values shown represent either individual datasets or the mean ± SE for groups. Statistical significance was tested by *t* test or ANOVA with *post hoc* Tukey test, as appropriate.

Results

Effect of steroid manipulation on plasma hormone levels and oxytocin binding within the forebrain

Animals that had undergone ovariectomy and a steroid replacement regimen in which progesterone was maintained throughout the study had plasma estradiol and progesterone levels that closely matched those seen on d 16 of pregnancy (Table 1). However, animals that underwent a steroid regimen that included progesterone withdrawal for the final days of the study had significantly lower plasma progesterone concentrations. The progesterone levels seen in this group were very similar to those measured on d 19 of pregnancy after luteolysis had occurred (Table 1).

Autoradiography for OTA binding revealed oxytocin receptor expression in selective areas of the limbic system and hypothalamus (Fig. 1, A and B) with patterns of expression similar to those reported previously (40, 41). Progesterone withdrawal had region-specific effects on oxytocin receptor densities within some of these areas (Fig. 1C). There was a significant increase in OTA binding in the LSV (Fig. 1Ci; $P < 0.025$) and the VMH (Fig. 1Cii, $P < 0.005$). as measured by the change in OD. However, there was no significant change in binding in either the BSTo (Fig. 1Ciii) or the CeA (Fig. 1Civ).

Effect of progesterone withdrawal on anxiety-related behavior

Animals placed on the plus-maze showed a characteristic pattern of behavior. They displayed a relatively high level of exploratory activity during the initial period of exposure to the maze, as shown by the total number of arm entries, before significantly reducing their activity during the next 15 min (Fig. 2A). Total activity levels, as indicated by the total number of arm entries on the maze, were similar between the two groups of animals (Fig. 2A); however, more subtle differences in where they chose to spend their time on the maze could be seen. Animals that had undergone progesterone withdrawal spent more time in the more exposed open arms of the plus-maze than those maintained on progesterone (Fig. 2B). This difference was significant over the first 6 min of the study ($P < 0.05$) when total exploratory activity was the greatest. The increase in the amount of time spent in the open arm of the maze was associated with an increase in the number of individual entries into this portion of the plus-maze (Fig. 2C), even when expressed as a percentage of the total number of arm entries (Fig. 2D). Both of these measures were significant over the first 3 min of the experiment. During the second 15 min of the study, when exploratory behavior had diminished, both groups spent the vast majority of time in the less-exposed closed arms of the maze.

After exposure to the plus-maze, c-*fos* mRNA expression was detected in many regions of the forebrain known to be activated by this anxiogenic stimulus (42). In most of these regions, including the dorsal hippocampus, dorsal dentate gyrus, piriform cortex, and VMH, c-*fos* mRNA levels were

TABLE 1. Plasma concentrations of estradiol-17β and progesterone in ovariectomized female rats with estradiol and progesterone replacement for 14 d (progesterone maintained), and those in which the progesterone replacement was substituted for oil over the last 3 d of treatment (progesterone withdrawn)

	Estradiol-17β (ng/ml)	Progesterone (ng/ml)
Progesterone maintained	126.0 ± 27.0 (6)	54.4 ± 2.7 (5)
Progesterone withdrawn	114.0 ± 24.5 (5)	30.8 ± 6.8[a] (6)
Pregnant (d 16)	132.7 ± 14.0 (8)	59.6 ± 11.9 (7)
Pregnant (d 19)	113.7 ± 9.3 (12)	34.5 ± 5.2[a] (6)

Data obtained from intact animals on d 16 and 19 of pregnancy are shown for comparison. Values shown are mean ± SE for the group sizes shown in *parentheses*.

[a] $P < 0.05$ *vs.* d 16 or progesterone-maintained group, respectively (Student's *t* test).

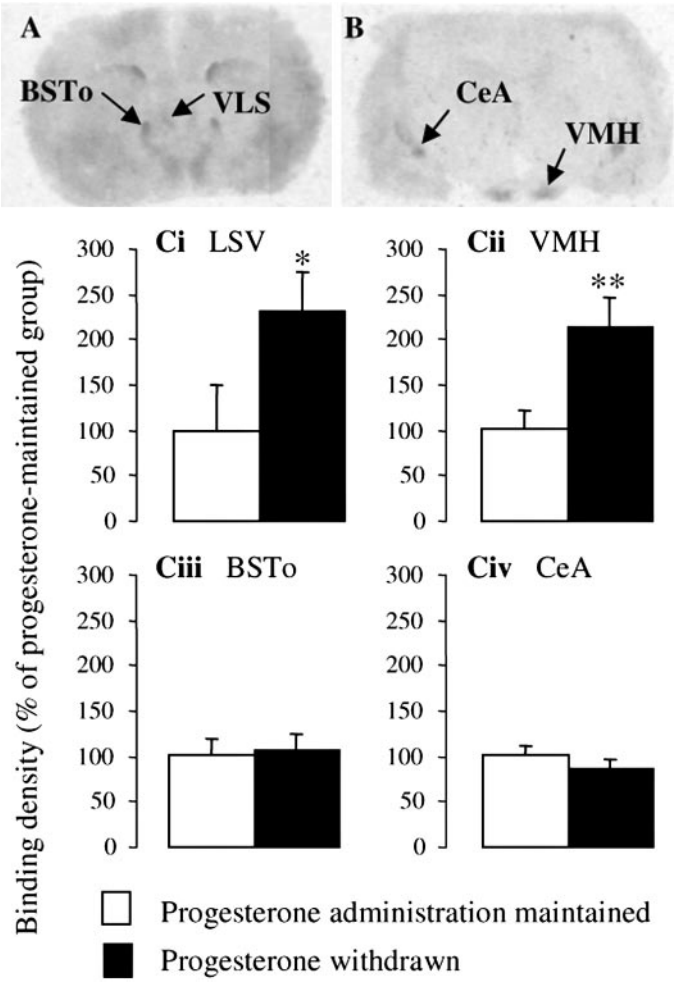

FIG. 1. Autoradiographs showing regional binding of OTA to the forebrain (A and B) and the relative change in binding in the LSV (Ci), the VMH (Cii), the BSTo (Ciii), and the CeA (Civ) of ovariectomized female rats that had undergone estradiol and progesterone replacement for 14 d (progesterone-maintained; □) and those in which progesterone replacement was substituted for oil for the last 3 d of treatment (progesterone-withdrawn; ■). Values are the mean ± SE OD measurements expressed as a percentage of the mean value in the progesterone-maintained group (n = 13–16). *, $P < 0.025$; **, $P < 0.005$ (compared with the progesterone-maintained group, by *t* test).

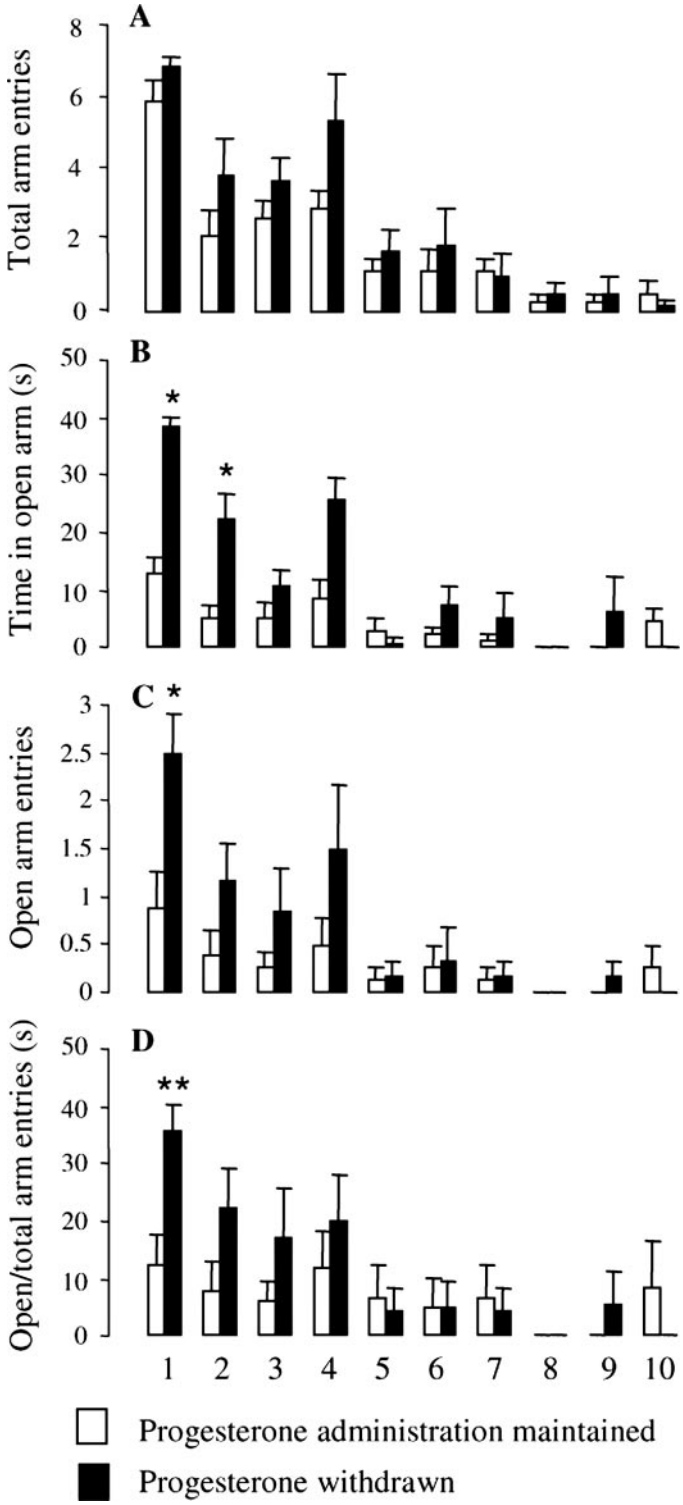

FIG. 2. The behavior of ovariectomized female rats that had undergone estradiol and progesterone replacement for 14 d (□; n = 8) and those in which progesterone replacement was substituted for oil for the last 3 d of treatment (■; n = 6) during a 30-min period on the elevated plus-maze. The parameters shown are: A, number of total arm entries; B, time spent in the open arm; C. number of open arm entries; and D, open-arm entries as a percentage of total arm entries. Each bar represents a 3-min time period. All values represent the mean ± SE. *, $P < 0.05$ compared with the progesterone-maintained group (by ANOVA with repeated measures and *post hoc* Tukey test).

comparable between the two groups (data not shown). However, in other areas, levels of appeared to be reduced by progesterone withdrawal. These areas included the LSV (Fig. 3A), PVN (Fig. 3B), and, most notably, ventral regions of the hippocampus (*e.g.* ventral CA1; Fig. 3C) and dentate gyrus (Fig. 3D). However, the large variance in the data meant that these differences failed to reach significance.

Effect of progesterone removal on the HPA axis

For the 2-h period before activation of noise stress, corticosterone concentrations were comparable in the two steroid-treated groups and were within the range expected at this time of day (Fig. 4). After the onset of noise, corticosterone concentrations rose rapidly in the progesterone-maintained group to reach a peak concentration of 243 ± 26 ng/ml at 30 min, before declining rapidly to the baseline by 50–60 min after the onset of noise stress (Fig. 4). The response in the progesterone-withdrawn group, although significant, was less than half that in the steroid-maintained group, with peak concentrations of only 138 ± 23 ng/ml. Overall, the corti-

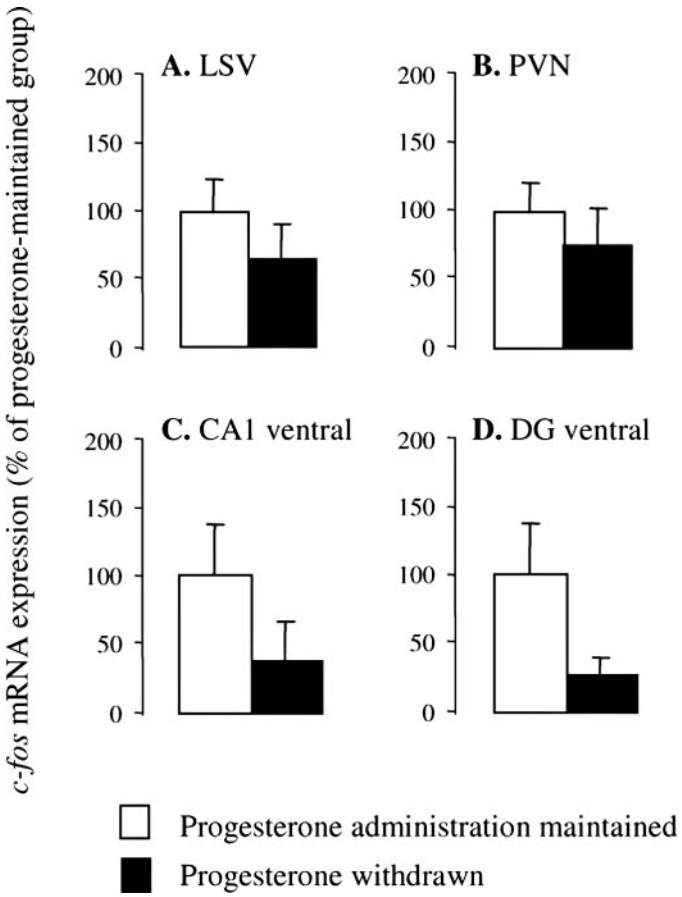

FIG. 3. The effect of 30-min exposure to the elevated plus-maze on relative expression of c-*fos* mRNA in the LSV (A), hypothalamic PVN (B), CA1 region of the ventral hippocampus (C), and ventral division of the dentate gyrus (D) of ovariectomized female rats that had undergone estradiol and progesterone replacement for 14 d (□) and those in which progesterone replacement was substituted for oil for the last 3 d of treatment (■). *Bars* show the mean ± SE OD expressed as a percentage of the mean value in the progesterone-maintained group (n = 4–8).

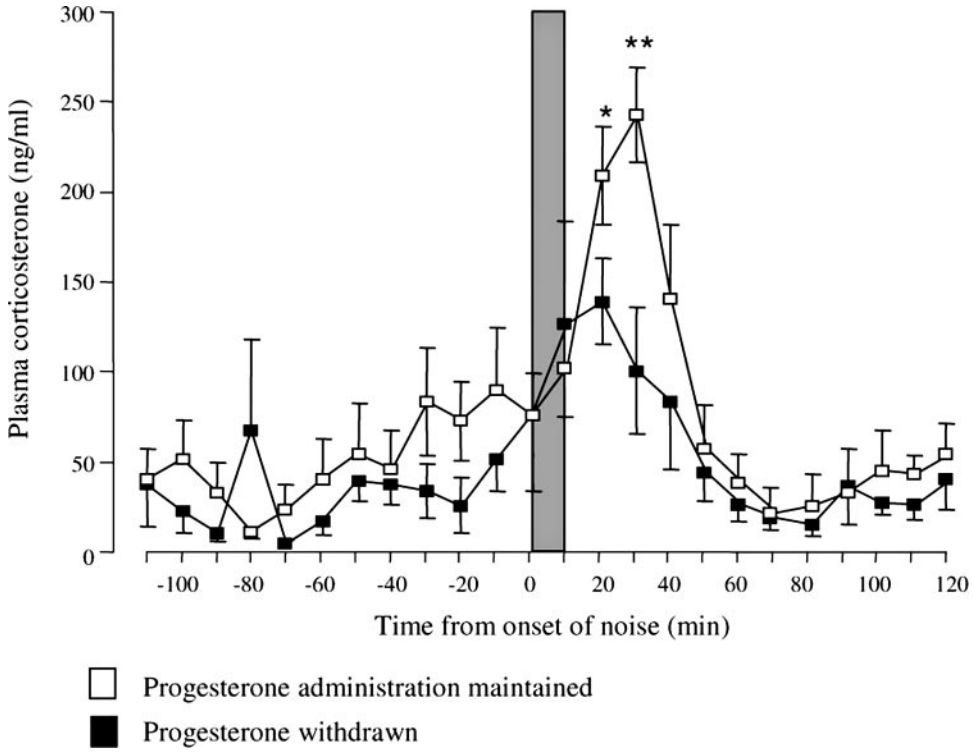

FIG. 4. Effect of noise stress (114 dB for 10 min, commencing at 0800 h; ▨) on plasma corticosterone release in ovariectomized female rats that had undergone estradiol and progesterone replacement for 14 d (□; n = 7) and those in which progesterone replacement was substituted for oil for the last 3 d of treatment (■; n = 7). Samples were taken every 10 min, and the values shown are the mean ± SEM for each. *, $P < 0.05$ compared with group in which progesterone was maintained (*post hoc t* test).

costerone response to noise was significantly smaller in this group at both 20 min ($P < 0.05$) and 30 min ($P < 0.01$) after activation of stress (Fig. 4).

Effect of oxytocin antagonists on HPA response to noise

In control animals receiving icv infusion of saline, animals that underwent progesterone withdrawal showed a significant reduction in the magnitude of the corticosterone response to noise stress compared with progesterone-maintained animals (Fig. 5A). The icv administration of the oxytocin antagonist OTA to animals that had undergone progesterone withdrawal failed to restore the magnitude of the response to noise stress; the overall response was very similar to that in the progesterone-withdrawn, icv saline-treated control group and was significantly attenuated when compared with the progesterone-maintained icv-treated saline controls (Fig. 5B). However, the selective oxytocin antagonist desGly-OTA completely reversed the effect of progesterone withdrawal on the HPA response to noise stress; the levels of corticosterone produced by noise stress in this group were significantly higher than those in progesterone withdrawn, icv-saline controls and comparable with those in the progesterone-maintained icv-treated saline control group (Fig. 5C). Neither antagonist affected basal (prestress) levels.

Discussion

It has been established that exogenous oxytocin has a potent effect in modulating anxiety and stress response (1–6). In the present study we show that a gonadal steroid model that induces endogenous oxytocin gene transcription (23–25) will up-regulate oxytocin-binding sites and has coincident effects on attenuating anxiety behavior and stress-induced HPA activity. The action of an oxytocin antagonist indicates that this stress-attenuating effect is in part dependent upon endogenous oxytocin. Thus, these data provide an important link between dynamic variations in gonadal steroid levels and anxiety behavior and stress responses, and thereby substantiate an important anxiolytic role for central oxytocin.

Steroid-induced oxytocin receptor binding

The model of estradiol/progesterone treatment with and without progesterone withdrawal produced plasma steroid levels similar to those measured in late pregnancy over the period of luteolysis. Previously, this model has been shown to increase the expression of oxytocin mRNA (23–25), and the present data show a region-specific increase in oxytocin receptor binding. Together, these two effects result in a marked increase in central oxytocinergic neurotransmission, with some degree of regional selectivity.

The ability of estradiol to increase oxytocin receptor expression in the brains of ovariectomized animals is well known (26–32). This effect has been shown to be region specific, with increased binding in the VMH (4, 26–29) and the principal nucleus of the BST (31, 32, 39), but much less or absent effects in the BSTo (41) and CeA (32). The present data show that progesterone withdrawal (with no change in estradiol administration) also causes selective induction of oxytocin binding in the VMH and LSV, while having no effect on the BSTo and CeA. Interestingly, progesterone receptor immunoreactivity (43) and mRNA (44) have been shown to be highly expressed in the VMH, but are relatively weakly expressed in other areas, including the amygdala and BST. Thus, the presence of a receptor on which progesterone can act may be the basis of this region-specific effect. Neverthe-

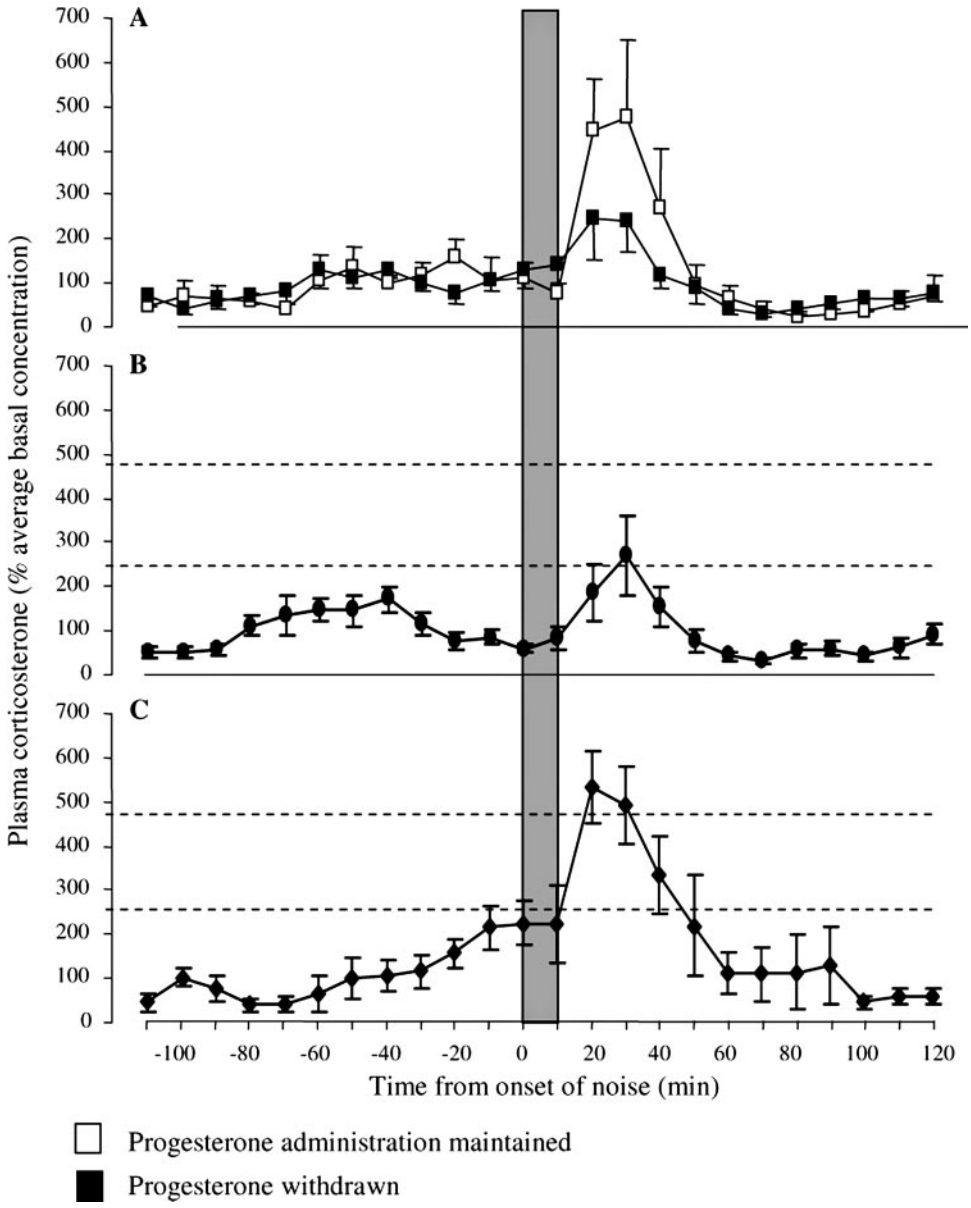

FIG. 5. Effect of icv administration of oxytocin receptor antagonists on the corticosterone response to noise stress (114 dB for 10 min, commencing at 0800 h; *outlined boxes*). All groups were ovariectomized and underwent estradiol replacement for a 14-d period before the study. A, Results are from control rats infused with 0.52 μl saline/h, icv, for 5 d. One group was maintained on progesterone replacement throughout (□; n = 5), whereas in the other group, progesterone replacement was substituted for oil for the last 3 d of treatment (■; n = 6). B, Effect of icv infusion of OTA (312 ng/h; n = 7) for 5 d in progesterone-withdrawn animals (●). C, Effect of icv infusion of desGly-OTA (312 ng/h; n = 5) in progesterone-withdrawn animals (♦). For comparison, in B and C, the maximal stress-induced corticosterone values measured in the progesterone-withdrawn and progesterone-maintained saline-infused groups from A are shown by the *upper* and *lower dotted lines*, respectively. Samples were taken every 10 min, and values shown are the mean ± SEM.

less, the mechanisms underlying this induction process are unclear. Studies of the uterine oxytocin receptor gene have suggested that progesterone treatment can either block the stimulatory action of estrogen (45) or have no effect (46). However, in the case of receptor expression in the brain, the ability of progesterone withdrawal to increase oxytocin binding is unlikely to be due to disinhibition, because previous studies have shown no difference between oxytocin binding in animals treated with estradiol and those treated with combined estradiol and progesterone in several areas, including the VMH (32).

Steroid modulation of anxiety behavior

The withdrawal of progesterone caused a marked increase in the proportion of open-arm entries in the elevated plus-maze, particularly during the initial exposure. Gonadal steroids have been shown to have variable effects on anxiety behavior depending on the steroid treatment and the anxiety test employed (47, 48). Recent reports have shown that estradiol has an antianxiety effect in ovariectomized animals tested on the elevated plus-maze when given as a single sc injection 48 h before the test (49) or as a pellet implant 6 d before testing (50). Interestingly, acute administration of progesterone also has an anxiolytic effect, which is believed to be mediated through conversion to metabolites that function as allosteric modulators of the γ-aminobutyric acid receptor. Thus, ovariectomized rats given estradiol and acute progesterone show greater exploration of the open-arm compared with nontreated ovariectomized animals (51), whereas progesterone injection 4–6 h before testing will increase the proportion of open-arm entries (52, 53). Conversely, others have shown that progesterone given to mice after 7-d treatment with estradiol will decrease open-arm entries (54). However, none of these treatments provides the sequential

changes in steroids that have been shown to induce the expression of oxytocin and its receptor (23–25). In the closest model to the present study, Stoffel and Craft (47) recently showed that 16 d of treatment with estradiol (2.5 μg/d) and progesterone (4 mg/d), followed by a high dose of estradiol alone (50 μg/d), had no significant effect on behavior on the elevated plus-maze 24 h after the last injection. However, testing occurred 8 d after the last injection of progesterone (compared with 3 d in the present study), and the effect on oxytocin expression may have been lost at this stage.

Steroid modulation of anxiety-induced c-fos mRNA expression

In addition to measuring anxiety behavior, this study provided evidence that areas of the brain exhibited differential c-*fos* mRNA expression after exposure to the elevated plus-maze. Control animals that had not been placed on the plus-maze were not included in our study design, and we assumed that most c-*fos* mRNA expression was induced by the anxiogenic stimulus. Thus, although we cannot exclude the possibility of different levels of basal gene expression between the two groups, our previous studies have shown that the level of c-*fos* mRNA is virtually undetectable in control animals not exposed to a stimulus, particularly in the PVN and LSV (6, 38). The elevated plus-maze has been shown to increase the expression of both c-*fos* mRNA (42) and Fos protein (55, 56) in discrete brain regions, including the hippocampus, PVN, and lateral septum, suggesting that these are components of an anxiety-related circuit. The present data showed that c-*fos* mRNA was expressed in a number of cortical and subcortical regions after exposure to the maze, and that in most of these areas, there was no difference in the level of gene expression between steroid groups. However, in the ventral hippocampus, there was a strong trend for a region-specific effect, with marked attenuation of mean c-*fos* mRNA levels after progesterone withdrawal. Although this decrease failed to achieve statistical significance ($P = 0.08$ for ventral DG), the large effect size suggests that this area may play an important role in processing of responses to the anxiety of the maze.

The CeA is another region implicated in the control of fear and anxiety behavior (57, 58), which possesses oxytocin-sensitive neurons (40). However, this region showed no steroid-induced change in oxytocin binding, consistent with previous reports of a lack of effect of estradiol (4, 32). Furthermore, c-*fos* mRNA measurements have shown no activation after either a stressful stimulus such as restraint (6) or an anxiogenic stimulus such as the plus-maze (present study). Despite this apparent lack of activation, Bale *et al.* (4) showed that local infusions of oxytocin into the CeA increase exploration of the central area of the open field, but have no effect on anxiety behavior in the elevated plus-maze. This suggests that oxytocin might affect different components of anxiety behavior through at least two mechanisms: one that involves the CeA and modulates open-field activity, and another that is steroid induced and affects plus-maze behavior, independent of CeA involvement. Additional studies will be required to substantiate this division.

Role of oxytocin in steroid modulation of HPA activation

In two separate studies we showed that progesterone withdrawal markedly attenuated the corticosterone response to noise stress, consistent with an antistress or anxiolytic effect. Although the effects of gonadal steroids on stress-induced HPA activity have been previously studied, most studies have employed steroid treatments that provide simple steroid replacement after ovariectomy. Thus, compared with ovariectomized rats, ACTH and corticosterone responses to a novel environment are enhanced by treatment with estradiol or combined estradiol and progesterone (59), and chronic (21 d) treatment with estradiol increases the corticosterone response to footshock or ether vapor (60). Likewise, we have recently shown that ovariectomized females had a significantly smaller corticosterone response to noise stress than intact females and that the response is restored by estradiol replacement (61). In contrast, using a more severe stimulus of 20 min of restraint, Viau *et al.* (62) showed that peak corticosterone levels were unaffected by gonadal steroids (a similar lack of effect was seen using the current steroid paradigm; data not shown), whereas others showed that the response to restraint may be reduced by estradiol treatment or combined estradiol and progesterone (63). However, despite these reports that estradiol can alter stress-induced HPA activity, it should be remembered that estradiol did not vary between treatments in the present studies. Indeed, this is the first report of stress responses using a sequential model of steroid treatments that model late pregnancy. In this respect, the observed effect of progesterone withdrawal contrasts with a report that pretreatment of rats with a single dose of progesterone (50 μg/kg) significantly attenuated the elevation of corticosterone after an emotional stress (2-min intermittent air puff stress) (64), suggesting that the pattern of steroid exposure plays a critical role in the effect.

Most importantly, we have shown that the effect of progesterone withdrawal could be reversed by simultaneous central infusion of an oxytocin antagonist, desGly-OTA. Previously, this antagonist has been shown to be effective in potentiating stress-induced HPA activity and anxiety behavior on the elevated plus-maze (7, 9). Interestingly, the antagonist that is widely used for the detection of oxytocin-binding sites (OTA) and has been shown to block oxytocin-induced excitatory responses *in vitro* (65) did not modify this stress-attenuating effect of progesterone withdrawal. This is surprising given the similar reported antioxytoxic potencies of the two antagonists when tested acutely both *in vitro* and *in vivo* (66). It is possible that the contrasting efficacy may relate to different physicochemical properties of the two antagonists.

The demonstration that the stress-attenuating effect of progesterone withdrawal are sensitive to an oxytocin antagonist substantiates the role of oxytocin in the stress response. In addition to the ability of exogenous oxytocin to reduce HPA responses (3, 6) and regional expression of c-*fos* mRNA after restraint (6), oxytocin has been shown to affect anxiety behavior (7–9), and female oxytocin knockout mice show reduced open-arm entry (increased anxiety) on the elevated plus-maze, which can be reversed by the infusion of oxytocin

into the lateral ventricle (10, 11). Conversely, infusion of the oxytocin receptor antagonist D-[D-Tyr(Et)^2,Thr^4]OVT (Atosiban) reduced open-arm entries, indicative of an effect of endogenous oxytocin in controlling anxiety behavior (10). Interestingly, this increased anxiety is associated with increased stress-induced Fos expression in the medial amygdala (11), an area that we have shown in the rat to be not sensitive to the effects of oxytocin (6). The present data indicate that this endogenous peptide activity may be under the regulation of dynamic variations in gonadal steroid levels.

Significance for pregnancy-related changes in stress responses

In addition to the overall conclusion that dynamic changes in gonadal steroid levels have an anxiolytic effect that is in part mediated through an effect of central oxytocin, it is tempting to speculate about the significance of this for adaptive changes occurring in late pregnancy. In the rat, levels of oxytocin mRNA in the hypothalamus (16, 17) and expression of oxytocin receptors (19, 20) increase toward the end of pregnancy, and this has been suggested to play a role in establishing the affiliation between mother and offspring (67, 68). At the same time, oxytocin may drive the decline in stress responses and anxiety behavior that occurs toward the end of pregnancy (13, 14, 38). The present data are consistent with these adaptive changes in stress and anxiety being induced by an effect of central oxytocin and triggered by the fall in progesterone levels that accompanies luteolysis (69). However, the fact that stress responses are unaffected by central administration of an oxytocin antagonist late, at the time of parturition (22), may suggest that multiple mechanisms contribute to the regulation of stress and anxiety throughout the periparturient period.

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