The neurosteroid allopregnanolone modulates oxytocin expression in the hypothalamic paraventricular nucleus

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A KEY FACTOR REGULATING the central expression of oxytocin (OT) in the rat is the pervading ovarian steroid milieu (1, 3, 7, 8, 17–20). If an ovariectomized rat is administered sequential estradiol (E2) and progesterone (P) followed by P withdrawal have increased hypothalamic oxytocin (OT) mRNA and peptide levels relative to sham-treated animals. This increase is prevented if P is sustained. In the central nervous system, P is metabolized to the neurosteroid allopregnanolone (3α-hydroxy-5α-pregn-20-one), which exerts effects by acting as a positive allosteric modulator of GABA<sub>A</sub> receptor/Cl⁻-channel complexes. In the present study, ovariectomized rats that received sequential E<sub>2</sub> and P for 2 wk followed by P withdrawal were administered allopregnanolone at the time of P withdrawal. Hypothalamic and plasma allopregnanolone concentrations, serum E<sub>2</sub> and P concentrations, and hypothalamic OT mRNA levels were measured at death. Steroid-induced increases in OT mRNA were attenuated in animals treated with allopregnanolone at the time of P withdrawal. The results suggest that allopregnanolone plays an important modulatory role in steroid-mediated increases in hypothalamic OT.

estrogen; gamma aminobutyric acid; progesterone
P levels in the rat (6), we hypothesized that GABA tone may be enhanced in the presence of P (which favors formation of A) and diminished with P withdrawal (which results in lower A concentrations). Diminished GABA tone may contribute to enhanced OT expression. To test this hypothesis, we administered A at the time of P withdrawal in estrogen-primed rats and determined its effects on hypothalamic OT mRNA concentrations.

MATERIALS AND METHODS

Animals. Rats were ovariectomized by the supplier (Harlan Sprague Dawley, Indianapolis, IN) 1 wk before shipment to our facility. To verify the completeness of ovariectomy, blood drawn by jugular venipuncture was assayed for E2. Only those animals with circulating E2 concentrations that were near or less than the lower limits of detectability of the assay (<5 pg/ml) were used in the experiments. At the time of arrival, animals were −10–11 wk of age (225–275 g body wt) and were acclimated to their surroundings for 7 days before a study. Animals were housed singly in stainless steel cages in a temperature (22°C)- and humidity (60%)-controlled room with automatic light-dark cycle (lights on at 0700 and off at 1900). Rats had free access to water and food (Prolab, Rat, Mouse, Hamster 300, PMI Feeds, St. Louis, MO).

Experiments 1 and 2 were performed on separate sets of rats. In experiment 1, the goal was to determine the dose of exogenous A to administer to an ovariectomized rat to achieve a plasma level equivalent to the level in a P-treated animal and to determine whether the plasma level of A correlated with the brain level of A. In experiment 2, once the appropriate dose of A was identified, we studied additional animals for the effect of A on hypothalamic PVN OT mRNA levels.

Experiment 1—blood and brain concentrations of A in rats. In this experiment, the dose of A (5 mg·kg⁻¹·day⁻¹) chosen was twice that which had previously been reported to induce anxiolysis in the rat (2–2.5 mg·kg⁻¹·day⁻¹ sc) (10). Brain tissue was removed at death, the hypothalami were extracted, and tissue A concentrations were measured by RIA. Trunk blood obtained at death was measured for A, E2, and P concentrations. Ovariectomized animals (n = 6 per group) were randomized to five treatments (Table 1). The sham group received empty capsules, and the A-alone group received A capsules. The remaining groups received E2 and P implants. In the E2/P+ group, the P implants remained in place until death. In both the E2/P– and E2/P–/A groups, P implants were removed 48 h before death. The E2/P+ group was included to verify that A concentrations declined following P removal, whereas the E2/P–/A was included to verify that A concentrations were sustained. The E2/P+ group was included to verify that A concentrations in animals in which P is sustained overlap levels in the A-supplemented group. The data were used to determine the dose of A to be used in experiment 2. P+ means P capsules were not removed, whereas P– means P capsules were removed. A+ means A capsules were not removed, whereas A– means A capsules were removed.

Experiment 2 effect of A on PVN OT mRNA. On the basis of the pilot data in experiment 1, the 5·mg·kg⁻¹·day⁻¹ dose of A appeared to be sufficient to achieve A concentrations equivalent to those in animals receiving sustained P implants. Additional animals were studied in this experiment to determine the effect of A on PVN OT mRNA. Trunk blood was collected at death, and serum E2 and P and plasma A concentrations were measured (n = 8 animals per group). The hypothalami (n = 8 per group) were processed for OT mRNA by in situ hybridization histochemistry (n = 32–35 tissue sections per group). Sufficient brain sections for in situ hybridization were obtained from four of the eight animals in each group. E2, P, and A concentrations of the animals from which brain sections were analyzed followed the trend of the entire treatment group. The E2/P+ group, which was included in experiment 1, was not included in experiment 2 because we have shown in prior publications that maintenance of P attenuates OT mRNA levels (1, 3, 7, 8, 17–20). In this experiment, we were testing whether A attenuates or abolishes the increase in OT mRNA levels that is induced by P withdrawal in the rat.

Preparation and administration of steroid hormone implants. Silastic tubing (ID 0.078 in., OD 0.125 in., Dow Corning, Midland, MI) cut to preselected length was packed with crystalline E2 or P (Sigma Chemical, St. Louis, MO) and prepared as previously described (7, 8). The doses used in the study are outlined in Table 1 and were chosen to replicate the concentrations of E2 and P achieved in prior experiments of a similar nature in this laboratory. A was purchased from Steraloids (Wilton, NH) and made into constant release capsules by Innovative Research (Sarasota, FL) at a dosage of 5 mg·kg⁻¹·day⁻¹. Sham capsules were prepared in the same manner but sealed empty. The subcutaneous placement and removal of capsules was done while animals received methoxyflurane inhalation anesthesia.

Table 1. Steroid treatments for experiments 1A and 1B

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>Insert one 2-mm sham implant</td>
<td>Insert three 30-mm sham implants</td>
<td>Remove three 30-mm sham implants, insert placebo</td>
</tr>
<tr>
<td>A</td>
<td>Insert one 2-mm sham implant</td>
<td>Insert three 30-mm sham implants</td>
<td>Remove 30-mm sham implants, insert placebo</td>
</tr>
<tr>
<td>E2/P–</td>
<td>Insert one 2-mm E2 implant</td>
<td>Insert three 30-mm P implants</td>
<td>Remove 30-mm P implants, insert A</td>
</tr>
<tr>
<td>E2/P+</td>
<td>Insert one 2-mm E2 implant</td>
<td>Insert three 30-mm P implants</td>
<td>Insert three 30-mm P implants, insert placebo</td>
</tr>
<tr>
<td>E2/P–/A</td>
<td>Insert one 2-mm E2 implant</td>
<td>Insert three 30-mm P implants</td>
<td>Insert placebo</td>
</tr>
</tbody>
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A, allopregnanolone; E2, estradiol; P, progesterone. Animals in each group were killed on day 16.
resulting in 50% formamide, 300 mM NaCl, and 80 mM Tris HCl, and were hybridized by application of 70 µl/slide of hybridization buffer. The slides were washed, and then lipidated in chloroform. After fixation, acetylation of the slides was performed in 0.25% acetic anhydride-0.1 M triethanolamine. The slides were fixed in 4% paraformaldehyde, rinsed with 0.1 M phosphate buffer, acetylated in 0.25% acetic anhydride-0.1 M triethanolamine, and mounted on gel-alum-coated slides, dried, and stored at 70°C until assay. Serum E2 and P concentrations were measured in duplicate by double-antibody RIA, using kits purchased from Diagnostic Products (Los Angeles, CA) and Diagnostic Systems Laboratories, (Webster, TX), respectively. The ovarian steroid treatments for Experiment 1 were 70°C until assay. The RIA had a minimum detectable concentration of A of 25 pg/ml and an intra-assay coefficient of variation of 8.5%. The assay has ~15% cross-reactivity with P (14). The A concentrations are corrected for this cross-reactivity.

For A determinations in tissue, brains were immediately placed in 1 ml MD TTT;2 1m MD TTT;2. 2 ml of 100% methanol and dried under nitrogen in a Savant centrifuge. Tail or trunk blood was collected into glass test tubes for E2 and P assays. Blood was centrifuged at 3,000 rpm for 15 min, and the serum was separated from the red blood cells (RBC) and stored frozen at −70°C until assay. Serum E2 and P concentrations were measured in duplicate by double-antibody RIA, using kits purchased from Diagnostic Products (Los Angeles, CA) and Diagnostic Systems Laboratories, (Webster, TX), respectively. The ovarian steroid treatments for Experiment 1 were 70°C until assay. The RIA had a minimum detectable concentration of A of 25 pg/ml and an intra-assay coefficient of variation of 8.5%. The assay has ~15% cross-reactivity with P (14). The A concentrations are corrected for this cross-reactivity.

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In situ hybridization histochemistry. Serial frozen coronal sections 20-µm thick were taken through the region of the hypothalamus extending from the rostral PVN through the caudal PVN. Tissue sections 100-µm apart were thaw-mounted on gel-alum-coated slides, dried, and stored at −70°C until hybridization. Thaw-mounted sections were fixed in 4% paraformaldehyde, rinsed with 0.1 M phosphate buffer, acetylated in 0.25% acetic anhydride-0.1 M triethanolamine HCl, pH 8.0, dehydrated through a series of ethanol washes, and dried under nitrogen in a Savant centrifuge. Tail or trunk blood was collected into glass test tubes for E2 and P assays. Blood was centrifuged at 3,000 rpm for 15 min, and the serum was separated from the red blood cells (RBC) and stored frozen at −70°C until assay. Serum E2 and P concentrations were measured in duplicate by double-antibody RIA, using kits purchased from Diagnostic Products (Los Angeles, CA) and Diagnostic Systems Laboratories, (Webster, TX), respectively. The ovarian steroid treatments for Experiment 1 were 70°C until assay. The RIA had a minimum detectable concentration of A of 25 pg/ml and an intra-assay coefficient of variation of 8.5%. The assay has ~15% cross-reactivity with P (14). The A concentrations are corrected for this cross-reactivity.

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the lower limit of detectability of the E2 assay (5 pg/ml). Serum P concentrations were 45 ± 4 ng/ml while P-filled implants were in place. After removal of P-filled implants, P concentrations declined to 9 ± 2 and 21 ± 3 ng/ml in the E2/P− and E2/P−/A groups, respectively. A concentrations in plasma and hypothalamus were higher in animals receiving A or P at the time of death (28–37 ng/ml and 4.9–7.9 ng/mg protein, respectively) than in animals receiving empty (sham) capsules or animals in which P capsules were removed before death (5–12 ng/ml and 1.1–1.8 ng/mg protein, respectively: ANOVA P < 0.0001; Fig. 2). Plasma and hypothalamic A concentrations were positively correlated (r = 0.72, P = 0.0006) as were serum P and plasma A concentrations (r = 0.94, P < 0.0001; Fig. 3).

Experiment 2. In this experiment, brains were removed from additional animals and the abundance of OT mRNA was measured in the PVN by in situ hybridization histochemistry. Differences in PVN OT mRNA abundance were found among groups, Kruskal-Wallis (P < 0.0001). Pairwise differences, Mann-Whitney U test, are shown in Fig. 4. Compared with ovariectomized rats receiving empty implants (sham), OT mRNA levels increased in both of the groups that received ovarian steroid-filled implants followed by removal of P-filled implants. However, the increase was attenuated (51% increase) in the rats receiving A at the time of P withdrawal (E2/P−/A group), whereas rats not receiving A at the time of P withdrawal (E2/P− group) had an increase of 87% relative to sham-treated controls. Compared with ovariectomized rats receiving empty implants, A alone was without effect on OT mRNA levels. Representative photomicrographs of the PVN from animals treated with sham, E2/P−, and E2/P−/A regimens are shown in Fig. 5, A and B. Increased grain density was identified in regions of the PVN that are known to contain both magnocellular as well as parvocellular OT-expressing neurons.

To be certain that there was release of the steroid from the implants, assays for E2, P, and A were performed on trunk blood collected at death from animals in this experiment. At death, plasma A concentrations were higher in animals receiving A- or P-filled implants (19–20 ± 3 ng/ml) than in animals receiving empty (sham) capsules or animals in which P capsules were removed before death (8–12 ± 1 ng/ml; ANOVA P = 0.0005). Pairwise differences were found among the following: A alone vs. sham (P = 0.006); A alone vs. E2/P− (P = 0.0003); E2/P−/A vs. sham (P = 0.01); and E2/P−/A vs. E2/P− (P = 0.0006, Fisher’s PLSD; Fig 3).

Mean (±SE) serum E2 concentrations after ovariectomy but before placement of E2-filled implants ranged from 4 to 6 ± 0.5 pg/ml, which is at or near the minimum detectable concentration of the E2 assay (5 ng/ml in the E2/P groups, P concentrations declined to 9

![Graphs of the PVN from animals treated with sham, E2/P−, and E2/P−/A regimens are shown in Fig. 5, A and B. Increased grain density was identified in regions of the PVN that are known to contain both magnocellular as well as parvocellular OT-expressing neurons.](http://ajpregu.physiology.org/DownloadedFrom)
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remained elevated above levels in both sham-treated animals and animals receiving only A.

One possible cause for the incomplete attenuation of OT may be insufficient administration of A, but this appears to be unlikely. We chose a dose of A (5 mg·kg\(^{-1}\)·day\(^{-1}\)) that is twofold greater than the dose that effectively induces anxiolysis (2–2.5 mg/kg) (10). A-induced anxiolysis is believed to be mediated by the GABA\(_A\) receptor within the CNS. Administration was via constant-release capsules, which result in steady-state levels of the steroid. We measured A concentrations in plasma and hypothalamic extracts. The methods of collection and extraction used in this RIA have been previously tested and found to minimize oxidation of the steroid. We measured A concentrations (9.8 ± 1.6 ng/ml) (6) a condition in which P levels are decreased, 19–20 ± 3 ng/ml) than in animals receiving empty ( sham) capsules or animals in which P capsules were removed. (8–12 ± 1 ng/ml), ANOVA P = 0.0005. Pairwise differences in plasma A were found between the following: A alone vs. sham P = 0.006; A alone vs. E\(_2\)/P\(-\)P, P = 0.0003; E\(_2\)/P\(-\)A vs. sham, P = 0.006; E\(_2\)/P\(-\)A vs. E\(_2\)/P, P = 0.0006, Fisher’s PLSD.

Previously reported levels in day 19 pregnant rats (9.8 ± 1.6 ng/ml) (6) a condition in which P levels are increased. The concentrations of A in plasma and hypothalamus were positively correlated, and both correlated with the serum P concentrations. Thus the dose of A appears to be sufficient to achieve concentrations equal to those of a high-P environment.

OT neurons in the PVN and SON are known to express \(\alpha_1\) and \(\beta_2\) subunits of the GABA\(_A\)-receptor complex (5, 9). Plasticity in the GABA\(_B_1\)-subunit composition within hypothalamic OT neurons has been reported in the pregnant and lactating rat (5) but not yet studied in the steroid-treated rat. In the pregnant rat, \(\alpha_1\)-subunit expression and plasma P and A concentrations rise from days 1 to 19 of pregnancy and then decline (5). As \(\alpha_1\)-subunit expression declines, \(\alpha_2\)-subunit expression increases (5). The ratio of \(\alpha_1\) to \(\alpha_2\) subunits is positively correlated with the sensitivity of the GABA\(_A\) receptor to allosteric modulation by A (5) and inversely with the activity of OT neurons. A relatively high level of A at a time when the GABA\(_A\) receptor is most sensitive to its allosteric modulation is likely to increase GABA-mediated inhibition of OT neurons. Both GABA\(_A\)-subunit expression and A influence OT expression. We have recently identified that the steroid regimen that increases OT mRNA and peptide also alters GABA\(_A\)-receptor binding.
in the SON of the steroid-treated rat (2). In future studies, we will measure GABA A subunits in animals receiving a variety of steroid paradigms.

Perspectives

In the rat, rising E 2 and declining P levels enhance OT mRNA and peptide levels in the PVN and SON (7, 8). This same ovarian steroid exposure also optimally stimulates maternal behavior (4), which typically has its onset immediately prepartum in the rat, when hypothalamic OT expression is at a maximum. P withdrawal without E 2 priming, or E 2 alone without P withdrawal, will not increase OT mRNA in the PVN and SON (8) and are suboptimal steroid paradigms for inducing maternal behavior (4). The data suggest that ovarian steroid-induced increases in PVN OT mRNA may be important for the events of late pregnancy. The ability of OT neurons in the PVN and SON to change from a quiescent pattern to one of increased synthesis during late pregnancy represents an example of functional plasticity within the CNS. Coincident with the changes in OT are cyclical changes in E 2 and P that appear to regulate OT expression. A is known to increase GABA A receptor currents in OT neurons of late-pregnant rats (5, 9), when high levels of A are present in the brain (6). Perhaps the increase in GABA-mediated mechanisms exerts an inhibitory influence on OT neurons. The decline in A concentrations coincident with the prepartum decline in P in the rat may disinhibit OT neurons and facilitate an increase in OT synthesis.

In summary, we conclude that A plays a modulatory role in the expression of hypothalamic OT in the virgin, ovariectomized steroid-treated rat. Sustained A in the absence of P partially attenuates steroid-induced increases in OT. Mechanistically, these effects are believed to be a result of the action of A as a positive allosteric modulator of the GABA A receptor. Conditions favoring high A levels result in increased GABA tone and OT inhibition, whereas conditions favoring low A levels result in decreased GABA tone and enhanced OT expression. These data provide evidence for an interaction between a peptide hormone and a major CNS neurotransmitter system. Understanding this system in the rat may provide insight into the neuroendocrine regulation of similar systems in the human.

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Fig. 5. Photomicrographs of right (A) and left (B) PVN from animals receiving sham or steroid regimens. Grain density is an index of relative abundance of OT mRNA.
REFERENCES


