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 prevailing 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, 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-pregnan-20-one), which exerts effects by acting as a positive allosteric modulator of GABA_A receptor/Cl⁻ channel complexes. In the present study, ovariectomized rats that received sequential E2 and P for 2 wk followed by P withdrawal were administered allopregnanolone at the time of P withdrawal. Hypothalamic and plasma allopregnanolone concentrations, serum E2 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

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oxidoreductase, may proceed in either direction.

**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 three 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 three 30-mm P 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 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>Remove three 30-mm P implants, insert placebo</td>
</tr>
</tbody>
</table>

A, allopregnanolone; E2, estradiol; P, progesterone. Animals in each group were killed on day 16.
Assays. 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 minimum detectable concentration of E2 and P in its respective assay was 5 pg/ml and 1 ng/ml. There is a 5% cross-reactivity of DHP, which can be reversibly metabolized from A (see Fig. 1) in the P assay (personal communication, Diagnostic Systems Laboratories). Synthetic A, in concentrations of 1–50 ng/ml, had no cross-reactivity in the P assay.

Plasma and hypothalamic A concentrations were measured using published methodology (13, 14). For plasma A determinations, blood was collected at death into polypropylene tubes containing 100 µl of EDTA (50 mg/ml) and immediately placed on ice. Within 10 min, the samples were centrifuged at 3,000 rpm at 4°C for 15 min. The plasma was separated from the RBCs, transferred to a clean polypropylene tube, and stored at −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 100 ml of 100% methanol and dried under nitrogen in a Savant (Savant Instruments, Farmingdale, NY). Dried samples were stored at −70°C until assay.

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 mammillary bodies. This thick slice of hypothalamus contains the entire paired PVN (7, 8). This coronal section was placed on an iced Petri dish, and the cortex was trimmed from the hypothalamus. The hypothalami were placed in pre-cooled polypropylene tubes containing 50% aqueous methanol and 1% acetic acid and homogenized. The homogenates were centrifuged at 1,200 rpm for 10 min at 4°C. The supernatant was applied to a C-18 silica cartridge (SEP-PAK, Waters Associates, Milford, MA) that had been equilibrated with 50% aqueous methanol and 1% acetic acid. The cartridge was then sequentially washed with 10-ml volumes of 50% aqueous methanol and 1% acetic acid, deionized water, and 50% aqueous methanol. The steroid fraction was eluted with 10 ml of 100% methanol and dried under nitrogen in a Savant condenser (Savant Instruments). Dried samples were stored at −70°C until assay.

For A determinations in tissue, brains were immediately placed in 100 ml of 100% methanol and dried under nitrogen in a Savant condenser (Savant Instruments). Dried samples were stored at −70°C until assay.

The ovarian steroid treatments for experiment 1 were made by post hoc Fisher’s protected least significant difference (PLSD) tests. For PVN OT mRNA data, a nonparametric test (Kruskal-Wallis) was used because of the variation in number of sections analyzed in each treatment group, and pairwise comparisons between groups were determined with the Mann-Whitney U test. Significance of correlations was determined with Fisher’s r to z transformation. All analyses were done on a Macintosh Power Computer with the StatView Statistics Package (Abacus Concepts, Berkeley, CA). A confidence level of P < 0.05 was considered significant.

RESULTS

Experiment 1. The ovarian steroid treatments for experiment 1 are illustrated in Table 1. Serum E2 concentrations after ovariectomy, but before placement of E2-filled implants, were 7 ± 2 pg/ml, which is near...
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 hypothalami 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 ovariian 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-filled capsules were removed (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

Fig. 2. Mean (±SE) concentrations of serum P (A), plasma A (B), and hypothalamic A (C) at death in experiment 1. Animals treated with P at time of death [E2 (estradiol)P+] had higher P concentrations than each of the other treatment groups [P < 0.0001, Fisher’s protected least-squares difference (PLSD)]. Plasma and hypothalamic A levels were higher in animals receiving A or P at time of death than in animals receiving empty (sham) capsules or animals in which P capsules were removed before death (ANOVA P < 0.0001). In particular, A levels achieved by treatment with A capsules were equal to or higher than A levels achieved in animals treated with P capsules only. Pairwise comparison with Fisher’s PLSD showed the following significant differences between treatment groups for plasma and hypothalamic A concentrations, respectively: sham and A alone, P < 0.0001 and P < 0.0001; sham and E2/P +, P = 0.002 and P = 0.0002; sham and E2/P –/A, P < 0.0001 and P = 0.001; A alone and E2/P +, P = 0.04 and P = 0.008; A alone and E2/P –, P < 0.0001 and P < 0.0001; E2/P + and E2/P –, P < 0.0001 and P < 0.0001; E2/P – and E2/P –/A, P < 0.0001 and P < 0.0001. In hypothalamus only, A alone was significantly higher than E2/P –/A, P = 0.001.
modulation of OT expression in the E2-primed rat. Ovariectomized steroid-treated rat to study the role of P

Mean (±SE) plasma P ranged from 34 to 36 pg/ml, thus verifying the completeness of ovariectomy. When implants were in place, serum E2 and P concentrations were higher in steroid-treated than in sham-treated animals (ANOVA P < 0.0001). Mean (±SE) serum E2 concentrations ranged from 20 to 26 ± 3 pg/ml in treatment groups receiving E2-filled capsules, and mean (±SE) plasma P ranged from 34 to 36 ± 3 ng/ml in treatment groups receiving P-filled capsules. Mean (±SE) serum P concentrations declined to comparable levels in E2/P- and E2/P-/A treatment groups following the removal of P-filled capsules to 10–13 ± 2 ng/ml.

**DISCUSSION**

Our findings support the hypothesis that A modulates OT mRNA levels in the hypothalamus of the virgin, ovariectomized steroid-treated rat. Our data indicate that PVN OT mRNA expression increased 87%, and A concentrations decreased 30–40% after P withdrawal in ovariectomized rats previously treated with E2 and P. Administration of A to E2/P-rats after P withdrawal decreased OT mRNA concentrations by 40% (P < 0.04; Fig. 3).

In our studies (1, 3, 7, 8, 17–20), we used the virgin, ovariectomized steroid-treated rat to study the role of P modulation of OT expression in the E2-primed rat. Steroid administration via subcutaneously implanted continuous-release capsules results in steady-state concentrations of steroid hormones and avoids the fluctua-

tions in hormone concentrations that may result with daily injections. The sequence and duration of steroids can be tailored to replicate the steroid milieu of pregnancy (4), a physiological condition in which the changes in OT have been linked to the steroid environment (7, 8). We have found that E2 priming (7, 8), declining P (3, 7, 8), and long duration of steroid exposure (1) favor increased OT expression, whereas omission of E2 (7, 8) or maintenance of P (3, 7, 8, 17–20) attenuate OT expression. The sequence and duration, rather than the absolute concentrations of E2 or P achieved during treatment, are the main factors modulating the relative expression of OT (1, 3, 7, 8). In the present study, suppressed E2 concentrations verified the completeness of ovariectomy, E2 and P measurements during capsule implantation quantified the release of the steroid, and P concentrations after removal of P-filled implants assessed the decrement in P that is essential for the increase in OT. Animals administered P-filled implants in this study had sustained P concentrations ranging from 32 to 46 ng/ml, which are typically higher than P concentrations across the estrous cycle. Serum P concentrations are <25 ng/ml during the rat estrous cycle (15), except during proestrus when P concentrations peak briefly (~12 h) at 60 ng/ml.

The slightly higher concentrations of P in the E2/P–A vs. E2/P– groups in experiment 1 are most likely due to the differences in the rate of decline in P between the two groups. We assayed synthetic A (purchased from Steraloids) in the P assay and found no cross-reactivity. Figure 1 also illustrates that A is not detected in the P assay because animals supplemented with A capsules (A alone) did not have significantly different levels of P than animals bearing empty capsules (sham). If A were detected in the P assay, then P levels should also be higher in the A-alone vs. sham group. Thus A cross-reactivity is not the cause for the discrepancy in P levels between E2/P–A– and E2/P– groups, and A is not detected in the P assay. The minor cross-reactivity of DHP in the P assay (~5%) is not likely the reason that levels of P are higher in animals treated with A. If this were so, then higher P concentrations should be found in every experiment in which animals are treated with A. This was not the case. For example, the P concentrations in animals treated only with A in experiment 1 were not different than sham animals. Moreover, there were no significant differences between P levels in the E2/P–A vs. E2/P– in experiment 2.

Declining P concentrations in the E2-primed rat increase hypothalamic OT expression, whereas maintenance of P attenuates this response. A concentrations are positively correlated with plasma P concentrations (6). When P implants are in place, A concentrations are higher than when P implants are removed. We determined the effect on OT mRNA levels of sustaining A at the time of P withdrawal. Animals treated with A (E2/P–A) at the time of P withdrawal had an attenuated OT response relative to cohorts that did not receive A (E2/P–). Although decreased, OT mRNA
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 plasma or hypothalamic proteins, respectively. The plasma concentration of A in these animals was also nearly fourfold greater than 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 α₁- and α₂- subunit mRNA (14). The antisera for RIA has been extensively tested for specificity to A (14), and the minor cross-reactivity with P was factored into the assay result. The goal was to achieve plasma A concentrations in the treated animals that were equivalent to the levels in P-treated animals. Animals treated with A constant-release implants achieved plasma and hypothalamic concentrations of A (35–38 ng/ml and 4.9–7.9 ng/mg of protein, respectively) that were equal to or greater than animals in which P was maintained (28 ng/ml and 5.5 ng/mg protein, respectively). The plasma concentration of A in these animals was also nearly fourfold greater than
in the SON of the steroid-treated rat (2). In future studies, we will measure \( \text{GABA}_A \) 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 \( \text{GABA}_A \)-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 disinherit 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 \( \text{GABA}_A \) 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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