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Central Opioid Inhibition of Neuroendocrine Stress Responses in Pregnancy in the Rat Is Induced by the Neurosteroid Allopregnanolone

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The hypothalamus–pituitary–adrenal (HPA) axis is the major neuroendocrine stress response system. Corticotropin-releasing hormone (CRH) neurons in the parvocellular paraventricular nucleus (pPVN) play a key role in coordinating responses of this system to stressors. The cytokine interleukin-1β (IL-1β), mimicking infection, robustly activates these CRH neurons via a noradrenergic input arising from the nucleus tractus solitarii (NTS). In late pregnancy, HPA axis responses to stressors, including IL-1β, are attenuated by a central opioid mechanism that auto-inhibits noradrenaline release in the PVN. Here we show that the neuroactive progesterone metabolite allopregnanolone induces these changes in HPA responsiveness to IL-1β in pregnancy. In late pregnancy, inhibition of 5α-reductase (an allopregnanolone-synthesizing enzyme) with finasteride restored HPA axis responses (rapidly increased pPVN CRH mRNA expression, ACTH, and corticosterone secretion) to IL-1β. Conversely, allopregnanolone reduced HPA responses in virgin rats. In late pregnancy, activity of the allopregnanolone-synthesizing enzymes (5α-reductase and 3α-hydroxysteroid dehydrogenase) was increased in the hypothalamus as was mRNA expression in the NTS and PVN. Naloxone, an opioid antagonist, restores HPA axis responses to IL-1β in pregnancy but had no additional effect after finasteride, indicating a causal connection between allopregnanolone and the endogenous opioid mechanism. Indeed, allopregnanolone induced opioid inhibition over HPA responses to IL-1β in virgin rats. Furthermore, in virgin rats, allopregnanolone treatment increased, whereas in pregnant rats finasteride decreased proenkephalin-A mRNA expression in the NTS. Thus, in pregnancy, allopregnanolone induces opioid inhibition over HPA axis responses to immune challenge. This novel opioid-mediated mechanism of allopregnanolone action may alter regulation of other brain systems in pregnancy.

Introduction

The brain activates the endocrine response to acute stress through multiple stressor processing pathways that converge onto corticotropin-releasing hormone (CRH) neurons in the parvocellular paraventricular nucleus (pPVN) of the hypothalamus (Buller et al., 2001; Choi et al., 2007). During stress, these neurons release CRH into the hypothalamo-hypophysial portal system, stimulating ACTH secretion from anterior pituitary corticotrophs, which drives glucocorticoid (corticosterone) synthesis and secretion by the adrenal cortex. Administration of the cytokine interleukin-1β (IL-1β), to mimic infection, robustly stimulates this hierarchical hypothalamus–pituitary–adrenal (HPA) axis (Buller et al., 2001; Brunton et al., 2005). IL-1β activates pPVN CRH neurons via a direct noradrenergic pathway from the nucleus tractus solitarii (NTS) (Rivier et al., 1989). Circulating IL-1β binds to IL-1 receptors on brain microvessel endothelium, triggering local prostaglandin synthesis (Zhang and Rivest, 1999; Rivest et al., 2000), which excites PVN-projecting noradrenergic A2 neurons (Ericsson et al., 1994).

In late pregnancy, reduced activation of pPVN CRH neurons by stressors leads to attenuated ACTH and corticosterone responses (Brunton et al., 2008). This adaptation may be important in altered metabolic and immune balance in pregnancy and in protecting fetuses from adverse programming (Brunton and Russell, 2008). In late pregnant rats, HPA axis responses to systemic IL-1β are essentially absent, through inhibition by an emergent endogeneous opioid mechanism (Brunton et al., 2005). Systemic IL-1β still activates NTS neurons in pregnancy, but opioid acting presynaptically blocks IL-1β-induced noradrenergic release in the PVN (Brunton et al., 2005). The source of the opioid peptide and receptor is evidently the NTS neurons that
project to the PVN: mRNA expression for proenkephalin-A (pENK-A) and the µ-opioid receptor (MOR) is increased in the NTS A2 region in late pregnancy (Brunton et al., 2005).

Here we sought to identify the pregnancy signal that induces opioid inhibition of the HPA axis. The female sex steroids estradiol and progesterone are produced in increasing amounts in pregnancy, but mimicking their pregnancy levels in virgin rats does not attenuate HPA axis stress responses (Douglas et al., 2000). However, allopregnanolone (3α,5α-tetrahydroprogesterone or 5α-pregn-3α-ol-20-one) (AP), the neuroactive steroid metabolite of progesterone, is abundant in the brain in pregnancy and can reduce stress-induced HPA axis activity in male rats (Concas et al., 1998).

Allopregnanolone is produced by sequential action of 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD), which are both expressed in the brain (Compagnone and Mellon, 2000). As well as allosterically modulating GABA<sub>A</sub> receptors, to enhance the effectiveness of GABA actions (Herd et al., 2007), allopregnanolone can also modulate neuropeptide gene expression (Bali and Kovács, 2003).

Here, we investigate a role for allopregnanolone in restraining HPA axis responses to IL-1β in late pregnancy by inhibiting allopregnanolone synthesis in late pregnant rats and by measuring HPA responses to IL-1β in nonpregnant rats treated with allopregnanolone. Furthermore, we test whether activity and gene expression of the allopregnanolone synthesizing enzymes are increased centrally in late pregnancy. Moreover, we seek evidence that allopregnanolone is involved in the induction and maintenance of the inhibitory opioid mechanism that emerges in pregnancy.

Materials and Methods

Animals. Female Sprague Dawley rats (initial body weight, 240–290 g; Charles River Laboratories) were housed under standard conditions of temperature (20–22°C) and lighting (12 h light/dark cycle, lights on at 7:00 A.M.) with food and water available ad libitum. Rats were initially housed in groups of five to six for 3 weeks and then caged individually after surgery. Pregnant rats were obtained by mating overnight with a sexually experienced male, and the pregnancy was confirmed by finding a vaginal plug of semen on the floor of the breeding cage the following morning (day 1 of pregnancy; day 22 is the expected day of parturition). Virgin rats selected at random stages of the estrus cycle were used as controls. The number of rats per group for each experiment is given in the figure legends. All procedures were performed in accordance with current United Kingdom Home Office regulations and with University of Edinburgh Ethical Committee approval.

Surgery: jugular vein cannulation. Changes in ACTH and corticosterone secretion were assessed by collecting serial venous blood samples. Five days before the experiment, virgin and day 16 pregnant rats were fitted with a SILASTIC jugular vein cannula (wall, 0.25 mm; internal diameter, 0.5 mm; filled with sterile heparinized 0.9% saline, 50 U/ml) under inhalational halothane anesthesia (2–3% halothane, in 1200 ml/min oxygen) for subsequent blood sampling and drug administration.

Blood sampling and tissue collection. On the morning of the experiment (between 7:30 and 9:00 A.M.), the jugular cannula was connected to polyvinyl chloride extension tubing (wall, 1 mm; internal diameter, 0.5 mm) filled with heparinized saline (50 U/ml 0.9% saline) and attached to a 1 ml syringe, for remote sampling. Rats were then left undisturbed for 90 min. After collection of two basal blood samples (at t = −31 and −1 min), rats were administered 500 mg/kg human recombinant IL-1β intravenously (1 µg/ml; dissolved in 0.2% bovine serum albumin in PBS; t = 0 min; R & D Systems). Additional blood samples were taken 15, 30, 60, 90, and 120 min after treatment. Blood samples (0.35 ml) were collected into 1 ml syringes containing 20 µl of 5% EDTA, chilled on ice, and centrifuged to separate plasma, which was frozen and stored at −20°C until assay. Each blood sample was replaced immediately with an equivalent volume of warmed sterile 0.9% saline. Rats were killed by conscious decapitation 4 h after IL-1β injection [the optimum time point for detecting peak changes in CRH mRNA expression after exposure to stress (Har buz and Lightman, 1989)]. Trunk blood was collected into tubes containing chilled 5% EDTA (0.2 ml/100 g body weight), and plasma was separated and stored as above. Brains were rapidly removed, frozen on dry ice, and stored at −70°C until sectioning. CRH neuron activation was evaluated by quantitative in situ hybridization (ISH) for CRH mRNA (see below). Pregnancy status was checked postmortem, and only pregnant rats with at least six viable fetuses were included in the study (actual range, 6–17 pups; mode, 14 pups).

Experiment 1: is allopregnanolone involved in suppressing HPA axis responses in pregnant rats? To investigate a role for allopregnanolone in suppressed HPA axis responses to IL-1β in late pregnancy, allopregnanolone production was blocked using finasteride (5α-reductase inhibitor). Blood samples and brains were collected (as above) from virgin and pregnant (day 21) rats treated with either finasteride (25 mg/kg, s.c.; Steraloids) or vehicle (15% ethanol in sesame oil; 0.5 ml/kg) 20 h before IL-1β (t = −20 and −2 h). A separate group of pregnant (day 21) rats were treated with finasteride or vehicle (as above) only (no IL-1β). Such finasteride treatment reduces brain allopregnanolone content by 90% in pregnant and 77% in nonpregnant rats, to similar actual levels (Concas et al., 1998).

Experiment 2: can the effects of pregnancy on HPA axis responses be mimicked in virgin rats with allopregnanolone treatment? To test whether suppressed HPA axis responses to IL-1β could be mimicked in virgin rats with allopregnanolone treatment, blood samples and brains were collected from virgin rats treated with AP (3 and 1 mg/kg, s.c., 20 and 2 h before IL-1β, respectively; Steraloids) or vehicle (15% ethanol in corn oil; 0.5 ml/kg). To verify that the effects of finasteride on HPA responses to IL-1β seen in pregnant rats were attributable to the actions of allopregnanolone, blood samples and brains were also collected from pregnant (day 20–21) rats treated with finasteride (as before) and either allopregnanolone or vehicle (as above).

Experiment 3: can the allopregnanolone precursors suppress HPA axis responses in virgin rats? To establish whether administration of the allopregnanolone precursors progesterone or dihydroprogesterone (DHP) can restrain HPA axis responses to IL-1β, virgin rats were treated with progesterone (20 and 4 mg/kg, s.c., 20 and 2 h before IL-1β, respectively; Steraloids) or vehicle (10% benzyl alcohol and 0.3% cresol in arachis oil; 0.5 ml/kg). A separate set of rats were treated with dihydroprogesterone (4 and 1 mg/kg, s.c. 20 and 2 h before IL-1β, respectively; Steraloids) or vehicle (15% benzyl alcohol and 0.3% cresol in arachis oil; 0.5 ml/kg). In both cases, blood and brains were collected to evaluate HPA axis activity.

Experiment 4: does the expression of mRNAs for the allopregnanolone-synthesizing enzymes increase in the brain during pregnancy? To test whether mRNA expression for 5α-reductase and/or 3α-HSD is increased in the brain in pregnancy, untreated virgin and pregnant (day 21) rats were killed by conscious decapitation. Brains, brainstems, and liver (positive control) were removed, frozen on dry ice, and stored at −70°C until processing for ISH.

Experiment 5: are the activities of the allopregnanolone-synthesizing enzymes increased in the brain during pregnancy? To investigate whether the activity of 5α-reductase and/or 3α-HSD is increased in the brain in pregnancy, untreated virgin and pregnant (day 21) rats were killed by conscious decapitation. Brains were removed, and the hypothalamus (cut rostrocaudally between the optic chiasm and mammillary bodies, laterally at the hypothalamic sulci and dorsally below the ventral thalamus) and medulla oblongata [bregma, −10 to −15 mm (Paxinos and Watson, 1998)] were dissected, frozen on dry ice, and stored at −70°C until subsequent enzyme assay.

Experiment 6: are the effects of allopregnanolone and endogenous opioids in pregnant rats interdependent? Pregnant rats (day 21) were pretreated with either vehicle or finasteride (as before). Immediately after the first basal blood sample was collected, rats were given either naloxone (5 mg/kg, i.v.); naloxone was used at this dose as a nonselective opioid receptor antagonist, as before (Brunton et al., 2005), because the opioid receptor type that mediates opioid inhibition of HPA axis in pregnancy has not been defined] or vehicle (0.9% saline, 0.5 ml/kg). After an addi-
tional two blood samples 15 and 30 min later, all rats were given IL-1β (as before). Additional blood samples were taken 15, 30, 60, 90, and 120 min after IL-1β, rats were killed at 4 h (as before), and brains were collected to assess CRH neuron activation by quantitative ISH for CRH mRNA. One group of control virgin rats were given oil vehicle and saline pretreatment before IL-1β and sampled as above. Separate groups of virgin and pregnant rats were blood sampled after treatment with only naloxone (i.e., with no subsequent IL-1β treatment) or saline.

Experiment 7: can allopregnanolone induce opioid inhibition over HPA axis responses in virgin rats? To establish whether allopregnanolone exerts its effects on HPA responsiveness by inducing inhibitory opioid tone, virgin rats were given either allopregnanolone or vehicle (as before). Immediately after the first basal blood sample was taken, rats were given either naloxone (5 mg/kg, i.v.) or vehicle (0.9% saline, 0.5 ml/kg). After an additional two blood samples 15 and 30 min later, all rats were given IL-1β (as before). Additional blood samples were taken 15, 30, 60, 90, and 120 min after IL-1β, rats were killed at 4 h (as before), and brains were collected to assess CRH neuron activation by quantitative ISH for CRH mRNA.

Experiment 8: does allopregnanolone alter central opioid expression? To investigate whether allopregnanolone upregulates central opioid expression virgin rats were treated with allopregnanolone or vehicle (as in experiment 2). Late pregnant rats were treated with finasteride or vehicle (as in experiment 1). Two hours after the second injection, rats were killed by conscious decapitation. Expression of pENK-A, proopiomelanocortin (POMC), and MOR mRNAs in selected nuclei in the hypothalamus and brainstem were evaluated by quantitative ISH.

Radioimmunoassays. ACTH concentrations were determined in unextracted plasma using a commercially available two-site immunoradiometric kit (Euro–diagnostica), whereas corticosterone (IDS Ltd.) and progesterone (Diagnostic Systems Laboratories) were measured using radioimmunoassay kits. The sensitivity and intra-assay variation for each of the assays were as follows: ACTH: sensitivity, 1 pg/ml and intra-assay variation, <7%; corticosterone: sensitivity, 0.4 ng/ml and intra-assay variation <8%; progesterone: sensitivity, 0.3 ng/ml and intra-assay variation <6%. All samples from any one experiment were assayed together.

In situ hybridization. Tissue was sectioned coronally on a cryostat at 15 μm and thaw mounted onto Poly/lysine slides. All sections from a particular experiment were processed in the same hybridization reaction. 35S-labeled oligonucleotide probes (MWG-Biotech) were used to detect CRH, pENK-A, POMC, and μ-opioid receptor mRNA expression. The sequences of each oligonucleotide probe are given in Table 1 and in situ hybridization using these probes was performed as described previously (Brunton et al., 2005).

[35S]UTP-labeled cRNA sense and antisense probes were synthesized from the linearized pBluescript SK− vector expressing a 344 bp (nucleotides 991–1334) DNA fragment encoding rat 5α-reductase type I (the predominant isoform expressed in adult brain (Compagnone and Mellon, 2000; Sánchez et al., 2008a,b); generously provided by Prof. Marcel Karperien, Leiden University Medical Center, Leiden, The Netherlands). The plasmid was linearized with XhoI and XbaI and transcribed using T7 and T3 polymerase (Promega) for the sense and antisense riboprobes, respectively. To detect 3α-HSD mRNA, [35S]UTP-labeled sense and antisense riboprobes were generated from the linearized pGem3 vector expressing a 982 bp (nucleotides −129 to +853) cDNA fragment encoding rat 3α-HSD (generously provided by Prof. Trevor Penning, University of Pennsylvania, Philadelphia, PA.). The plasmid was linearized with SalI and SphI, and sense and antisense cRNAs incorporating [35S]UTP were transcribed from the T7 and SP6 promoters, respectively.

For in situ hybridization using riboprobes, tissue was first fixed in 4% paraformaldehyde, acetylated in triethanolamine/acetic anhydride solution, and then partially dehydrated in ethanol before incubation in prehybridization buffer [50% deionized formamide (v/v), 1 X Denhardt’s solution, 1 ml EDTA, 0.5 mg/ml salmon testes DNA, 600 μg/ml sodium chloride, 10 mg Tris, and 125 μg/ml yeast tRNA] at 50°C for 120 min. Riboprobes incorporating [35S]-labeled UTP were mixed with hybridization buffer [50% deionized formamide (v/v), 1 X Denhardt’s solution, 10% dextran sulfate (w/v), 15 μg dithiothreitol, 1 ml EDTA, 0.1 mg/ml salmon testes DNA, 600 μg/ml sodium chloride, 10 mg Tris, and 125 μg/ml yeast tRNA], heated for 10 min at 70°C, quenched on ice, and applied to the tissue (650,000 cpm/section). To aid tissue penetration, the radiolabeled 3α-HSD riboprobe was fragmented by alkaline hydrolysis and neutralized before hybridization. Sections were hybridized at 55°C and 60°C (for 5α-reductase and 3α-HSD mRNAs, respectively) for 18–19 h. Sections of rat liver treated as above were used as positive controls. Brain and liver sections serving as negative controls to ensure probe specificity were hybridized with 35S-labeled UTP-labeled cDNA sense probes. The hybridization signal over tissue hybridized with the sense probe was not different from background.

After hybridization, the slides were rinsed briefly (to remove coverslips) in 2x SSC and then washed three times for 5 min in 2x SSC at room temperature. Next, sections were incubated in buffer (1 ml EDTA, 0.1 mg/ml salmon testes DNA, 10 mg Tris) containing 15 μg/ml RNAse A for 60 min at 37°C. Sections were then briefly rinsed in 2x SSC at room temperature before additional stringency washing (for 5α-reductase, once in 2x SSC at 37°C and twice at 55°C for 30 min each, followed by two 60 min washes in 0.2x SSC at 55°C and for 3α-HSD, 60 min each in 2x SSC at 50°C and in 0.2x SSC at 55°C and then 60°C). After the posthybridization washes, tissue was dehydrated in an ascending series of ethanol containing 300 mM ammonium acetate, air dried, dipped in autodiagnostic emulsion (Ilford K-5), and exposed at 4°C. Exposure times for each mRNA species were as follows: CRH, 10 weeks; 3α-HSD, 10 weeks; pENK-A, 6 weeks; POMC, 5 weeks; μ-opioid receptor, 6 weeks; and 5α-reductase, 7 weeks.

Slides were developed (Kodak D-19; Sigma), fixed (Hypam rapid fixer; Ilford), and counterstained with hematoxylin and eosin. Autoradiographs were quantified using three methods. (1) The area of each region of interest (e.g., PVN or NTS) and the silver grain area overlying the region were measured (NIH Image software, version 1.62) and are presented as grain area/brain area (mm2/mm2). Background measurements were made over areas adjacent to the region of interest and subtracted. (2) The number of cells positively hybridized in a particular brain region was counted manually in emulsion-dipped sections. A positive cell was defined as one with more overlying silver grains than the mean over 10 cells lateral to the region of interest (background) + 3 SDs. Data are presented as the number of positive cells per brain area (mm2). (3) Grain density per cell data were determined by measuring the silver grain area over ~240 cells per area per rat (Openlab version 2 software; Improvison). Background measurements were made over equivalently sized cells in the neuropil adjacent to the region of interest and subtracted. In each case, measurements were made over a minimum of 12 PVN or NTS profiles (bilateral measurements in six consecutive sections). Integrated density was calculated by multiplying the mean number of positive neurons in a specific brain region section by the mean grain area per cell (data given in arbitrary units). For all ISH measurements, average values for each rat were used to calculate group means ± SEM.

Enzyme assays and thin-layer chromatography. Brain tissue was weighed, and a 10% (w/v) homogenate (−12.5 mg protein/ml) was prepared in buffer, pH 7.22, containing 100 mM potassium phosphate, 1 mM EDTA, and 0.32 mM sucrose using a glass homogenizer. Protein concentration of the homogenates was measured using the Bradford protein assay. Homogenates were preincubated at 37°C for 10 min in 100 mM potassium phosphate buffer containing 50 μM EDTA, 5 mM dithiothreitol, and the appropriate cofactor (100 μM NADPH for 5α-reductase and 100 μM NADPH and 100 μM NADH for 3α-HSD) before addition of 20 μCi [1,2,6,7-3H(N)]-progesterone (specific activity, 115 Ci/mmol). The
preparations were incubated in a shaking water bath for 40 min at 37°C. Control reactions were included for each assay and contained all of the components (given above) except for the tissue homogenate. The reaction was terminated by transferring the tubes to ice and by the addition of ice-cold methanol/chloroform (1:1). Tubes were agitated for 5 min, and the different phases were separated by centrifugation (6000 rpm for 1 min). Progesterone metabolites were separated by thin-layer chromatography (TLC) using silica gel 60 F254 TLC plates (20 × 20 cm; Merck) previously activated by heating at 100°C for 15 min. Fifty microliters of each extract or unlabeled standard [1 mg/ml (w/v) progesterone, dihydroprogesterone, and allopregnanolone in ethanol] were spotted onto the bottom right-hand corner of the TLC plates. Each plate was run in a two-dimensional system: twice in solvent system 1 (benzene/methanol, 19:1, v/v); then plates were turned 90° and run twice in solvent system 2 (cyclohexane/ethylacetate, 1:2, v/v). Steroids were visualized by first exposing the TLC plates to 30% H2SO4 in ethanol, heating to 140°C for 15 min, and then by ultraviolet illumination. The spots corresponding to the substrate (progesterone) and metabolites were scraped from the plates and the radioactivity counted in a β-scintillation counter. The quantity of the metabolites converted from progesterone is expressed as a percentage of the total radioactivity and is indicative of the enzyme activities.

**Statistical analysis.** SigmaStat (version 3.1; Systat Software) and SPSS software (version 17; SPSS) were used to analyze the data. The specific statistical tests used are given in Results. Data from *in situ* hybridization studies and enzyme activity data were analyzed by ANOVA or Student’s *t* test; when we had predicted direction of change, a one-tailed Student’s *t* test was used. To analyze the effects of treatment across time in plasma hormone data, two-way repeated measures ANOVA (two-way RM ANOVA) (SigmaStat) were used when there were two treatment groups. In cases in which three or more treatment groups were to be analyzed, a repeated measures mixed-model ANOVA (mixed ANOVA) (SPSS) was used, followed by a two-way RM ANOVA to critically test our original hypotheses with planned comparisons when appropriate. Specific differences were isolated using Student–Newman–Keuls (SNK) multiple comparison *post hoc* tests. In each case, values shown are group means ± SEM. *p* values <0.05 were considered statistically significant.

**Results**

**Effect of blocking allopregnanolone production on HPA axis responses to IL-1β**

There was a significant effect of pregnancy (*F*(1,23) = 12.0, *p* = 0.002, mixed ANOVA) and time (*F*(1,23) = 43.7, *p* < 0.0001) on plasma ACTH responses to IL-1β (Fig. 1a). Within the virgin groups (“virgin”), finasteride (“FIN”) treatment had no significant effect on plasma ACTH concentration (*F*(1,84) = 0.675, *p* = 0.427, two-way RM ANOVA), but there was a significant effect of finasteride treatment within the pregnant groups (“preg”) (*F*(1,77) = 7.547, *p* = 0.019, two-way RM ANOVA). Basal plasma ACTH concentrations did not differ between virgin and pregnant rats pretreated with vehicle (“veh”) or finasteride (Fig. 1a). IL-1β significantly increased ACTH secretion (3.2-fold; *p* < 0.001, SNK test) in the vehicle-pretreated virgin rats, but IL-1β had no significant effect in the vehicle-pretreated pregnant rats (*p* = 0.839, SNK test). Finasteride pretreatment had no effect on the amplitude of the ACTH response to IL-1β in virgin rats. In late pregnant rats, finasteride significantly restored an ACTH response to IL-1β (*p* = 0.002 vs basal levels, SNK test) to 77% at peak of that observed in vehicle-treated virgin rats.

There was a significant effect of pregnancy (*F*(1,20) = 6.0, *p* = 0.023, mixed ANOVA) on plasma corticosterone concentrations (Fig. 1b). Finasteride treatment had no significant effect on plasma corticosterone concentration within the virgin groups (*F*(1,44) = 0.176, *p* = 0.683, two-way RM ANOVA), but there was a significant effect of finasteride treatment within the pregnant groups (*F*(1,42) = 4.922, *p* = 0.048). Basal plasma corticosterone concentration was greater in the pregnant rats (this only reached statistical significance in the preg/veh group; *p* = 0.02, SNK test), but finasteride treatment had no effect in either virgin or pregnant rats (basal plasma corticosterone concentrations: virgin/veh, 50.1 ± 5.3 ng/ml; virgin/FIN, 54.3 ± 6.5 ng/ml; preg/veh, 159.9 ± 27.6 ng/ml; preg/FIN, 120.4 ± 17.5 ng/ml). As expected, IL-1β significantly increased plasma corticosterone concentra-

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**Figure 1.** Effect of inhibiting allopregnanolone synthesis on HPA axis responses to IL-1β in virgin and pregnant rats. Virgin and day 21 pregnant (preg) rats were treated with either finasteride (FIN; 25 mg/kg, s.c.) or vehicle (veh) 20 and 2 h before IL-1β. After two basal (B) blood samples (at *t* = −31 and −1 min), all rats were administered IL-1β (500 ng/kg, i.v.) at *t* = 0 min. Additional blood samples were taken 15, 30, 60, 90, and 120 min after treatment. Trunk blood was collected at *t* = 240 min. n = 6–7 rats per group. a, Plasma ACTH concentration. *p* < 0.03 versus basal values in the same group; *p* < 0.05 versus all other groups at the same time point. b, Plasma corticosterone concentration. *p* < 0.04 versus basal values in the same group; *p* < 0.04 versus preg/veh group at the same time point. Rats were killed 4 h after IL-1β administration, and brains were processed by *in situ* hybridization. c, Quantification of CRH mRNA expression in the pPVN. CRH mRNA expression levels (expressed as arbitrary units [a.u.]) were achieved by multiplying the grain density per neuron by the number of neurons positively expressing CRH mRNA. *n* = 5–7 rats per group. *p* < 0.001 versus virgin/veh group; *p* < 0.003 versus virgin/FIN group; *p* < 0.05 versus preg/veh group. Values are group means ± SEM. d, CRH mRNA: representative dark-field autoradiographs of coronal pPVN sections from the following: i, virgin/veh; ii, virgin/FIN; iii, pregnant/vehicle; and iv, pregnant/FIN. 3V, Third ventricle. Scale bar, 200 μm.
Treatment with finasteride alone (i.e., no IL-1β) had no significant effect on basal CRH mRNA expression in the pPVN of late pregnant rats (Fig. 1c,d). IL-1β-evoked increases were, however, significantly attenuated in the allopregnanolone-treated group (57% of vehicle-treated group, *p* < 0.001, SNK test) and the allopregnanolone-pretreated virgin rats (Fig. 2a). IL-1β significantly increased ACTH secretion within 15 min in both the vehicle-treated virgins (4.0-fold increase; *p* < 0.001, SNK test) and the allopregnanolone-treated virgins (2.0-fold increase; *p* < 0.001); however, the response was markedly reduced in the allopregnanolone-treated group (57% of the peak response in the vehicle group; *p* < 0.001).

There was also a significant effect of allopregnanolone treatment on corticosterone secretion in the virgin rats (*F*<sub>1,66</sub> = 13.2, *p* = 0.003, two-way RM ANOVA) on ACTH secretion in the virgin rats (Fig. 2a). There was no difference in basal plasma ACTH concentration between vehicle and allopregnanolone-pretreated virgin rats (Fig. 2a). IL-1β significantly increased ACTH secretion within 15 min in both the vehicle-treated virgins (4.0-fold increase; *p* < 0.001, SNK test) and the allopregnanolone-treated virgins (2.0-fold increase; *p* < 0.001); however, the response was markedly reduced in the allopregnanolone-treated group (57% of the peak response in the vehicle group; *p* < 0.001).

In the late pregnant rats, there was a significant effect of drug treatment (*F*<sub>1,58</sub> = 6.2, *p* = 0.034, two-way RM ANOVA) on plasma ACTH concentration (Fig. 2b). Neither finasteride alone nor combined finasteride and allopregnanolone treatment affected basal plasma ACTH concentration in late pregnant rats (Fig. 2b). As before, blocking allopregnanolone production with finasteride restored a significant ACTH response to IL-1β in late pregnant rats (Fig. 2b). This
Effect was prevented in the pregnant rats by coadministration of allopregnanolone. There was a significant effect of drug treatment ($F_{(1,40)} = 20.6, p = 0.002$, two-way RM ANOVA) on corticosterone secretion in the pregnant rats. Accordingly, finasteride treatment restored a corticosterone response to IL-1β in late pregnant rats (Fig. 2d), which was attenuated by combined allopregnanolone treatment.

CRH mRNA expression in the pPVN 4 h after systemic IL-1β was significantly less in virgin rats pretreated with allopregnanolone compared with those pretreated with vehicle (Fig. 2e) ($p < 0.001$, one-tailed Student’s $t$ test). Furthermore, CRH mRNA expression in the pPVN 4 h after systemic IL-1β was significantly less in pregnant rats treated with a combination of finasteride and allopregnanolone compared with pregnant rats treated with finasteride alone (Fig. 2f) ($p = 0.01$). Treatment of virgin rats with allopregnanolone only for 20 h had no effect on basal CRH mRNA expression in the pPVN (Table 2).

**Effect of progesterone or dihydroprogesterone on HPA axis responses to IL-1β in virgin rats**

As expected, subcutaneous progesterone injections significantly increased plasma progesterone concentrations (before injection, 11.9 ± 1.1 ng/ml vs after injection, 54.6 ± 7.5 ng/ml; $F_{(1,22)} = 18.3, p < 0.001$, two-way RM ANOVA). There was no significant effect of progesterone treatment on either plasma ACTH (Fig. 3a) or corticosterone concentrations (Fig. 3b), but there was a significant effect of IL-1β administration on ACTH ($F_{(1,26)} = 11.4, p < 0.001$) and corticosterone ($F_{(5,39)} = 9.6, p < 0.001$) secretion. Systemic IL-1β significantly increased both plasma ACTH (Fig. 3a) and corticosterone (Fig. 3b) concentrations compared with basal levels in the vehicle- and progesterone-treated rats, but there were no differences between groups. Furthermore, progesterone pretreatment had no significant effect on CRH mRNA expression in the pPVN after IL-1β (Table 2).

DHP treatment had no significant effect on either plasma ACTH (Fig. 3c) or corticosterone (Fig. 3d) concentrations, but IL-1β administration did have a significant effect (two-way RM ANOVA: ACTH, $F_{(1,21)} = 18.4, p < 0.001$; corticosterone, $F_{(5,47)} = 5.7, p = 0.001$). IL-1β increased plasma ACTH (Fig. 3c) and corticosterone (Fig. 3d) concentrations similarly in the vehicle- and DHP-treated virgin rats. As with progesterone, DHP treatment had no significant effect on CRH mRNA expression in the pPVN after IL-1β (grain area was 0.20 ± 0.03 mm²/mm² in the vehicle group vs 0.29 ± 0.04 mm²/mm² in the DHP group; $p = 0.08$, two-tailed Student’s $t$ test).

**Central expression of mRNA for the progesterone converting enzymes in late pregnancy**

Both 5α-reductase and 3α-HSD are expressed in white matter (Melcangi et al., 1990; Lauber and Lichtensteiger, 1996). There was no difference in mRNA expression for either enzyme in the pyramidal tract [brain area (μm²/μm²²): 5α-reductase mRNA, 66.6 ± 8.3 in virgin rats vs 74.2 ± 14.1 in day 21 pregnant rats, $p > 0.05$; 3α-HSD mRNA, 8.9 ± 1.2 in virgin rats vs 8.2 ± 0.7 in day 21 pregnant rats, $p > 0.05$ two-tailed Student’s $t$ test; $n = 6$ per group]. There was no difference in 5α-reductase mRNA ex-

### Table 2. Virgin rats were treated with AP (3 and 1 mg/kg, s.c.) or veh at $t = -20$ and $-2$ h and then killed by conscious decapitation at $t = 0$ h ($n = 6–7$ rats per group).

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Brain region</th>
<th># Positive cells</th>
<th>Grain density per cell (μm²)</th>
<th>AP</th>
<th># Positive cells</th>
<th>Grain density per cell (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENK-A</td>
<td>NTS A2</td>
<td>115 ± 3</td>
<td>34.0 ± 0.3</td>
<td>114 ± 10*</td>
<td>38.4 ± 0.9**</td>
<td></td>
</tr>
<tr>
<td>pENK-A</td>
<td>mgPVN</td>
<td>21.8 ± 4.9</td>
<td>45.1 ± 1.0</td>
<td>17.5 ± 3.6</td>
<td>45.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>pENK-A</td>
<td>Arc</td>
<td>46.5 ± 4.5</td>
<td>30.6 ± 0.5</td>
<td>51.2 ± 1.3</td>
<td>30.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>POMC</td>
<td>Arc</td>
<td>46.8 ± 2.7</td>
<td>19.0 ± 0.4</td>
<td>47.0 ± 1.9</td>
<td>18.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>MOR</td>
<td>NTS A2</td>
<td>117.1 ± 12.2</td>
<td>19.2 ± 0.6</td>
<td>108.5 ± 7.5</td>
<td>18.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>pPVN</td>
<td>131.1 ± 11.7</td>
<td>49.3 ± 6.6</td>
<td>131.1 ± 6.9</td>
<td>44.6 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>

mgPVN, Magnocellular subdivision of the paraventricular nucleus; Arc, arcuate nucleus. *$p < 0.05$, **$p < 0.001$ versus veh group. Values are mean ± SEM.
Expression in the PVN between virgin and late pregnant rats (Fig. 4a), but 5α-reductase mRNA levels were significantly greater in the A2 region of the NTS of the late pregnant group compared with the nonpregnant group (Fig. 4b) (p = 0.047, one-tailed Student’s t test). There was a modest but significant increase in 3α-HSD mRNA expression in the PVN in late pregnant rats compared with virgin rats (Fig. 4c) (p = 0.042), but a significant difference in the level of 3α-HSD mRNA expression in the A2 region of the NTS was not detected between virgin and pregnant rats (Fig. 4d) (p = 0.069).

Activity of the progesterone converting enzymes in the brain in late pregnancy
5α-Reductase activity was significantly greater in the hypothalamus in late pregnant rats compared with virgin rats ([3H]progesterone conversion into DHP was 2.6 ± 0.5% in the virgin group, n = 8 vs 5.6 ± 0.8% in the late pregnant group, n = 6; p = 0.033, one-tailed Student’s t test). 3α-HSD activity also tended to be greater in the hypothalamus in pregnant rats, but this was not statistically significant ([3H]progesterone conversion into AP was 0.3 ± 0.1% in the virgin group, n = 8 vs 0.7 ± 0.3% in the late pregnant group, n = 6; p = 0.07). No difference was detected in the activities of either enzyme in the medulla oblongata ([3H]progesterone conversion into DHP was 2.6 ± 0.7% in the virgin group vs 3.2 ± 0.8% in the late pregnant group, p > 0.05; [3H]progesterone conversion into AP was 0.3 ± 0.1% in the virgin group vs 0.4 ± 0.1% in the late pregnant group, p > 0.05).

Effect of blocking allopregnanolone synthesis and endogenous opioid action on HPA axis responses to IL-1β in late pregnant rats
There was a significant effect of finasteride (F(1,20) = 13.9, p < 0.001, mixed ANOVA) and naloxone (F(1,20) = 4.4, p = 0.048) treatment on ACTH responses to IL-1β in the late pregnant groups (Fig. 5a). Neither finasteride nor naloxone altered basal plasma ACTH concentration in virgin or pregnant rats (Fig. 5a). IL-1β significantly increased plasma ACTH concentration (3.2-fold increase) (Fig. 5a,b) in the vehicle-pretreated virgin rats but had no significant effect in the vehicle-pretreated pregnant rats (Fig. 5a,b). Both finasteride only and naloxone only pretreatment restored an ACTH response to IL-1β in the late pregnant rats to 71 and 65% of the response observed in the virgin controls, respectively (Fig. 5a,b). However, combined finasteride and naloxone pretreatment had no additional effect on the ACTH response to IL-1β in late pregnant rats compared with either naloxone or finasteride treatment alone (Fig. 5a,b). In a separate experiment, naloxone (5 mg/kg, i.v., with no subsequent IL-1β treatment) had no effect on plasma ACTH concentration over a 90 min period in either virgin or late pregnant rats (Fig. 5c) (p < 0.05, mixed ANOVA).

Plasma corticosterone concentrations before IL-1β administration were as follows: virgin/oil/saline, 106.6 ± 11.5 ng/ml; pregnant/oil/saline, 121.4 ± 8.9 ng/ml; pregnant/oil/naloxone, 129.2 ± 10.1 ng/ml; pregnant/finasteride/saline, 107.0 ± 9.7 ng/ml; pregnant/finasteride/naloxone, 117.4 ± 8.9 ng/ml. There was an overall effect of drug treatment (F(4,158) = 4.6, p = 0.006, two-way RM ANOVA) on corticosterone responses to IL-1β. IL-1β significantly increased plasma corticosterone concentration in the vehicle-pretreated virgin but not pregnant rats (Fig. 5d,e). Pretreatment with finasteride alone, naloxone alone, or combined finasteride and naloxone all restored corticosterone responses to IL-1β in the pregnant groups, with no significant differences between groups or compared with the vehicle-pretreated virgin rats (Fig. 5d,e).

There was a significant effect of pregnancy status (F(1,31) = 25.9, p < 0.001, two-way ANOVA) and drug treatment (F(3,31) = 5.0, p = 0.006) on CRH mRNA expression. CRH mRNA expression in the pPVN 4 h after systemic IL-1β was significantly less in vehicle-pretreated pregnant rats than in vehicle-pretreated virgin rats (Fig. 5f). Pretreatment with finasteride alone, naloxone alone, or combined finasteride and naloxone all resulted after...
IL-1β in similar levels of CRH mRNA expression in the pPVN of late pregnant rats that were significantly greater than in the vehicle-treated pregnant group (Fig. 5f). Naloxone alone (i.e., no subsequent IL-1β treatment) had no effect after 4 h on basal CRH mRNA expression in the pPVN (same rats as in Fig. 5e); the mean ± SEM number of CRH mRNA-expressing cells in the pPVN was as follows: virgin/saline, 228.1 ± 13.4 (n = 10); virgin/naloxone, 221.2 ± 12.2 (n = 5); pregnant/saline, 176.5 ± 16.4 (n = 10); pregnant/naloxone, 176.6 ± 18.3 (n = 6). There was no significant effect of naloxone on CRH mRNA in the virgin or the pregnant rats (p > 0.05, two-way ANOVA), but there was an effect of pregnancy (F(1,27) = 8.3, p = 0.008). As detailed above, finasteride alone had no effect on CRH mRNA expression in the pPVN of late pregnant rats.

Effect of allopregnanolone treatment on induction of inhibitory opioid tone over HPA axis responses to IL-1β in virgin rats

There was a significant effect of the allopregnanolone treatment (F(1,19) = 6.2, p = 0.023, two-way RM ANOVA) within the saline-treated rats on plasma ACTH concentrations (Fig. 6a). Moreover, within the allopregnanolone-treated rats, there was a significant effect of naloxone treatment (F(1,10) = 4.0, p = 0.048). There was no difference in basal ACTH secretion among vehicle- and allopregnanolone-pretreated virgin rats with or without naloxone treatment (Fig. 6a). In the vehicle (oil)-pretreated groups, IL-1β significantly increased plasma ACTH concentration, with no effect of previous naloxone treatment (Fig. 6a,b). As before (Fig. 2), IL-1β induced a significant increase in ACTH secretion in the AP/saline-treated group (2.2-fold increase), but the amplitude of the response was attenuated compared with the virgin control group (3.8-fold increase) (Fig. 6b). Moreover, the suppressive effect of AP on the ACTH response to IL-1β was completely reversed in the presence of naloxone (Fig. 6a,b). Similarly, CRH mRNA expression in the pPVN 4 h after IL-1β administration was less in the virgin rats treated with AP alone compared with the controls (Fig. 6c) (F(1,36) = 5.5, p = 0.025, two-way ANOVA). This effect of AP was reversed in rats coadministered naloxone (Fig. 6c).

Effect of allopregnanolone treatment on central opioid gene expression in virgin rats

Mimicking pregnancy in virgin rats with allopregnanolone treatment significantly increased pENK-A mRNA expression in the A2 region of the NTS (Fig. 7a,b) (p < 0.001, one-tailed Student’s t test). This was a consequence of both an increase in the number of neurons expressing pENK-A mRNA and an increase in the
levels of expression per neuron (Table 2). Allopregnanolone treatment had no significant effect on pENK-A mRNA expression in the pPVN, magnocellular division of the PVN or the arcuate nucleus (Table 2). There were also no significant differences in POMC mRNA expression in the arcuate nucleus or µ-opioid receptor mRNA expression in the NTS (Table 2). In late pregnant rats, 20 h finasteride treatment resulted in a significant reduction in pENK-A mRNA expression in the A2 region of the NTS (Fig. 7c) (p = 0.003).

Discussion
Our results demonstrate that allopregnanolone restrains HPA axis responses to immune challenge in late pregnancy and implicates mediation of allopregnanolone actions by activation of endogenous opioid expression in the NTS.

Role of allopregnanolone
HPA responses to IL-1β were suppressed in late pregnancy as found previously (Brunton et al., 2005). Blocking allopregnanolone production with finasteride [previously shown to decrease cortical allopregnanolone content by 90% (Concas et al., 1998)] restored HPA axis responses to IL-1β in pregnant rats, reflected by increased CRH mRNA expression in the pPVN, and ACTH and corticosterone secretion, whereas concurrent allopregnanolone treatment prevented these finasteride effects. Furthermore, we mimicked reduced pregnancy-type HPA axis responses to IL-1β in nonpregnant rats with short-term (2–20 h) allopregnanolone treatment. Central effects were shown because, in pregnant rats finasteride enhanced and in virgin rats allopregnanolone treatment reduced the increase in pPVN CRH mRNA expression after systemic IL-1β. These treatments did not affect basal pPVN CRH mRNA expression or basal plasma ACTH or corticosterone concentrations. Hence, the neurosteroid manipulations specifically altered HPA responses to the systematic IL-1β challenge.

Systemic IL-1β activates pPVN CRH neurons via brainstem noradrenergic A2 neurons (Rivet et al., 2000) but is ineffective in late pregnancy (Brunton et al., 2005). It is unclear where in the brain allopregnanolone acts to exert its suppressive effects on HPA responses to IL-1β. Allopregnanolone may prevent stimulation of CRH neurons in the PVN by noradrenaline (Patchev et al., 1994) or it may inhibit CRH neurons by modulating GABA inputs to the PVN by its actions on GABAA receptors. For example, GABA neurons in the peri-PVN area mediate glucocorticoid negative feedback (Di et al., 2009), whereas GABA neurons in the bed nucleus of stria terminalis (Cullinan et al., 2008) can modulate HPA responses to IL-1β (Crane et al., 2003).

The NTS contains intrinsic GABA neurons (Fong et al., 2005; Bailey et al., 2008), GABA-containing terminals, and GABAA receptors (Terai et al., 1998; Saha et al., 2001), and GABA release in the NTS is increased by depolarization (Sved and Curtis, 1993). Effects of GABA on NTS neurons identified as projecting to the PVN are not reported, nonetheless GABA inhibits the electrical activity of A2 neurons (Moore and Guynet, 1983) and
activity in the hypothalamus can be regulated by ovarian hormones (Bertics et al., 1987), and estrogen, but not progesterone, can increase hippocampal 3α-HSD mRNA expression (Mitev et al., 2003).

5α-Reductase gene expression in the female forebrain is stimulated by dihydrotestosterone (Torres and Ortega, 2006); however, whether other 5α-reduced steroids, such as allopregnanolone can regulate expression, is not known. In pregnancy, increased levels of prolactin may play a role in upregulating 5α-reductase gene expression in the forebrain (Sánchez et al., 2008).

Endogenous opioids Reduced HPA responses to IL-1β in late pregnancy involve opioid inhibition of noradrenaline release in the PVN, potentially by met-enkephalin coproduced in NTS neurons projecting to the PVN (Beaulieu et al., 1996), because NTS pENK-A and MOR mRNA expression increase in late pregnancy (Brunton et al., 2005). Here, combined finasteride and naloxone treatment had no greater effect in restoring HPA axis responses to IL-1β in late pregnancy than either drug alone. Finasteride and naloxone, alone or combined, fully restored the corticosterone profile but not the ACTH levels to the virgin values. Enhanced sensitivity to ACTH as a result of high estrogen levels in pregnancy can explain such lack of fidelity between the ACTH and corticosterone responses (Figueiredo et al., 2007). The capacity of the anterior pituitary to generate an ACTH response to stress is not reduced in late pregnancy (Ma et al., 2005), indicating that some central HPA elements remain hypoactive even after finasteride and naloxone treatment. Nonetheless, lack of additive effects of finasteride and naloxone indicated interlinked allopregnanolone and opioid mechanisms. Indeed, allopregnanolone induced opioid inhibition over HPA axis responses to IL-1β in virgin rats. Moreover, allopregnanolone treatment for 20 h upregulated pENK-A mRNA expression in the NTS of virgin rats by 35%, consistent with the 31% increase reported in late pregnancy (Brunton et al., 2005). Furthermore, finasteride reduced pENK-A mRNA expression in the NTS of late pregnant rats, likely explaining how finasteride restores HPA axis responses to IL-1β.

Regulation of opioid expression in the NTS by allopregnanolone might involve interaction with GABA	extsubscript{A} receptors, but such action inhibits PVN oxytocin mRNA expression (Blyth et al., 2000) and direct GABA	extsubscript{A} receptor activation inhibits pPVN pENK-A, CRH, and vasopressin mRNA expression (Borsook et al., 1999; Bali and Kovács, 2003).

Changes in pENK-A mRNA expression in the NTS after treatment with allopregnanolone in virgins or finasteride in pregnant rats were seen within 20 h. Rapid increases in pENK-A mRNA expression have been described after acute stress in the NTS (Boone and McMillen, 1994) and in the pPVN (García-Garcia et al., 1998), which are dependent on increased corticosterone, at least in the case of the PVN (García-Garcia et al., 1998). Here, the changes in pENK-A mRNA in the NTS were not related to basal plasma corticosterone levels, because these were unaltered by pregnancy or by allopregnanolone or finasteride treatment.

Allopregnanolone treatment did not alter MOR mRNA expression in the NTS, which is increased by day 10 of pregnancy (Brunton et al., 2005). Upregulation of MOR may rely on increased levels of circulating estrogen in pregnancy (Zhu and Pfaff, 1995; Eckersell et al., 1998; Priest and Roberts, 2000). It remains to be shown whether allopregnanolone induction of pENK-A mRNA in the NTS leads to preterminal inhibition of noradrenaline release in the pPVN after systemic IL-1β as in pregnancy (Brunton et al., 2005).
We conclude that the emergence and maintenance of endogenous opioid inhibition of HPA axis responses to stress in late pregnancy depends on central actions of increased levels of allopregnanolone. This novel allopregnanolone-induced opioid mechanism may regulate other neuroendocrine mechanisms in pregnancy (Brunton and Russell, 2008).

References


