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# ROLE OF VARIOUS NEUROTRANSMITTERS IN MEDIATING THE LONG-TERM ENDOCRINE CONSEQUENCES OF PRENATAL ALCOHOL EXPOSURE

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## Abstract

Adult rats and mice born to dams exposed to alcohol (FAE) exhibit enhanced activity of their hypothalamic-pituitary-adrenal (HPA) axis when exposed to stressors. However, the mechanisms responsible for this phenomenon remain uncompletely understood. Here we review two possibilities: one that pertains to nitric oxide (NO), an unstable gas that stimulates the HPA axis; and one that focuses on catecholamines, which also stimulate this axis. We did not observe significant alterations in levels of NO synthase, the enzyme responsible for NO formation, in the PVN of FAE rats. However, the stimulatory influence of this gas on the HPA axis was enhanced in these animals, thereby providing a mechanism likely to participate in the neuroendocrine hyperactivity that is the hallmark of this model. We also recently showed that while the ability of catecholamines to release ACTH was comparable in control rats and rats exposed to alcohol during embryonic development, there was a significant upregulation of the C1 brain stem region when these latter animals were exposed to mild footshocks. As this region sends prominent projections to the PVN, its increased activity may participate in the HPA axis hyperactivity observed in FAE offspring. Finally, we used microarray technology to search for potential differences in genes present in the brains of control and FAE mice. When these brains were collected on day 17.5 of embryonic development, several genes were upregulated while others were downregulated, which may provide potential new candidates that mediate the influence of prenatal alcohol on the HPA axis of adult offspring.

### Keywords

alcohol; CRF; ACTH; nitric oxide; catecholamines

The ability of homeostatic challenges present during embryonic development to cause long-term changes in adult offspring is now well recognized. For example, reprogramming of behavioral, metabolic and endocrine functions has been described in rodents born to dams exposed to various stressors, drugs, immune stimuli or food restriction. As elegantly discussed by Simerly,[1] “the brain retains the effect of early experience well into adulthood through permanently altered wiring”. This article will address some of the findings obtained in our laboratory in rodents exposed to alcohol during embryonic development, focusing specifically on the ability of this drug to induce a permanent hyperactivity of the hypothalamic-pituitary-

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adrenal (HPA) axis. (Please note that the quoted literature is only meant as illustrative, and not as fully representative of the topics discussed.)

The HPA axis, sometimes called "stress axis", consists of neurons present in the paraventricular nucleus (PVN) of the hypothalamus, the anterior pituitary and the adrenal glands. As abundantly described elsewhere,[2–7] the PVN harbors cell bodies that synthesize the peptides corticotropin-releasing factor (CRF) and vasopressin (VP) that, upon release from terminals in the median eminence, act on cells in the pituitary corticotrophs that release ACTH (Fig. 1). This is followed by secretion of glucocorticosteroids (GC; corticosterone in rats and mice) from the adrenal cortex. Many stimuli, whether they are consciously aversive or represent internal homeostatic threats, activate the HPA axis and if this response is sustained, upregulate the synthesis of CRF and/or VP. The magnitude and duration of HPA axis activation is very tightly controlled by a series of feedback and feedforward mechanisms, and any prolonged dysregulation of these mechanisms results in pathologies.[8–10] In the case of CRF, the importance of this peptide in appropriate behavioral responses to stressors,[11] in the regulation of sympathetic activity,[12, 13] reproductive functions[14, 15] and gastrointestinal (GI) functions, [16, 17] among others, means that its persistent elevations can lead to mood disorders, disturbances of sympathetic activity, disrupted fertility and diseases of the GI tract. In addition to the consequences of elevated hypothalamic CRF levels, abnormal ACTH/GC secretion due to increased CRF production[18] can in themselves disrupt immune cells activity, metabolism, protein synthesis and reproductive functions.

Work carried out in a number of laboratories, including ours, has shown that rats[19–24] or mice[25] born to dams exposed to alcohol during gestation exhibit abnormally elevated ACTH and corticosterone responses to various stimuli while displaying no or little changes under basal conditions. In the rat, we reported that the drug was most effective when delivered during the second week of embryonic development,[26] which corresponds to peak neurogenesis of hypothalamic CRF neurons,[27, 28] and that this treatment resulted in increased ACTH release when the adult male or female offspring was exposed to mild footshocks, a pro-inflammatory cytokine or an endotoxin challenge (Fig. 2). This phenomenon is not restricted to offspring born to dams exposed to alcohol vapors, but is also found when the dams were fed alcohol[29] (Fig. 3). Finally, in order to show that the ability of prenatal alcohol to alter the offspring' HPA axis activity was a general phenomenon, i.e., not one restricted to a particular experimental model, we demonstrated that it was also found in mice[25] (Fig. 4).

What are the mechanisms that mediate the influence of this drug? Alcohol is a unique drug because it appears to have multiple primary targets that include ligand-gated ion channels such as those associated with GABA, NMDA and serotonin, transporters, neurotransmitters, peptides and steroids. As alcohol readily crosses the placenta, a better understanding of how it activates the HPA axis in naïve animals (i.e., not exposed to the drug during fetal development) may be useful because it may provide a basis for understanding how the drug modifies the HPA axis of the developing fetuses. We have found that acute injection of alcohol elevated plasma ACTH levels and upregulated PVN CRF and VP heteronuclear (hn) RNA gene expression[30, 31] (Fig. 5). As removal of endogenous CRF or blockade of its receptors virtually abrogated alcohol-induced ACTH release[32] (Fig. 6) as well as alcohol-induced POMC synthesis,[33] we concluded that this peptide was required for these responses. While several neurotransmitters likely mediate the sitmulatory influence of alcohol on the HPA axis {see for example [34]}, it is also possible that the drug acts directly on the CRF gene. Indeed, we recently reported that a moderate dose of alcohol increased CRF release by cultured hypothalamic cells (Fig. 7A) as well as CRF mRNA levels in these cells, as demonstrated by RT-PCR[35] (Fig. 7B).

At present, we do not know whether alcohol induces permanent changes in the fetuses' HPA axis through a direct or indirect effect on PVN CRF, and our studies have therefore focused

on the mechanisms through which the adult offspring' HPA axis was activated. We had found that in adrenal-intact fetal alcohol exposed (FAE) adult offspring, pituitary responsiveness to CRF or VP was unaltered.[36] On the other hand, PVN CRF hnRNA gene expression was significantly increased in response to stressors, compared to controls (Fig. 8). Studies of the influence of maternal alcohol on the rat fetus's developping brain have uncovered a vast array of possible mechanisms, including changes in overall CNS development,[37, 38] cell proliferation[39, 40] or survival,[41] synaptic plasticity,[42] levels of retinoids[43, 44] and neurotransmitters,[45–49] electrophysiological,[50] behavioral[51] or memory-linked events,[52] and poor nutrition,[53, 54] to quote only a few. We based our own studies on what we knew about some of the critical signals that influence PVN CRF neuronal activity, and will illustrate two such mechanisms here. The first pertains to nitric oxide (NO), an unstable gas that acts as a transmitter in many parts of the brain, including the hypothalamus.[55, 56] We had shown that the injection of NO donors into the brain lateral ventricles rapidly increased plasma ACTH levels, and that this response depended on endogenous CRF.[57–59] As there was some evidence that prenatal alcohol might alter brain NO levels in the offspring,[46, 60–62] we tested the hypothesis that our FAE model was accompanied by upregulated hypothalamic levels of this gas or by an altered the HPA axis response to NO. We did not measure significant changes in gene expression levels of NO synthase, the enzyme responsible for NO formation, in the PVN of FAE rats.[63] On the other hand, we observed that these animals displayed an altered HPA axis response to NO.[63] Indeed, as illustrated in Fig. 9, not only did the NO donor SIN-1 cause larger increases in plasma ACTH levels in FAE rats, compared to controls, it also caused a more robust PVN response in terms of CRF neurons.

The second hypothesis we tested was that the HPA axis of FAE rats might be more responsive to catecholamines, and/or that adrenergic inputs to the PVN might be upregulated. In agreement with other investigators, we had previously shown that both alpha and beta-adrenergic agonists stimulated the HPA axis in naïve rats {for ref. see 64}. However, when we investigated the potential role of these amines in our prenatal alcohol model, we found that the ACTH response of FAE rats to phenylephrine or propranolol was comparable to that of controls.[65] Similarly, the ability of footshocks to stimulate adrenergic neurons in the locus coeruleus, a region with critical adrenergic innervation of the PVN,[66] or to elevate the number of PVN cells positive for tyroxine-hydroxylase (TH), a rate-limiting enzyme in catecholamine synthesis, was also comparable in FAE and control rats. In contrast, we made the unexpected observation that the C1 adrenergic region of the brain stem was significantly more activated by shocks in rats exposed to alcohol during embryonic development.[65] The C1 brain stem region, which is illustrated in Fig. 10, contributes to the adrenergic innervation of the PVN, in particular those of CRF cells.[67] Its influence on PVN neuronal activity is further supported by the fact that lesions transecting the adrenergic projections between the brain stem medulla oblongata and the hypothalamus prevent increased catecholaminergic activity in the brain stem.[68] These observations suggest a functional role of C1 neurons on the areas of the PVN that drive the HPA axis. Thus while at present the precise mechanisms through which increased activity of the C1 region participates in the HPA axis hyperactivity observed in our prenatal alcohol model, it is possible that it augments adrenaline release onto CRF-expressing neurons in the parvocellular region of the PVN, thus potentiating footshock-induced increases in the ACTH response observed in FAE rats. This being said, it should be noted that C1 adrenergic neurons have also been shown to influence ACTH via changes in arterial pressure.[69, 70] Therefore, increased activity of these neurons could potentially contribute to the enhanced ACTH response to footshocks via changes in afferents to the PVN as well as via altered cardiovascular activity. Regardless of the mechanisms that will be shown important in the FAE model, our results indicate that brainstem adrenergic neurons may exert more influence on the HPA axis than previously understood.

As it is likely that prenatal alcohol exposure alters many neurotransmitter systems (see above), we recently decided to use microarray analysis to search for candidate genes that might provide us with new hypotheses. A previous study conducted in mice had identified 75 genes that were down-regulated by exposure to alcohol on days 7 and 9 of fetal development, but none that were up-regulated.[71] The genes whose levels were altered are linked to cell proliferation, differentiation and apoptosis, and are in general considered to contribute to tissue growth and survival. In our own studies, we used the brain area containing the hypothalamus and the thalamus of 17.5 day-old mice embryos, and compared tissues obtained from controls as well as from animals exposed to alcohol vapors from day 8 of gestation[25] (Table 1). Possibly relevant for our model was the finding of slightly upregulated POMC gene expression, which may be linked to stimulation of the fetal HPA axis. Messenger RNA levels for the CRF-related peptide Urocortin 2 (Ucn 2) were significantly decreased by prenatal alcohol while those for the corresponding receptor, CRFR2, were upregulated. However, these results are difficult to interpret because our preliminary findings do not support a role for these receptors in our prenatal alcohol model (S. Lee and C. Rivier, in preparation). Prenatal alcohol also altered levels of the three known vasopressin receptors.[72] Among these receptors, the R1b type[73] is most relevant for ACTH because it mediates the pituitary effect of its cognate peptide.[74] On the other hand, in the periphery, VPR1a[75] mediates the vascular influence of vasopressin[76] while VPR2[77] mediates its antidiuretic effects in the kidney.[77] While all three receptors have been found in the rat brain, including the hypothalamus,[78–86] their role in the central nervous system remains ill defined. Thus, the significance of prenatal alcohol-induced changes in gene expression of these receptors will need to be investigated, and may well extend beyond their possible involvement within the HPA axis. Finally, it was of interest to note that mRNA levels for phenylethanolamine N-methyltransferase (PNMT), an enzyme that converts norepinephrine to epinephrine,[67] and mRNA levels for dopamine-β-hydroxylase (DBH), which is required for norepinephrine formation after dopamine side chain hydroxylation and is a marker of epinephrine/norepinephrine neurons, were significantly lowered in the brain of mouse embryos exposed to alcohol. Whether this phenomenon is linked to altered adrenergic influence on the HPA axis, is presently unknown.

In conclusion, we have uncovered two possible mechanisms that may mediate the hyperactivity of the HPA axis of adult FAE rats. The first one pertains to the NO system, a gas whose stimulatory influence is stronger on the HPA axis of FAE, compared to control rats. The second mechanism is represented by a more robust C1 neuronal response to stressors, which may, in turn, induce a stronger activation of PVN cell bodies. As briefly described in the Introduction, brain CRF on one hand, and circulating GC on the other, exert important influences on key responses of the body to homeostatic challenges. Consequently, deregulation of their release is likely to induce significant pathogeneses. Children born to mothers who abused alcohol during pregnancy can display, for example, increased occurrence of infections, disorders in which abnormally elevated GC levels often play a role. These children also often show increased activity levels, attention deficits, higher incidence of drug abuse and/or depression.[51, 87] While none of these disorders have been unambiguously demonstrated to result from elevated CRF levels in humans, the influence of this peptide on behavior, drug consumption and mood disorders is well documented in pre-clinical studies (see above). In view of the effects of GC on the mobilization of sugars and fat reserves, as well as the peptides that regulate food intake,[8, 88] it is also likely that individuals who consistently release too much cortisol in response to various stimuli may develop metabolic disorders. Experiments carried out in mice lacking CRF or its receptors, or the use of CRF antagonists in human medicine, may allow us to determine the role played by CRF in disorders caused by prenatal alcohol exposure, and to develop therapies that are palliative or restorative.

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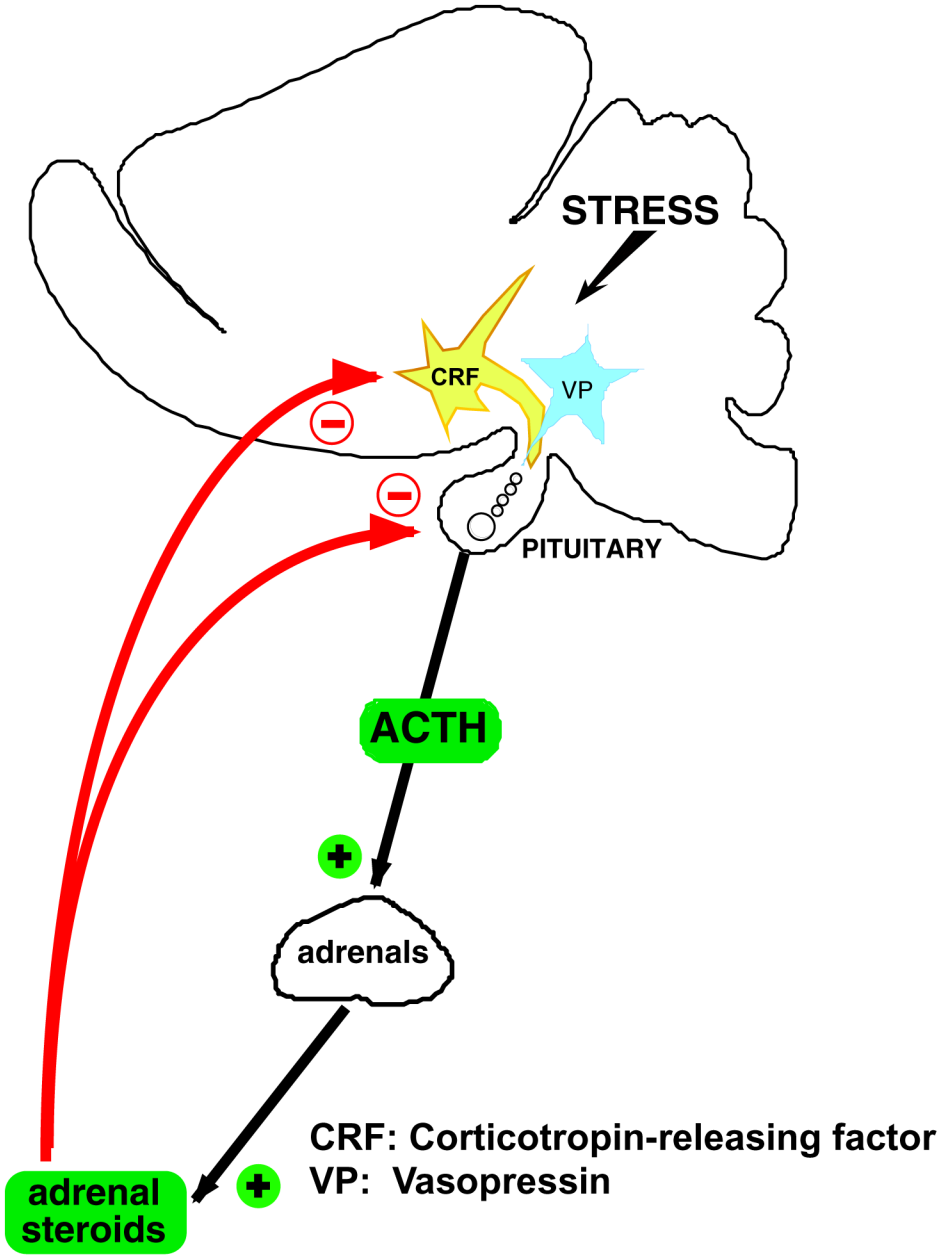


**Figure 1.**
Cartoon illustrating the HPA axis.

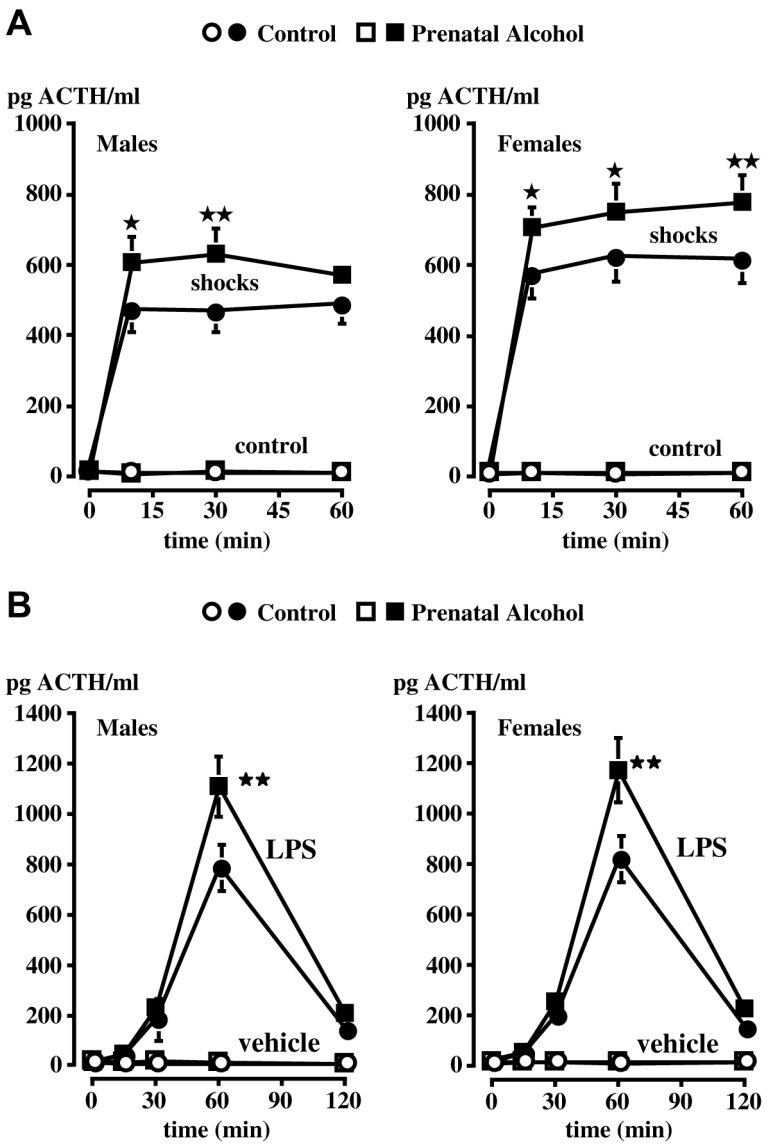


**Figure 2.**

Upregulated ACTH release in adult male FAE rats exposed to (A) shocks or (B) endotoxin (LPS). In these experiments, the dams were exposed to alcohol vapors during the second week of gestation. A. Effect of the exposure to a 60-min session of mild electrofootshocks (0.5 mA, 1-s duration, 2 shocks/min) on plasma ACTH levels in control (C) and alcohol (E) male or female rats. B. Effect of the iv injection of the vehicle or LPS (0.4 μg/kg) on plasma ACTH levels in C and E male or female rats. Each point represents mean ± SEM of 5–7 animals. *, $P<0.05$; **, $P<0.01$ vs C/shocks or C/LPS rats. {Modified by permission from Lee *et al*.[36]}

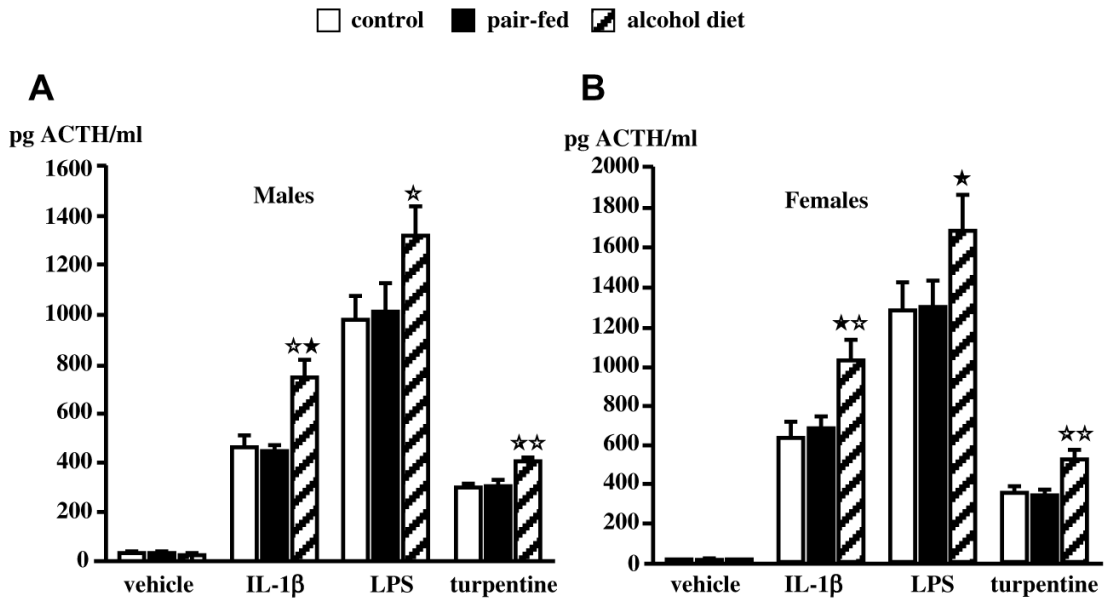


**Figure 3.**

Upregulated ACTH response to immune stimuli in adult FAE male rats. In these experiments, the dams were fed a liquid alcohol diet during gestation. ACTH released by IL-1β, LPS or turpentine in intact control, pair-fed and alcohol male (A) and female (B) rats tested at 9 weeks of age. Each bar represents the mean ± SEM of 6 animals. Blood samples were obtained 30 min after IL-1β administration, 60 min after LPS injection and 8 h after induction of tissue damage by turpentine. *, $P<0.05$; **, $P<0.01$ vs corresponding controls. {Modified by permission from Lee *et al.*[22]}

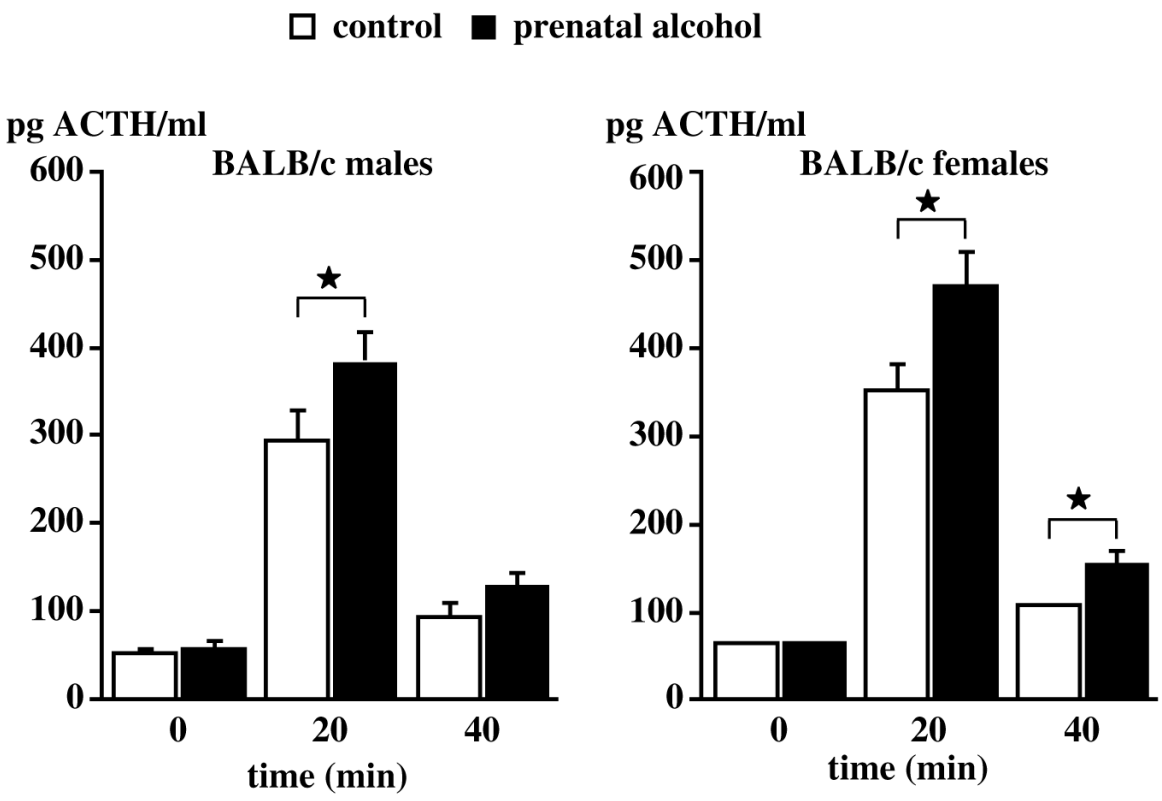


**Figure 4.**

Upregulated ACTH response to footshocks in adult male mice born to dams exposed to alcohol vapors during the second week of gestation. Male and female mice (8–9 week old) of the BALB/c strain that were born to control dams or dams that were exposed to alcohol during gestation were exposed to a 20 min session of footshocks (0.3 mA, 1-sec duration, 2 shocks/min). The mice were decapitated at the end of the shock session. Each bar represents the mean ± SEM of six (controls) or eight (shocked) animals. *, $P<0.05$ vs corresponding controls. {Adapted by permission from Kang *et al*.[25]}

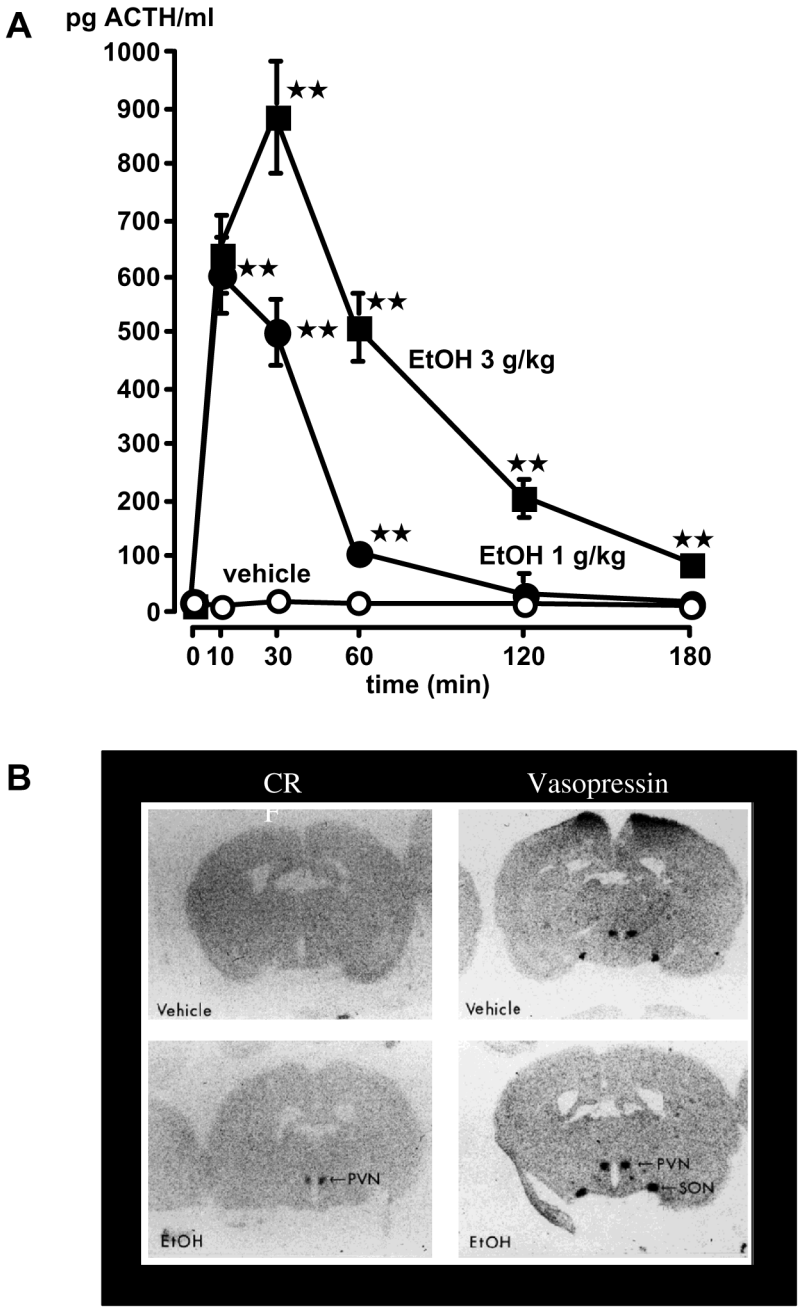


**Figure 5.**

Acute alcohol releases ACTH (A) and upregulates PVN CRF and VP hnRNA gene expression (B). Results were obtained in adult male rats as the results of their first exposure to the drug. A. The vehicle or alcohol was injected ip and blood samples were removed serially for ACTH measurement. Each point represents the mean ± SEM of 5 rats. **, P<0.01. B. Effect of alcohol on PVN CRF and VP heteronuclear transcript (hnRNA). Left panel: rostrocaudal coronal sections of the PVN of rats injected with the vehicle or alcohol (3 g/kg) that display signals on X-ray film for CRF hnRNA measured 20 min post-injection. Right panel: rostrocaudal sections showing VP hnRNA measured in the PVN 5 min after vehicle or alcohol treatment. Sections

were exposed directly to X-ray film for 3 days. {Modified by permission from Rivier *et al*.[30]}

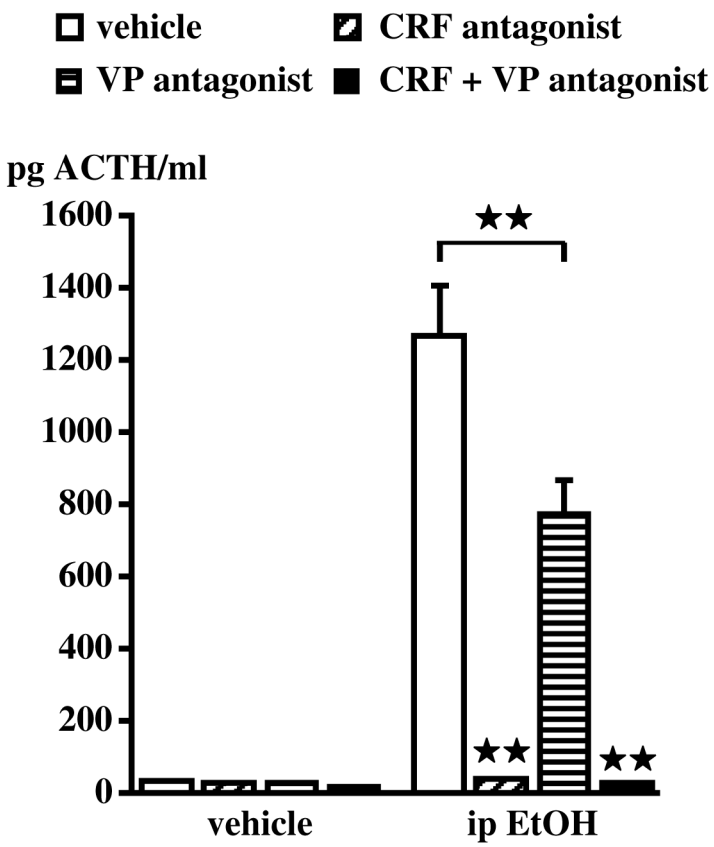


**Figure 6.**

Blockade of CRF or VP significantly decreases the ACTH response to acute alcohol. Results were obtained in adult male rats as the results of their first exposure to the drug. The CRF antagonist astressin (3 mg/kg) or the VP antagonist dPTyr(Me)VP (0.25 mg/kg) were injected iv and blood samples were obtained 30 min after alcohol administration (3 g/kg, ip). Each bar represents the mean ± SEM of 5–6 animals. **, P<0.01. {Modified by permission from Rivier *et al*.[30] and Ogilvie *et al*.[89]}

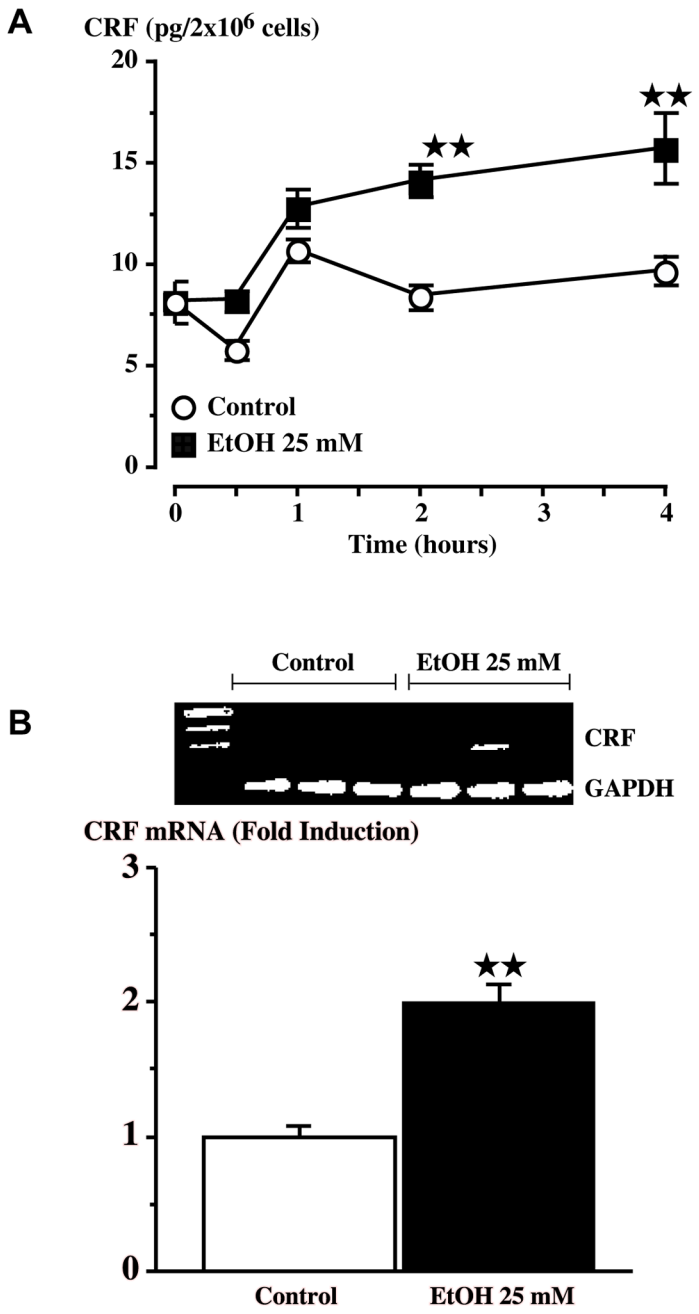


**Figure 7.**

Alcohol (25 mM) increases CRF release (A) or mRNA levels (B) in primary hypothalamic cell cultures. (A) CRF peptide secretion was detected by RIA after treatment of cells with alcohol. Each point represents the mean ± SEM. (B) RT-PCR analysis of CRF mRNA expression after treatment with 25 mM alcohol. **, P<0.01 vs control. {Modified by permission from Li *et al.*[35]}

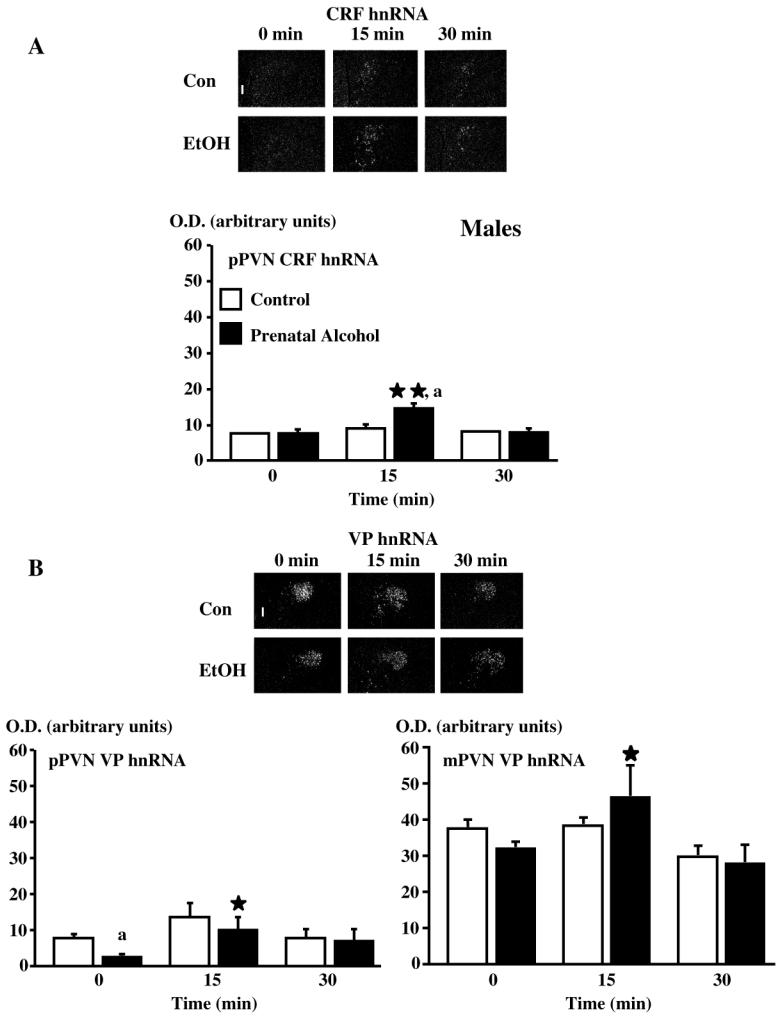


**Figure 8.**

The PVN CRF neuronal response in response to footshocks is larger in adult adult male FAE offspring, compared to controls. Dark-field photographs of a representative coronal section through the midportion of the PVN of C or E male rats exposed to a 30-min session of mild electrofootshocks and showing increases in CRF hnRNA (A) levels in the pPVN at 15 min and no overall changes in VP hnRNA (B) levels of the pPVN and the mPVN at 15 and 30 min after shocks. Statistical analysis of the data expressed as arbitrary units for optical density (OD) for mRNA levels. Each point represents the mean ± SEM of 4–5 rats. *, P <0.05 and **, P<0.01 vs t=0; a, P<0.01 vs C at the corresponding time. pPVN, parvocellular division of the paraventricular nucleus, mPVN, magnocellular division of the paraventricular nucleus. Magnification, 220×, III, third ventricle. {Adapted by permission from Lee *et al.*[36]}

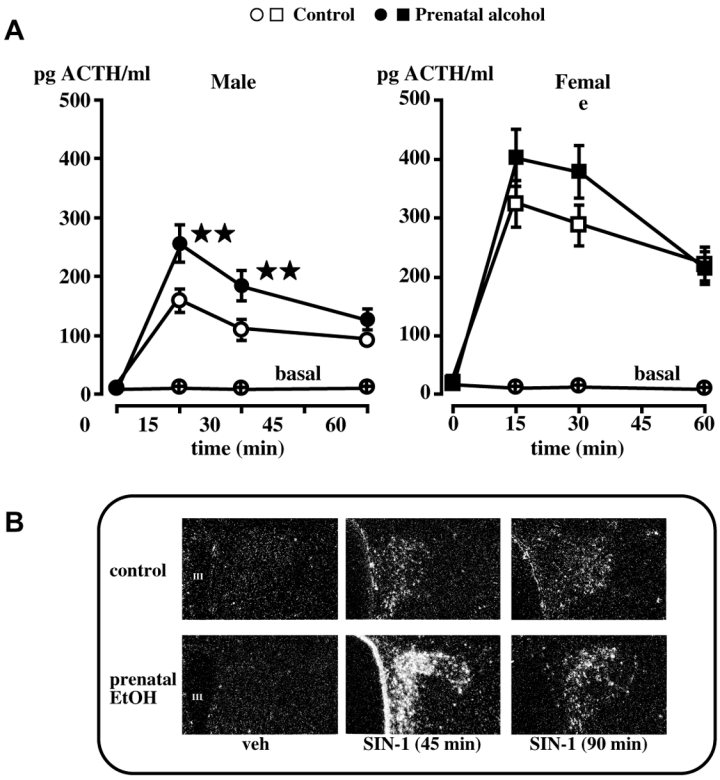


**Figure 9.**

Compared to controls, male FAE rats release more ACTH when injected with SIN-1 icv (A) and display enhanced PVN CRF neuronal activity (B). A. Effect of the icv injection of SIN-1 (20 μg) or vehicle on the ACTH response of male and female C and E rats. **, P<0.01. B. Representative dark-field photomicrographs illustrate the immediate early gene NGFI-B transcripts measured before SIN-1 icv injection as well as 45 and 90 min later. III, 3rd ventricle. Magnification: 340×. {Adapted by permission from Lee *et al.*[63]}

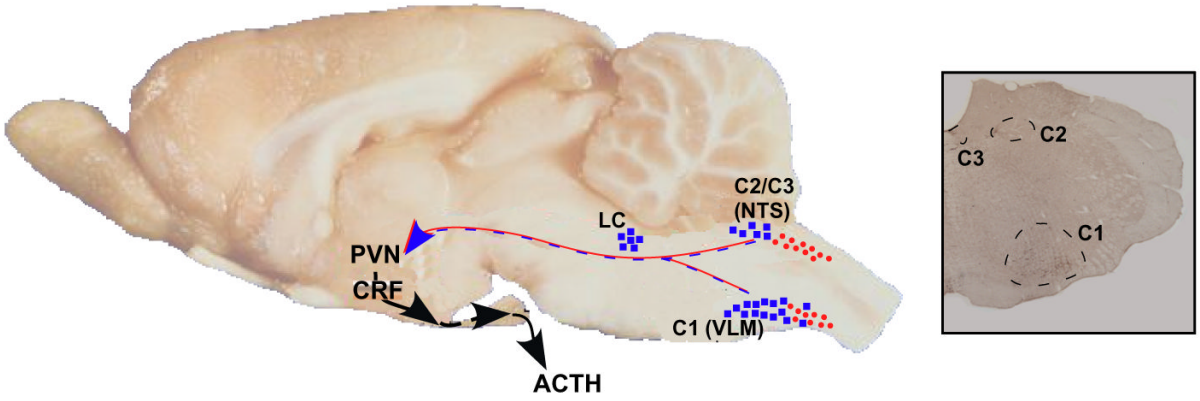


**Figure 10.**

Cartoon illustrating portions of the ascending catecholaminergic innervation of the PVN. Left panel, sagittal view of the rat brain illustrating the adrenergic (closed squares) and noradrenergic (closed circles) cell groups of the locus coeruleus (LC), ventrolateral medulla (VLM) and nucleus of the solitary tract (NTS). Right panel, coronal section through the rat brain stem illustrating the C1-C3 adrenergic cell populations. (Based on templates published in [3, 90–92].)

**Table 1**

Microarray of fetal hypothalami and thalami area (E 17.5) in control or alcohol-exposed mice.

| Gene | Ratio (EtOH/CTL) |
|---|---|
| CRF | ND |
| CRFR1 | 1.007 |
| CRFR2 | 3.725 |
| UCN | ND |
| UCN2 | 0.329 |
| VP | 0.788 |
| VPR1a | 0.171 |
| VPR1b | 0.57 |
| VPR2 | 7.753 |
| VPi1 | 1.08 |
| POMC1 | 1.633 |
| TH | 1.032 |
| DBH | 0.581 |
| PNMT | 0.369 |
| NPY | 1.051 |
| NPYR1 | 0.534 |
| NPYR2 | 0.911 |
| NPYR5 | 0.507 |
| NPYR6 | 2.185 |