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CRH gene expression in the fetal rat is not increased after pharmacological adrenalectomy

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A regimen of twice daily metyrapone injections (100 mg/kg), resulted in pharmacological adrenalectomy of pregnant rats and fetuses in utero, i.e. depression of plasma corticosterone and elevation of plasma adrenocorticotropic hormone (ACTH). Toxicity was minimal on days 14–17 of pregnancy, and increased with higher maternal weight and pregnancy progression. Corticotropin releasing hormone (CRH) messenger RNA abundance in the pregnant adults increased significantly within 48 h of metyrapone initiation. No change in CRH gene expression in the paraventricular nucleus of fetuses (days 17–18) was seen, even after 72 h of the regimen. This is compatible with the independence of CRH gene expression of glucocorticoid feedback in the fetal rat.

The mechanisms controlling corticotropin releasing hormone (CRH) gene expression in the developing rat brain have not been defined. The onset of messenger RNA (CRH-mRNA) synthesis occurs on the 17th fetal day, and after increasing in the late fetal period, decreases markedly around the time of birth [2, 7]. Whether the onset and the hiatus in CRH gene expression are intrinsic, constitutively regulated phenomena, or are modulated via negative feedback by circulating adrenocorticotropic hormone (ACTH) or corticosterone, is unknown. This study was designed to examine the effects of eliminating the negative corticosterone (CORT) feedback on the brain–adrenal axis in the fetal rat. Our goal was to study the effects of functional fetal adrenalectomy on CRH gene expression in the fetal rat, in comparison with the pregnant adult.

Forty timed-pregnancy Sprague–Dawley derived rats, weighing less than 350 g, were kept on a 12 h light/dark cycle and allowed unlimited access to lab chow and water. Pregnancy was dated by the presence of a vaginal plug (day 0). Gestation lasted for 21 days. Pharmacological adrenalectomy schedule [14] was started on the 14th–18th day of pregnancy, and continued for 48 or 72 h, as previously described [4]. Briefly, pregnant rats were injected subcutaneously with dimethylsulfoxide (DMSO) vehicle or 100 mg/kg metyrapone (Sigma, St. Louis, MO), every 12 h. On the morning of sacrifice, metyrapone was followed, at 09.15 h, by 100 mg/kg aminoglutethimide.

Rats were decapitated at 10.00 h and uteri quickly harvested. Maternal and pooled (n = 5) fetal trunk blood was obtained as described previously, and assayed for CORT and ACTH [4]. Adult brains and fetal heads were frozen immediately on powdered dry ice and kept frozen at −80°C.

Frozen brains were cut into 20 μm coronal sections and mounted on gelatin-coated slides. Brain regions were identified by established landmarks [1]. Prior to in situ hybridization (ISH), sections were brought to room temperature, air-dried and fixed for 20 min in fresh 4% buffered PBS-paraformaldehyde. Sections were dehydrated through increasing ethanol concentrations, rehydrated, exposed to 0.5% acetic anhydride–0.1 M triethanolamine (pH=8), then dehydrated through 100% ethanol [2, 3].

Details of oligonucleotide probe generation and of ISH, modified from Young [19], have been described [2, 3]. Briefly, prehybridization for 1 h in hybridization buffer lacking dextran sulfate (0.2 ml/slice), was followed by a 20 h ISH at 40°C [2, 3]. Reaction volume was 0.03 ml/slice, under a coverslip in a humidity chamber. Following serial washes, hybridized sections were processed for autoradiography [2, 3]. A 60 nucleotide synthetic probe corresponding to the codons for the 20

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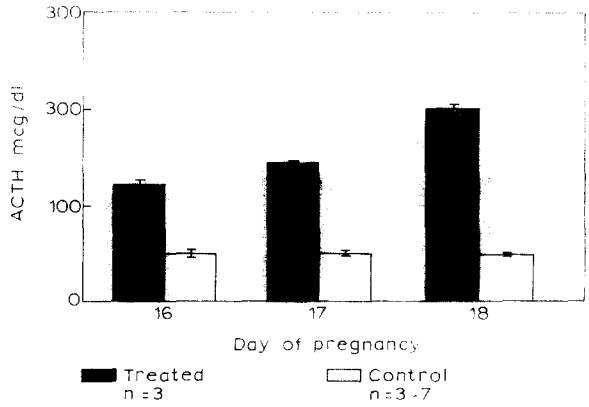

Fig. 1. Plasma ACTH in pregnant rats treated with metyrapone for 48 h. Pharmacological adrenalectomy was achieved by metyrapone injections (see text). ACTH levels of treated animals were significantly different from those of controls: $P<0.05$.

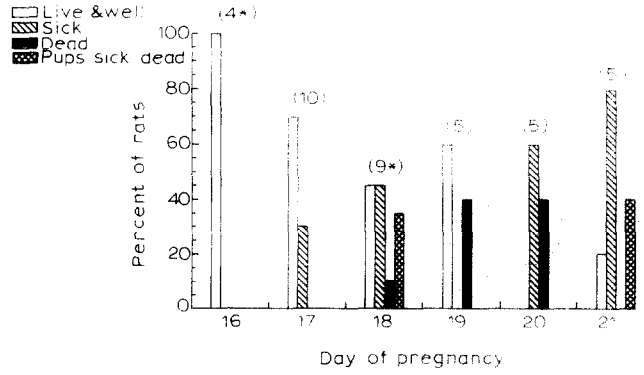

Fig. 2. Toxicity of metyrapone, given for 48–72 h, as a function of gestational day in pregnant rat and fetuses. Day of sacrifice is indicated. Number of rats are in brackets. +, an additional rat in this group was not pregnant.

COOH-amino acids of CRH [17] was labelled on the 3' end with dATP-^{35}S. Specific activity of labelled probes was $5–8 \times 10^8$ dpm/μg. The complementary 'sense' strand was similarly generated and labelled.

Serial sections of the hypothalamus at the level of the paraventricular nucleus (PVN) were examined. For quantitative studies, 5 sections with the maximal CRH–mRNA, derived from at least 3 rats each from 2 separate litters (for fetuses), were compared [2, 3]. Images were obtained and optical density (O.D.) determined over the PVN and over the parietal cortex as described previously [2, 3]. To allow for variability in background (cortex) O.D., the O.D. ratio of PVN to parietal cortex was utilized as a measure of CRH–mRNA abundance, and means and standard deviations for 10 ratios were determined. For adult rats, sections derived from comparable levels in the PVN were also dipped in emulsion (NTB-2, Kodak), and exposed for 1 week. They were subjected to darkfield microscopy and analysis. Brainpaste standards were used to ascertain that measurements were in the linear range [2]. Analysis of variance (Minitab, University Park, PA), was used to assess the significance of differences between experimental groups.

Effect of pharmacological adrenalectomy on maternal and fetal levels of CORT and ACTH. Plasma ACTH increased significantly ($P<0.05$) in metyrapone-treated rats (Fig. 1). This effect persisted for 72 h. The metyrapone regimen decreased plasma CORT levels 85—95%, e.g. plasma CORT in treated adults was 5.9 ± 1.6 μg/dl, compared with 30.2 ± 3.7 μg/dl in controls 48 h after initiation of the regimen [4]. The reduction in CORT levels was maximal by 24 h, and persisted for at least 72 h [4].

Toxicity of the metyrapone regimen as a function of pregnancy day. Metyrapone injections were well tolerated by rats on the 14th–17th pregnancy days. Rats weighing less than 350 g by day 17 of pregnancy (11/14), as well as the two animals found not to be pregnant, appeared well (Fig. 2). Abnormal fetal appearance or activity, fetal demise or poor maternal activity were seen in heavier animals (3/10, day 17) and worsened with progression of pregnancy (Fig. 2). Therefore, following these preliminary results, no experiments were conducted beyond the 18th gestational day, to avoid animal stress and malaise. Furthermore, CRH-mRNA was analyzed *only* in adult and fetal rats which appeared healthy and indistinguishable from DMSO-injected controls in terms of weight, activity etc. Only treated adult and fetal rats with (pooled, for fetuses) CORT levels lower than 5 μg/dl were analyzed [18].

Effects of metyrapone treatment in CRF-mRNA in fetal and pregnant rats. At the end of 48 h of metyrapone treatment, CRH-mRNA in the PVN of pregnant rats increased significantly (Fig. 3). In fetal rats aged 17–18 days, subjected to 48 or 72 h of the regimen, no change in CRH-mRNA abundance was evident. Fig. 3 demonstrates the quantitative analysis of CRH-mRNA abundance in pregnant and fetal rats. Metyrapone 'pharma-

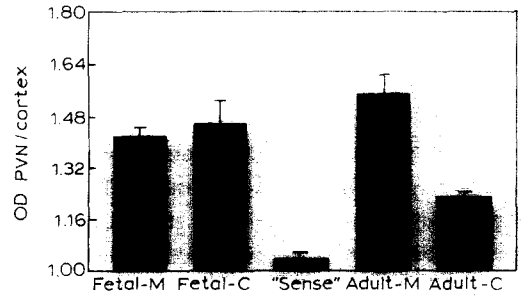

Fig. 3. Effect of metyrapone-injection regimen on CRH-mRNA in PVN of pregnant and fetal rats. Metyrapone was initiated 48 h prior to sacrifice. Bars indicate means and standard errors. Control (C) and treated (M) fetal values are not statistically significant. Adult treated group (M) differs significantly ($P<0.05$) from control (C). 'sense' denotes oligonucleotide complementary to CRH probe.

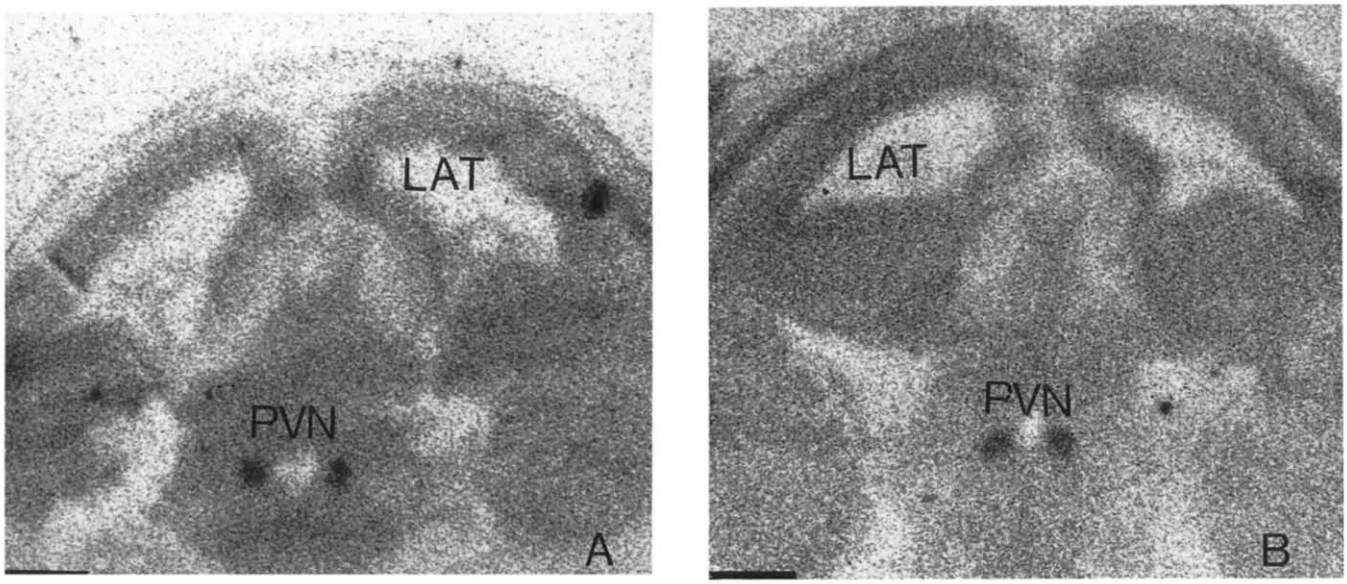

Fig. 4. Photomicrographs of the PVN of 18 day fetal rats following ISH with an oligonucleotide complementary to CRH-messenger RNA. A: treated: 48 h of metyrapone administration. B: control. PVN, paraventricular nucleus. Bar = 1 mm. See text for details of ISH.

cological adrenalectomy' was initiated on the 16th gestational day, and animals were sacrificed 48 h later. The significant ($P<0.05$) increase in CRH-mRNA abundance in the pregnant adults rats is contrasted with the lack of change in CRH-gene expression in the 18 day fetuses. Fig. 4 shows photomicrographs of coronal brain sections at the level of the PVN, of fetal rats subjected to the same regimen. In Fig. 5, control and treated adult PVN are shown. Darkfield micrographs of comparable PVN levels mainly show an increase in intensity of CRH-mRNA signal, and not an increase in number of cells expressing CRH.

These experiments provide evidence that significant reduction of plasma CORT levels in the fetal rat does not result in the expected enhancement of CRH gene expression. This is in contradistinction to the reported effect in the adult [9, 11, 18, 19], as well as to our findings in pregnant rats. CORT levels decreased to 5 μg/dl, levels reported in surgically adrenalectomized rats, and resulting in enhanced CRH gene expression in adult animals [18]. Specifically, Swanson and Simmons [18] have shown that in the adult rat, adrenalectomy results in increased CRH-mRNA in the PVN within 48 h, while other investigators, using either ISH or Northern blot analysis, have documented elevation of CRH gene expression in the PVN a week following adrenalectomy [9, 11, 18].

Swanson and Simmons [18] found that CORT levels greater than 5 μg/dl were necessary for inhibition of adrenalectomy-induced increase in CRH-mRNA. Beyer

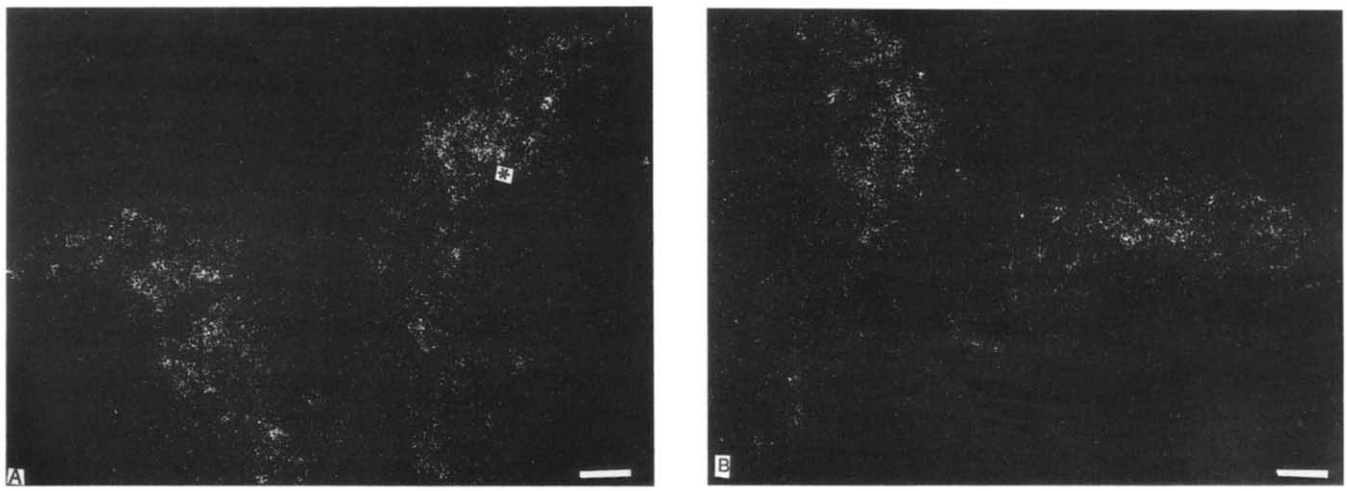

Fig. 5. Darkfield photomicrographs of comparable levels of the paraventricular nucleus of treated (A) and control (B) pregnant rats subjected to ISH for CRH-mRNA. Increased number of grains per cell is seen in the treated animal (star). Bar = 100 μm.

et al. [5] reported restoration of control PVN-CRH-mRNA with lower CORT levels (2.9 μg/dl), but utilized Northern blot analysis of whole PVN.

The levels of plasma CORT achieved with the metyrapone regimen should result in minimal occupation of GC receptors [15]. The enhanced CRH gene expression in the pregnant rats suggests that metyrapone-induced GC depletion was adequate to eliminate its negative feedback on CRH synthesis. Furthermore, non-specific stress secondary to the regimen would be expected to increase fetal CORT and CRH-mRNA [12] – neither of which was observed.

Metyrapone administration is associated with the stress of injection and handling [8] which, present also in vehicle-injected controls, is unlikely to account for increased CRH-mRNA in the pregnant rats. Metyrapone has been shown to accelerate the response of the adrenocortical system to adrenalectomy [8], but this effect, significant within hours of adrenalectomy, was not observed 2–3 days later [8]. The effective metyrapone dose in pregnant rats (100 mg/kg) is significantly lower than in adult males (200–300 mg/kg) [14]. This fact, as well as the correlation of morbidity and body weight, is probably secondary to the altered pharmacokinetics of the drug during late gestation in the pregnant rat.

We found no temporal alteration of CRH gene expression by pharmacological adrenalectomy in the fetal rat. Metyrapone administration starting on the 14th or 15th fetal day did not result in an onset of CRH-gene expression prior to the reported [2, 7] 17th fetal day (not shown).

Our findings are compatible with the lack of a GC-negative feedback effect on CRH-gene expression in the fetal rat. GC receptors have been demonstrated by Northern blot analysis of total fetal brain RNA [10]. The lack of negative feedback may thus be a consequence of decreased receptor function [13], or the immaturity of transduction mechanisms beyond the steroid–receptor complex interaction.

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