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CRF Administered to Pregnant Rats Alters Offspring Behavior and Morphology[1]

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WILLIAMS, M. T., M. B. HENNESSY AND H. N. DAVIS. *CRF administered to pregnant rats alters offspring behavior and morphology.* PHARMACOL BIOCHEM BEHAV **52**(1) 161-167, 1995.—Pregnant rats injected with 20 μg of corticotropin-releasing factor (CRF) from day 14 through 21 gained less weight during gestation than did saline-injected controls. The offspring of CRF-injected females differed from the offspring of control females in several ways: males and females weighed less during the first 2 weeks of life, males had shorter anogenital distances at birth, and males and females emitted more ultrasonic vocalizations during isolation in tests at 6 and 14 days of age. These effects are similar to those that have been observed following exposure of pregnant females to stressors, and provide support for the notion that CRF and/or CRF activation of the hypothalamic-pituitary-adrenal axis mediate effects of gestational stress.

CRF Stress Prenatal Ultrasonic vocalizations (USV)

STRESSING female rats during pregnancy with, for instance, electric shock or restraint affects the behavior, physiology, and morphology of the offspring. In early studies, the offspring typically were tested while isolated in a novel environment (e.g., open field) and were generally found to be less active and slower to initiate behavior, and to defecate more than control animals [for review see (5)]. These results were commonly interpreted as reflecting heightened "emotionality" in the offspring of females stressed during pregnancy (hereafter referred to as "prenatally stressed" offspring) (40,59).

Ward (63) showed that male offspring of female rats subjected to heat, light, and restraint during the last trimester of pregnancy exhibited less masculine and more feminine sexual behavior in standardized tests than did males of normal pregnancies. Subsequent research established that various morphological and physiological as well as other behavioral, sexually dimorphic characteristics of males could be feminized/demasculinized if their mothers were stressed during pregnancy. These effects include: a reduced anogenital distance (AGD) (3,15,38), reductions in the size of the medial preoptic area (3,4,30) and of certain spinal nuclei (23), higher levels of basal corticosterone (58), decreased play behavior (57,64), and a greater likelihood of engaging in maternal behavior (31). Furthermore, prenatally stressed females exhibit some evidence of masculinized/defeminized effects. These females have been shown to have longer latencies to initiate maternal behavior (21,31), and neurotransmitter levels in the entorhinal cortex and periventricular nucleus that are comparable to normal male levels (41), but greater than normal female levels.

To date there has been no clear indication of the mechanism of action that leads to the offsprings' altered behavior, morphology, or physiology. However, Ward and Weisz (65) showed that male offspring of mothers that underwent a heat/light/restraint stressor during the last trimester had an early peak in testosterone levels on gestational day 17 and lower levels of testosterone on days 18 and 19, whereas normal males had their peak around day 18 or 19 of gestation. This early peak may influence masculine traits, but does not explain changes that occur in the female offspring.

Hormones of the hypothalamic-pituitary-adrenal (HPA) axis [i.e., corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), beta-endorphin (B-END), and glucocorticoids] may be involved in mediating prenatal stress effects. The HPA system is activated during times of stress, and glucocorticoids (6,68) and B-END (50) are known to cross the placental barrier. Furthermore, hormones of the HPA axis can produce profound and wide-ranging physiological and behavioral effects, not the least interesting of which is the apparent ability of glucocorticoids (7,37), B-END (2), and CRF (19,60,61) to suppress testosterone production by the testes.

[1] These results were originally presented at the Second Annual International Behavioral Neuroscience Society Conference held in Clearwater Beach, FL, USA, from April 22-25, 1993.

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Administration of either ACTH (47,52,54) or B-END (29) to pregnant rats during the last trimester decreases masculine sexual behavior or increases female sexual behavior in male offspring. However, other parameters affected by stress during pregnancy [e.g., AGD at birth (52,54) and spinal nuclei (29)] are either unaffected or affected differently by ACTH and B-END. A possible reason for such discrepant results is that a single hormone of the HPA axis may play a role in affecting a certain behavior or other outcome, but not be responsible for the entire range of effects seen after prenatal stress.

CRF is a 41-amino acid peptide that is released by the hypothalamus and stimulates secretion of ACTH and B-END (48,62). CRF can also produce effects independent of the other hormones of the HPA axis, including, but not limited to: decreased sexual behavior, increased and/or decreased locomotor activity, autonomic activation, and increased neurotransmitter release [for reviews see (16,45)]. Thus, because CRF initiates the cascade of HPA hormones, and also has effects independent of the HPA system, we reasoned that CRF may, either directly or indirectly, mediate changes seen after prenatal stress. In the current study we administered CRF during the last trimester to pregnant females and examined the morphology and behavior of the offspring. One earlier study found that the administration of CRF to pregnant females during the last trimester reduced the body weight of offspring on the day of birth (67), an outcome consistent with the effects of gesational stress (8,26,35,46,47).

In this study, we measured AGD to detect possible feminization/demasculinization effects of CRF on morphology. Further, because stress during pregnancy has been shown to reduce the weight of the pregnant female (8,24,32) as well as her offspring (8,26,35,46,47), we weighed pregnant females during the last trimester and offspring both at birth and at various intervals until 2 weeks of age. Because shock during pregnancy has been shown to reduce the emission of ultrasonic vocalizations (USVs) in male offspring (56), our measure of behavior was the number of USVs that rat pups emitted while isolated during the first 2 weeks of life. USVs of pups are often regarded as reflecting distress, particularly at older ages (27). Further, USVs appear to be a sexually dimorphic characteristic, both in pups [(42); Harvey, personal communication] and in adults (9). Consequently, USVs can be viewed as both an "emotional" and sexually dimorphic behavior. The rectal temperature of pups was obtained at the conclusion of USV tests for comparison with pup weight and USV data.

The injection of any substance into a pregnant female could have detrimental effects on the developing fetus; therefore, length of gestation, litter size (including number of males and females), and the number of dead animals were recorded for each mother. A dose of 20 μg of CRF was selected based on pilot work. Multiple, daily injections were given during the last trimester of gestation because this regime has been commonly used in studies demonstrating effects of prenatal stress [e.g., (63)].

METHOD

Animals and Experimental Conditions

Thirty-six primiparous Sprague–Dawley female rats (Harlan, Indianapolis, IN) and their offspring were used as subjects. The females were time-mated in our laboratory by placing an estrous female with a sexually active male until the occurrence of two ejaculations. Females were weighed after mating and housed singly. Day of conception was designated postconception day 0 (PC-0). Twelve impregnated females were randomly assigned to each of three conditions: noninjected (NI), saline injected (SAL), and 20 μg CRF injected (CRF). Pups are designated by their mother's injection condition (i.e., NI, SAL, or CRF).

On PC-21, females were given nesting material. Starting at PC-22, all females were checked once in the morning and once in the evening for the presence of a litter. The day that a litter was discovered was designated postnatal day 0 (PN-0). The litter was left undisturbed until PN-1. The colony room was maintained on a reversed 12L : 12D cycle (lights on at 2200 h). Food and water were available ad lib, and all procedures were approved by Wright State University's Laboratory Animal Care and Use Committee.

Injections

CRF (1–41 rat/human; Sigma Chemical Company, St. Louis, MO) was dissolved in saline, and an aliquot of 20 μg in a volume of 0.1 ml was obtained for each dose. The doses were stored at -70°C until the time of administration. CRF injections were given within 5 min of thawing. SAL animals received 0.1 ml saline per injection. All injections were administered subcutaneously into the nape of the neck.

On the mornings of days PC-14 through PC-21, each pregnant female was removed in her home cage from the colony room, brought into a separate room, weighed, injected if required, and returned to the colony room. Animals in the two injection conditions underwent the same procedure in the afternoon, but were not weighed. The interval between injections was never less than 6 h.

Postnatal Procedures

On PN-1, the litter was sexed and the number of live males and females and the number of dead animals were recorded for each litter. Each pup was weighed and its AGD was measured. All measurements were obtained without knowledge of the mother's injection condition. Weights were calculated to the nearest tenth of a gram using a Mettler PS1200 scale (Mettler Instrument Corp, Highstown, NJ). AGDs were measured to the nearest tenth of a millimeter using a vernier caliper. All litters were then culled to six.

A male and female from each litter were randomly selected for inclusion in a test for USVs during isolation. On PN-1, India ink was injected into the rear, right foot pad of these two pups for identification purposes. On PN-6, PN-10, and PN-14, each of these animals was removed in turn from its mother and littermates, brought to a nearby secluded room, and placed into an empty, clear, uncovered, Pyrex container (190 mm in diameter $\times$ 100 mm in height) that served as the test arena. A Mini-2 bat detector (UltraSound Advice, London, UK) was positioned 12 cm above the test arena. The bandwidth of the detector was adjusted to approximately 42 $\pm$ 4 kHz, which we and others have found to be the predominate range of rat pup vocalizations at the ages tested (25, 43,44). Earphones were used to preclude any effects of feedback from the ultrasound detector on the pup. The number of USVs per minute was recorded using a hand counter during a 6-min test period. The animal was then removed from the test arena, weighed, and its rectal temperature measured using a temperature probe (YSI model 520, Yellow Springs, OH) and thermometer (YSI 4000-a, on loan from YSI, Yellow Springs, OH). The test arena was wiped clean with a wet sponge after each test, and all testing was performed between 1000 and 1400 h during the dark phase of the cycle.

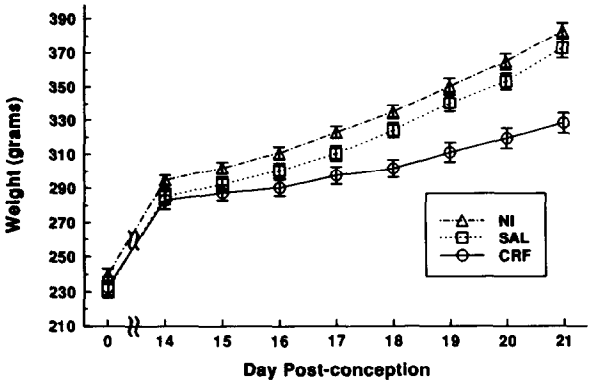

FIG. 1. Means and SEs (vertical lines) of body weights of pregnant females on day of conception and days 14 through 21 postconception.

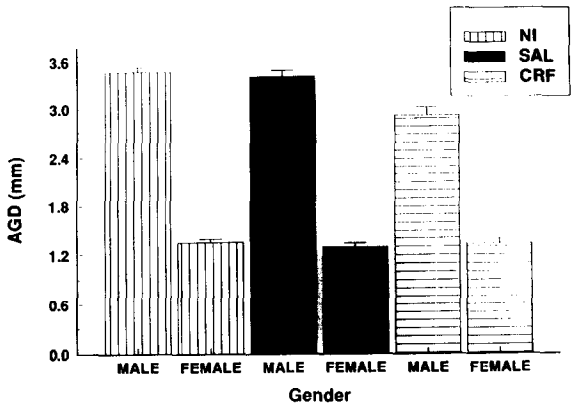

FIG. 2. Means and SEs (vertical lines) of anogenital distances of male and female rats on postnatal day 1.

Statistics

Weights of adult females on the day of mating were analyzed with a one-way (condition) analysis of variance (ANOVA). Weight gain of these females from PC-14 through PC-21 was analyzed with a mixed-factor design ANOVA (condition × day) with day as the repeated measure. The length of gestation, number of stillborns, and total number of live males and females were analyzed using the appropriate ANOVA (i.e., condition or condition × sex). Body weight and AGD of pups on PN-1 were analyzed with two-way ANOVA (condition × sex) using the mean of the measurements of pups of each gender within a litter as the unit of statistical analysis (1). Pup weight, rectal temperature, and USVs on PN-6, PN-10, and PN-14 were analyzed using mixed-design ANOVA with condition and sex as between-subject variables and either day alone (pup weight, rectal temperature) or day and minute (USVs) as repeated measures. For all repeated measures, epsilon was determined, and when deviations from orthogonal sphericity were detected, Greenhouse-Geisser adjustments were used to control for type I error. Significant interactions were analyzed with tests for simple effects as detailed by Winer (66). Pairwise comparisons were performed using the Tukey method. A 0.05 level of significance (two-tailed) was used for all tests.

RESULTS

Weight of Adult Females and Length of Gestation

The weights of females on the day of mating did not differ among conditions. However, for weight gain during gestation, the main effects of condition, $F(2, 33) = 11.74$, $p < 0.001$, and of day, $F(7, 231) = 1093.94$, $p < 0.001$, as well as the interaction of condition × day, $F(14, 231) = 40.99$, $p < 0.001$, were significant. Weight gain was reduced by CRF injections during the treatment period (Fig. 1). Specifically, CRF adult females weighed less than did NI adult females on PC-16 and PC-17 ($ps < 0.01$) and less than both NI and SAL mothers on PC-18 through PC-21 ($ps < 0.01$). The weight gain of NI and SAL adult females did not differ statistically. The length of gestation did not differ among conditions (Table 1).

Measures on PN-1

There were no differences among conditions in the number of stillborns, number of live animals per litter, or the number of males and females per litter (Table 1). Males weighed more than females on PN-1, $F(1, 66) = 6.76$, $p < 0.02$. There also was a significant effect of condition on pup weight, $F(2, 66) = 27.54$, $p < 0.001$. Pups in the NI condition (mean = 6.83 ± 0.13) weighed more than SAL pups (mean = 6.24 ± 0.16; $p < 0.01$), which weighed more than CRF pups (mean = 5.46 ± 0.11; $p < 0.01$).

As expected, the males' AGDs were longer than those of females, $F(1, 66) = 1188.93$, $p < 0.001$. The effect of condition, $F(2, 66) = 9.73$, $p < 0.001$, and the interaction of condition × sex were also significant, $F(2, 66) = 10.0$, $p < 0.001$. Further analysis of the interaction showed that the AGDs of CRF males were shorter than those of NI and SAL males ($ps < 0.01$), which did not differ from one another. Females from all conditions had similar AGDs (Fig. 2).

Measures on PN-6, PN-10, and PN-14

ANOVA of the weight of those animals tested for USVs revealed significant effects of condition, $F(2, 66) = 15.26$, p

TABLE 1

GESTATIONAL LENGTH, NUMBER OF STILLBORNS, AND NUMBER OF LIVE PUPS

Condition	N	Length of Gestation (Days)	No. of Stillborns	Live Pups/Litter	No. of Males	No. of Females
NI	12	22.83 ± 0.11	1.25 ± 0.58	11.92 ± 0.63	6.33 ± 0.47	5.58 ± 0.53
SAL	12	22.75 ± 0.13	0.83 ± 0.39	13.42 ± 0.77	6.58 ± 0.48	6.83 ± 0.55
CRF	12	22.83 ± 0.17	1.08 ± 0.50	12.33 ± 0.61	6.42 ± 0.43	5.92 ± 0.48

Values are mean ± SEM.

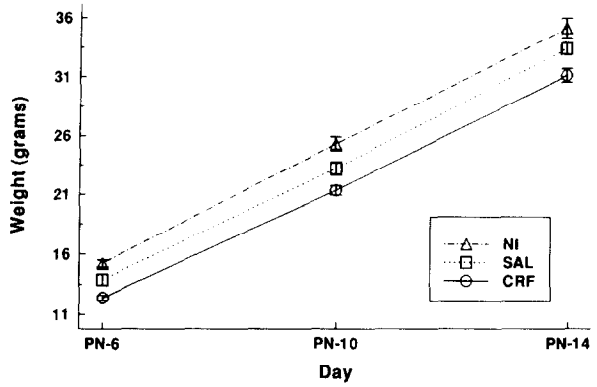

FIG. 3. Means and SEs (vertical lines) of body weights of pups on postnatal days 6, 10, and 14.

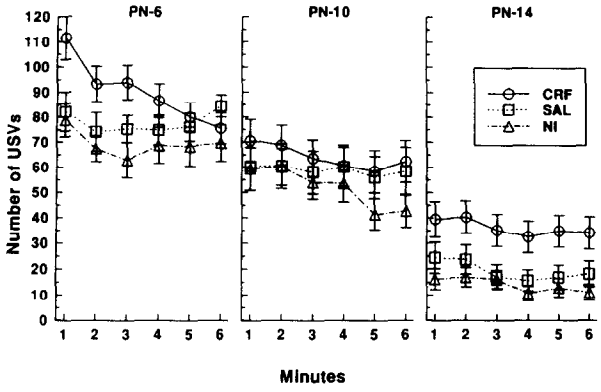

FIG. 4. Means and SEs (vertical lines) of the number of ultrasonic vocalizations emitted per minute after isolation on postnatal days 6, 10, and 14.

< 0.001, and of day, $F(2, 132) = 3172.89$, $p < 0.001$. As at PN-1, NI pups weighed more than SAL pups, which weighed more than CRF pups ($ps < 0.01$) (Fig. 3). The rectal temperature of all animals in the USV test increased over day as expected with maturational changes, $F(2, 132) = 233.57$, $p < 0.001$. No other effects were significant for rectal temperature (Table 2).

For USVs, all main effects were significant: condition, $F(2, 66) = 5.61$, $p < 0.01$; sex, $F(1, 66) = 4.75$, $p < 0.05$; day, $F(2, 132) = 99.42$, $p < 0.001$; minute, $F(5, 330) = 11.01$, $p < 0.001$. These effects indicated that overall: 1) CRF pups vocalized more than did NI and SAL pups ($ps < 0.05$), which did not differ; 2) males (mean = 1049.56 ± 65.10) vocalized more than females (mean = 871.94 ± 55.86); 3) USVs declined over days; and 4) vocalizing diminished across the 6-min test.

However, the condition × day × minute interaction was also significant, $F(20, 660) = 2.09$, $p < 0.05$ (Fig. 4). To further assess this three-way interaction, tests for simple interaction effects (condition × minute) were conducted at each day. On PN-6, the condition × minute interaction was significant. Follow-up pairwise comparisons indicated that CRF pups emitted more USVs at minute 1, 2, and 3 than did NI pups ($ps < 0.01$) and SAL pups ($ps < 0.01$, 0.01, and 0.05, respectively). Furthermore, CRF pups emitted more USVs at minute 4 than did NI pups ($p < 0.05$). NI pups and SAL pups did not differ at any minute. The analysis at PN-10 revealed no main or interactive effects of condition. On PN-14, the main effect of condition was significant; CRF pups vocalized more than NI and SAL pups ($ps < 0.01$), which did not differ. In summary, CRF administration increased vocalizing overall, but effects were statistically reliable only at certain minutes and days.

DISCUSSION

The current results show that the administration of CRF to pregnant females has effects similar to those of prenatal stress. Specifically, we found that CRF administered during the last trimester produced: 1) a reduction in the weight of pregnant females during gestation; 2) a reduction in the weight of the offspring; 3) a shorter AGD in the male offspring; and 4) an increase in the emission of offspring USVs during isolation. A reduction in the body weight of both adult females (8,24,32) and their offspring (8,26,35,46,47), and in the AGD of male offspring (3,15,38) has previously been reported following gestational stress. USVs have also been shown to be altered by prenatal stress (i.e., shock), though the direction of the effect differed from the effect observed following CRF treatment [cf., (56)].

It is possible that the decline in weight gain of adult females during gestation was due to an anorectic effect of CRF. Central administration of CRF inhibits the intake of food, independent of the HPA axis (28,39). But, whether the peripheral route of CRF administration in this study had anorectic effects similar to those found with central administration is unclear. We did not monitor food intake of animals in the current study; therefore, we are unable to say if the decreased body weight was related to a decrease in food consumption or to some other factor(s). The reduced body weight of the adult females might account for the low weight of CRF pups at birth. The persistence of the effect of CRF on pup weight during the first 2 weeks of life might be attributed to a lasting impact of CRF on the mother (e.g., on milk production). However, we have observed the weight deficit of CRF pups to continue beyond PN-40 (unpublished), well after the permanent removal of the mothers. Thus, it appears the influence of

TABLE 2

RECTAL TEMPERATURES (°C) AFTER 6-MIN USV TEST ON PN-6, 10, 14

Condition	N	PN-6	PN-10	PN-14
NI	24	31.17 ± 0.32	33.30 ± 0.18	34.77 ± 0.20
SAL	24	30.31 ± 0.40	33.20 ± 0.19	34.78 ± 0.19
CRF	24	30.34 ± 0.34	32.42 ± 0.21	34.72 ± 0.13

Values are mean ± SEM.

CRF, whether a direct effect of CRF or an indirect effect mediated through the mother (e.g., milk production), is nonetheless long lasting.

Perhaps the most striking of the present findings was the selective reduction of the AGD of CRF males. AGD is a prominent, androgen-dependent, sexually dimorphic anatomical characteristic present at birth (13), and is affected by prenatal stress in the same fashion as by CRF here (3,15,38). Faber and Hughes (20) found that the relative size of the AGD in females at birth correlated positively with the size of the sexually dimorphic nucleus of the medial preoptic area (SDN-MPOA) in adulthood. This effect was thought to reflect differences in the degree of exposure of the females to androgens during the fetal period. Furthermore, the size of the SDN-MPOA in males is reduced by prenatal stress (3,4,30). Therefore, the reduction in the length of AGD may be an indicator not only of the peripheral anatomical effects of CRF administration to the pregnant female, but also of central nervous system effects as well. This possibility is currently being investigated in our laboratory.

Our USV data indicate that the behavior of pups after birth can also be altered by CRF treatment during pregnancy. The offspring of females administered CRF during the last trimester vocalized more during isolation than did control pups. Takahashi et al. (56) observed that the number of USVs emitted by male offspring (females were not tested) while isolated during daylight hours was reduced by a prenatal shock treatment that was administered every other day throughout gestation. This discrepancy between Takahashi's and our data in the direction of effects could be due to the differences in the timing of the shock treatments/injections or the phase in the light/dark cycle when testing occurred. Previous research has found that the timing of prenatal stress (e.g., first, second, or third trimester) is an important factor in determining effects (22,40,49,55).

It is possible that the reduction in USVs by CRF pups was secondary to their lighter body weight. Smaller pups have a larger surface-to-volume ratio and therefore exhibit more rapid cooling when removed from the warmth of the nest. When a pup becomes cool, nonshivering thermogenesis occurs and increases body temperature, and USV production can be a by-product of this process (11,12). However, weight differences do not appear to be capable of completely accounting for the greater number of USVs by CRF pups because the weight difference between NI and SAL pups and between SAL and CRF pups on each day of testing was similar, but the number of USVs produced by the NI and SAL groups was not different. Furthermore, the rectal temperature of animals in the three prenatal conditions did not differ at the conclusion of the 6-min test period on any test day.

We showed, as did Naito and Tonoue (42), that males vocalized more than females regardless of prenatal treatment. This sex difference in USV production would caution against the common practice in studies of USV in young rats of assigning male and female pups to experimental groups without consideration of gender [e.g., (10,14,18,27,44)].

This study provides evidence for the involvement of CRF and/or other hormones of the HPA axis in the effects of prenatal stress. At this time, it is uncertain if CRF crosses the placental barrier; it is known that the rat placenta does not produce CRF in the manner that the human placenta produces CRF during gestation (51). However, whether HPA hormones of the mother might act directly on the fetus to produce effects such as those observed in the present study, or if maternal HPA hormones or other factors might increase fetal HPA hormone secretion, which in turn might mediate effects, is unclear. Administration of a single HPA hormone, whether one known to cross the placental barrier [i.e., B-END (50) or glucocorticoids (6,68)] or one known not to cross the barrier [e.g., ACTH (17,36)] has limited or contradictory effects when compared with the entire range of prenatal stress outcomes. It may be that the entire range of effects observed after prenatal stress is dependent on the combined action of various HPA axis hormones, and possibly on other stress-induced physiological changes as well.

Exposure to stressors throughout ontogeny has long been known to have many immediate as well as lasting consequences (33,34,53,63). Studies have often revealed a remarkable similarity in the short-term effects of stressors and of CRF administration [for reviews see (16,45)]. These studies and related research suggest an involvement of the HPA axis, and more specifically of CRF, in the mediation of many of the short-term effects of stress (16). The current study is among the first to investigate the long-term effects of CRF administration. Therefore, at this general level, the present results are also noteworthy because they demonstrate that exogenous CRF can have lasting effects and that these effects are similar to those seen following exposure to stressors.

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