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Effects of Centrally Administered Corticotropin-Releasing Factor (CRF) and α-Helical CRF on the Vocalizations of Isolated Guinea Pig Pups

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HENNESSY, M. B., D. R. O'NEIL, L. A. BECKER, R. JENKINS, M. T. WILLIAMS AND H. N. DAVIS. *Effects of centrally administered corticotropin-releasing factor (CRF) and α-helical CRF on the vocalizations of isolated guinea pig pups.* PHARMACOL BIOCHEM BEHAV **43**(1) 37–43, 1992.—Intraventricular corticotropin-releasing factor (CRF) was administered to guinea pig pups both with a freehand injection technique and via indwelling cannula. Behavioral effects depended upon the technique used. The highest dose of CRF (5 μg) inhibited the vocalizing of pups in a subsequent isolation test only when CRF was given by freehand injection. The possibility that disturbance attendant to the freehand procedure can account for this difference is discussed. To determine the effect of endogenous CRF in the absence of additional disturbance, the CRF antagonist α-helical CRF (ahCRF) was administered with the indwelling cannula procedure. ahCRF enhanced vocalizing during the first 10 min, and enhanced locomotor activity during the last 10 min, of a 30-min isolation test. Overall, the results indicate that endogenous CRF reduces vocalizing and locomotion during social isolation and that under certain injection conditions exogenous CRF can exacerbate the behavioral effect. The results also demonstrate the potential impact of the technique used to administer exogenous CRF. Further, the prevailing view, that CRF mediates stress-related behavioral responses, is supported only if behavioral inhibition, rather than vocalizing or locomotor activity, is viewed as the stress-related response in this situation.

CRF α-Helical CRF Maternal separation Isolation Vocalizations Cortisol Guinea pig

CORTICOTROPIN-releasing factor (CRF) released from the hypothalamus appears to be the primary stimulus for pituitary secretion of corticotropin (ACTH) and β-endorphin (11,22). CRF and its receptors are also present in many extrahypothalamic brain sites (1,19). Central administration of CRF can produce a variety of effects, including sympathetic activation (7), stimulation of central noradrenergic activity (9), and various behavioral changes, including suppression of feeding and exploratory behavior, increased grooming, and potentiation of the acoustic startle and defensive withdrawal responses (18,26,27,29). It has been suggested that CRF may be an early mediator or coordinator of the body's diverse physiological and behavioral reactions in times of stress (5,10).

Most studies of CRF's behavioral effects have been conducted with adult animals. The limited work that has been performed with younger subjects has focused primarily on the influence of CRF on the vocalizing of infants during periods of social separation. As might be expected given CRF's general facilitatory influence on stress-related behaviors in adults, Panksepp et al. (20) found that ICV CRF increased the vocalizing of isolated chicks. However, in separated rhesus monkeys, Kalin et al. (16) observed a decrease in locomotor activity but no change in vocalizations following ICV CRF. Further, Insel and Harbaugh (15) found that ICV CRF inhibited the ultrasonic vocalizing of isolated 5- to 6-day-old rat pups, whereas the CRF antagonist, α-helical CRF (ahCRF), enhanced vocalizing. Recently, we reported that *peripherally* administered CRF inhibited the vocalizing of guinea pig pups during isolation (12). This inhibition could not be produced with exogenous ACTH and was not naloxone reversible, suggesting that it was not mediated by CRF receptors in the pituitary. Although peripheral CRF is not thought to readily cross the blood-brain barrier, we cannot rule out the possibility that the peripherally injected CRF acted directly on the brain to

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produce its behavioral effects, perhaps via the circumventricular organs (26), which lie outside the protection of the blood-brain barrier.

In light of the inconsistent findings for ICV CRF on vocalizations in the young of other species, and the possibility of a direct central effect of peripherally injected CRF in young guinea pigs, the present study examined the effect of ICV CRF and ahCRF on the vocalizations of isolated guinea pig pups. Locomotor activity was also observed because a reduction in locomotor activity was found to accompany the suppression of vocalizations induced by peripheral administration of CRF in our earlier study (12). Because ICV CRF has been reported to reduce body temperature in separated rhesus monkeys (16), and because reduced body temperature is associated with vocalizing in the young of some rodents (25), the rectal temperature of pups was also measured. Finally, since ICV CRF has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis in other species (8,16) plasma cortisol was monitored following CRF administration for comparison with behavioral results.

EXPERIMENT 1

In this experiment, a freehand injection procedure was used to administer either 0.05, 0.5, or 5 μg CRF to guinea pig pups prior to isolation testing.

METHOD

Animals

Albino guinea pigs (*Cavia porcellus*) of the Hartley strain were bred in our laboratory. Each lactating female and her litter were housed in a clear polycarbonate maternity cage (48.3 × 38.1 × 20.3 cm). Water (fortified with ascorbic acid) and guinea pig chow were freely available. Diets were supplemented with alfalfa. The colony room was maintained on a 12L : 12D cycle (light on at 0700h). Pups were 20–23 days old at the time of testing.

Central Injections

Central injections were made under ether anesthesia with the aid of skull landmarks that could be felt under the scalp. Due to the incomplete calcification of the skull at this age, a 21-ga needle was easily inserted through it. The needle was then used as a guide for a 26-ga needle attached to a Hamilton microliter syringe. A needle stop limited penetration of the 26-ga needle to the depth of the lateral ventricle. Then, 5 μl test solution was injected with the Hamilton syringe with 0.5 μl of methylene blue dye as a marker. Injection rate was approximately 11 s/μl, and the entire apparatus was held in place for an additional 30 s following delivery to allow for equilibration. The pup was then returned to the home cage until isolation testing 90 min later. Previous studies have found behavioral and physiological effects of centrally administered CRF to persist for at least several hours [e.g., (17,18)].

Conditions and Test Procedure

Separate groups of pups were assigned to each of five conditions: no injection, vehicle (saline) injection, or injection of 0.05, 0.5, or 5 μg CRF. Pups in the no-injection condition were not disturbed prior to isolation testing. Pups in the other four conditions were injected ICV with either saline or CRF (rat/human, Sigma Chemical Co., St. Louis, MO) in saline. Individual aliquots of each dose were maintained at −80°C and injected within 5 min of thawing.

For isolation testing, the pup was transported (< 10 s) in a carrying cage from the colony room to the test room located in the same laboratory suite as the colony room. Here, the pup was placed into a clean, clear, empty, uncovered polycarbonate cage (47.5 × 23.8 × 20.0 cm) for 30 min. The cage was located on a table under standard fluorescent room lighting. An observer behind one-way glass recorded the number of pup vocalizations ["whistles," (2)] on a hand counter. Lines divided the floor of the test cage into quarters, and the number of line crossings was recorded on a checksheet to assess locomotion. Behavior was scored separately for each of the three 10-min blocks of the 30-min isolation period. Following testing, rectal temperature of the pup was immediately recorded with a microprobe thermometer (BAT-4, Bailey Instruments, Saddlebrook, NJ), after which the pup was lightly anesthetized with ether and decapitated. Trunk blood was collected in a heparinized tube for cortisol analysis. For animals in the four injection conditions, the brain was then removed and the ventricles were examined. Only animals with dye in the ventricles and without damage to underlying brain structures were included in the experiment. Ten pups (five males, five females), each from a different litter, were included in each of the five conditions. No more than two pups from the same litter were tested on the same day. All testing was conducted between 1300–1800h.

Cortisol Determination

Blood samples were centrifuged to separate plasma, which was then frozen until analyzed for cortisol. Duplicate aliquots were assayed using a radioimmunoassay kit ("Coat a Count," Diagnostic Products Corp., Los Angles, CA). Intra- and inter-assay coefficients of variation were calculated at 3 and 7%, respectively.

Data Analysis

Similar patterns of effects were observed for males and females. Therefore, for ease of presentation, gender is not included as a variable here. Because of violations of assumptions for parametric analyses, behavioral data were analyzed with nonparametric tests. These tests were performed on data from the entire 30-min test. For the vocalization measure, this was done because similar patterns of results occurred in each time block; for line crossings, summing scores reduced the large number of scores of zero in individual time blocks. The Kruskal–Wallis analysis of variance (ANOVA) by ranks was used to examine overall group differences. Posthoc analysis was performed with the Mann–Whitney *U*-test. Body temperature and cortisol data were analyzed with parametric ANOVA and the Newman–Keuls posthoc test.

RESULTS

The number of vocalizations emitted by pups varied across conditions, $H = 18.05$, $p < 0.005$ (Fig. 1). The 5-μg dose of CRF reduced vocalizing relative to the saline comparison group ($p < 0.02$). Pups receiving 5 μg CRF also vocalized less than did pups in the 0.5 μg CRF condition ($p < 0.05$). In addition, the anesthesia and/or injection procedure itself greatly reduced vocalizing, as can be seen in the higher rate of vocalizing in the no-injection condition than in the saline condition ($p < 0.001$). Noninjected pups also emitted more vocalizations than did pups receiving either 0.5 μg ($p < 0.001$)

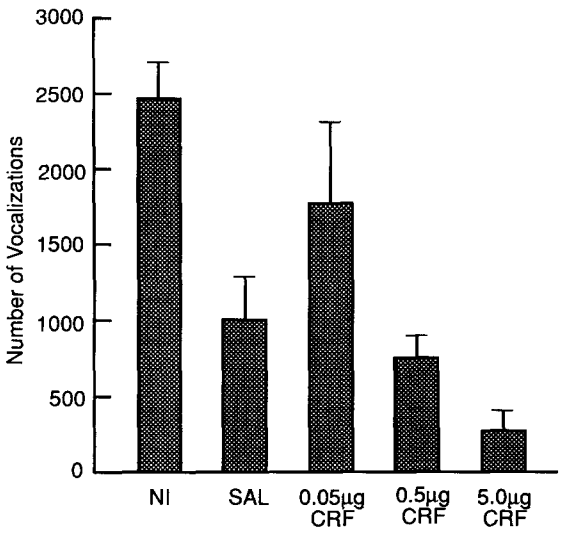

FIG. 1. Mean number of vocalizations emitted by pups during 30 min of isolation following either no injection (NI) or injection of either saline (SAL) or CRF in Experiment 1. Vertical lines indicate SEMs.

or 5 μg ($p = 0.005$) CRF. Scores in the 0.05-μg CRF group displayed considerable variability. The presence of clusters of both very high and very low scores accounts for the lack of significant difference between this condition and any other condition in the experiment. No difference across conditions was found for the number of line crossings. Locomotor activity was low overall, particularly among injected pups, that is, 2 of 10 noninjected, 6 of 10 saline-injected, and 17 of 30 CRF-injected pups had locomotion scores of zero for the entire 30-min test.

Plasma cortisol levels differed significantly across conditions, $F = 5.11$, $p < 0.01$ (Fig. 2). The levels in the no-injection condition were similar to levels previously observed in untreated pups following 30 min of separation [e.g., (13)]. Significantly higher levels were seen in pups receiving 0.05 μg ($p < 0.05$), 0.5 μg ($p < 0.01$), and 5 μg ($p < 0.01$) CRF. Rectal temperature following testing did not differ across conditions ($\bar{X}$s = 35.5 ± 0.4°C–36.2 ± 0.4°C).

EXPERIMENT 2

In Experiment 1, 5 μg CRF reduced the vocalizing of isolated guinea pig pups. However, the anesthesia and/or injection procedure also had an impact on outcome measures, as seen in the 58% decrease in vocalizations and 55% increase in plasma cortisol levels of saline-injected pups as compared to noninjected controls. Moreover, most saline-injected pups exhibited no line crossings, which may have created a floor effect precluding detection of a CRF influence on this response measure. To reexamine the effects of CRF under conditions in which disturbance at the time of testing was minimized, CRF was administered via indwelling cannula in Experiment 2. Two doses of CRF were given: 5 μg, which suppressed vocalizing in the first experiment; and 0.05 μg, which had variable, and therefore ambiguous, effects on vocalizing in Experiment 1.

METHOD

Animals and Surgical Procedures

Animals were bred and maintained as described for Experiment 1. At either 15 or 16 days of age, a 26-ga cannula (Plastics One, Inc., Roanoke, VA) was surgically implanted, aimed 0.5 mm above the left lateral ventricle [AP-5.0 mm (bregma), L + 3.0 mm, DV-2.5mm], using stereotaxic procedures under sodium pentobarbital anesthesia. The cannula was secured to the skull with three small stainless steel screws and acrylic. A threaded obturator was inserted into the cannula and then replaced daily. All pups gained weight, and interactions with the mother in the home cage appeared normal.

Injection and Test Procedures

Pups were assigned to either the 0.05- or 5-μg CRF test condition. Each animal received two injections, one of the appropriate dose of CRF and the other of saline vehicle, in a counterbalanced order. The first injection was made 5–7 days following cannula implantation (i.e., 20–23 days of age), and the second was made 4 days later. For each pup, the two injections were made at the same approximate time of day (5-h range).

Injections were made with a Hamilton microliter syringe. A length of flexible tubing connected the syringe to the needle to give the pup freedom of movement during injection. The needle was cut so that it extended beyond the end of the cannula and into the lateral ventricle. Infusions (5 μl volume) were made at a rate of approximately 18 s/μl and the needle was left in place for an additional 30 s. During infusion, the pup was maintained unrestrained in a 30.5 × 17.8 × 12.7 cm polycarbonate cage. There was no obvious behavioral reaction

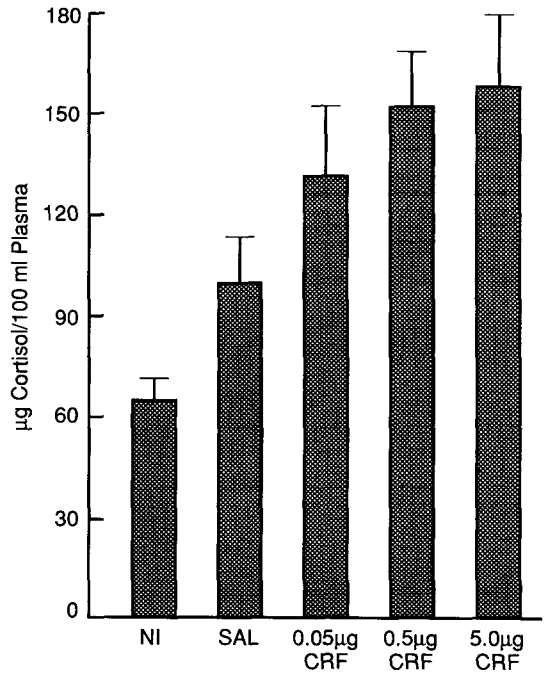

FIG. 2. Mean plasma cortisol levels of pups after 30 min of isolation following either no injection (NI) or injection of either saline (SAL) or CRF in Experiment 1. Vertical lines indicate SEMs.

to the infusion. After infusion, the animal was returned to its home cage. Because animals did not need to recover from anesthesia in Experiment 2, the interval from injection to testing was reduced from the 90 min used in Experiment 1 to 60 min to be consistent with our earlier work with peripherally injected CRF (12).

During isolation testing, behavior was scored as in Experiment 1. At the conclusion of the test, rectal temperature was taken, and immediately following each pup's second test, methylene blue dye (3 μl) was infused using the same procedures as for injection of CRF or saline. The pup was then lightly anesthetized with ether, decapitated, and trunk blood collected for cortisol analysis. The brain was removed and the ventricles inspected to verify cannula placement using the same criteria as in Experiment 1. In all, 9 pups were included in the 0.05-μg CRF group (6 males and 3 females from 7 litters) and 10 in the 5-μg CRF group (5 males and 5 females from 9 litters). All other methodological details concerning testing and cortisol analysis were as for Experiment 1.

Data Analysis

Patterns of results for males and females were similar. Vocalizations were analyzed with two (drug:CRF vs. saline) × three (time block) repeated-measures ANOVAs. For line crossings, data were summed across the three 10-min blocks to reduce the number of scores of zero. For this measure, as well as for cortisol and rectal temperature, comparisons between CRF and saline were made with *t*-tests for dependent measures.

RESULTS

For vocalizations, ANOVAs revealed significant effects only for time block [0.05 μg: $F(2, 16) = 3.62$, $p < 0.05$; 5 μg: $F(2, 18) = 7.79$, $p < 0.005$], reflecting a reduction in vocalizing over the course of the isolation test. The tendency seen in Fig. 3 for CRF to reduce vocalizing was not statistically reliable ($p > 0.10$ for all main and interaction effects involving the factor of drug). The number of line crossings was unaffected by either dose of CRF (Table 1) and similar to the number of crossings ($\bar{X} = 23.0$) made by noninjected pups in Experiment 1.

Mean plasma cortisol levels of pups following injection of saline and of the low dose of CRF were virtually identical (Fig. 4) and comparable to those of the noninjected pups of Experiment 1. Following administration of the large dose of CRF (5 μg), a 76% increase in cortisol levels was observed, although this elevation only approached significance, $t(8) = 1.81$, $p = 0.105$, with the small sample sizes ($n = 5$) available to assess cortisol differences here (i.e., trunk blood was collected only after each animal's second test). Rectal temperature was not affected by CRF (Table 1).

EXPERIMENT 3

In the first two experiments, only the highest dose of exogenous CRF was found to affect vocalizing and this influence was observed only with the technique that involved the most disturbance prior to isolation. However, all animals, including controls, probably experienced considerable endogenous CRF release in response to isolation as judged by the marked elevations of plasma ACTH and cortisol levels this isolation procedure produces (13,14). To assess the effect of this endogenous CRF release on pup vocalizations, ahCRF was administered prior to isolation testing with the indwelling cannula technique in Experiment 3. This antagonist appears to have little or no intrinsic effect in rodents [e.g., (6,23)], so any effect of ahCRF demonstrated here might be attributed to a reversal of the influence of endogenous CRF. The dose chosen (25 μg) was based upon work with adult rats of the same approximate size as the guinea pig pups tested here [~200–300 g; (27–29)].

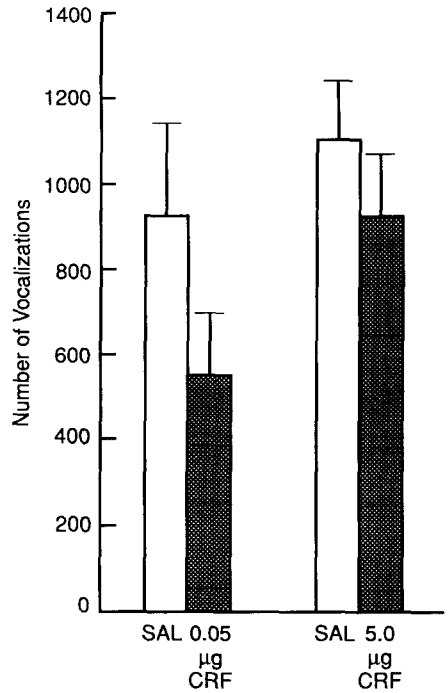

FIG. 3. Mean number of vocalizations emitted by pups during 30 min of isolation following infusion through an indwelling cannula of either saline (SAL) or CRF in Experiment 2. Vertical lines indicate SEMs.

METHOD

ahCRF (Sigma) and vehicle were injected via an indwelling cannula using the same methods as in Experiment 2 except distilled water (pH = 6.6) was used as a vehicle to promote solubility and plasma was not assayed for cortisol. Eight pups (five males and three females from six litters) were included in the study. Behavioral data were analyzed with two-way ANOVA, followed by tests for simple main effects. Rectal temperature was analyzed with a *t*-test.

RESULTS

ANOVA of vocalizations yielded a significant effect for time block, $F(2, 14) = 8.51$, $p < 0.005$, and a significant drug × time block interaction, $F(2, 14) = 4.78$, $p < 0.05$. The interaction was due to ahCRF increasing the number of vocalizations emitted by pups during the first 10 min of the isolation test ($p < 0.01$, Fig. 5, top).

For line crossings, there was a marginally significant effect for time block, $F(2, 14) = 3.65$, $p = 0.052$, and a significant drug × time block interaction, $F(2, 14) = 8.41$, $p < 0.005$. The interaction was due to ahCRF increasing the locomotor activity of pups during the last 10 min of the isolation test ($p < 0.01$, Fig. 5, bottom). Rectal temperature following ahCRF ($\bar{X} = 36.1 \pm 0.3$°C) and vehicle ($\bar{X} = 36.5 \pm 0.3$°C) did not differ.

TABLE 1

MEAN (±SE) NUMBER OF LINE CROSSINGS AND RECTAL TEMPERATURE OF PUPS IN THE TWO CONDITIONS OF EXPERIMENT 2 FOLLOWING INFUSION OF EITHER SALINE OR CRF THROUGH AN INDWELLING CANNULA

	Condition			
	0.05 μg CRF		5 μg CRF	
Measure	Saline	CRF	Saline	CRF
Number of line crossings	25.3 ± 7.2	24.3 ± 10.8	19.5 ± 8.8	19.6 ± 8.1
Rectal temperature (°C)	36.9 ± 0.4	36.7 ± 0.4	36.4 ± 0.4	36.8 ± 0.4

GENERAL DISCUSSION

ahCRF increased vocalizing during the first 10 min of the isolation test and increased locomotor activity during the last 10 min. These results indicate that *endogenous* CRF suppresses these two behaviors during isolation, although the timing of the influence varies with the behavior. Inspection of Fig. 5 suggests that high levels of both behaviors are moderated by endogenous CRF, that is, it appears that the timing of CRF's effect is determined by the period during isolation that the particular behavior would occur most frequently in the absence of endogenous CRF.

Five micrograms of exogenous CRF suppressed vocalizing relative to saline-injected controls when CRF was administered with the freehand technique of Experiment 1 but not when it was given via the indwelling cannula of Experiment 2. It is likely that the anesthesia and possibly the insertion of the needle guide in Experiment 1 induced substantial endogenous CRF release prior to isolation. This is suggested by the finding of 55% higher plasma cortisol concentrations in saline-injected as compared to noninjected pups in Experiment 1. It may be that the 5-μg dose of CRF was sufficient to suppress vocalizing only when combined with this additional endogenous CRF release prior to isolation. It is also possible that the anesthesia and/or injection procedure of Experiment 1 activated other neural or endocrine systems that interacted

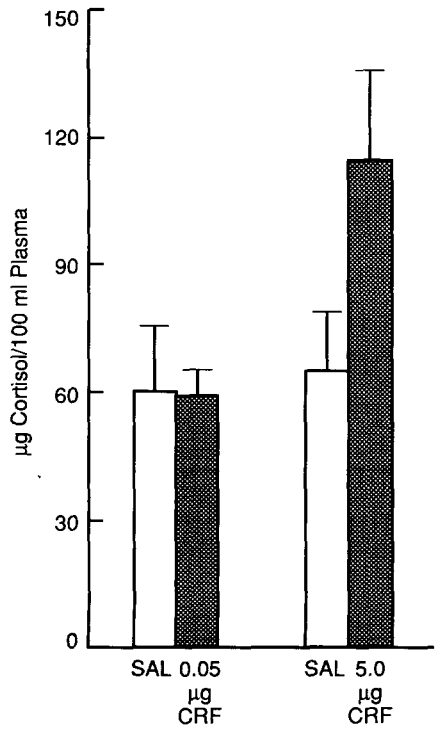

FIG. 4. Mean plasma cortisol levels of pups after 30 min of isolation following infusion through an indwelling cannula of either saline (SAL) or CRF in Experiment 2. Vertical lines indicate SEMs.

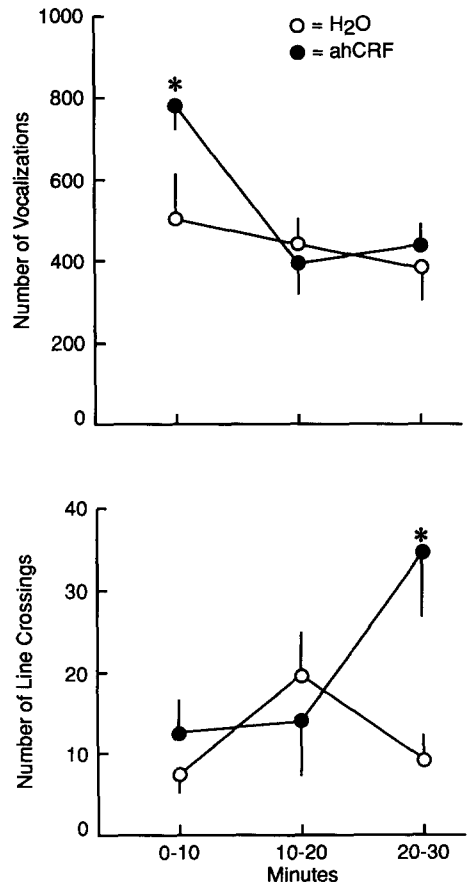

FIG. 5. Mean number of vocalizations (top) and line crossings (bottom) of pups in three 10-min blocks of the isolation test following infusion through an indwelling cannula of either water or ahCRF in Experiment 3. Vertical lines indicate SEMs. *$p < 0.01$ compared to water control.

with the 5 μg exogenous CRF to suppress vocalizing. Whatever the reason for the difference in the effect of the 5-μg dose in the two experiments, the results point out the potential impact of the injection technique employed in studies of ICV CRF administration.

In Experiment 1, we also found that the anesthesia and/or injection procedure had a suppressive effect on vocalizing in the absence of exogenous CRF, as seen in the comparison of noninjected and saline-injected pups. This effect might seem most easily attributable to the anesthesia or trauma induced by the injection procedure, but the results of Experiment 2 suggest otherwise. Although no anesthesia was required in Experiment 2, and pups were infused through an indwelling cannula while unrestrained, the number of vocalizations emitted following saline injection in this experiment was similar to that seen in saline-injected pups in Experiment 1 and much lower than that observed in noninjected controls in the first experiment (see Figs. 1 and 3). Perhaps, just the handling and several minutes of isolation required for injection in each of the experiments was sufficient to inhibit vocalizing during the isolation test 60–90 min later. Alternatively, injection of vehicle may have had an inhibitory effect on the vocalizing of pups.

In light of the prevailing view that CRF acts as a mediator of stress-related behavior (5,10), it is of interest that we found no evidence that CRF enhanced vocalizing during separation. Of course, we cannot be certain that some other dose of CRF or its antagonist could not have yielded such evidence. Yet, across conditions ranging from the 0.05-μg dose of CRF in Experiment 2, which was insufficient to produce any discernable change in cortisol levels, to the 5-μg dose of Experiment 1, which produced the highest mean plasma cortisol levels we have yet observed in guinea pigs, either no change in vocalizations or a suppression of vocalizing was observed during isolation. Moreover, administration of the CRF antagonist enhanced, rather than reduced, vocalizing. In adult animals, central CRF repeatedly has been found to elicit or enhance a variety of behaviors associated with stress or heightened arousal and its antagonist to diminish the occurrence of these behaviors (10). However, studies in young animals of the most prominent behavioral response during social isolation, vocalizing, have yielded very different results. In the mammalian species that have been examined to date—rhesus monkeys (16), Norway rats (15), and now guinea pigs—ICV CRF has been found to be without effect or to actually suppress the vocalizing of preweaning subjects during isolation; further, ICV ahCRF has been observed to stimulate the vocalizing of isolated rat (15) and guinea pig pups. Only in chicks has ICV CRF been observed to enhance vocalizing in an isolation paradigm (20).

One might interpret these findings as indicating that CRF's role in mediating behavioral responses to stressors does not develop in some mammalian species until about the time of weaning. However, it is probably an error to regard the vocalizations emitted by young animals during isolation simply as "stress responses." The complexity of factors that can affect these vocalizations is illustrated by recent evidence suggesting that, in many cases, ultrasonic vocalizations emitted by isolated rat pups are produced as a result of a respiratory mechanism serving to enhance oxygen uptake (3). Further, it is relevant that "freezing," that is, behavioral inhibition, often is seen in stressful conditions, such as following electric shock in adult rats (4), and ICV CRF can potentiate freezing (24). Therefore, the inhibition of vocalizing by pups receiving the highest dose of exogenous CRF in Experiment 1, and the suppressive effect of endogenous CRF on vocalizing and locomotion evident in Experiment 3, may represent CRF's mediation of stress-related behavior in the present study.

Previously, peripherally administered CRF was demonstrated to greatly suppress the vocalizing of isolated guinea pig pups (12). This was among the first behavioral effects of peripheral CRF to be reported. The similarity in the direction of the central CRF effects observed here is consistent with the possibility that the peripherally injected CRF acted at a central receptor. Although peripheral peptides do not generally pass the blood–brain barrier readily, CRF could potentially enter the brain at a circumventricular organ that is devoid of a blood–brain barrier. Further, although the guinea pig is a precocial species it is still possible that the blood–brain barrier is not fully mature in guinea pig pups at the ages tested in the present study. On the other hand, the difficulty in demonstrating a suppressive effect of ICV CRF on vocalizing here, and the finding that the ICV dose required to suppress vocalizing in Experiment 1 was almost as great as the peripheral dose required in our earlier study, suggest that the peripherally administered CRF did not act at a central receptor. Furthermore, since peptides may pass the blood–brain barrier from brain to periphery much more rapidly than vice versa (21) it is conceivable that some portion of the effects of CRF and ahCRF seen here were due to action at peripheral CRF receptors, such as those known to be located in the adrenal medulla (1). Additional research will be required to sort out these possibilities.

In summary, the present study provides evidence for a suppressive action of CRF on the behavior of isolated guinea pig pups. Inhibition of vocalizations and locomotor activity appear to be mediated by endogenous CRF, and under certain conditions, exogenous ICV CRF administration can further suppress vocalizing.

ACKNOWLEDGEMENTS

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