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Effects of infusion of corticotropin-releasing factor antagonist into the locus coeruleus on freezing behavior and brain catecholamines in rats

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Abstract. Administration of 0.2 μg of alpha-helical CRF_{9-41} (corticotropin-releasing factor receptor antagonist, $ahCRF_{9-41}$) into the locus coeruleus (LC) region significantly reduced footshock-induced freezing behavior in adult male rats. Changes in the concentrations of noradrenaline (NA), dopamine and their catabolites in cortex, hippocampus and hypothalamus of footshocked rats were reminiscent of those observed in stressed animals. Rats injected with ahCRF prior to footshock displayed cerebral catecholamine responses that were not different from controls injected with vehicle. The results confirm earlier findings that CRF receptors at the LC region may mediate freezing and behavioral expression of fear. However, the results also suggest that though CRF receptors within the LC region mediate footshock-induced behavior, they are not necessarily involved in the short-term catecholamine response to footshock.

Key words: corticotropin-releasing factor antagonist, locus coeruleus, freezing behavior, noradrenaline, stress, rats

INTRODUCTION

Hypothalamic corticotropin releasing factor (CRF) plays a crucial role in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. CRF is also widely distributed throughout the whole brain (Imaki et al. 1991) and available evidence suggest that extrahypothalamic CRF may serve as a neurotransmitter modulating autonomic and behavioral responses in stress (Dunn and Berridge 1990). Several experiments demonstrated that intracerebroventricular (i.c.v.) administration of CRF and its antagonist, alpha-helical CRF_{9-41}, modified expression of stress-induced behavioral patterns (Berridge and Dunn 1987, Butler et al. 1990, Kalin et al. 1988, Sherman and Kalin 1988, Swerdlow et al. 1986, Takahashi et al. 1989). Also, it was found that i.c.v. infusions of CRF changed catecholamine turnover in several forebrain areas (Dunn and Berridge 1987, Matsuzaki et al. 1989). These effects resembled those observed with a variety of stressors such as footshock or restraint and suggested that behavioral effects of CRF could be produced by interaction of CRF system with cerebral catecholamines (Dunn 1988, Dunn and Berridge 1987, Emoto et al. 1993). The largest brain cluster of almost exclusively noradrenergic neurons that has been implicated in stress, fear and arousal responses (Foote et al. 1983) is locus coeruleus (LC). It has been established that stress increases noradrenaline turnover in these brain structures whose sole source of NA arises from the LC (Cassens et al. 1980). It has also been found that the LC region contains CRF binding sites and displays an increase in CRF-like immunoreactivity in stress (Chappell et al. 1986, De Souza 1987, Imaki et al. 1991). Experiments by Valentino and co-workers demonstrated that CRF increased, whereas its antagonist decreased rate of discharge of the LC noradrenergic neurons (Valentino et al. 1983, Valentino et al. 1991). According to Korf et al. (1973) altered firing frequency of the LC neurons could lead to changes in CA turnover in structures receiving impulses from the LC. Indeed, administration of CRF or its antagonist into the LC seems to affect turnover of catecholamines in brain areas that receive projections from the locus coeruleus (Butler et al. 1990, Palamarchouk et al. 2000, 2002). Studies with administration of the CRF antagonist into the locus coeruleus region demonstrated a reduction in footshock-induced freezing behavior in rats (Świergiel et al. 1992).

The aim of the present experiment was simultaneous examination of effects of antagonism of a CRF system within the locus coeruleus region on footshock-induced behavioral, catecholamine and HPA axis responses.

METHODS

Animals

Three-month-old rats male Harlan Sprague-Dawley were purchased from Harlan Sprague Dawley, Madison, WI. Rats were housed four per cage (60 x 45 x 19 cm) with free access to water and Teklad Diets, Madison, WI, standard rat chow.

Apparatus

The shock box (45 x 20 x 20 cm) was constructed from Plexiglas front, rear and cover and aluminum side walls. Scrambled shock from a constant current shock generator was delivered *via* the metal rods floor. The shock box was housed in a larger enclosure with observation window made from a one-way mirror. A small fan and a 10-W light bulb were positioned on the side wall of the larger enclosure.

Procedure

LOCUS COERULEUS CANNULATION AND INFUSION

Rats weighing 300-350 g were anesthetized with sodium pentobarbital and implanted bilaterally with 22-gauge guide cannulae aimed at the locus coeruleus. The following coordinates were applied to a skull leveled between bregma and lambda: AP = -1.2 mm from the interaural line, ML = ± 1.2 mm and VD = -6.8 mm from the skull surface. The animals were allowed to recover from surgery for a minimum of 7 days. Bilateral infusions were made with 28 gauge injectors projecting 1.0 mm beyond the tips of guide cannulae. CRF receptor antagonist, alpha-helical CRF_{9-41} (Peninsula Lab. Inc., Belmont, CA) was dissolved in physiological saline (0.1 µg/1 µl), adjusted to pH 6.7 and 1 µl per cannula was infused into the LC over a 2-min period. Each animal thus received a total dose of 0.2 µg of the CRF antagonist. Injectors were left in place for additional 60 s. Immediately after removal of the injectors rats were tested in a shock box. Control animals received infusions of vehicle.

EXPERIMENTAL DESIGN AND BEHAVIORAL TESTING

Four groups of animals were used in the experiment. Two groups received infusions of either vehicle or the CRF antagonist into the LC prior to footshock (vehicle-footshock and CRF antagonist-footshock groups). The animals were individually placed in the shock box and allowed to habituate for 120 s. Following habituation, 3 footshocks (1 mA, 1 s duration) were delivered at 20 s intervals. Immediately after the last footshock, rats were observed for 15 min. The total duration of freezing, grooming behavior and number of ultrasonic vocalizations were recorded. Freezing was defined as an immobile stationary posture, with cessation of all skeletal and vibrissae movements except during respiration. Ultrasounds emitted by rats were converted into the human audible range using a bat detector tuned to a frequency range of 28 kHz. The remaining two groups received the infusions, were placed in the shock box but did not receive footshocks (vehicle-quiet and antagonist-quiet groups). The shock box was washed after each rat.

COLLECTION OF TISSUES AND CATECHOLAMINES AND ACTH ASSAYS

Rats were decapitated immediately after behavioral testing. Trunk blood was collected in tubes containing 4 mg of EDTA and centrifuged at 4°C for ACTH radioimmunoassay. The brains were removed from the skull and placed on ice-cold glass. The brain stem containing the locus coeruleus region was separated and placed in 10% formalin. Cortex, hippocampus and hypothalamus were dissected similarly to the method of Glowinski and Iversen (1966). The dissected tissues were frozen on dry ice, weighed, and stored at -70°C for later catecholamine assays. Supernatants of homogenized tissues were injected directly into the high performance liquid chromatography system employing electrochemical detection (HPLC-EC). The rate of recovery of the system determined by the addition of an internal standard - 3,4-dihydroxybenzylamine approached 90%. Amounts of noradrenaline (NA), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), dopamine (DA), and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined using standard calibration curves based on pure catecholamines: NA and DA provided by ESA Inc., MHPG by Calbiochem, DHPG by Aldrich Chemical Co., and DOPAC from Sigma Corp. Amounts of catecholamines are expressed in picograms (pg) per mg of fresh tissue.

PLASMA ACTH ASSAY

ACTH concentration was measured in plasma by a radioimmunoassay. Specific antibody raised against ACTH was supplied by IgG Corp., Nashville, TN, and the radioactive tracer by Nichols Institute Diagnostics, San Juan Capistrano, CA. The assay was calibrated using ACTH obtained from Penisula Labs. Inc., Belmont, CA, as the standard. The sensitivity of the ACTH assay was 0.3 pg/tube, and the intra-assay coefficient of variation was 6.0%.

HISTOLOGY

Brain coronal sections 50 μm thick were cut on a freezing microtome and stained with thionine. Placements of the infusion cannulae tips were determined and marked on the plates from rat brain stereotaxic atlas (Paxinos and Watson 1986) by two independent examiners unaware of the animals' treatment. Data were analyzed only from rats in which cannula tips were located either entirely within the locus coeruleus or along the medial region of the LC adjacent to the Barrington's nucleus. Infusion sites not considered to be within the LC region were located in the parabrachial nuclei, laterodorsal tegmental area, the region of the central gray pons, and deep below the locus subcoeruleus. The histological examination revealed 11, 10, 9 and 14 animals that had cannulae placed within the LC in the vehicle-quiet, CRF antagonist-quiet, vehicle-footshock, and CRF antagonist-footshock group, respectively.

DATA ANALYSIS

Results from animals with injection sites within the locus coeruleus were analyzed using repeated measures analysis of variance (ANOVA) and unpaired *t*-test for *a priori* designed pair wise comparisons.

All procedures were approved by the Ethics Committee of the University of Wisconsin, Madison, WI and are in accordance with the standards of National Institute of Health guidelines for the treatment of laboratory animals.

RESULTS

Plasma ACTH

Plasma concentrations of ACTH (Fig.1) were significantly higher ($F_{3,40}$=12, P<0.01) in the animals subjected to footshock than in the quiet groups. No significant difference occurred between the vehicle--footshock and the antagonist-footshock groups.

Behavior

Repeated measures ANOVA revealed that the CRF antagonist significantly ($F_{1,40}$=9.04, P<0.01) decreased freezing (total time - vehicle: 802 ± 35 s compared to antagonist: 510 ± 61 s). In addition, effect of time was found: duration of freezing was significantly ($F_{1,40}$=6.82, P<0.05) decreasing with elapsing of time since the CRF antagonist infusion. There was no significant interaction between the drug treatment and time that elapsed since delivering the footshocks (Fig. 2). Footshock evoked ultrasonic vocalization and grooming but neither vocalization nor grooming behavior were affected by the infusion of the CRF antagonist into the LC.

Noradrenergic system. Cortex, hippocampus and hypothalamus

Footshock increased DHPG/NA and MHPG/NA ratios in all examined regions. There was no statistically significant ($F_{3,40}$=1.24, P<0.05) effect of administration of the CRF antagonist on footshock-induced changes in NA, DHPG, MHPG concentrations and DHPG/NA and MHPG/NA ratios in the cortical tissue. The level of unconjugated MHPG in hypothalamus was below the detection level of the HPLC system (Table I).

Dopaminergic system

There were no significant differences between the groups in concentration of DA. Footshock increased concentration of DOPAC and the DOPAC/DA ratios in cortical, hippocampal or hypothalamic tissues. These responses were not affected by administration of the CRF antagonist (data not shown).

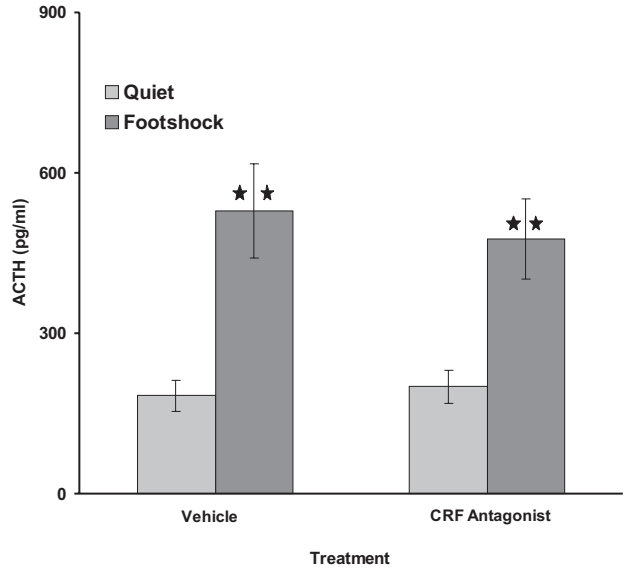

Fig. 1. Effect of footshock and infusion of alpha-helical CRF_{9-41} into locus coeruleus on concentration of ACTH (pg/ml) in blood plasma. **, Significantly different from quiet groups, P<0.01. Values are means ± SEM.

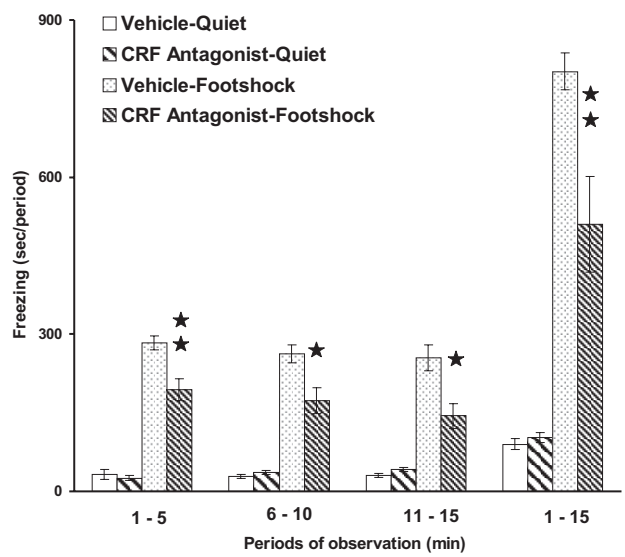

Fig. 2. Effect of alpha-helical CRF_{9-41} on the duration of freezing behavior induced by electric footshock. (Two-way ANOVA: effect of antagonist: P<0.01; effect of time: P<0.05). *; **, Significantly different from the vehicle group, P<0.05; 0.01, respectively. Values are means ± SEM.

DISCUSSION

We studied the effects of administration of the alpha-helical CRF_{9-41} antagonist into the locus coeruleus on stress-induced behavior and activity of neurotransmitter systems possibly involved in mediating behavioral responses. The results implicate extrahypothalamic CRF and its receptors in behavioral responses in stress.

Table I

Effect of footshock and infusion of alpha-helical CRF_{9-41} into locus coeruleus on NA, MHPG and DHPG concentrations (pg/mg tissue), and the ratios of MHPG/NA and DHPG/NA in cortex, hippocampus and hypothalamus. Values are means ± SEM

Brain region	Quiet and vehicle	Quiet and CRF antagonist	Footshock and vehicle	Footshock and CRF antagonist
Cortex				
NA	300 ± 16	315 ± 22	290 ± 14	295 ± 16
MHPG	4.7 ± 1.3	5.1 ± 0.3	11.2 ± 0.9*	14.6 ± 1.2*
MHPG/NA	0.016 ± 0.006	0.016 ± 0.007	0.039 ± 0.002*	0.048 ± 0.004*
Hippocampus				
NA	390 ± 26	415 ± 33	360 ± 21	320 ± 13*
MHPG	8.6 ± 1.4	10.3 ± 1.1	12.8 ± 1.3	15.9 ± 1.1
MHPG/NA	0.022 ± 0.003	0.025 ± 0.002	0.035 ± 0.002	0.050 ± 0.003*
Hypothalamus				
NA	2,420 ± 79	2,120 ± 35	2,173 ± 68*	2,224 ± 50*
DHPG	35 ± 3	27 ± 8	60 ± 4*	66 ± 5*
DHPG/NA	0.014 ± 0.001	0.013 ± 0.001	0.028 ± 0.002*	0.030 ± 0.002*

*, Significantly different from the respective quiet group, $P<0.05$.

The behavioral effects of the antagonist were similar to those observed in our previous study (Świergiel et al. 1992). A low dose of the CRF antagonist infused into the LC region reliably decreased freezing, apparently reversing stress-induced response. The decrease in duration of defensive freezing suggests that antagonism of CRF receptors in the LC or surrounding structures mitigate effects of stress on behavior, possibly by affecting neuronal activity of NA cells in the LC of stressed rats (Curtis et al. 1993).

Freezing is a species-specific defensive behavioral response triggered by a threatening environmental stimulus (Fanselow 1986). Stress-induced freezing behavior in rats has been proved to be a dependable index of stress and fear (Bolles 1970, Bolles and Collier 1976, Bouton and Bolles 1980, Fanselow and Bolles 1979, Świergiel et al. 1992). The behavior, conditions for its occurence and its acquisition are well described for rats, but its underlying neural mechanisms are not well known (Blanchard and Blanchard 1969, Bolles 1970, Bolles and Collier 1976, Bouton and Bolles 1980, Fanselow 1980). Previous results implicated CRF system of the central nervous system (CNS) in mediating freezing displayed both immediately after shock and after re-exposure of animal to the environment associated with shock. Those studies revealed that intracerebroventricular infusions of CRF potentiated freezing, whereas competitive antagonist of CRF, alpha-helical CRF_{9-41}, administered either i.c.v. or into the LC, reduced its occurence (Kalin et al. 1988, Sherman and Kalin 1988, Świergiel et al. 1992, Takahashi et al. 1989). In addition, our recent observations suggest that duration of freezing can be affected by pharmacological alterations of the locus coeruleus noradrenergic system, that is administration of alpha-2 noradrenergic agonist or chemical lesions of the LC projections (Świergiel, unpublished). Taken together with findings that CRF and its antagonist affect rate of discharge of the LC neurons (Takahashi et al. 1990, Valentino et al. 1983, Valentino et al. 1991) and catecholamine activity in the LC terminal fields (Smagin et al. 1994), the results suggest that changes in duration of freezing might be mediated by the LC-noradrenergic system.

Because changes in the activity of CNS noradrenergic system are observed in stress (Dunn 1988, Glavin 1985) we hypothesized that the CRF antagonist would result in differences in noradrenergic turnover between the footshocked, vehicle treated animals and the

footshocked, antagonist treated ones. As it turned out, there were no differences between the groups. The results of footshock resemble those caused by stressful conditions, i.c.v. infusions of CRF, or electrical stimulation of the LC (Dunn 1988, Dunn and Berridge 1987, Korf et al. 1973) and are contrary to what might be expected of the effects of the CRF antagonist. CRF receptors in LC can be considered to be excitatory to the activity of LC noradrenergic neurons (Valentino et al. 1983, Valentino et al. 1991). Therefore, administration of the CRF antagonist should result in less activity of the LC (Valentino et al. 1991) and, as a consequence, a decrease in CA turnover in projection areas of the LC. The pattern of changes and the affected brain structures suggest that the dorsal NA pathway from the LC might be involved, though of course, a possibility that the footshock activated cortex, hippocampus and hypothalamus independently of each other, can not be excluded.

A possible explanation for our findings is as follows. Small amounts of the CRF antagonist infused directly into the LC could selectively affect stress- or extrahypothalamic CRF-modified discharge of the LC neurons. Such a response might have been quite sufficient to attenuate or even reverse stress-induced behavior but not necessarily sufficient to affect catecholaminergic turnover in brain areas that we studied. It is well recognized that behavioral patterns are very sensitive to any pharmacological manipulations and usually much lower doses of drugs are required to affect behavior than to elicit well defined autonomic or neurochemical changes. It has been observed previously that considerably higher doses of CRF were needed to affect catecholamine turnover in brain than to modify behavior (Butler et al. 1990, Dunn and Berridge 1987, Matsuzaki et al. 1989). Also, stress exposure increases catecholaminergic activity in many brain regions and whether it is only locus coeruleus that mediates such responses is not known. Moreover, since it is known that i.c.v. infusions of CRF do activate noradrenergic and dopaminergic systems (Butler et al. 1990, Dunn and Berridge 1987) and since stress increases the amount of CRF in brain (Chappell et al. 1986), it can be reasonably expected that direct effect of endogenous cerebral CRF on neurons could overwhelm much weaker inhibitory input from the LC.

Another possibility is, of course, that CRF system of the LC region is involved in modulating short-term noradrenergic responses in stress. Recent studies with mice lacking the gene for CRF (CRF knockout mice) suggest that CRF and its receptors are not involved in the catecholamine responses to a brief period of footshock (Dunn 2000) (and see Borsody and Weiss 1995, Weiss et al. 1994).

Finally, it can not be excluded that the noradrenergic systems of brain areas considered in the presented study are not involved in modulating footshock-induced freezing behavior. Although both cortex and hippocampus are implicated in arousal, fear and contextual freezing, and hypothalamus in autonomic and endocrine responses, other structures, amygdala in particular, could be involved in freezing elicited by a direct physical stimulus (Świergiel et al. 1993).

CONCLUSIONS

Thee present behavioral and neurochemical observations support the idea that the CRF system of the locus coeruleus region play a role of a modulator of stress--evoked behavior.

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