The Impact of the Nonpeptide Corticotropin-Releasing Hormone Antagonist Antalarmin on Behavioral and Endocrine Responses to Stress*

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ABSTRACT

The nonpeptide CRH antagonist antalarmin has been shown to block both behavioral and endocrine responses to CRH. However, it's potential activity in blunting behavioral and endocrine sequelae of stressor exposure has not been assessed. Because antagonism of central CRH by α-helical CRH attenuates conditioned fear responses, we sought to test antalarmin in this regard. In addition, it remains unclear as to whether this is a result of receptor blockade during conditioning or during testing. Thus, we explored whether CRH mediates the induction or expression of conditioned fear (freezing in a context previously associated with 2 footshocks; 1.0 mA, 5 sec each).

Furthermore, because rats previously exposed to inescapable shock (IS; 100 shocks, 1.6 mA, 5 sec each), demonstrate enhanced fear conditioning, we investigated whether this effect would be blocked by antalarmin. Antalarmin (20 mg/kg·2 ml ip) impaired both the induction and expression of conditioned fear. In addition, antalarmin blocked the enhancement of fear conditioning produced by prior exposure to IS. Despite the marked behavioral effects observed in antalarmin-treated rats, antalarmin had no effect on IS-induced rises in ACTH or corticosterone. However, antalarmin did block the ACTH response produced by exposure to 2 footshocks. (*Endocrinology* **140:** 79–86, 1999)

CRH IS A 41-amino acid peptide initially identified as a hypothalamic factor responsible for stimulating ACTH from the anterior pituitary (1). Stressors induce the synthesis and release of CRH from cells of the paraventricular nucleus, into the portal blood, initiating the hypothalamo-pituitary-adrenal (HPA) response to stressors (2–4). CRH is also involved in the mediation of autonomic and behavioral sequelae of exposure to stressors. The intracerebroventricular (icv) administration of CRH produces autonomic activation (5) and many of the same behavioral (6–9), neurochemical (10), and electrophysiological (11) alterations that are produced by stressors. Furthermore, the icv administration of CRH antagonists, such as α-helical CRH$_{9-41}$ and D-Phe CRH$_{12-41}$, can blunt or block these stress-induced alterations in behavior and autonomic activity (12–15). Many of these effects can be obtained by infusing CRH or it's antagonists into nonhypothalamic sites, such as the locus coeruleus and amygdala (16–19), and these effects persist in hypophysectomized and dexamethasone-treated subjects (12). These facts, together with the wide extrahypothalamic distribution of high-affinity CRH receptors (20, 21) and CRH-like immunoreactivity (22–24), suggest that CRH functions as a neu-

rotransmitter, as well as a hormone, and that it mediates stress-related behavioral responses by action at extrahypothalamic sites (25, 26).

The critical role of brain CRH in mediating stress-related phenomena (along with the possible importance of brain CRH) in a number of human disorders, such as depression, posttraumatic stress disorder, and bulimia (27–30), has motivated the recent development of a nonpeptide CRH receptor antagonist capable of readily crossing the blood-brain barrier. The compound, first synthesized by Chen (31), is a pyrrolopyrimidine and has been called CP-154,526 (32) and antalarmin (33). It has high affinity for the CRH receptor, with selectivity for the Type 1 receptor, CRHR-1. The compound, here to be called antalarmin, blocked or blunted the effects of CRH on adenylate cyclase in membranes from rat cortex and pituitary, plasma ACTH increases in response to iv CRH, locus coeruleus electrical activity in response to icv CRH, and the potentiation of the startle reflex by icv CRH (32). In addition, antalarmin blunted the increase in startle produced by a light that had been paired with footshock (32) and a carageenin-induced sc inflammation (33).

The purpose of the present experiments was both to further explore the effects of this nonpeptide CRH antagonist and to investigate the role of CRH in several phenomena. One phenomenon was fear conditioning. The term fear conditioning refers to the fact that both discrete and contextual cues that are present during exposure to a stressor such as footshock come, themselves, to elicit behavioral and physi-

ological responses, such as freezing, inhibited appetitive behavior, potentiated startle, increased autonomic, HPA activity, etc. (34). Brain CRH systems have been shown to be important in mediating the fear responses observed in fear-conditioning experiments. Rats and other organisms freeze when placed in an environment in which they have previously received an aversive stimulus, such as footshock; and freezing has been shown to be a measure of fear conditioned to the environment by the aversive stimulus (35). It is to be noted that freezing is not simply an absence of movement but rather an active defensive response consisting of no movement beyond that required for respiration (including the absence of vibrissae movement), typically accompanied by a hunched posture and muscular rigidity. icv α-helical CRH reduced the freezing that occurred when rats were exposed to the environment in which they had previously received footshock (24 h earlier) (36), as well as the potentiation of startle produced by a light that had previously been paired with shock (15). In these experiments, the CRH antagonist was administered before the behavioral testing, rather than before the fear conditioning (the exposure to shock in the apparatus, the pairing of the light with shock), which occurred 24 h or more earlier. Thus, it is clear that CRH receptor blockade reduces the expression of fear, but it is not known whether it would blunt the conditioning or development of the conditioned fear. One purpose of the present experiments was to determine whether the effect reported by Kalin and Takahashi (36), using α-helical CRH, would be duplicated by antalarmin. An additional goal was to determine whether antalarmin would reduce the development of conditioned fear and its expression.

A second phenomenon explored was learned helplessness. This term refers to the fact that rats and other organisms, exposed to inescapable (uncontrollable) shock (IS), later fail to learn to escape in a different environment in which escape is possible, whereas animals originally exposed to exactly equal amounts and intensities of escapable shock (controllable), later learn normally (37). Exposure to IS also leads to potentiated fear conditioning in a different environment 24 h later (38). The potential involvement of CRH in these phenomena has not been studied, but it is of interest because the IS paradigm and the behavioral sequelae that occur have been argued to constitute an animal model of depression (39).

Finally, the ability of antalarmin to blunt the pituitary-adrenal response to stressors has not been previously explored. Antalarmin has been shown to blunt ACTH and corticosteroid (CORT) responses to CRH administration, but its efficacy, with regard to stressors, is unknown. This was also tested in the present experiments.

Materials and Methods

Subjects

Adult male Sprague Dawley rats (350–400 gms; Harlan Sprague Dawley, Inc., Indianapolis, IN) were individually housed in suspended wire cages (24.5 cm × 19 cm × 17.5 cm) with food and water available *ad libidum*. Colony conditions were maintained at 22 C on a 12-h light, 12-h dark cycle (lights on, 0600–1800 h). Rats were given at least two weeks to habituate to the colonies before experimentation. Care and use of the animals were in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee.

Group size in all experiments was eight rats per group. All testing was performed during the light cycle.

Drug

Antalarmin was dissolved in a sterile, lipid soluble fat emulsion (Liposyn II, Abbott Laboratories , Chicago, IL) and was administered ip (20 mg/kg·2 ml). Two hours before experimentation, rats were injected with antalarmin or equivolume vehicle.

Procedures

Fear conditioning. During fear conditioning, subjects were individually placed in shuttleboxes measuring 46.0 × 20.7 × 20.0 cm. The end walls were aluminum, and the sidewalls and tops were clear Plexiglas. The floor was constructed of stainless steel rods, 0.3 cm in diameter and spaced 1.4 cm apart. An aluminum wall, with a 5.5 × 7.5 cm archway cut out of it, divided the shuttleboxes into two equal compartments. The shuttleboxes were housed within sound- and light-attenuating enclosures equipped with a ventilation fan and a 28-V houselight. The front of each enclosure was left open during behavioral observation. Scrambled shocks were delivered to the grid floors by shockers modeled after the Grason Stadler Model 700. During the fear conditioning session, the subjects were first placed in a 5-min acclimatization period in the shuttleboxes. This was followed by two 5-sec shocks of 1.0 mA in intensity. There was 1 min between the two footshocks. The subjects were removed from the shuttleboxes 30 sec after the last shock.

Testing for the amount of fear conditioned to the cues of the shuttleboxes occurred 24 h later. The rats were placed in the shuttleboxes, and freezing was assessed for 20 min. The experimenter observed each rat, every 8 sec to a signal, and scored it as freezing or not freezing. To be scored as freezing, a rat had to have all four paws on the grids, and there had to be an absence of all movement of the body and vibrissae beyond that required for respiration. The experimenter was always unaware of group membership. Interrater reliability on this measure is greater than .90.

Blood sampling procedure

If blood samples were taken after conditioning or testing, the rat was removed from the apparatus and gently restrained in a towel. If blood samples were to be taken during the IS session, a tail nick was made without disturbing the subject, because it's tail protruded from the rear of the apparatus. A small nick was made in a lateral tail vein with a scalpel (no. 15 blade), and the tail was gently stroked until a volume of approximately 300–400 μl of whole blood was obtained in microfuge tubes precoated with EDTA. All samples taken from animals in their home cages were obtained within 2 min of approaching the cage. Samples were spun in a refrigerated centrifuge immediately, and plasma was aliquoted and stored at −20 C until the time of assay.

IS

ISs were administered while the subjects were restrained in Plexiglas tubes, 17.5 cm long and 7.0 cm in diameter. The rat's tail extended from the rear of the tube and was taped to a plexiglas rod. Shock was administered to the tail through fixed electrodes and was 1.6 mA in intensity. One hundred 5-sec shocks were administered on a variable intertrial interval ranging from 30–90 sec. These are the minimal parameters necessary to produce learned helplessness effects (40).

Fear and escape testing after IS

Twenty-four hours after IS or control treatment, the subjects were placed in shuttleboxes as described above. Freezing was measured for 10 min. The subjects then received 2 escapable footshocks in the shuttleboxes in which each shock could be terminated by the rat's crossing to the other compartment. Shock intensity was 0.8 mA. There was 1 min between the shocks. Freezing was then measured for 20 min, as described above. This was followed by 3 further escape trials requiring a single crossing of the shuttleboxes (FR-1). These trials were then followed by 25 trials that required a back-and-forth crossing of the shuttleboxes (FR-2) to terminate footshock. It is these FR-2 trials that typically

reveal an IS-induced escape deficit (*e.g.* Ref. 41). Shocks terminated automatically after 30 sec if an escape response had not occurred.

Assays

ACTH. Plasma levels of ACTH were determined by RIA, according to the procedure outlined by INCSTAR Corp. (catalog no. 24130; Stillwater, MN).

Corticosterone

Total plasma CORT levels were measured by RIA using rabbit antiserum (antibody B21–42; Endocrine Sciences, Inc., Tarzana, CA). This antiserum has very low cross-reactivity with other glucocorticoids and their metabolites. The assay sensitivity was 1 μg/dl. Interassay and intraassay coefficients of variation for both ACTH and CORT were all less than 9%.

Results

Fear conditioning

On day 1, four groups received two footshocks in the apparatus as described in *Subjects and Methods*. Two received antalarmin, as described in *Subjects and Methods*, 120 min before the conditioning session; and the other two received injection of vehicle. Freezing in the conditioning apparatus was measured 24 h later, as described in *Subjects and Methods*. One of the groups that had received antalarmin before conditioning on day 1 received antalarmin again 120 min before the day-2 test session, whereas the other group that had received antalarmin was administered vehicle. One of the groups that had received vehicle before conditioning on day 1 received antalarmin 120 min before the day-2 test session, whereas the other group that had received vehicle was again administered vehicle. A fifth group did not receive shocks in the apparatus on day 1 and was given vehicle before both days.

The results are shown in Fig. 1. The group that had not received shock on day 1 (No Shock-Veh/Veh) provides a baseline of freezing against which to assess fear conditioning. Clearly, there was virtually no freezing in the apparatus unless shock had been given the previous day. The two footshocks conditioned a large amount of fear to the cues of the apparatus. The maximun freezing score possible is 15 (freezing was observed every 8 sec, and the data are presented in 2-min blocks), and the Shock-Veh-Veh group showed maximal freezing, which extinguished over the 20-min testing period. Antalarmin produced a potent reduction of the conditioning, both when given before the footshock session on day 1 and when administered before the day-2 test. Further, these effects summated, so that the most potent reduction occurred in the group (Shock-Ant/Ant) that had received antalarmin before both conditioning and testing. These conclusions are confirmed by ANOVA [F(4,35) = 17.1293, $P <$ 0.0001].

Escape failure and potentiated fear conditioning after IS

Two groups were given IS on day 1, as described in *Subjects and Methods*, and two remained in their home cages as home cage controls (HCC). Twenty-four hours later, all groups were tested, as described above, for fear conditioning and shuttlebox escape learning. One of the IS groups was given antalarmin, 120 min before both the ISs and 120 min before the day-2 testing. One HCC group received antalarmin on both days, and the other received vehicle on both days.
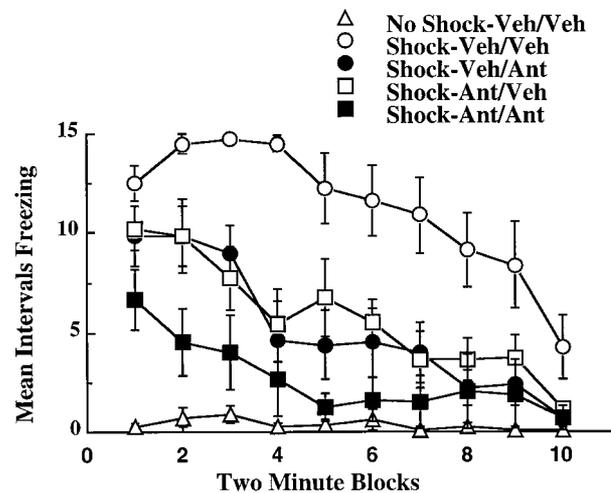

FIG. 1. In Exp 1, rats (Harlan Sprague Dawley, Inc.; n = 8 per group) were injected with either vehicle or antalarmin (20 mg/kg·2 ml ip). Two hours later, rats were placed in the shock chamber for 5 min. Rats then received two footshocks (1.0 mA, 5 sec each) or an equivalent amount of time in the box. Rats were removed from the box 30 sec after the termination of the second foot shock and were returned to their home cages. The next day, rats were once again injected with vehicle or antalarmin and (2 h later) were placed in the chamber for 20 min. Freezing was scored by a blind observer, every 8 sec, and is presented as blocks of 2 min. Virtually no freezing was evident in the No Shock-Veh/Veh controls. Antalarmin, given before shock or before testing, attenuated freezing to the contextual cues and seems to have an additive effect if given both before conditioning and before testing ($P <$ 0.05).

Antalarmin was given before IS and testing to maximize the possibility of detecting an effect.

There was no observable freezing before the shocks in the shuttlebox; and so, data from before the first shock are not shown. Figure 2a shows freezing for the 20-min testing period. There was conditioning of fear in all of the groups. A comparison of the group given prior IS and vehicle (Shock-Veh) with the group that remained in their home cage on day 1 and received vehicle (No Shock-Veh) indicates the typical potentiation of fear conditioning by prior IS. Antalarmin eliminated this potentiation, as well as reducing the overall level of fear conditioning, as in the prior experiment [F(1,27) = 10.459, $P <$ 0.01].

The latencies to escape during escape training are depicted in Fig. 2b. As in previous experiments (42), IS did not alter escape responding when only a single crossing of the shuttlebox was required. However, a large escape deficit appeared on the double-crossing (FR-2) trials [F(1,26) = 46.447, $P <$ 0.0001]. The maximum possible latency was 30 sec, and the 20-sec latencies that occurred were produced by the majority of animals that had received IS failing completely to escape. Antalarmin had no effect whatsoever on either the escape deficit produced by IS or on escape behavior in control subjects [F(4,104) = .510, $P >$ 0.05]. Escape proceeded normally.

CORT after IS

Blood samples were collected, as described in *Subjects and Methods*, from the animals in the above experiment before IS, immediately after the shock session, 60 min later, and 24 h later (before the shuttlebox test). Plasma CORT levels are
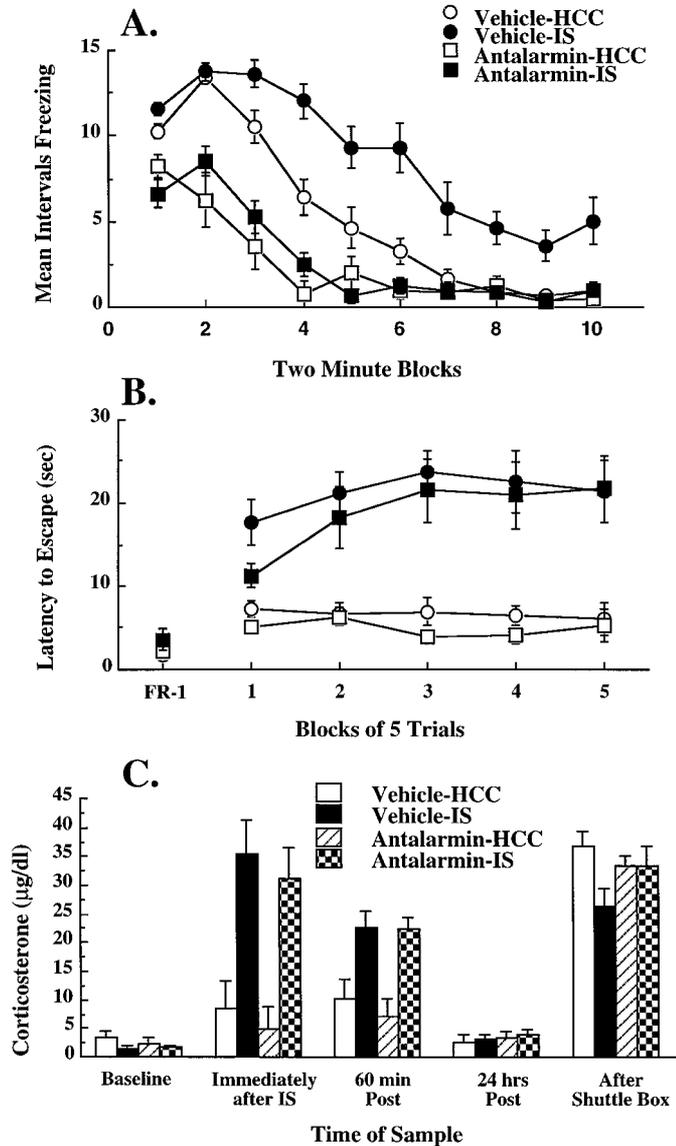
FIG. 2. After a baseline blood sample was taken, rats were injected with either vehicle or antalarmin (20 mg/kg·2 ml ip). Two hours later, rats were exposed to 100 inescapable tailshocks (1.6 mA, 5 sec each) or remained in their home cages. On day 2, rats were injected with vehicle or antalarmin (20 mg/kg·2 ml ip). Two hours later, freezing was observed by a blind observer, and shuttle box escape behavior was measured. Antalarmin blunted freezing to contextual cues, although it is unclear whether this effect is on the induction or the expression of the conditioned fear response. Importantly, antalarmin also blocked the enhancement of fear conditioning produced by IS (*P* < 0.05 (A). Antalarmin had no effect on the escape deficits produced by IS exposure (B). Antalarmin also had no effect on plasma CORT levels at the timepoints observed (C).

shown in Fig. 2c. The IS produced a large increase in CORT, which was still present, in reduced magnitude, 60 min later. Antalarmin showed no tendency to blunt this CORT response [$F_{(3,81)} = 0.6$, $P > 0.05$].

ACTH and CORT during the IS session

Numerous explanations for the failure of antalarmin to blunt the CORT response after IS are possible. One hundred inescapable tailshocks is a very potent stressor, and a ceiling effect may have prevented an effect from being detected. In addition, ACTH has often been argued to be a more sensitive measure of HPA activation. Thus, in the next experiment, ACTH and CORT were measured from blood samples taken after 5, 50, and 100 shocks, as well as 30 min after the session. Because the subject's tail protrudes from the rear of the tube, repeated samples can be readily obtained. In terms of controls, HCC would be problematic, because they would have to be repeatedly removed from their cages for blood sampling. The controls here were instead confined to the loose restraining tubes in which the ISs are administered. This was done in the animal's colony rooms to minimize disturbance. We have found that this loose restraint during the inactive part of the rats light-dark cycle in the colony rooms produces little or no ACTH or CORT increase. Baseline values were obtained 2 days before the experiment. Four groups were employed. Two were restrained and 2 received ISs. One of each was given antalarmin, 120 min before treatment; and one of each received vehicle.

Figure 3a shows that ACTH values did indeed remain at baseline in the restrained controls. ACTH was elevated after 5 shocks, reached maximum by 50 shocks, remained at this level at 100 shocks, and was still elevated 30 min later. Antalarmin had no effect on ACTH in controls. Antalarmin did not reduce the ACTH response to IS, even after only 5 shocks. Furthermore, an effect of antalarmin did not emerge 30 min after treatment, at which point the ACTH values were submaximal [$F_{(4,84)} = 0.566$, $P > 0.05$]. The results for CORT (Fig. 3b) mirror those for ACTH [$F_{(4,84)} = 0.998$, $P > 0.05$].

ACTH and CORT, after two footshocks and testing for fear

The next experiment determined whether antalarmin might blunt the ACTH or CORT response to a briefer or milder stressor. On day 1, two footshocks were delivered as above. A blood sample was obtained immediately after the session. Plasma ACTH (Fig. 3c) was increased by the 2 footshocks, relative to merely being placed in the apparatus (comparison of Shock-Veh/Veh with No shock-Veh/Veh). Antalarmin given before the footshock session did blunt the ACTH response to the footshocks and, indeed, brought ACTH to control levels [$F_{(2,22)} = 9.7464$, $P < 0.001$]. A similar pattern was also observed for CORT; however, this trend was not significant ($P > 0.05$). No reliable conditioned increase in ACTH or CORT was observed on day 2; and so, any effect of antalarmin could not be examined (data not shown).

Discussion

Prior testing of antalarmin has primarily involved determination of whether it would reduce the effects of CRH. The present studies extend the investigation of this drug by demonstrating that the peripheral administration of antalarmin potently blocks several effects of a stressor. Similar to the results reported by Kalin and Takahashi (36), using icv administration of α-helical CRH, antalarmin reduced the freezing that occurred when the subjects were placed into the environment in which footshocks had occurred 24 h earlier. This freezing is generally regarded as a reflection of fear
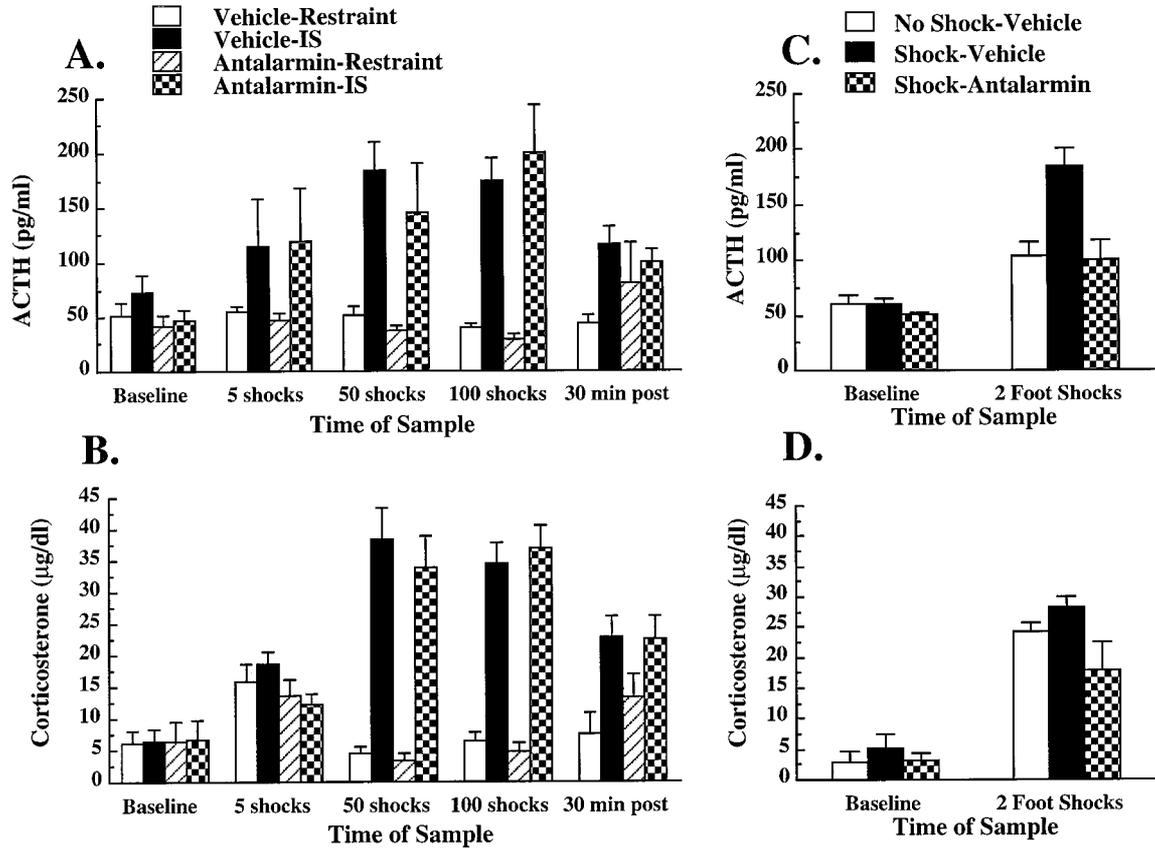
FIG. 3. After a baseline blood sample was taken, rats were injected with vehicle or antalarmin (20 mg/kg·2 ml ip). Two hours later, rats were exposed to 100 ISs (1.6 mA, 5 sec each, 60 sec ITI) or were loosely restrained in their home colony room. Blood samples were taken after 5, 50, and 100 shocks, and 30 min after the termination of IS. IS exposure significantly increased plasma ACTH (A) and plasma CORT (B); $P < 0.05$. Pretreatment with antalarmin had no effect on the IS-induced increase in plasma ACTH or CORT. In Exp 4, a baseline blood sample was taken, and rats were injected with vehicle or antalarmin (20 mg/kg·2 ml). Two hours later, rats received 2 footshocks or an equivalent time in the shock chamber. Thirty seconds after the second footshock, rats were removed from the chamber, and a blood sample was taken. Antalarmin blocked the footshock-induced rise in ACTH, reducing them to control levels (C; $P < 0.05$). Although there was a trend for antalarmin to reduce the plasma CORT response, this effect was not significant (D; $P > 0.05$).

conditioned to the contextual cues of the shock environment because it only occurs if the organism has been previously shocked in that particular environment (35). Freezing does not occur if the rat has been shocked in a different environment. Thus, the present results suggest that antalarmin is capable of interfering with the expression of fear that has been conditioned to a set of contextual environmental cues.

The present data extend knowledge concerning CRH systems by revealing that blockade of CRH receptors with antalarmin interferes with the development of fear conditioning, as well as with the expression of fear. Antalarmin given before fear conditioning, rather than before the later testing, reduced the fear measured 24 h later. Prior studies have not separated these two possibilities because CRH antagonists have typically been administered before the testing, rather than before the stressor. Furthermore, in those few experiments in which a CRH antagonist was given before the stressor, the behavioral testing occurred immediately or very soon after the stressor (43). Thus, CRH receptors were occupied by the antagonist, during both the stressor and the behavioral testing for fear; and so, the impact of the drug on the conditioning process itself could not be assessed. In the present experiment, subjects who had been given antalarmin

before the footshocks showed diminished freezing when exposed to the shock environment 24 h later. It might be noted that these results also counter the argument that the drug merely inhibits freezing itself rather than influencing fear, because here the drug was not present when freezing was measured.

It might be argued that antalarmin did not interfere with the fear conditioning process *per se*, but rather reduced the aversiveness of the footshocks. If the footshocks were experienced as less aversive, then of course reduced freezing would be expected to develop to the environmental cues present. Indeed, there is very little prior research directed at measurement of whether CRH antagonists alter the unconditioned aversiveness of noxious stimuli, in addition to blunting the organisms's emotional reaction to the stimulus. The aversiveness of a stimulus is generally assessed by measuring the organism's propensity to escape from the stimulus. Escape from footshock was studied in the present experiments, and antalarmin had no impact whatsoever on the escape responding to footshock measured in a shuttlebox. Rats that had been administered the very same dose of antalarmin before shuttlebox escape testing as was used in the fear conditioning study escaped with normal latencies on

both the single-crossing and double-crossing trials. If the drug reduced the aversiveness of the footshocks, then escape responding should have been slowed. In fact, the results were (nonsignificantly) in the other direction. Consistent with the finding that antalarmin failed to alter responsivity to footshock, as assessed by escape behavior, icv α-helical CRH has been reported to have no effect on pain thresholds (44). There is thus no support for the possibility that antalarmin interfered with the development of freezing to the contextual cues in which footshock occurred because it reduces the aversiveness of footshock. Rather, antalarmin would seem to have acted on the fear conditioning process itself.

Peripherally administered antalarmin has access to CRH receptors in both the periphery and brain. However, it is not likely that the reduction in fear produced by antalarmin was mediated by action on the pituitary-adrenal system. This is because: 1) peripheral immunoneutralization of CRH does not reduce fear behavior to stressors (45); 2) inhibition of pituitary-adrenal responses to CRH by dexamethasone does not blunt the behavioral changes produced by icv CRH (12); and 3) icv α-helical CRH sufficient to blunt fear responses to stressors does not reduce the pituitary-adrenal response to the same stressor (46).

The amygdala plays a key integrative role in both the development of fear conditioning and the expression of fear-related behavior. Lesions in basolateral regions of the amygdala (47) or microinjection of N-methyl-D-aspartate (NMDA) antagonists (48) in this region prevent the development of fear conditioning. In contrast, infusion of NMDA antagonists into the central nucleus of the amygdala does not retard fear conditioning (48). However, NMDA antagonists, injected either into the amygdala (49) or icv (50), have no effect on the expression of fear that has been previously conditioned. Lesions of the central nucleus, however, block the expression of fear conditioning (47). This pattern of data has led to the view that the association between the sensory cues that precede the stressor and the stressor itself are formed in basolateral regions of the amygdala and critically involves NMDA receptors. The information then flows to the central nucleus of the amygdala, which functions in the expression of fear as a final common path to initiate the behavioral and physiological manifestations of fear (34). NMDA receptors do not seem to play a role in this central nucleus expression mechanism.

The amygdala contains CRH immunoreactive cells and fibers (51), and both the type 1 and type 2 CRH receptors are widely distributed in both the basolateral region and central nucleus (52). Exposure to a stressor has been reported to increase CRH mRNA in the amygdala (53), and microinjection of α-helical CRH into the central nucleus decreases the expression of conditioned fear (43), as well as other stressor-induced behavioral changes (46). Thus, previous research has implicated NMDA-related processes in the basolateral amygdala in the development (but not expression) of fear conditioning, and CRH in the central nucleus, in the expression of fear. The potential role of CRH in the development of fear conditioning has not been explored. The present results suggest that CRH is important in both development and expression of conditioned fear, and it would be of interest to determine whether the critical site of CRH action in the development of fear is the basolateral amygdala.

The impact of CRH antagonists on the behavioral consequences of IS has only recently received attention. Mansbach *et al.* (54) have recently demonstrated that CP-154,526 (antalarmin) blocked the escape deficit produced by prior exposure to inescapable footshock. The present data are clearly not in accord with those of Mansbach *et al.* (54). In fact, antalarmin had no effect whatsoever on the escape deficits produced by IS in the present experiment, despite the fact that the dose used here was in a dose range comparable with that of Mansbach *et al.* (54). This discrepancy may be a result of procedural differences between the two studies. Mansbach's group induced learned helplessness by exposing rats to inescapable footshocks on 3 consecutive days and tested for escape deficits with footshock in the same chamber on the 4th day. On the other hand, the present experiment induced learned helplessness by a single exposure to inescapable tailshock, and escape responding was assessed in a novel environment 24 h later. Learned helplessness is not the only potential cause of poor escape learning; and so, the escape deficits observed by Mansbach *et al.* and the present studies may reflect different phenomena. Learned helplessness is generally inferred as the cause of poor escape learning, using some protocol or procedure only after the demonstration that the poor escape learning follows inescapable (uncontrollable) but not escapable (controllable) shock. Such sensitivity to stressor controllability has been demonstrated with the procedures and parameters used here (38, 42, 55) but has not been reported for the procedures and parameters used by Mansbach *et al.* (54). For example, habituation to footshock could also produce poor escape responding to footshocks when the rats are then tested in the same apparatus. The inescapable nature of the footshocks would increase the likelihood of this possibility.

In addition to escape responding, fear conditioning after exposure to two footshocks was also assessed in the present experiments. It has been shown that freezing, measured immediately after footshock, reflects fear conditioned to the contextual cues by the footshocks, not a reaction to the shock itself (50). Consistent with the fear-conditioning experiment, antalarmin reduced the freezing observed after the two footshocks in control subjects. As in prior research, exposure to IS potentiated the fear conditioning occurring 24 h later. Antalarmin also blocked this potentiation of fear conditioning. The HCC and IS groups that had been given antalarmin did not differ, indicating that the potentiation of fear produced by IS was blocked. It is not possible to determine whether the critical point of drug action was during the IS, during the fear conditioning, or both.

Interestingly, antalarmin did not even slightly reduce the deficit in escape learning produced by prior IS, even though the drug was given before both IS and shuttlebox escape testing and reduced fear in the same subjects. This suggests that the IS-produced escape failure is mediated by a mechanism fundamentally different from fear and the potentiation of fear, one that does not involve CRH as a critical element. Of course, it is possible that a larger dose might have been effective, but the dose used potently reduced fear and was already far in excess of minimally effective dosage (32).

Consistent with the present data, lesions of basolateral and central nuclei of the amygdala have produced the same pattern as did antalarmin (blockade of fear but not the escape deficit) (42). Thus, the mechanism responsible for escape deficits after IS would seem to be independent of the amygdala and CRH. Furthermore, escape itself was not influenced by antalarmin, suggesting some specificity with regard to stressor actions. Amygdala lesions also do not interfere with escape behavior (42); and so, CRH involvement in behavioral sequelae of stressors may be restricted to behaviors that are, at least partly, mediated by the amygdala. Indeed, the potential clinical usefulness of antalarmin may be enhanced by its specificity and failure to interfere with escape behavior, an outcome that might be undesirable.

Although antalarmin blocked one of the behavioral effects of IS, it had no detectable impact on the ACTH or CORT response to IS. It is perhaps not surprising that antalarmin did not reduce CORT or ACTH measured at the end of the IS session. Although CRH is thought to be the major secretagogue in stimulating ACTH secretion in response to stressors, arginine vasopressin (AVP), catecholamines, and possibly other factors also play a role (56). Perhaps 100 ISs is a stressor that is sufficiently potent, so that these other secretagogues drive the system to its maximum, leaving CRH receptor blockade ineffective. However, antalarmin did not reduce the ACTH or CORT response measured after only 5 ISs, nor did it alter the time course of recovery after the IS session. It cannot be argued that antalarmin is simply ineffective in regulating the pituitary-adrenal response to stressors, because the ACTH increase produced by two footshocks was prevented by the drug. Whether the critical determinant was the number of shocks, type of shocks, etc., cannot be determined from the present data. Although it has most frequently been argued that AVP plays a secondary role, potentiating the actions of CRH (57), there is increasing evidence that AVP and CRH production and release from parvocellular neurons within the PVN are under independent regulation (58). Thus, it is possible that, under certain circumstances, AVP may play a dominant role in mediating the pituitary-adrenal response to stressors.

In sum, the peripheral administration of antalarmin produced effects consistent with it's ability to cross the blood-brain barrier. Both stress-induced behavioral changes that are mediated by central CRH receptors, as well as pituitary-adrenal responses that are mediated by peripheral CRH receptors, were blunted. The present results also further support the notion that there is considerable selectivity in the mechanisms that mediate both the behavioral and the endocrine consequences of stressor exposure.

Acknowledgment

References

1. **Vale W, Spiess J, Rivier C, Rivier J** 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. Science 231:1394–1397
2. **De Souza EB, Grigoriadis DE** 1995 Corticotropin-releasing factor: physiology, pharmacology and role in central nervous system and immune disorders. In: Bloom FE, Kupfer DJ (eds) Psychopharmacology: The Fourth Generation of Progress. Raven Press, New York, pp 505–517
3. **Owens MJ, Nemeroff CB** 1991 Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev 43:425–473
4. **Rivier C, Rivier J, Vale W** 1982 Inhibition of adrenocorticotropic hormone secretion in the rat by immunoneutralization of corticotropin-releasing factor. Science 218:377–379
5. **Brown MR, Fisher LA, Rivier J, Spiess J, Rivier C, Vale W** 1982 Corticotropin-releasing factor: effects on the sympathetic nervous system and oxygen consumption. Life Sci 30:201–210
6. **Berridge CW, Dunn AJ** 1986 Corticotropin-releasing factor elicits naloxone sensitive stress-like alterations in exploratory behavior in mice. Regul Pept 16:83–93
7. **Koob GF, Britton KT** 1990 Behavioral effects of corticotropin-releasing factor. In: De Souza EG, Nemeroff CB (eds) Corticotropin-releasing Factor: Basic and Clinical Studies of a Neuropeptide. CRC Press, Boca Raton FL, pp 253–266
8. **Kalin NH, Shelton SE, Kraemer GW, McKinney WT** 1983 Corticotropin-releasing factor administered intraventricularly to rhesus monkeys. Peptides 4:217–220
9. **Takahashi LK, Kalin NH, Vanden Burgt JA, Sherman JE** 1989 Corticotropin-releasing factor modulates defensive-withdrawal and exploratory behavior in rats. Behav Neurosci 103:648–654
10. **Dunn AJ, Berridge DW** 1987 Corticotropin-releasing factor administration elicits a stress-like activation of cerebral catecholaminergic systems. Pharmacol Biochem Behav 27:685–691
11. **Valentino RJ, Foote SL, Aston-Jones G** 1983 Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus. Brain Res 270:363–367
12. **Britton KT, Lee G, Vale W, Rivier J, Koob F** 1986 Corticotropin-releasing factor (CRF) receptor antagonist blocks activating and 'anxiogenic' actions of CRF in the rat. Brain Res 369:303–306
13. **Kalin NH, Sherman JE, Takahashi LK** 1988 Antagonism of endogenous CRH systems attenuates stress-induced freezing behavior in rats. Brain Res 457:130–135
14. **Korte SM, Korte-Bouws GA, Bohus B, Koob GF** 1994 Effect of corticotropin-releasing factor antagonist on behavioral and neuroendocrine responses during exposure to defensive burying paradigm in rats. Physiol Behav 56:115–120
15. **Swerdlow NR, Britton KT, Koob GF** 1989 Potentiation of acoustic startle by corticotropin-releasing factor (CRF) and by fear are both reversed by alpha-helical CRF (9–41). Neuropsychopharmacology 115:141–146
16. **Butler PD, Weiss JM, Stout JC, Nemeroff CB** 1990 Corticotropin-releasing factor produces fear-enhancing and behavioral activating effects following infusion into the locus coeruleus. J Neurosci 10:176–183
17. **Kalivas PW, Duffy P, Latimer LG** 1987 Neurochemical and behavioral effects of corticotropin-releasing factor in the ventral tegmental area of the rat. J Pharmacol Exp Ther 242:757–764
18. **Liang KC, Lee EHY** 1988 Intra-amygdala injections of corticotropin releasing factor facilitate inhibitory avoidance learning and reduce exploratory behavior in rats. Psychopharmacology 96:232–236
19. **Wiersma A, Baauw AD, Bohus B, Koolhaas JM** 1995 Behavioural activation produced by CRH but not alpha-helical CRH (CRH-receptor antagonist) when microinfused into the central nucleus of the amygdala under stress-free conditions. Psychoneuroendocrinology 20:423–432
20. **De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ** 1985 Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. J Neurosci 5:3189–3203
21. **Wong ML, Licinio J, Pasternak KI, Gold PW** 1994 Localization of CRH receptor in RNA in adult rat brain by in situ hybridization histochemistry. Endocrinology 135:2275–2278
22. **Cummings S, Elde R, Ells J, Lindvall A** 1983 Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: an immunohistochemical study. J Neurosci 3:1355–1368
23. **Olschowka JA, O'Donohue TL, Mueller GP, Jacobowitz DM** 1982 The distribution of corticotropin-releasing factor-like immunoreactive neurons in rat brain. Peptides 3:995–1015
24. **Sawchenko PE, Swanson LW** 1990 Organization of CRF immunoreactive cells and fibers in the rat brain: immunohistochemical studies. In: De Souza EB, Nemeroff CB (eds) Corticotropin-releasing Factor: Basic and Clinical Studies of a Neuropeptide. CRC Press, Boca Raton FL, pp 29–52
25. **Dunn AJ, Berridge CW** 1990 Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety of stress responses? Brain Res Rev 15:71–100
26. **Koob GF** 1990 Behavioral responses to stress-focus on corticotropin-releasing factor. In: Brown MR, Rivier C, Koob G (eds) Neurobiology and Neuroendocrinology of Stress. Marcel Dekker, New York, pp 255–271
27. **Gold PW, Wong J-L, Chrousos GP, Licinio J** 1996 Stress system abnormalities in melancholic and atypical depression: molecular, pathophysiological, and therapeutic implications. Mol Psychiatry 1:257–264
28. **Krahn DD, Gosnell BA** 1989 CRH: possible role in eating disorders. Psychiatry Med 7:235–245
29. **Nemeroff CG** 1996 The corticotropin-releasing factor (CRF) hypothesis of depression: new findings and new directions. Mol Psychiatry 1:336–342

30. **Grillon C, Southwick SM, Charney DS** 1996 The psychobiological basis of posttraumatic stress disorder. Mol Psychiatry 1:278–297
31. **Chen YPL** 1994 International Patient No. wo 94/13676, June 23, 1994
32. **Schulz DW, Mansbach RS, Sprouse J, Braselton JP, Collins J, Corman M, Dunaiskis A, Faraci S, Schmidt AW, Seeger T, Seymour P, Tingley III FD, Winston EN, Chen YL, Heym J** 1996 CP-154,526: a potent and selective nonpeptide antagonist of corticotropin releasing factor receptors. Proc Natl Acad Sci USA 93:10477–10482
33. **Webster EL, Lewis DB, Torpy DJ, Zachman EK, Rice KC, Chrousos GP** 1996 In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinology 137:5745–5750
34. **Davis M** 1992 A neural systems approach to the study of the amygdala, fear, and anxiety. In: Elliott JM, Marsden DJ, Marsden CA (eds) Experimental Approaches to Anxiety and Depression. John Wiley & Sons, Chichester, pp 45–73
35. **Fanselow MS, Lester LS** 1988 A functional behavioristic approach to aversively motivated behavior: predatory imminence as a determinant of the topography of defensive behavior. In: Bolles RC, Beecher MD (eds) Evolution and Learning. Erlbaum, Hillsdale NJ, pp 185–212
36. **Kalin NH, Takahashi LK** 1990 Fear-motivated behavior induced by prior shock experience is mediated by corticotropin-releasing hormone systems. Brain Res 509:80–84
37. **Maier SF, Seligman MEP** 1976 Learned helplessness: theory and evidence. J Exp Psychol Gen 105:3–46
38. **Maier SF** 1990 The role of fear in mediating the shuttle escape learning deficit produced by inescapable shock. J Exp Psychol Anim Behav Proc 16:137–150
39. **Weiss JM, Simson PG** 1985 Neurochemical mechanisms underlying stress-induced depression. In: Field TM, McCabe PM, Schneiderman N (eds) Stress and Coping. Lawrence Erlbaum Associates, Hillsdale NJ, pp 93–117
40. **Minor TR, Pelleymounter MA, Maier SF** 1988 Uncontrollable shock, forebrain NE, and stimulus selection during choice escape learning. Psychobiology 16:135–146
41. **Maier SF, Wiertelak EP, Martin D, Watkins LR** 1993 Interleukin-1 mediates the behavioral hyperalgesia produced by lithium chloride and endotoxin. Brain Res 623:321–324
42. **Maier SF, Grahn RE, Kalman BA, Sutton LC, Wiertelak EP, Watkins LR** 1993 The role of the amygdala and dorsal raphe nucleus in mediating the behavioral consequences of inescapable shock. Behav Neurosci 107:377–388
43. **Swiergiel AH, Takahashi LK, Kalin NH** 1993 Attenuation of stress-induced behavior by antagonism of corticotropin-releasing factor receptors in the central amygdala in the rat. Brain Res 623:229–234
44. **Kita A, Imano K, Nakamura H** 1993 Involvement of CRH in the antinociception produced by interleukin-1 on mice. Eur J Pharmacol 237:317–322
45. **Pich EM, Heinrichs SC, Rivier C, Miczek KA, Fisher DA, Koob GF** 1993 Blockade of pituitary-adrenal axis activation induced by peripheral immuno-neutralization of corticotropin-releasing factor does not affect the behavioral response to social defeat stress in rats. Psychoneuroendocrinology 18:495–507
46. **Heinrichs SC, Pich EM, Miczek KA, Britton KT, Koob GF** 1992 Corticotropin-releasing factor antagonist reduces emotionality in socially defeated rats via direct neurotropic action. Brain Res 531:190–197
47. **Campeau S, Davis M** 1995 Involvement of subcortical and cortical afferents to the lateral nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. J Neurosci 15:2301–2311
48. **Fanselow MS, Kim JJ** 1994 Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. Behav Neurosci 108:210–212
49. **Miserendino MJ, Sananes CB, Melia KR, Davis M** 1990 Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala. Nature 345:716–718
50. **Kim JJ, Fanselow MS, DeCola JP, Landeira-Fernandez J** 1992 Selective impairment of long-term but not short-term conditional fear by the N-methyl-D-aspartate antagonist APV. Behav Neurosci 106:591–596
51. **Swanson LW, Sawchenko PE, Rivier J, Vale W** 1983 The organization of ovine CRF-innunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36:165–186
52. **Chalmers DT, Lovenberg TW, De Souza EB** 1995 Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci 15:6340–6350
53. **Kalin NH, Takahashi LK, Chen FL** 1994 Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. Brain Res 656:182–186
54. **Mansbach RS, Brooks EN, Chen YL** 1997 Antidepressant-like effects of CP-154,526, a selective CRF1 receptor antagonist. Eur J Pharmacol 323:21–26
55. **Maier SF, Ryan SM, Barksdale CM, Kalin NH** 1986 Stressor controllability and the pituitary-adrenal system. Behav Neurosci 100:669–674
56. **Tilders FJ, Berkenbosch F, Vermes I, Linton EA, Smelek PG** 1985 Role of epinephrine and vasopressin in the control of the pituitary-adrenal response to stress. Fed Proc 44:155–168
57. **Scaccianoce S, Muscolo LA, Cigliana G, Navarra D, Nicolai R, Angelucci L** 1991 Evidence for a specific role of vasopressin in sustaining pituitary-adrenocortical stress response in the rat. Endocrinology 128:3138–3143
58. **De Goeij DC, Kvetnansky R, Whitnall MH, Jezova D, Berkenbosch F, Tilders FJ** 1991 Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. Neuroendocrinology 53:150–159