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The Role of Corticotropin-Releasing Hormone in the Dorsal Raphe Nucleus in Mediating the Behavioral Consequences of Uncontrollable Stress

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Inescapable shock (IS) produces subsequent interference with escape behavior and increased fear conditioning that has been linked to increased activity and release of serotonin (5-HT) from neurons within the caudal dorsal raphe nucleus (DRN) both at the time of IS and later behavioral testing. Extrahypothalamic corticotropin-releasing hormone (CRH) has been implicated in many stress-related phenomena and has recently been shown to increase DRN 5-HT activity in the same caudal DRN area at which IS increases 5-HT activity. The current set of studies therefore examined the role of CRH in mediating the behavioral sequelae of IS. Intra-DRN microinjection of the nonselective CRH receptor antagonist D-Phe CRH (12–41) blocked the IS-induced behavioral changes when administered before IS but not when administered before later behavioral testing. Furthermore, intra-DRN administration of CRH in the absence of IS dose-dependently mimicked the effects of IS and interfered with escape behavior and increased fear conditioning 24 hr later. This effect was specific to injection of CRH into the caudal DRN and was not produced by microinjection into the rostral DRN. Intracerebroventricular CRH produced escape deficits and potentiated fear conditioning 24 hr later at only much higher doses, further confirming the site specificity of the effects. The potential role of the caudal DRN in states of anxiety is discussed.

Key words: corticotropin-releasing hormone; dorsal raphe nucleus; learned helplessness; serotonin; rats; shock

Corticotropin-releasing hormone (CRH) plays a key role in integrating neural, endocrine, and behavioral responses to stressful stimuli (Dunn and Berridge, 1990; Owens and Nemeroff, 1993). Although endocrine consequences of stressors are mediated by CRH-secreting cells in the hypothalamus, behavioral and neurochemical sequelae of stressor exposure are regulated by extrahypothalamic CRH (Liang et al., 1992; Lee and Davis, 1997).

Serotonin (5-HT) systems are also involved in mediating reactions to stressors, and CRH has been shown to interact with 5-HT systems (Kirby et al., 2000; Lowry et al., 2000). There are CRH-immunoreactive fibers associated with 5-HT neurons in the raphe nuclei (Cummings et al., 1983; Austin et al., 1997), as well as CRH receptor mRNA expression, immunoreactivity, and binding (Cummings et al., 1983; DeSouza, 1985; Chen et al., 2000). Although the effects of CRH on 5-HT neuronal firing have been reported to be primarily inhibitory in the rostral dorsal raphe nucleus (DRN; Kirby et al., 2000), Lowry et al. (2000) have recently identified a population of 5-HT neurons in the caudal DRN that are potently excited by CRH.

Interestingly, the caudal region of the DRN has been implicated in the behavioral consequences of exposure to uncontrollable stressors that have been called "behavioral depression" (Weiss et al., 1981) or "learned helplessness" (Maier and Seligman, 1976). These terms refer to the general finding that stressors over which an organism has no behavioral control produce changes that do not occur if the organism can control the stressor, although they are physically identical (Maier and Seligman, 1976). Inescapable shock (IS) or uncontrollable shock, relative to escapable or controllable shock, produces intense activation of 5-HT cells in the caudal DRN (Grahn et al., 1999), as well as a large accumulation of extracellular 5-HT within the DRN (Maswood et al., 1998) and its projection regions (Amat et al., 1998a,b). Moreover, pharmacological blockade of this caudal DRN 5-HT activation blocks the behavioral consequences of IS (Maier et al., 1995a), whereas pharmacological induction of caudal DRN 5-HT activation produces the usual behavioral consequences of IS in the absence of shock (Maier et al., 1995b).

The inputs that selectively activate 5-HT cells in the caudal DRN during IS are unknown. Because CRH has been shown to activate 5-HT neurons in the caudal DRN (Lowry et al. 2000), CRH input is an obvious possibility. Indeed, a recent report by Ronan et al. (2000) indicated that intracerebroventricular administration of CRH mimicked a typical behavioral effect of IS. It should be noted that although animals escaped poorly immediately after low doses of CRH, a high dose of CRH (10.0 μg) was required to induce escape deficits 24 hr later. To determine whether CRH activation of the caudal DRN plays a role in the production of behavioral depression and learned helplessness, the present experiments determined whether (1) intra-DRN microinjection of a CRH antagonist either before IS or before later behavioral testing would block the effects of IS, and (2) intra-DRN microinjection of CRH would induce behavioral changes characteristically produced by IS.

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MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (Harlan, Madison, WI) weighing 275–325 gm were used in all experiments. Rats were single-housed and maintained on a 12 hr light/dark cycle. Food and water were provided *ad libitum*. Behavioral testing was performed between 8 A.M. and 12 P.M. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

Apparatus

Rats given IS were placed into Plexiglas tubes measuring 17.5 × 7.0 cm (length × diameter). The rat's tail extended from the rear of the tube and was attached with tape to a Plexiglas rod. Electrodes were fixed to the tail, and computer-controlled 1.0 mA shocks were created by shock sources modeled after the Grason-Stadler model 700 shock source.

For behavioral testing, rats were placed into shuttle boxes measuring 46 × 20.7 × 20 cm (length × width × height). Scrambled 0.5 mA foot shocks were delivered through stainless steel grids on the floor of the apparatus. The shuttle box was divided into two halves by an aluminum wall containing an archway that allowed passage from one side to the other.

Surgery

Rats were anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ) and implanted with guide cannulas into the region of the caudal DRN, an area 2.2 mm lateral to the caudal DRN for site-specificity control studies, the rostral DRN, or the third ventricle. Twenty-six gauge stainless steel cannulas that were 13 mm long were implanted stereotaxically based on coordinates from the atlas of Paxinos and Watson (1986) and aimed 1 mm dorsal to the target region of the DRN to prevent damage to the area. Caudal DRN coordinates were anteroposterior (AP), +0.7 mm; dorsoventral (DV), +4.3 mm; and mediolateral (ML), 0 mm. Off-placement control coordinates were AP, +1.0 mm; DV, +4.5 mm; and ML, +2.2 mm. Rostral DRN coordinates were AP, +1.7 mm; DV, +5.2; and ML, 0 mm. Third ventricle coordinates were AP, 10.3 mm; DV, 5.5 mm; and ML, 0 mm. All coordinates used interaural zero as a reference. For all studies, the bite bar was set at −3.5 mm.

Procedure

DRN D-Phe CRH (12–41). One week after DRN cannulation, rats were randomly assigned to one of four groups: IS plus D-Phe CRH, IS plus vehicle, home cage plus D-Phe CRH, and home cage plus vehicle. Each rat was handheld in a towel during the injection procedure. The stylet was removed, and rats were injected by hand through the guide cannula with 50 ng of the CRH receptor antagonist D-Phe CRH (12–41) (Bachem, King of Prussia, PA) or an equivalent volume (1 μl) of saline vehicle. The injector extended 1 mm below the end of the guide cannula into the DRN. Injectors were constructed of 33 gauge stainless steel tubing (Small Parts, Miami Lakes, FL) that was connected to a 50 μl Hamilton (Reno, NV) syringe with a length of PE-20 tubing. The flow of drug was measured with a small air bubble created in the tubing. Injectors were left in place for 2 min to allow drug diffusion into brain tissue.

At 15 min after injection, IS rats were given 100 5 sec tail shocks delivered on a 1 min variable-interval schedule. Home cage rats were returned to their home cages after injection.

All subjects received behavioral testing 24 hr later. Immediately before behavioral testing, rats were injected with either 50 ng of D-Phe CRH (12–41) or vehicle in the same manner as described above. After 15 min, both conditioned fear and shuttle box escape learning were tested using a procedure described previously (Maier et al., 1993). Freezing was measured for the first 5 min after placement in a shuttle box. Each subject's behavior was scored every 8 sec as being either freezing or not freezing. Freezing was defined as the absence of all movement except that required for respiration. The observer was blind with regard to treatment condition, and inter-rater reliability has been calculated to be >0.92.

This observation period was followed by two foot shocks, which could be terminated by crossing to the other side of the shuttle box [fixed ratio-1 (FR-1) trials]. IS does not alter FR-1 shuttle box escape latencies (Maier et al., 1993); therefore, IS and home cage subjects were exposed to shocks of equal duration. These two shocks were followed by a 20 min observation period in which freezing was scored. Previous work has indicated that this freezing is a measure of fear that has been conditioned to the contextual cue of the shuttle box (Fanselow and Lester, 1988). This observation period was followed by 3 additional FR-1 escape trials and then 25 FR-2 escape trials. The subjects were required to cross to the other side and then back to terminate the shock on the FR-2 trials; it is here that IS-induced escape deficits are typically revealed. Each shock terminated after 30 sec if an escape response had not occurred.

To examine site specificity, the same experiment was repeated, except that the guide cannula was placed 2.2 mm lateral to the DRN. All procedures for the off-placement experiment were identical, except that D-Phe CRH (12–41) was injected 2.2 mm lateral to the DRN.

DRN CRH. One week after caudal DRN cannulation, rats were microinjected with 0.1, 0.5, or 1.0 μg of rat or human CRH in 1.0 μl (Sigma, St. Louis, MO) or 1.0 μl of saline vehicle. Immediately after injection, rats were placed into separate plastic bins in a different room from their home cages for 2 hr. Rats were then returned to their home cages. After 24 hr, rats were behaviorally tested for conditioned fear and shuttle box escape performance as described above. The choice of these large doses was based on pilot experiments and a report by Ronan et al. (2000). This issue will be addressed in Discussion.

To examine site specificity, 1.0 μg of CRH or saline was injected into an off-placement control site 2.2 mm lateral to the DRN, as described above. In an additional experiment, 0.0, 1.0, 10.0, or 20.0 μg of CRH in 1.0 μl of saline was administered intracerebroventricularly. The 20.0 μg dose was administered in two injections separated by 1 hr. In a final experiment addressed at the issue of site specificity, 0.8 μg of CRH in 0.25 μl of vehicle or vehicle alone was injected into either the rostral or the caudal DRN. After injection, all procedures were identical to those described for the initial DRN CRH experiment.

A final experiment was designed to determine whether multiple lower doses of CRH would produce learned helplessness and behavioral depression 24 hr later. Fifty nanograms of CRH in 0.25 μl of vehicle was administered into the caudal DRN once an hour for 3 hr. Testing was conducted 24 hr later as above.

Histology

At the completion of each study, cannulated rats were anesthetized and injected through the guide cannula with Evans blue dye (1 μl). After 15 min, rats were perfused, and their brains were removed and fixed in a 10% formalin, 30% sucrose solution. Brains were then sectioned on a cryostat and stained with cresyl violet. Cannula verifications of the sections were conducted under a light microscope.

Data analysis

Data were analyzed with repeated measures ANOVA and followed with Newman–Keuls analysis (α set at 0.05), which made all possible pairwise comparisons.

RESULTS

DRN D-Phe CRH (12–41)

Injecting D-Phe CRH (12–41) into the DRN before IS blocked both the interference with FR-2 escape responding and potentiated fear conditioning (Fig. 1) normally observed 24 hr later. As can be seen, previous IS led to very poor FR-2 escape behavior, with no reduction in escape latency occurring across trials. The intra-DRN administration of D-Phe CRH (12–41) before IS completely blocked this effect. With regard to fear conditioning, there was no freezing in any of the groups before the two foot shocks in the shuttle box. However, all groups showed substantial freezing after the foot shock, and this measure of conditioned fear was extinguished across the 20 min of testing. Previous IS potentiated the amount of freezing, and intra-DRN D-Phe CRH (12–41) given before IS blocked this potentiation. Injecting D-Phe CRH (12–41) into the DRN 15 min before testing had no effect on either measure.

D-Phe CRH (12–41) injected lateral to the DRN

As in the previous study, exposure to IS led to poor escape behavior and exaggerated fear conditioning (Fig. 2) 24 hr later. However, in contrast to the previous experiment, here the admin-

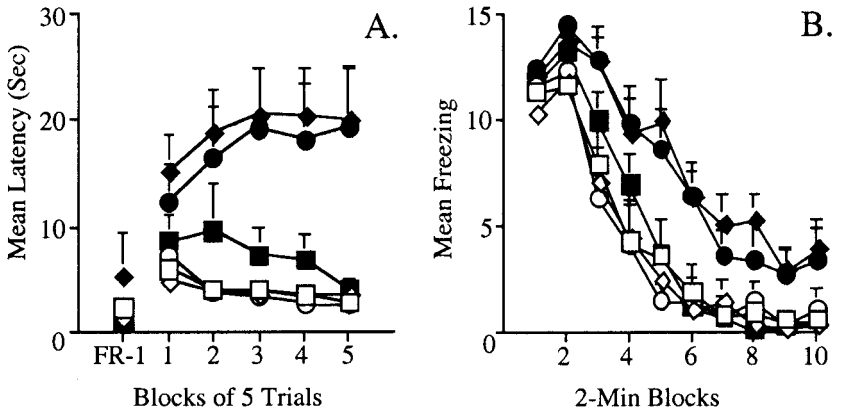

Figure 1. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Forty either received IS or were left in their home cages 24 hr earlier. Rats also received intra-DRN injection of the CRH antagonist D-Phe CRH (12–41) or vehicle immediately before treatment with IS or home cage, immediately before testing in the shuttle box, or neither. For FR-2 escape latencies, there was a significant effect of group ($F_{(5,34)} = 7.383$; $p < 0.05$) and a significant interaction between group and FR-2 trials ($F_{(20,136)} = 2.054$; $p < 0.05$). Newman–Keuls analysis revealed that IS rats injected with vehicle significantly differed from all groups, except IS rats that were injected with antagonist before testing. IS rats that were injected with antagonist before testing also differed from all groups except the IS vehicle-injected rats. All remaining groups did not reliably differ. *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in a shuttle box. Rats received either IS or were left in their home cages 24 hr earlier. Rats also received intra-DRN injection of the CRH antagonist D-Phe CRH (12–41) immediately before treatment with IS or home cage, immediately before testing in the shuttle box, or neither. There was a significant effect of group ($F_{(5,32)} = 4.809$; $p < 0.05$) and a significant interaction between group and 2 min blocks of freezing ($F_{(45,288)} = 1.799$; $p < 0.05$). IS rats injected with vehicle significantly differed from all groups except IS rats that were injected with antagonist before testing. IS rats that were injected with antagonist before testing also differed with all groups except IS vehicle-injected rats. All remaining groups did not reliably differ. □, Home cage, D-Phe before treatment, vehicle before test; ◇, home cage, vehicle before treatment and test; ○, home cage, vehicle before treatment, D-Phe before test; ■, IS, D-Phe before treatment, vehicle before test; ◆, IS, vehicle before treatment and test; ●, IS, vehicle before treatment, D-Phe before test.

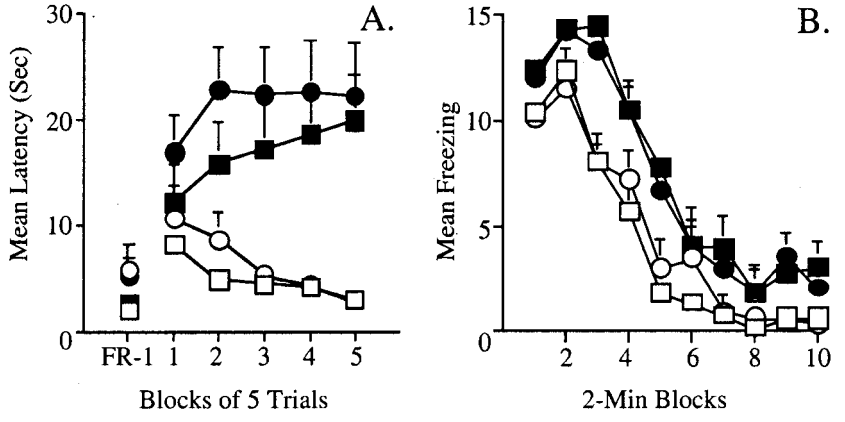

Figure 2. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Twenty-eight rats either received IS or were left in their home cages 24 hr earlier. Rats were also injected 2.2 mm lateral to the dorsal raphe nucleus with the CRH antagonist D-Phe CRH (12–41) or saline vehicle immediately before treatment with IS or home cage. There was an effect of shock ($F_{(1,24)} = 22.803$; $p < 0.05$) but no effect of drug ($F_{(1,24)} = 1.201$; $p > 0.05$) or interaction between shock and drug ($F_{(1,24)} = 0.319$; $p > 0.05$). Although shock interacted with trials ($F_{(4,96)} = 10.172$; $p < 0.05$), drug did not ($F_{(4,96)} = 0.969$; $p > 0.05$). There was no interaction among trials, shock, and drug ($F_{(4,96)} = 0.093$; $p > 0.05$). A Newman–Keuls analysis revealed a significant effect of shock, but no other comparisons were reliable. *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats either received IS or were left in their home cages 24 hr earlier. Rats were also injected 2.2 mm lateral to the dorsal raphe nucleus with the CRH antagonist D-Phe CRH (12–41) or saline vehicle immediately before treatment with IS or home cage. There was an effect of shock ($F_{(1,24)} = 23.519$; $p < 0.05$) but no effect of drug ($F_{(1,24)} = 0.0003$; $p > 0.05$) or interaction between shock treatment and drug ($F_{(1,24)} = 0.4993$; $p > 0.05$). Although shock interacted with trials ($F_{(9,216)} = 3.476$; $p < 0.05$), drug did not ($F_{(9,216)} = 0.576$; $p > 0.05$). There was no interaction among trials, shock, and drug ($F_{(9,216)} = 0.491$; $p > 0.05$). A Newman–Keuls analysis revealed a significant effect of shock, but no other comparisons were reliable. □, Home cage, D-Phe; ○, home cage, vehicle; ■, IS, D-Phe; ●, IS, vehicle.

istration of D-Phe CRH (12–41) lateral to the DRN before IS did not affect the behaviors observed 24 hr later.

Caudal DRN CRH

CRH injected into the caudal DRN dose-dependently increased FR2 escape latencies and conditioned fear (Fig. 3) 24 hr later. A dose of 0.1 μg had a very small effect on escape latencies but no effect at all on the freezing produced by foot shock; 0.5 μg increased escape latencies but not by as much as does IS (Figs. 1, 3); and 0.5 μg also had a small effect on freezing, but again, the effect was not as prominent as that produced by IS (Figs. 2, 4). However, 1.0 μg produced both interference with escape behavior and potentiation of fear conditioning as sizable as that produced by IS.

CRH (1.0 μg) injected lateral to the DRN

Whereas 1.0 μg CRH into the caudal DRN produced large interference with escape behavior and potentiation of fear conditioning, injecting the same dose of CRH lateral to the DRN did not alter FR-2 escape latencies or freezing behavior (Fig. 4) 24 hr later.

Intracerebroventricular CRH

Intracerebroventricular CRH dose-dependently interfered with escape behavior and potentiated fear conditioning (Fig. 5) 24 hr later. Importantly, the 1.0 μg dose that was effective intra-DRN had no effect at all intracerebroventricularly on either measure. Indeed, even the dose of 10.0 μg CRH had no effect at all on escape behavior and only a small effect on fear conditioning. A dose of 20 μg administered in two injections of 10.0 μg separated by 1 hr was required to produce effects as large as those produced by 1.0 μg administered intra-DRN.

Rostrocaudal Intra-DRN CRH

The injection of 0.8 μg of CRH was here made in 0.25 μl rather than in 1.0 μl as above, but injection into the caudal DRN still produced interference with escape behavior and potentiation of fear conditioning (Fig. 6). These effects were as pronounced as

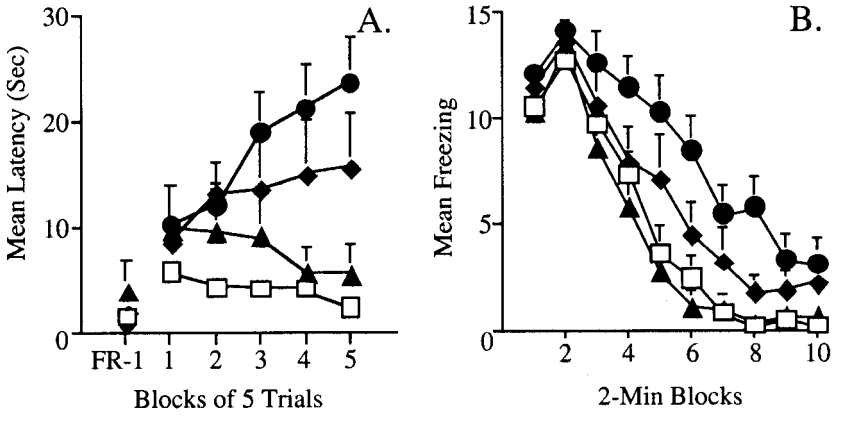

Figure 3. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Twenty-eight rats received 0.1, 0.5, or 1 μg of CRH or saline vehicle injected into the dorsal raphe nucleus 24 hr earlier. There was an effect of drug ($F_{(3,24)} = 4.458$; $p < 0.05$) and an interaction between drug and trials ($F_{(12,96)} = 6.139$; $p < 0.05$). Only the 1.0 μg dose of CRH was reliably different from vehicle. *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats either received 0.1, 0.5, or 1.0 μg of CRH or saline vehicle injected into the dorsal raphe nucleus 24 hr earlier. As for previous IS, intra-DRN CRH did not lead to any freezing before the foot shocks in the shuttle box. For postshock freezing behavior, there was a reliable effect of drug ($F_{(3,23)} = 5.801$; $p < 0.05$) and an interaction between drug and time ($F_{(27,207)} = 2.005$; $p < 0.05$). The 1.0 μg dose of the CRH group was reliably different from vehicle and the 0.1 μg dose groups. □, Vehicle; ▲, 0.1 μg of CRH; ♦, 0.5 μg of CRH; ●, 1.0 μg of CRH.

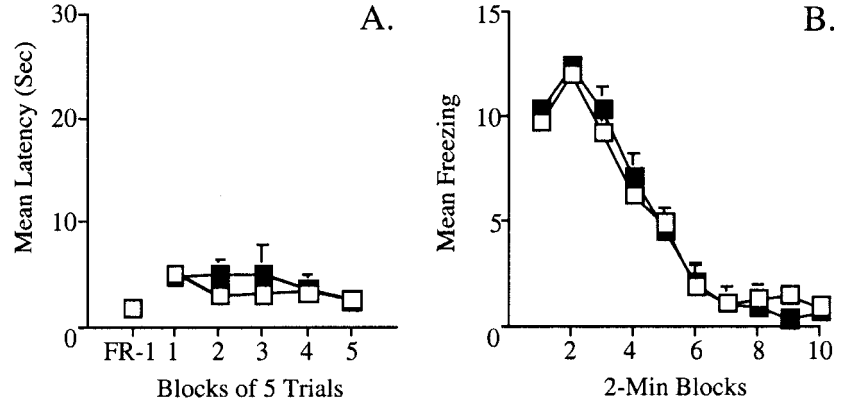

Figure 4. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Sixteen rats either received 1.0 μg of CRH or saline vehicle injected 2.2 mm lateral to the dorsal raphe nucleus 24 hr earlier. There was no effect of drug ($F_{(1,14)} = 0.344$; $p > 0.05$) or interaction between drug and trials ($F_{(4,56)} = 0.725$; $p > 0.05$). *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats either received 1.0 μg or saline vehicle injected 2.2 mm lateral to the dorsal raphe nucleus 24 hr earlier. There was no effect of drug ($F_{(1,14)} = 0.027$; $p > 0.05$) or interaction between drug and time ($F_{(9,126)} = 0.653$; $p > 0.05$). □, Vehicle; ■, 1.0 μg of CRH.

they were with the larger injection volume. In contrast, injection of CRH into the rostral DRN had little, if any, effect on either behavioral measure. Effective and ineffective injection sites are shown in Figure 7.

Multiple Intra-DRN CRH

The caudal intra-DRN injection of 50 ng once an hour for 3 hr produced interference with escape behavior and potentiation of fear conditioning (Fig. 8) 24 hr later. These effects were much larger than those produced by the single intra-DRN injection of 0.5 μg and as large as those produced by the single injection of 1.0 μg.

DISCUSSION

Pharmacological blockade of CRH receptors within the DRN during IS blocked the potentiation of fear conditioning and poor escape learning normally observed 24 hr later. Interestingly, blockade of CRH receptors during behavioral testing did not alter the effects of previous IS, nor did it have an effect on escape behavior or fear conditioning in non-IS controls.

Previous work has indicated that activation of 5-HT neurons in the caudal DRN during IS is critical to the production of the behavioral sequelae of IS, and the present data therefore suggest that CRH input to the DRN is importantly involved in this activation. Consistent with this blockade of learned helplessness and behavioral depression produced by intra-DRN D-Phe CRH (12–41) administered at the time of IS, the administration of CRH into the caudal DRN produced potentiated fear conditioning and poor escape behavior 24 hr later, just as IS does.

Two issues concerning the effects of CRH require discussion. The first concerns site specificity. Possibilities are that CRH leaked into either the ventricles or aqueduct and exerted its effects at a distant site, or that the CRH diffused to nearby tissue and exerted its effects at that local region. To test the possibility that leakage into the ventricles was responsible, CRH was injected intracerebroventricularly. The effective intra-DRN dose had no effect intracerebroventricularly, and behavior was not altered until the dose was substantially increased. Because CRH injected into the rostral DRN had no effect, an intra-aqueduct injection was not necessary (both rostral and caudal sites are equally close to the aqueduct). Moreover, CRH injected lateral to the DRN or into the rostral DRN did not induce behavioral effects, and even 0.25 μl injections were effective in the caudal DRN. As a group, these data suggest that the behavioral effects of CRH were mediated in the caudal DRN.

The second issue concerns dose. Large amounts of CRH were needed to produce interference with escape and potentiation of fear conditioning 24 hr later. A dose of 1.0 μg was required intra-DRN, and 20.0 μg was needed intracerebroventricularly. However, exposure to IS elevates extracellular levels of 5-HT in the DRN for at least 4 hr and perhaps longer (Maswood et al., 1998). A single administration of CRH is not likely to have this type of prolonged effect on DRN 5-HT neurons, and it may be that a large dose is required to maintain the presence of CRH, the activation produced by CRH, or both for a sufficiently prolonged period. The fact that three hourly injections of only 50 ng of CRH produced behavioral effects 24 hr later equal to those that followed 1.0 μg of CRH is consistent with this argument.

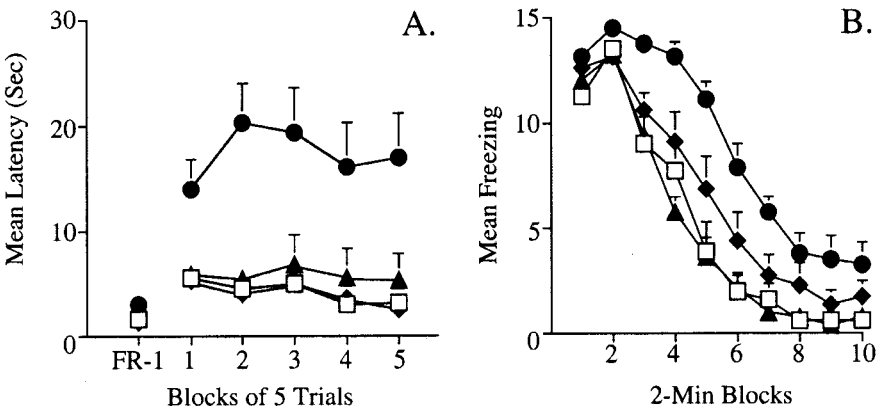

Figure 5. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Thirty-two rats received vehicle or 1.0, 10.0, or 20.0 μg of CRH intracerebroventricularly 24 hr before behavioral testing. There was an effect of drug ($F_{(3,28)} = 10.124$; $p < 0.05$) but no effect of trials ($F_{(4,112)} = 2.288$; $p > 0.05$) and no interaction between drug and trials ($F_{(12,112)} = 1.383$; $p > 0.05$). The 20.0 μg group was reliably different from all other groups, but no other comparisons were reliable. *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats received vehicle or 1.0, 10.0, or 20.0 μg of CRH intracerebroventricularly 24 hr before behavioral testing. There was an effect of drug ($F_{(3,28)} = 9.974$; $p < 0.05$), an effect of trials ($F_{(9,252)} = 224.401$; $p < 0.05$), and an interaction between drug and trials ($F_{(27,252)} = 2.814$; $p < 0.05$). The 20.0 μg group was reliably different from all other groups, but no other comparisons were reliable. □, Vehicle; ▲, 1.0 μg of CRH; ◆, 10.0 μg of CRH; ●, 20 μg of CRH.

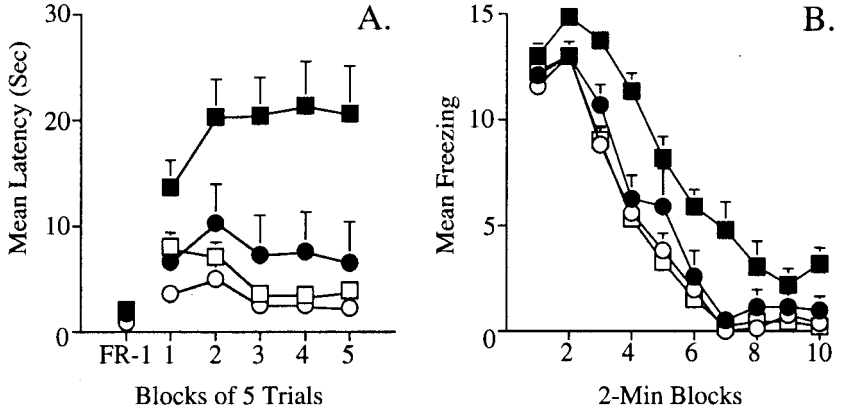

Figure 6. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Twenty-five rats received either 1.0 μg of CRH in 0.25 μl or vehicle injected into the caudal or rostral DRN 24 hr before behavioral testing. There was a reliable difference between groups ($F_{(3,21)} = 5.917$; $p < 0.05$) and a significant interaction between group and FR-2 trials ($F_{(12,84)} = 2.367$; $p < 0.05$). Rats injected into the caudal DRN with CRH reliably differed from rostrally injected CRH rats, rostrally injected vehicle rats, and caudally injected vehicle rats. No other comparisons were reliable. *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats received either 1.0 μg of CRH in 0.25 μl or vehicle injected into the caudal or rostral DRN 24 hr before behavioral testing. There was a reliable difference between groups ($F_{(3,21)} = 9.498$; $p < 0.05$), a significant effect of trials ($F_{(9,189)} = 199.843$; $p < 0.05$), and an interaction between group and FR-2 trials ($F_{(27,189)} = 1.788$; $p < 0.05$). Rats injected into the caudal DRN with CRH reliably differed from rostrally injected CRH rats, rostrally injected vehicle rats, and caudally injected vehicle rats. No other comparisons were reliable. ●, Rostral CRH; ○, rostral vehicle; ■, caudal CRH; □, caudal vehicle.

Although it is clear that both 5-HT and CRH are involved in the mediation of stress effects and that 5-HT and CRH systems interact, the nature of the interaction is complex. Low doses of intracerebroventricular and intra-DRN CRH have been reported to generally inhibit DRN 5-HT discharge rates and extracellular levels of 5-HT in the striatum and lateral septum (Price et al., 1998; Kirby et al., 2000; Price and Lucki, 2001), whereas at higher doses, CRH becomes either neutral or excitatory with both measures. In addition, Lowry et al. (2000) have recently reported excitatory effects of CRH on DRN 5-HT neurons. Although there are numerous differences between these studies [e.g., *in vivo* recording by Kirby et al. (2000) vs *in vitro* recording by Lowry et al. (2000)], Lowry et al. (2000) argued that the recording site within the DRN might be a key factor. The DRN is composed of subregions that receive unique, topographically organized afferent input (Peyron et al., 1998). This is noted because neurons that responded in an excitatory manner in the study by Lowry et al. (2000) were clustered in a small region of the caudal DRN between the medial longitudinal fasciculi at the caudal interface between the DRN and the median raphe nucleus. Indeed, these neurons were almost exclusively −8.0 to −8.5 mm from bregma, and the neurons recorded by Kirby et al. (2000) were more rostrally located (−7.5 mm from bregma). Moreover, subregions of the DRN send topographically organized efferent projections (Van Bockstaele et al., 1993), and it is the more rostral regions that project to striatum and lateral septum (Imai et al., 1986; Vertes, 1991), the regions in which extracellular 5-HT is reduced by intracerebroventricular CRH. It is thus noteworthy that (1) in the present study CRH was microinjected into the caudal DRN; (2) IS selectively activates DRN 5-HT neurons only in the mid to caudal DRN (Grahn et al., 1999); and (3) cannula placement in all of the studies in which pharmacological manipulation of DRN neurons has modulated learned helplessness and behavioral depression has been in the caudal DRN (−8.0 from bregma or beyond).

The effectiveness of D-Phe CRH (12–41) in blocking the behavioral consequences of IS when given before IS contrasts with a previous report from our laboratory (Deak et al., 1999) in which peripheral administration of the nonpeptide CRH antagonist antalarmin had no effect on the escape deficit produced by IS. Mansbach et al. (1997) did find antalarmin to be active in a quite different model also labeled as learned helplessness, but using the very same procedures, D-Phe CRH (12–41) and antalarmin had contrasting effects. There are numerous potential explanations for this difference. However, an intriguing possibility concerns the receptor selectivity profiles of these two compounds. Although D-Phe CRH (12–41) is nonselective with regard to the type I and II CRH receptors, antalarmin is selective for CRH I (Webster et al., 1996). The DRN is one of the few regions in which the type II receptor is found in high density (Chalmers et al., 1996), and

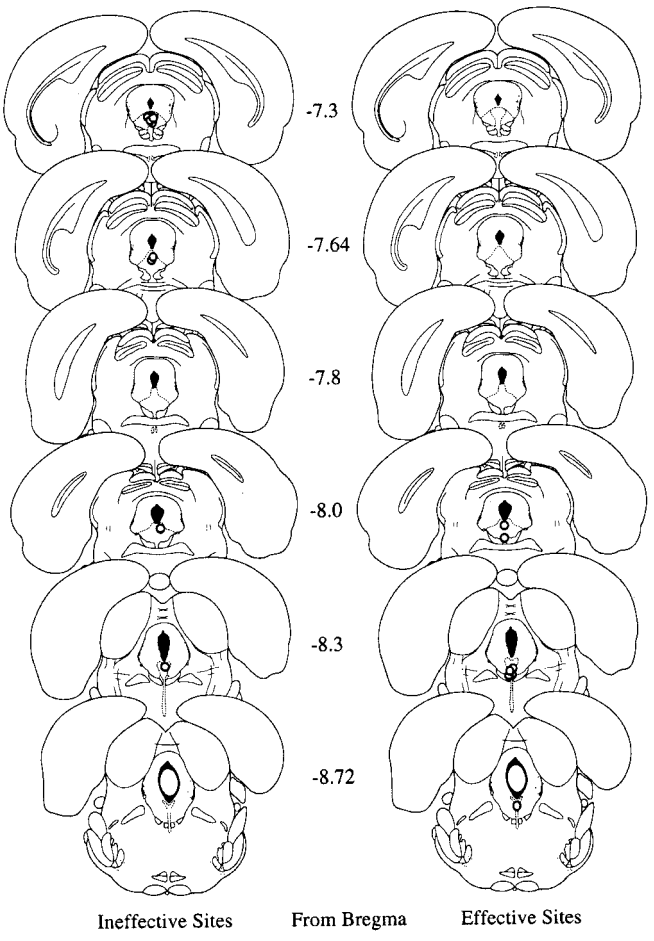

Figure 7. Ineffective and effective CRH injection sites in the DRN (○). Rats received either 0.8 μg of CRH in 0.25 μl or vehicle injected into the caudal or rostral DRN 24-hr before behavioral testing. For effective sites, CRH injection caused failures (maximum escape latency of 30 sec) in at least 8 of the last 10 escape trials tested 24 hr later.

Kirby et al. (2000) found antalarmin to block the inhibitory effect of intracerebroventricular CRH on neuronal discharge in the rostral DRN. This raises the intriguing possibility that the type I receptor might mediate the inhibitory effects of CRH on DRN 5-HT neurons, whereas the type II receptor is involved in excitatory interactions. This suggestion is consistent with the electrophysiological and neurochemical data from Valentino et al. (2001) and Price and Lucki (2001) in that most of their studies have used ovine CRH, which has approximately eightfold selectivity for the CRH type I receptor. Thus, low doses may affect only CRH I, with CRH II being recruited as the dosage increases. Indeed, this possibility has been suggested by Kirby et al. (2000). In addition, Valentino et al. (2001) have reported that there are heterogeneous interactions of CRH terminals with neuronal processes in different subregions of the DRN, thereby providing a basis for different effects of CRH on 5-HT activity within the DRN.

The effectiveness of D-Phe CRH (12–41) in blocking the behavioral consequences of IS when given before IS but not before behavioral testing supports the argument that CRH activation of DRN neurons during IS is essential in the production of behavioral depression and learned helplessness. This suggests that CRH input to the DRN is required to produce the alterations in DRN neurons that are required for the expression of learned helplessness and behavioral depression, but that CRH input to the DRN at the time of escape training and fear conditioning is not involved in the interference with escape performance or the exaggeration of fear conditioning that follow IS. Previous work has indicated that the intense activation of caudal DRN 5-HT neurons by IS sensitizes these neurons for a period (Amat et al., 1998b), and that this sensitization is necessary for the production of the behavioral sequelae of IS (Maier et al., 1995a). Thus, once DRN neurons are sensitized, a process that would appear to require CRH activity at the DRN, CRH at the DRN no longer plays a role. This is consistent with data that indicate that DRN 5-HT is not involved in fear conditioning (Maier et al., 1993) or in escape learning per se (Maier et al., 1993). The argument is that the DRN sensitization produced by IS modulates fear conditioning and escape learning, which are themselves mediated by other neural structures. Indeed, DRN lesions prevent the interference with escape and the potentiation of fear conditioning produced by IS but have no effect whatsoever on escape performance or fear conditioning in non-IS control subjects (Maier et al., 1993). However, pharmacological blockade of DRN 5-HT activity before the behavioral testing does completely block the interference with escape and exaggeration of fear conditioning produced by IS. Thus, DRN 5-HT activity is required for the expression of behavioral depression and learned helplessness, but CRH would seem to be uninvolved in initiating this activity during escape training or fear conditioning.

Lowry et al. (2000) have suggested that the caudal DRN and its projections to limbic and cortical structures might form a mesocorticolimbic 5-HT system that is importantly involved in the

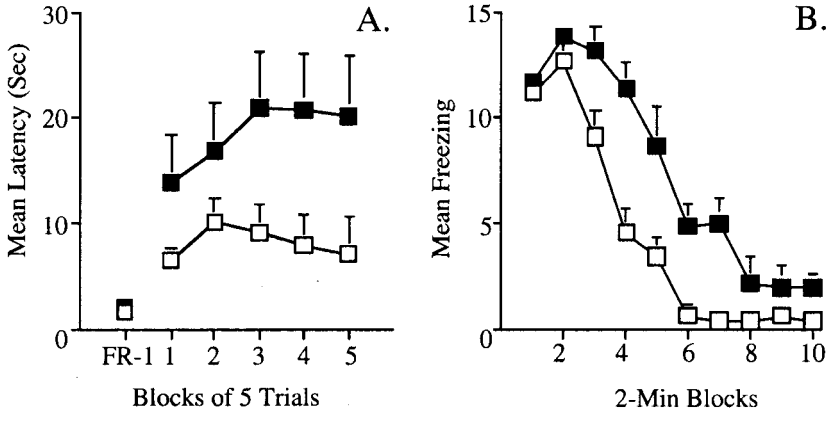

Figure 8. *A*, Mean shuttle box escape latencies for FR-1 trials and five blocks of FR-2 trials. Fourteen rats received either three caudal intra-DRN injections of 50 ng of CRH or vehicle 24 hr before behavioral testing, There was a significant effect of drug injection ($F_{(1,12)}$ = 4.986; $p < 0.05$) but no reliable effect of FR-2 trials ($F_{(4,48)}$ = 1.584; $p > 0.05$) and no interaction between drug and FR-2 trials ($F_{(4,48)}$ = 1.089; $p > 0.05$). *B*, Mean number of 8 sec periods in which freezing occurred across 2 min blocks after two shocks in the shuttle box. Rats received either three caudal intra-DRN injections of 50 ng of CRH or vehicle 24 hr before behavioral testing. There was a significant effect of drug injection ($F_{(1,12)}$ = 13.06; $p < 0.05$), a reliable effect of trials ($F_{(9,108)}$ = 80.749; $p < 0.05$), and an interaction between drug and trials ($F_{(9,108)}$ = 4.04; $p < 0.05$). □, Vehicle; ■, 50 ng of CRH three times.

mediation of anxiety. These experiments are in agreement with this hypothesis and suggest that CRH input to the DRN may be critical to the activation of this mesocorticolimbic 5-HT system by anxiogenic stimuli such as IS. The present experiments also suggest that the behavioral consequences of uncontrollable stressors are mediated by this circuit and that uncontrollability might be a key factor in the activation of this circuit.

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