

Research report

Low doses of corticotropin-releasing hormone injected into the dorsal raphe nucleus block the behavioral consequences of uncontrollable stress

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Abstract

The behavioral consequences of uncontrollable stress that are collectively called learned helplessness (LH) are mediated in part by increased levels of serotonin (5-HT) activity in the dorsal raphe nucleus (DRN) and its projection regions. Recently, corticotropin-releasing hormone (CRH) within the DRN has been implicated in the development of LH because intra-DRN CRH produces LH at very high doses, and because intra-DRN antagonists for the CRH 2 receptor (CRHR2) block LH. Since these behavioral effects are mediated by both 5-HT excitation and CRHR2 activation, we have suggested that CRHR2 mediates excitation of DRN 5-HT neurons. However, CRH has been shown to inhibit DRN 5-HT neurons at low doses that are expected to bind to CRHR1. Since CRHR1 antagonists were ineffective in blocking LH, we have further suggested that CRHR1 might mediate the inhibition of DRN 5-HT neurons. In support of this hypothesis, although low doses of CRH that preferentially bind CRHR1 inhibit DRN 5-HT activity, higher doses at which CRH would be expected to bind both receptor subtypes no longer inhibit DRN 5-HT. In addition, high doses of CRH are required to produce LH, which is known to be mediated by 5-HT excitation, and the CRHR2 agonist urocortin II (UCN II) produces LH at much lower doses than does CRH. The present studies show that intra-DRN CRH microinjection blocks the behavioral effects produced by DRN UCN II, but only at doses that have been shown to inhibit DRN 5-HT activity. Indeed, a higher dose of CRH that has been shown to no longer inhibit DRN 5-HT activity did not affect the behavioral consequences of DRN UCN II. In a separate experiment, the effective dose of CRH blocked the usual behavioral consequences of uncontrollable stress.

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1. Introduction

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide that mediates the endocrine, autonomic and behavioral responses to stress [8,25]. CRH has been found in several extra-hypothalamic brain areas [36,41], where the peptide coordinates behavioral responses to stress by modulating the activity of other neurotransmitter systems. CRH alterations of noradrenergic activity in the locus coeruleus [45] and serotonergic (5-HT) activity in the dorsal raphe nucleus (DRN) [14,19,31] are examples.

Recently, CRH action at the DRN has been implicated in the mediation of the behavioral effects of uncontrollable stress [12]. Exposure to uncontrollable stressors such as inescapable tailshock (IS) produces a variety of behavioral and physiological outcomes that do not occur if the stressor is controllable (i.e. escapable tailshock (ES)), a phenomenon that has been termed behavioral depression [47] and learned helplessness (LH) [24]. Although a number of neural structures are involved in the mediation of behavioral depression/LH, the DRN seems to play a pivotal role. The most relevant findings are that (a) IS produces a greater activation of serotonergic (5-HT) neurons in the caudal region of the DRN than does ES [10], (b) lesion of the DRN [22] or pharmacological blockade of DRN activation during IS [23]

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blocks the development of behavioral depression/LH, and (c) pharmacological activation of DRN 5-HT neurons without the presentation of IS duplicates the behavioral effects of IS [21].

Although the importance of the activation of DRN 5-HT neurons in the production of behavioral depression/LH is clear, the inputs to the DRN that lead to the selective activation of these neurons by IS are largely unknown. CRH input is an obvious candidate since the DRN expresses CRH immunoreactivity [2,19], contains CRH receptors [5], and receives projections from a number of extra-hypothalamic brain regions in which CRH-containing cell bodies are localized [28]. Consistent with this possibility, microinjection of high doses of CRH into the caudal DRN induces behavioral depression/LH 24 h later, and intra-DRN microinjection of the CRH antagonist D-Phe CRH (12–41) blocks the behavioral effects of IS [12].

The actions of CRH in mammals are mediated by two receptor subtypes, aptly named CRH R1 and R2. Both subtypes are 7-transmembrane G_s -protein coupled receptors that when bound by an agonist activate adenylate cyclase and increase intracellular cAMP. R1 binding sites and mRNA [7,29,48] have been found throughout the CNS, whereas R2 binding and mRNA has a more limited distribution [5, 33]. Although numerous studies have supported a role for R1 in mediating the effects of stress, fear and anxiety [6,20, 38,39,43], the role for R2 in these behaviors is less clear. There is evidence to suggest both an anxiolytic [4,15,44], and anxiogenic [3,13,27,34,42] role for R2, depending on the type of R2 manipulation and behavioral model.

It is to be noted that most research concerning the role of R1 and R2 in stress-related phenomena has not studied the function of these receptors in particular structures, but rather has utilized manipulations that involve all or much of the brain. Thus, R1 and R2 selective pharmacological agents have most often been administered intracerebroventricularly (ICV), as have antisense oligonucleotides. Similarly, R1 and R2 knockouts have not been regionally selective. The DRN is one of the few regions of the brain in which R2 is densely expressed, and indeed, R2 outnumbers R1 in this structure [5].

It thus seemed conceivable that CRH R2 within the DRN might play a critical role in the production of behavioral depression/LH. This was also suggested by the high doses of intra-DRN and ICV CRH (0.5–1.0 μg r/h CRH and 5.0–10.0 μg , respectively) required to mimic the behavioral consequences of IS [12]. R/h CRH binds preferentially to R1, and 1.0 μg is a far higher dose than is required to produce fear/anxiety behavior when microinjected into other brain regions. Indeed, ICV injections of less than 1.0 μg CRH produce amygdala-mediated fear/anxiety phenomena (e.g. Ref. [17]), which appear to be R1 mediated [18].

To explore the role of DRN R1 and R2, Hammack et al. [13] administered intra-DRN urocortin II (UCN II). This endogenous CRH-like peptide has a much higher affinity for R2 than R1 [35], and produced behavioral depres-

sion/LH 24 h later at a dose 100–1000-fold lower than CRH on a molar basis. Furthermore, the selective R2 antagonist anti-sauvagine-30 dose dependently blocked the behavioral effects of IS, while the selective R1 antagonist NBI 27914 was without effect at any dose tested.

These data are consistent with the idea that CRH or a related peptide activates DRN 5-HT neurons to produce behavioral depression/LH. However, the nature of CRH/5-HT interactions within the DRN is complex. In general, both intra-DRN and ICV injections of CRH *inhibit* rather than excite DRN 5-HT neurons as measured by unit responding and release of 5-HT in projection regions of the DRN [14,31,32]. Interestingly, in all of these studies the inhibitory effects of CRH were only evident at low doses (0.3–1.0 μg oCRH ICV and 1.0–10.0 ng intra-DRN) and *diminished* as dose increased. Indeed, CRH tended to become *excitatory* as dose increased further (3.0 μg oCRH ICV and 30.0 ng intra-DRN). Given that oCRH is selective for R1 and only binds R2 at higher doses [33], an interpretation that is consistent with these data is that R1 inhibits and R2 excites DRN 5-HT neurons, a possibility noted by Kirby et al. [14]. This antagonistic arrangement would occur if R1 are expressed predominantly on the GABAergic interneurons within the DRN that inhibit 5-HT cells [40], with R2 being expressed on the 5-HT neurons themselves. Given the relative potency of CRH at R1 and R2, low doses would be expected to activate predominantly R1 and so be inhibitory. As dose increases CRH should then activate R2 as well, thereby shifting the net effect on 5-HT neurons to neutrality, and finally to excitation. An additional complexity is that there may be subregions within the DRN in which 5-HT neurons respond differently to CRH. This possibility was suggested by Lowry et al. (2001) who reported CRH to be inhibitory in the rostral DRN and excitatory in the caudal DRN. As noted above, Grahn et al. (1999) found IS to selectively activate DRN 5-HT neurons only in the caudal DRN. In addition, Hammack et al. (2001) found that microinjection of CRH into the caudal DRN produced the usual behavioral consequences of IS 24 h later, whereas microinjection within the rostral DRN was without effect.

The present experiments were designed to further explore the possibilities that behavioral depression/LH is mediated by action at R2 within the DRN and that ligands selective for R1 and R2 have antagonistic effects. As described above, UCN II is highly selective for R2 and intra-DRN UCN II administration produces the same behavioral outcomes (e.g. poor escape behavior and exaggerated fear conditioning) 24 h later as does IS. The ideas to be tested here would suggest that low doses of CRH, which act primarily at R1, should *block* the effects of UCN II on behavior. If a submaximal UCN II dose is used, this blockade should diminish as CRH dose increases since CRH would now act at R2 as well as R1. In addition, rather than synergizing with IS, low doses of intra-DRN CRH should *block* the effects of IS, whether administered before IS or before later behavioral testing.

2. Materials and methods

2.1. Subjects

Adult male Sprague–Dawley rats (250–325 g Harlan, Madison, WI) were single-housed and maintained on a 12-h light/dark cycle. Food and water were provided ad libitum, and all behavioral testing was performed between 08:00 a.m. and 12:00 p.m. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

2.2. Apparatus

Uncontrollable shock occurred in Plexiglas tubes measuring 17.5 cm × 7 cm (length × diameter). Rats were placed in the tubes and their tails were secured with tape to a Plexiglas rod that extended from the end of the tube. An electrode was fixed to each tail with tape and computer-controlled 5-s 1.0 mA shocks were administered. Shocks were created by shock sources modeled after the Grason-Stadler model 700 shock source.

Behavioral testing occurred in shuttle boxes measuring 46 cm × 20.7 cm × 20 cm (length × width × height). Scrambled 0.5 mA foot shocks were administered through a grid floor made of stainless steel. The shuttle box was divided into two equal halves with an aluminum partition that contained an archway that allowed passage from one side to the other.

2.3. Surgery

Rats were anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ) and implanted with guide cannulae aimed at the region of the caudal DRN. Thirteen millimeters long cannulae were constructed from 26 gauge stainless steel tubing (Small Parts, Miami Lakes, FL), and were implanted stereotaxically based on coordinates from the atlas of Paxinos and Watson (1986) [26]. The tip of the guide cannula was implanted 1 mm dorsal to the DRN to prevent damage to the area, and a small stylet was placed inside the cannula to maintain the patency of the cannula lumen. The bite bar was set at –3.5 mm. Caudal DRN coordinates were 1 mm anterior, 4.3 mm dorsal, and 0 mm lateral to interaural zero.

2.4. Drugs

UCN II (8.7 ng, Phoenix Pharmaceuticals, Inc., Belmont, CA) was dissolved in sterile saline. Aliquots were frozen and thawed once on treatment day. Ovine CRH (0.5, 5, 50 ng; Sigma Chemical Co., St. Louis, MO) was also dissolved in sterile saline, aliquoted and frozen.

2.5. Procedure

2.5.1. CRH + UCN II

One week after DRN cannulation, rats were randomly assigned to one of eight groups each of which received

two intra-DRN microinjections: vehicle/vehicle, vehicle/UCN II, 0.5 ng CRH/vehicle, 0.5 ng CRH/UCN II, 5.0 ng CRH/vehicle, 5.0 ng CRH/UCN II, 50 ng CRH/vehicle, and 50 ng CRH/UCN II. All rats microinjected with UCN II received 8.7 ng. Thus, the design was a 4 × 2 factorial, with the first injection being either vehicle or 1 of 3 doses of oCRH, and the second injection being either vehicle or a dose of UCN II previously shown to produce submaximal behavioral depression/LH. There were 9–11 animals per group. Each rat was gently wrapped in a towel and handheld during the injection procedure. CRH injection always occurred just prior to UCN II injection. The stylet was removed from the cannula and rats were injected by hand through the guide cannula with 0.5 μl of drug or equivolume vehicle. The injector was constructed of 33 gauge stainless steel tubing (Small Parts, Miami Lakes, FL) that was attached to a 50 μl Hamilton (Reno, NV) syringe with a length of PE-20 tubing. The injector extended 1 mm below the end of the guide cannula into the DRN. The flow of drug was measured with a small air bubble created in the tubing. Injections took approximately 60 s and injectors were left in place for 2 min to allow drug diffusion into brain tissue. Immediately after injection, rats were placed into separate plastic bins in a separate room that was different than the testing room. Two hours later, rats were returned to their home cages.

All subjects received behavioral testing 24 h later using a procedure described previously [22]. Rats were placed in the shuttle box apparatus and freezing was assessed for 5 min. Freezing was defined as the absence of all movement except that required for respiration, and was scored every 8 s as being either freezing or not freezing. The observer was blind with regard to treatment condition, and inter-rater reliability has been calculated to be >0.92.

After this initial observation period, rats were administered two 0.5 mA foot shocks that were delivered on a 1 min variable-interval schedule and could be terminated by crossing to the other side one time [fixed ratio-1 (FR-1) trials]. FR-1 trial scores did not differ between groups ($F < 1.0$), therefore all groups were exposed to shocks of equal duration. Following the two FR-1 trials, rats were assessed for freezing for 20 min. Previous work has indicated that this freezing is a measure of fear that has been conditioned to the contextual cues of the shuttle box [9]. This observation period was followed by three more FR-1 trials, and then 25 FR-2 trials during which rats were required to cross the shuttle box twice to terminate the shock. It is during the FR-2 trials that the escape deficits produced by IS are normally revealed. All shocks were terminated after 30 s if the proper escape response did not occur.

2.5.2. CRH before uncontrollable shock

One week after cannulation, rats were randomly assigned to one of four groups: vehicle/shock, CRH/shock, vehicle/

home cage, CRH/home cage, and microinjected with 5 ng CRH in 0.5 μ l saline or equivolume vehicle. There were 9–11 animals per group. Fifteen minutes after injection, shocked rats were given 100 5-s tailshocks delivered on a 1 min variable-interval schedule. Home cage rats were returned to their home cages after injection. Thus, the design was a 2×2 factorial, with rats receiving either low dose CRH or vehicle before either IS or control treatment. Behavioral testing occurred 24 h later in the manner described above.

2.5.3. CRH before behavioral testing

One week after cannulation, rats were randomly assigned to one of four groups: vehicle/shock, CRH/shock, vehicle/home cage, CRH/home cage. There were 9–11 animals per group. Shock and home cage treatment occurred on Day 1 as described above. Twenty-four hours after treatment, rats were microinjected with 5 ng CRH in 0.5 μ l saline

or equivolume vehicle. Fifteen minutes after injection, behavioral testing was conducted in the manner described above.

2.5.4. Histology

When each study was completed, cannulated rats were anesthetized and injected through the guide cannula with Evans blue dye (0.5 μ l). Fifteen minutes later, 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 placements were verified under a light microscope.

2.5.5. Data analysis

All data were analyzed by repeated measures factorial analysis of variance followed by Newman–Keuls multiple comparisons with the alpha set at 0.05.

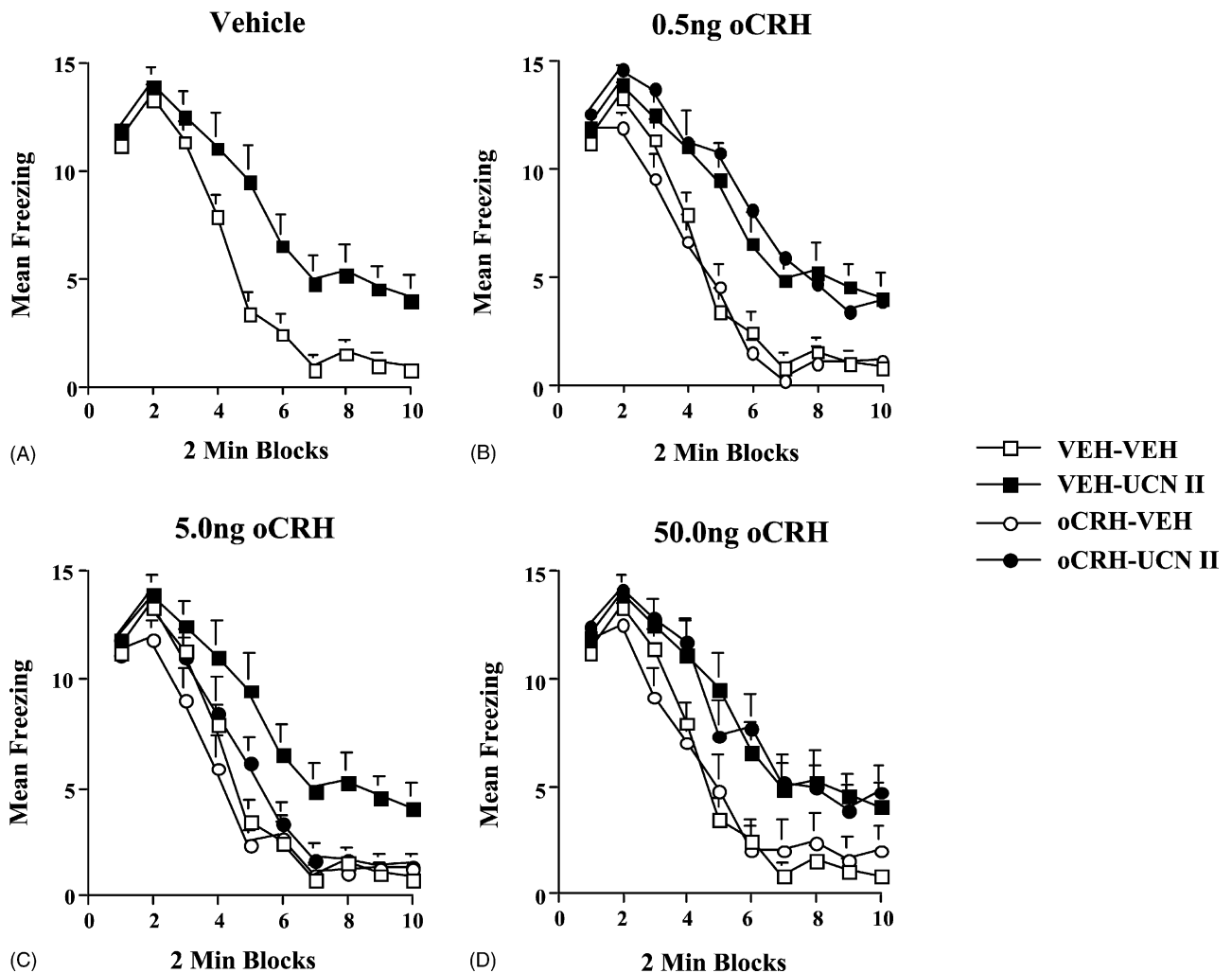


Fig. 1. Mean levels of freezing across blocks of 2 min following exposure to two footshocks. Panel A depicts data for groups that received vehicle before either vehicle or UCN II 24 h before testing. Panel B repeats the data for these groups and adds data for groups that received 0.5 ng oCRH before either vehicle or UCN II. Panel C again repeats the data for the control groups and adds data from groups that received 5.0 ng oCRH before either vehicle or UCN II. Panel D also again repeats the data for the control groups and adds data from groups that received 50.0 ng oCRH before either vehicle or UCN II.

3. Results

3.1. CRH + UCN II

No subjects were eliminated due to cannulae misplacement. There was no measurable freezing before the two footshocks in the shuttlebox. Mean levels of freezing after the two footshocks, across blocks of 2 min, are shown in Fig. 1. For ease of inspection the data are presented in four panels, with each panel including the data for the UCN II and vehicle groups that had received one of the CRH doses. The vehicle/vehicle and vehicle/UCN II groups are presented in Panel A and repeated in Panels B–D as baselines. As can be observed, the administration of UCN II exaggerated the level of freezing produced by the footshocks administered 24 h later. 0.5 ng CRH did not alter the effects of the UCN II, whereas 5.0 ng CRH blocked the enhanced freezing produced by UCN II. Interestingly, 50.0 ng CRH did not reduce the effects of UCN II. These conclusions

were supported by repeated measures analysis of variance. The effects of UCN II [$F(1, 66) = 26.32, P < 0.0001$], Time [$F(9, 595) = 241.56, P < 0.0001$], the interaction between Time and UCN II [$F(9, 595) = 5.97, P < 0.0001$], and Time, UCN II, and CRH [$F(27, 595) = 2.38, P < 0.05$] were significant. Newman–Keuls tests indicated that the vehicle/UCN II group differed from the vehicle/vehicle group, the 0.5 ng CRH/UCN II group differed from the 0.5 ng CRH/vehicle group, and that the 50.0 ng CRH/UCN II group differed from the 50.0 ng CRH/vehicle group. None of the CRH/vehicle groups differed from each other, and only the 5.0 ng CRH/UCN II group differed from the rest.

Mean FR-2 escape latencies, across blocks of five trials, are shown in Fig. 2. The general pattern is similar to that above. UCN II produced poor escape responding 24 h later, and this was blocked only by the 5.0 ng dose of CRH. Analysis of variance yielded significant effects of UCN II [$F(1, 66) = 162.17, P < 0.0001$], CRH [$F(3, 66) = 4.59, P < 0.006$], the interaction of UCN II and CRH

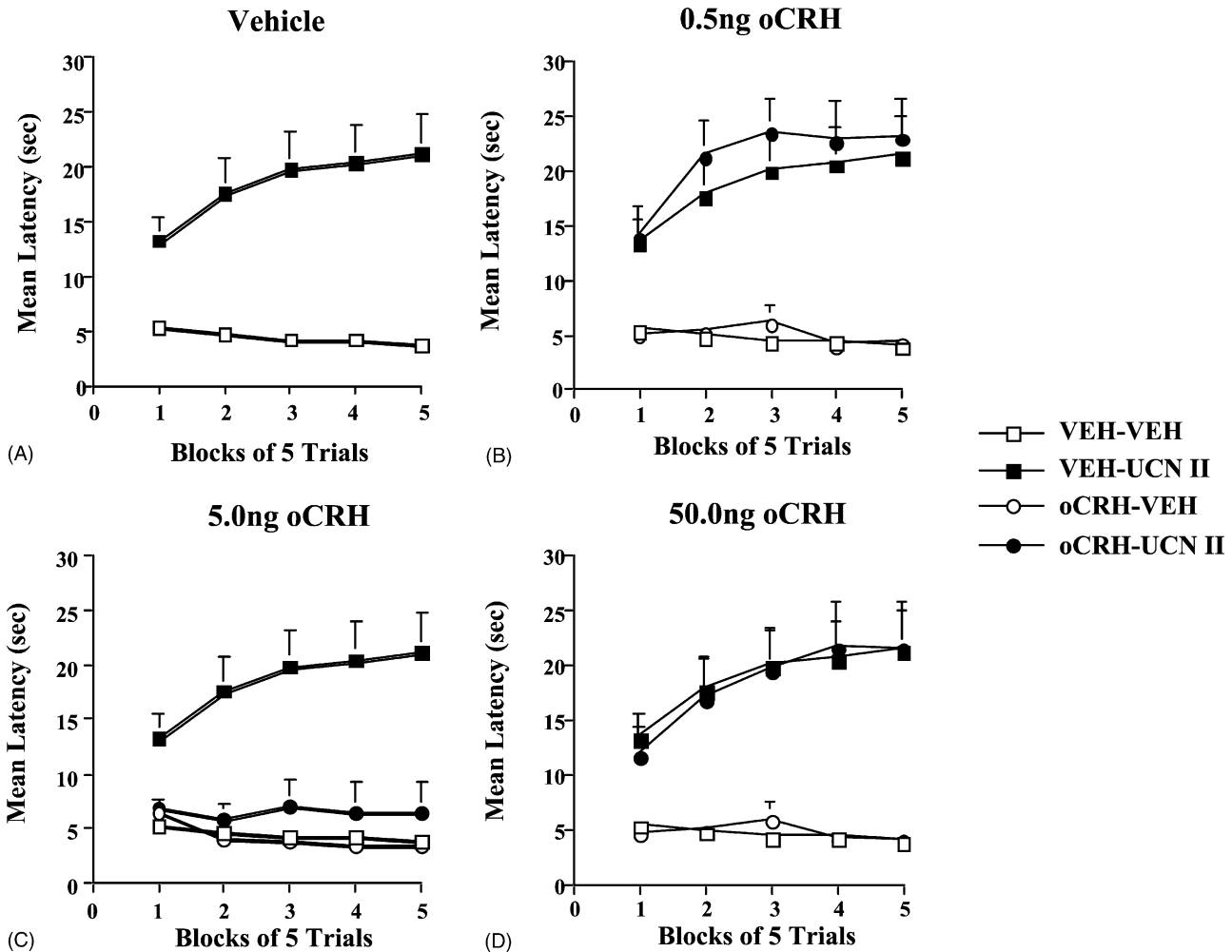


Fig. 2. Mean shuttlebox escape latencies across blocks of five FR-2 trials. Panel A depicts data for groups that received vehicle before either vehicle or UCN II 24 h before testing. Panel B repeats the data for these groups and adds data for groups that received 0.5 ng oCRH before either vehicle or UCN II. Panel C again repeats the data for the control groups and adds data from groups that received 5.0 ng oCRH before either vehicle or UCN II. Panel D also again repeats the data for the control groups and adds data from groups that received 50.0 ng oCRH before either vehicle or UCN II.

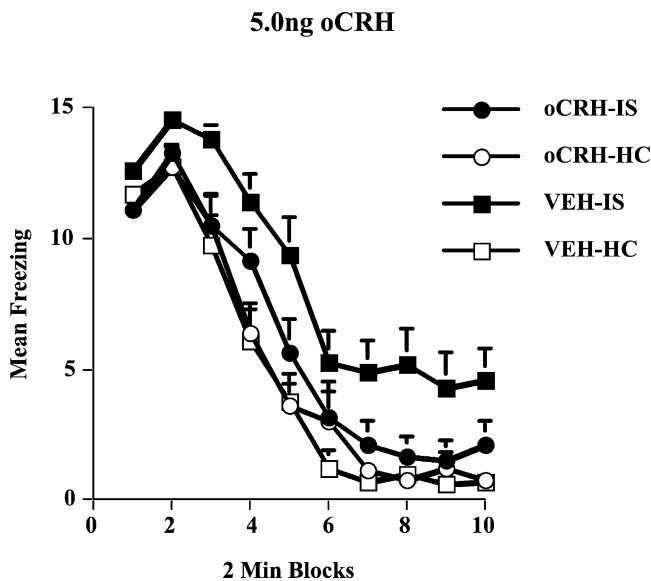


Fig. 3. Mean levels of freezing across blocks of 2 min following exposure to two footshocks for groups that 24 h earlier had received either 5.0 ng oCRH or vehicle before either IS or home cage control treatment (HC).

[$F(3, 66) = 4.04, P < 0.02$], Trial Block [$F(4, 260) = 8.60, P < 0.0001$], the interaction of UCN II and Trial Blocks [$F(4, 260) = 27.80, P < 0.0001$], and the interaction between CRH and Trial Blocks [$F(12, 260) = 2.85, P < 0.001$]. As above, Newman–Keuls tests indicated that the vehicle/UCN II group differed from the vehicle/vehicle group, the 0.5 ng CRH/UCN II group differed from the 0.5 ng CRH/vehicle group, and that the 50.0 ng CRH/UCN II group differed from the 50.0 ng CRH/vehicle group. None of the CRH/vehicle groups differed from each other, and only the 5.0 ng CRH/UCN II group differed from the rest.

3.2. CRH before uncontrollable shock

Two subjects were eliminated for incorrect cannula placement, leaving sample sizes of 8–10 per group. There was no freezing before the two footshocks in the shuttlebox. The freezing data following the two footshocks are shown in Fig. 3. As is typical, IS potentiated the level of freezing produced by the footshocks. Five nanograms CRH did not alter the level of fear conditioning in controls, but it blocked the effects of IS. The effects of IS [$F(1, 33) = 11.59, P < 0.002$], the interaction between CRH and IS [$F(1, 33) = 4.23, P < 0.05$], Time [$F(9, 288) = 78.2, P < 0.0001$], and the interaction of IS and Time [$F(9, 288) = 2.80, P < 0.004$] were significant. Newman–Keuls post hoc comparisons indicated that the IS group that received vehicle differed from all of the other groups, which did not differ among themselves.

Escape latencies are shown in Fig. 4. IS produced poor escape behavior, and this effect was blocked by CRH. Analysis of variance yielded significant effects of IS [$F(1, 34) =$

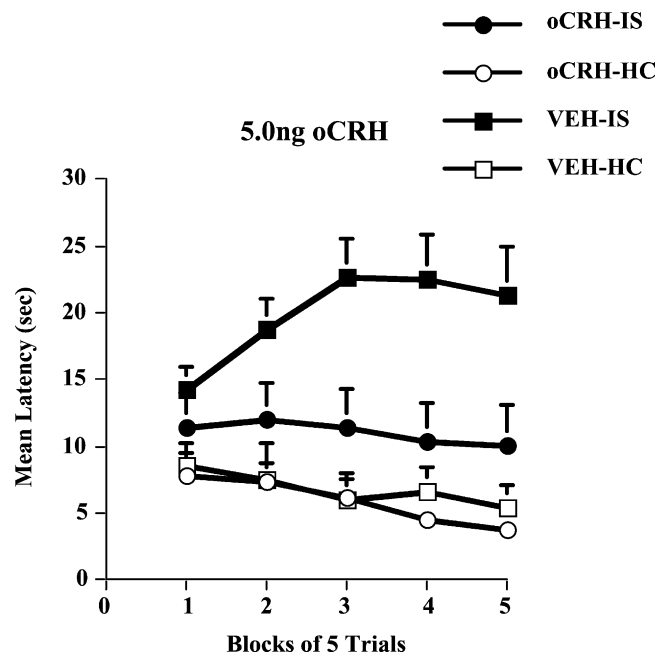


Fig. 4. Mean shuttlebox escape latencies across blocks of five FR-2 trials for groups that 24 h earlier had received either 5.0 ng oCRH or vehicle before either IS or home cage control treatment (HC).

$20.20, P < 0.0001$], CRH [$F(1, 34) = 5.82, P < 0.03$], the interaction of IS and CRH [$F(1, 34) = 4.34, P < 0.05$], the interaction of CRH and Trials [$F(4, 136) = 3.32, P < 0.02$], the interaction of IS and Trials [$F(4, 136) = 5.44, P < 0.0005$], and the interaction between CRH, IS, and Trials [$F(4, 136) = 2.28, P < 0.07$]. Newman–Keuls post hoc comparisons indicated that the IS group that received vehicle differed from all of the other groups, which did not differ among themselves.

3.3. CRH before behavioral testing

Two subjects were eliminated for incorrect cannula placement, leaving sample sizes of 9–10 per group. As is typical, there was very little freezing before the footshocks. Post-shock freezing is shown in Fig. 5. IS again potentiated fear conditioning as measured by freezing, and CRH given before testing reduced, but did not entirely eliminate this effect of IS. The effects of IS [$F(1, 36) = 11.94, P < 0.002$], Time [$F(9, 298) = 46.17, P < 0.0001$] and the interaction of IS and Time [$F(9, 298) = 2.49, P < 0.01$] were significant. Newman–Keuls comparisons indicate that the IS group given vehicle differed from both home cage groups. No other differences were significant.

Escape latencies are depicted in Fig. 6. IS interfered with later escape behavior, and this effect was reduced, but not eliminated, by CRH given before testing. The effects of IS [$F(1, 36) = 32.89, P < 0.0001$], CRH [$F(1, 36) = 6.05, P < 0.02$], the interaction between IS and CRH [$F(1, 36) = 4.83, P < 0.04$], Trials [$F(4, 144) = 5.17, P < 0.0006$], the interaction between

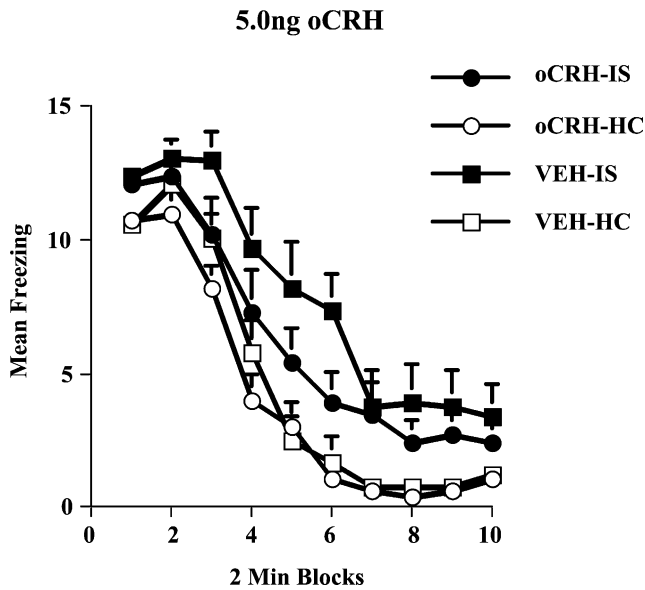


Fig. 5. Mean levels of freezing across blocks of 2 min following exposure to two footshocks for groups that had received either IS or home cage control treatment (HC) 24 h before testing, and either 5.0 ng oCRH or vehicle 15 min before behavioral testing.

IS and Trials [$F(4, 144) = 22.53, P < 0.0001$], and the interaction between IS, CRH and Trials [$F(4, 144) = 5.05, P < 0.0008$] were all significant. Post hoc Newman–Keuls comparisons indicate that the IS group given vehicle differed from all of the other groups, that the IS group given CRH differed from both home cage groups.

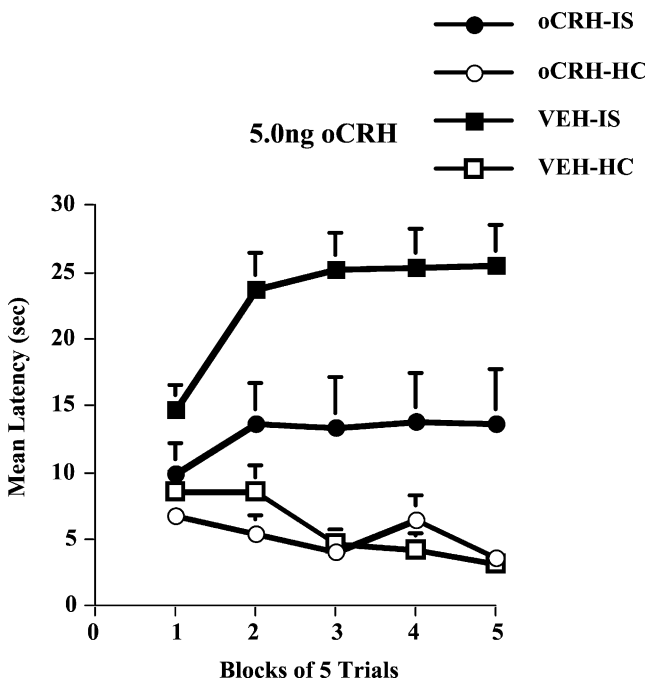


Fig. 6. Mean shuttlebox escape latencies across blocks of five FR-2 trials for groups that had received either IS or home cage control treatment (HC) 24 h before testing, and either 5.0 ng oCRH or vehicle 15 min before behavioral testing.

4. Discussion

Intra-DRN microinjection of the R2 agonist UCN II decreased escape latencies and increased fear conditioning 24 h later, replicating an earlier report [13]. The intra-DRN injection of CRH just prior to UCN II injection blocked the effects on UCN II on these LH behaviors, but only at a low CRH dose that has been previously shown to inhibit DRN 5-HT neurons [14,31,32]. Indeed, CRH lost its ability to block or even reduce the effects of UCN II at a higher CRH dose. Thus the dose–response pattern of CRH on UCN II-induced behavioral changes was U-shaped, as 0.5 ng was ineffective, 5.0 ng was effective, and 50.0 ng was without effect. Moreover, 5.0 ng of CRH injected into the DRN was able to block the behavioral changes induced by uncontrollable stress when administered before this stressor, and able to reduce these behavioral changes when given before testing.

These data further support a critical role for the DRN in mediating the behavioral effects of uncontrollable stressors and are consistent with the idea that activation of 5-HT neurons within the DRN during exposure to the stressor and during later behavioral testing are key events. This is because a dose of CRH known to be in the range of CRH dosages that inhibit DRN 5-HT neurons blocked or reduced the behavioral effects of IS, while doses known to either have no effect on DRN activity or even to excite DRN activity did not [14,31,32].

We have previously shown that CRH receptor blockade before behavioral testing does not affect LH behaviors [12], whereas in the present set of experiments presumed CRH R1 receptor activation before testing reduced these behavioral changes. Although it might seem that these two findings are inconsistent, they are not. The argument has been that excessive release of 5-HT during testing is the proximate cause of escape deficits and potentiated fear conditioning, and that this occurs because the escape and fear conditioning experiences act on a sensitized DRN produced by IS. The data further suggest that the release of a CRH-like ligand within the DRN during IS is involved in the sensitization process. However, there has been no presumption that the testing conditions release CRH within the DRN, or that CRH is involved in the activation of the DRN for the escape or fear conditioning procedures. Thus, CRH receptor blockade at this time should be without effect. However, any procedure that leads to the inhibition of DRN 5-HT activity at the time of testing should reduce escape deficits and potentiated fear, and this is the hypothesized effect of CRH R1 stimulation.

A concern with the present set of experiments may be that the site of action of CRH receptor activation was not the DRN. However, we have previously shown that CRH injected 2.2 mm lateral to the DRN or into the rostral extent of the DRN is ineffective in producing LH behaviors [12]. Moreover, ICV injection of CRH was only effective at a dose over 10 times greater than that required in the caudal DRN [12]. Taken together, these results suggest that the

critical site for the modulation of LH behaviors by CRH is the caudal DRN.

The view that motivated the present studies is that multiple CRH receptor subtypes mediate the effects of CRH on DRN 5-HT and behaviors mediated by DRN 5-HT, and that activation of R2 within the DRN promotes the behavioral effects of IS, while activation of R1 inhibits these effects. Indeed intra-DRN UCN II, which is selective for R2, produces behavioral effects that are associated with DRN 5-HT excitation [13]. In the present experiments, a low dose of oCRH, which preferentially binds to R1 [33], blocked the behavioral effects produced by intra-DRN UCN II, suggesting that R1 may indeed mediate DRN 5-HT inhibition and thus block the behavioral effects produced by UCN II-mediated 5-HT excitation.

The possibility that two CRH receptor subtypes mediate these effects readily explains the U-shaped dose–response curve produced by CRH on DRN 5-HT activity [14,31,32] and UCN II-induced behavioral changes (present data). At lower doses, CRH preferentially binds R1 [33], and would produce DRN 5-HT inhibition. However, as dose increases, and R1 sites become saturated, CRH would begin to bind R2, counteracting the DRN 5-HT inhibition until, at very high CRH doses, DRN 5-HT neurons would become excited. Furthermore we have previously reported that very high doses of CRH produce behavioral changes that are associated with DRN 5-HT excitation [12]. Here we found that low doses of CRH block these behavioral changes associated with DRN 5-HT excitation. It should be noted that all of the procedures in these studies were identical, except that the former used r/h CRH, which is even less selective for R1 than the oCRH [33] used in the present studies.

Both R1 and R2 are G_s-protein coupled receptors that activate adenylate cyclase. Despite the similar actions of these receptor subtypes, there are arrangements by which they could have opposite effects on DRN 5-HT. For example, DRN 5-HT neurons are under tonic inhibition from GABAergic interneurons [40]. One possibility is that R2 is predominantly expressed by DRN 5-HT neurons, while R1 is predominantly expressed by the GABAergic inhibitory interneurons.

A variety of research has implicated CRH in mediating the effects of stress, fear and anxiety. However, much of this research has suggested a heavy involvement of R1 in mediating these effects. Indeed R1 antagonists suppress CRH-induced acoustic startle [38], are anxiolytic on the elevated-plus maze [11,20], and block CRF induced defensive withdrawal [1]. These effects are, in part, mediated by R1 receptors in the amygdala [18]. In addition, the endocrine response to stress is mediated by R1 receptors in the anterior pituitary.

The potential role of R2 in mediating the effects of stress, fear and anxiety is less clear. There is evidence for both an anxiogenic [3,13,27,34,42] and anxiolytic [4,15,44] effect of this receptor on several behavioral tests. For example, R2 knockout mice show increased levels of anxiety [4,15], suggesting an anxiolytic role for the R2 receptor. How-

ever, interpretation of these data is difficult since a compensatory mechanism, such as an upregulation of R1, may have changed the phenotype of the adult mouse. In addition, genetic knockout has removed R2 from all regions of the brain, and the role of R2 in different regions may be quite different. Here we address the role of R2 and R1 only within the caudal DRN, and the function of these receptors in other brain regions, such as the amygdala may be quite different. This point applies equally to studies in which CRH antagonists or antisense oligonucleotides have been administered systemically or ICV. Any effects observed will be the summed result of the actions of the drugs at different regions and will depend on which regions of the brain that mediate the behaviors under study. For example, the behavioral consequences of uncontrollable stress assessed here depend on the DRN and not on the amygdala. DRN lesions block the ability of IS to produce later poor escape learning, but even large lesions of the amygdala do not [22]. In contrast, fear conditioning depends on the amygdala but not the DRN, with DRN lesions having no effect on fear conditioning (DRN lesions prevent IS from potentiating fear conditioning but do not alter fear conditioning in controls), but amygdala lesions preventing fear conditioning [37]. Thus, non-regional manipulations of CRH will likely be mediated by the local circuitry present in the amygdala if conditioned fear is measured as the outcome, and the DRN if LH/behavioral depression is measured.

Although the present data further confirm the importance of R2 stimulation in mediating the behavioral consequences of uncontrollable stress, they do not indicate whether R1 plays a role, nor do they suggest the nature of the endogenous CRH-like peptide involved or its source. An intriguing possibility is that since ES fails to have any effect on extracellular levels of 5-HT within the DRN or its projection regions relative to controls even though it is quite “stressful,” that the element of control might actively inhibit DRN 5-HT neurons. Clearly, this would occur if ES were to lead to a low level of CRH release within the DRN that would bind only R1. Perhaps controllable shock produces low levels of DRN CRH release, suppressing DRN 5-HT activity, with CRH release within the DRN increasing to levels that strongly excite DRN 5-HT neurons when the stressor is uncontrollable.

The source of DRN CRH or other CRH-like peptide induced by uncontrollable stress is not currently known, although the DRN receives projections from several forebrain and limbic structures that contain CRH cell bodies. These include the amygdala and bed nucleus of the stria terminalis (BNST) [28]. Both structures have been implicated in mediating fear and anxiety states and may do so by modulating DRN 5-HT activity or by being modulated by DRN 5-HT activity, or both. However, the amygdala is not a likely source of IS-induced CRH since large lesions of the regions of the amygdala that contain CRH cell bodies fail to reduce the behavioral consequences of IS [22]. It is also still unclear whether the endogenous ligand that activates DRN CRH receptors during IS is CRH, or one or more of the

several CRH-related peptides including UCN I [46], UCN II [35], or UCN III [16]. All of these peptides are found in regions that project to the DRN.

In sum, these data suggest that DRN R1 and R2 receptors act in an antagonistic manner to generate IS-induced behavioral outcomes, with R1 mediating 5-HT inhibition and R2 receptors mediating 5-HT excitation. An antagonistic relationship between R1 and R2 has been suggested before [30], although the nature of the antagonistic relationship was quite different. It may be that R1 and R2 are generally antagonistic, with the direction and nature of the relationship varying with the neural structure involved.

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