ROLE OF CORTICOTROPIN-RELEASING HORMONE IN THE AMYGDALA AND BED NUCLEUS OF THE STRIA TERMINALIS IN THE BEHAVIORAL, PAIN MODULATORY, AND ENDOCRINE CONSEQUENCES OF OPIATE WITHDRAWAL

G. P. MCNALLY* and H. AKIL
Mental Health Research Institute, The University of Michigan, Ann Arbor, MI, USA

Abstract—The extra-hypothalamic actions of corticotropin-releasing hormone (CRH) have been accorded an important role in coordinating responses to stressors and contributing to the consequences of drug abuse. Recent proposals suggest that CRH actions in the bed nucleus of the stria terminalis coordinate responses to tonic/unpredictable stressors whereas these actions in the central nucleus of the amygdala coordinate responses to phasic/predictable stressors. We used in situ hybridization histochemistry and site-specific microinjections of a CRH receptor antagonist to study the role of CRH in opiate withdrawal. Rats undergoing opiate withdrawal displayed clear behavioral and autonomic changes accompanied by hyperalgesia and increased plasma corticosterone. In situ hybridization of CRH mRNA revealed significant increases in the central nucleus of the amygdala but not in the bed nucleus of the stria terminalis among rats either chronically pre-treated with morphine, given an injection of naloxone, or both (precipitated withdrawal). An increase of CRH mRNA in the parventricular nucleus of the hypothalamus was specific to rats undergoing withdrawal. Intracerebroventricular microinjection of the CRH receptor antagonist, hCRH4-41, reduced the severity of opiate withdrawal. Microinjections of hCRH4-41 into the central nucleus of the amygdala also reduced the severity of withdrawal whereas bed nucleus of the stria terminals microinjections of hCRH4-41 were without effect. These experiments provide evidence for a role of amygdala, but not bed nucleus of the stria terminalis, CRH in opiate dependence. We propose a specific role for down-regulation of opiate receptor signaling in increased expression of the CRH gene in the amygdala. Moreover, we suggest that the roles accorded to CRH in the bed nucleus of the stria terminalis versus amygdala in coordinating responses to stressors may need to be reconsidered to distinguish between external and internal/interoceptive stressors. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: drug dependence, stress, fear, anxiety, hyperalgesia, in situ hybridization.

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide controlling the secretion of adrenocorticotropin hormone from the anterior pituitary, and thus is integral to hypothalamic–pituitary–adrenal (HPA) axis activity following exposure to a stressor (Vale et al., 1981). Intracerebral infusions of CRH also produce marked alterations in autonomic, immune, and behavioral function, which are similar to those observed following exposure to a stressor (Dunn and Berridge, 1991; Owens and Nemeroff, 1991), and have been taken as evidence for an extra-hypothalamic role for CRH in responding to stressors (for reviews see Akil and Morano, 1995; De Souza and Grigoriadis, 1995). These extrahypothalamic actions of CRH also contribute to the consequences of substance abuse, including withdrawal from a variety of drug classes (Menzaghi et al., 1994; Rassnick et al., 1993; Richter and Weiss, 1999; Rodriguez de Fonseca et al., 1997). The role of CRH during opiate withdrawal is of particular interest because it has been implicated in both aversive motivational and profound behavioral, as well as autonomic, changes associated with withdrawal (Brugger et al., 1998; Heinrichs et al., 1995; Iredale et al., 2000). Thus, systemic or intracerebral administrations of a CRH receptor antagonist reduced the aversions to contexts produced by explicit pairings with morphine withdrawal (e.g., Heinrichs et al., 1995), and also reduced the severity of the somatic withdrawal syndrome (Brugger et al., 1998; Iredale et al., 2000; Lu et al., 2000). Whereas the anatomical locus for the role of CRH in the aversive motivational effects of morphine withdrawal has been studied (e.g., Heinrichs et al., 1995), the anatomical locus for this role of CRH in opiate withdrawal is unknown. The central nucleus of the amygdala (CeA) and bed
nucleus of the stria terminalis (BNST) may be important sites for the actions of systemic or i.c.v. CRH receptor antagonists during opiate withdrawal. These structures display considerable anatomical and neurochemical symmetry and are considered parts of the ‘extended amygdala’ (e.g., Alheid et al., 1995; De Olmos and Heimer, 1999). Both are rich in cells producing the opioid and CRH systems (e.g., Day et al., 1999; De Souza and Grigoriadis, 1995; Mansour et al., 1995), and lesions or microinjections of various receptor antagonists into these regions reduce many of the consequences of morphine withdrawal (e.g., Calvino et al., 1979; Delfs et al., 2000; Heinrichs et al., 1995; Koob et al., 1992; Maldonado et al., 1992). None the less, several lines of evidence suggest that CRH actions in the BNST and CeA serve distinct roles. It has been suggested that CRH in the BNST coordinates responding to unconditioned stimuli or stressors (e.g., footshock) whereas CRH in the amygdala coordinates responding to learned or conditioned stimuli rendered dangerous via their association with such aversive stimuli or stressors (Davis and Shi, 1999). Among the evidence supporting this distinction are demonstrations that lesions of the BNST but not CeA block the ability of CRH to potentiate the rat’s acoustic startle response, and demonstrations that lesions of the CeA prevent potentiation of startle by conditioned fear whereas lesions of the BNST prevent potentiation of startle by unconditioned fear (Lee and Davis, 1997; Walker and Davis, 1997). Other evidence also supports this distinction. For example, CRH actions in BNST but not amygdala mediate the ability of footshock to reinstate extinguished drug seeking (Erb and Stewart, 1999). Because withdrawal from opiates is an unconditioned stimulus or stressor it follows from the literature reviewed above that any CRH contribution to this withdrawal might be mediated by actions in the BNST rather than the amygdala.

We studied the roles of BNST and CeA CRH in opiate withdrawal. First, we used in situ hybridization histochemistry to study the effect of chronic opiate administration and naloxone-precipitated withdrawal on CRH mRNA in the amygdala, BNST, and hypothalamus. Second, we studied the effects of infusions of a CRH receptor antagonist into the lateral ventricle, CeA, or BNST on behavioral, pain modulatory, and endocrine responses to opiate withdrawal. The results are internally consistent and lead us to a revised view of the role of the extended amygdala in opiate dependence.

EXPERIMENTAL PROCEDURES

Subjects

Male Sprague–Dawley rats (Charles River, Portage, MI, USA) weighing between 250 and 300 g were housed in a colony room maintained on a 12-h light/12-h dark cycle (lights on 7.00 h). Prior to surgery rats were housed in groups of three and after surgery they were singly housed. Food and water were freely available. There were eight to 10 rats per group at the start of each experiment other than Experiment 2, which had five or six rats per group. The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996).

Surgery and drugs

Rats were anesthetized using sodium pentobarbital (50 mg/kg, i.p.) supplemented where necessary by methoxyflurane. Stereotaxic coordinates were defined in millimeters relative to bregma (antero-posterior (AP), dorso-ventral (DV), and mediolateral (ML)). A 22-gauge guide cannula was lowered unilaterally into the lateral ventricle (AP, –0.5; ML, –0.8; DV, –4.0), CeA (AP, –2.4; ML, –4.1, and DV, –7.4), or the BNST (AP, –0.5; ML, –3.7; and DV, –5.6 at 15° from vertical). The guide cannula was anchored to the skull using jeweler’s screws and dental cement. A 28-gauge dummy cannula was kept in the guide cannula at all times other than during infusion. There were 5 days recovery following surgery. For pellet implantation rats were anesthetized using methoxyflurane and two 75-mg morphine or placebo pellets (NIDA, Bethesda, MD, USA) were implanted s.c. in the lower back. An s.c. injection of 1 mg/kg naloxone hydrochloride (Sigma, St. Louis, MO, USA) was used to precipitate withdrawal in Experiments 1 and 4, and 3 mg/kg to precipitate withdrawal in Experiment 2. A higher dose of naloxone was used in Experiment 2 to maximize any changes in CRH mRNA associated with morphine withdrawal. The variation in naloxone dose between Experiment 2 and the remaining experiments does not preclude comparisons between them. Rather, if the results converge on some common theme (e.g., a role for one structure but not another) despite variations in naloxone dose, then the generality and robustness of the effect will be enhanced.

oteCH2±41 (Peninsula Laboratories, San Diego, CA, USA) was dissolved in artificial cerebrospinal fluid (aCSF) glucose 7.1 mM, NaCl, 126.6 mM; NaHCO3, 27.4 mM, KCl 2.4 mM, KH2PO4 0.5 mM, NaHPO4 0.48 mM, MgCl2 0.8 mM, CaCl2 0.89 mM; pH 7.4), and infused at doses of 1 µg, 3 µg, or 5 µg. aCSF was used for control infusions. A 28-gauge microinjection cannula, which projected a further 1 mm ventral to the tip of the guide cannula, was inserted and connected to a 5-µl glass syringe operated by an infusion pump. Infusions into the lateral ventricle were delivered in a volume of 3 µl and infusions into the CeA and BNST in a volume of 1 µl, across a 2-min period. The microinjection cannula was left in place for a further minute to permit diffusion.

Apparatus

Plastic buckets (24 cm diameter × 50 cm height) were used as behavioral testing chambers. A 10% alcohol solution was used to clean the buckets between animals. The tailflick apparatus consisted of a waterbath (47 cm long × 26 cm wide × 21 cm high) whose temperature was controlled at 51°C (± 0.5°C).

Protocols

Experiment 1: Characterization of the behavioral, pain modulatory, and corticosterone responses during naloxone-precipitated morphine withdrawal. On day 1 rats were implanted with morphine or placebo pellets. Twice on each of days 3 and 4 rats were familiarized with the handling procedures and test apparatus. On day 5 rats were placed in the plastic buckets and injected 5 min later with naloxone or saline. This design yielded four groups: placebo–saline, morphine–saline, placebo–naloxone, and morphine–naloxone. Rats were then observed for the following signs of opiate withdrawal: escape jumps, wet dog shakes, teeth chattering, diarrhea, profuse salivation, secretions from the eyes or nose, ptosis, ejaculation, postural abnormalities, and irritability on handling. The number of escape jumps was recorded whereas for the other signs of withdrawal the observer simply indicated their presence or absence. Fifteen minutes later rats were tested for tailflick latency by immersing the distal 4-cm portion of the tail in the waterbath. Tailflick testing was repeated a further three times at 30, 45, and 60 min follow-
ing naloxone injection. At 7.30 h on day 8, rats were briefly removed from their home cages, injected s.c., and returned to those cages. Rats that had been injected previously (day 5) with s.c. saline were now injected with naloxone, whereas rats that had previously been injected with s.c. naloxone were now injected with saline. This crossover design was used to ensure that measures of corticosterone for rats in the withdrawal group (groups morphine-naloxone) were obtained from animals during their first episode of withdrawal rather than from rats with a history of withdrawal episodes. Blood was collected via nicking the tail vein 15 min, 30, 60, and 240 min later.

**Experiment 2: Changes in CRH mRNA associated with morphine withdrawal.** On day 1 rats were implanted with morphine base or placebo pellets. On day 5 rats were briefly removed from their home cages, injected with naloxone or not injected, and returned to their home cages. This design also yielded four groups: placebo alone (n = 6), morphine alone (n = 6), placebo-naloxone (n = 5) and morphine-naloxone (n = 6). Four hours later all rats were rapidly decapitated directly after being removed from their home cages. Brains were removed and frozen in isopentane cooled to between −80°C and −50°C and were then stored at −80°C until processing for *in situ* hybridization histochemistry.

**Experiments 3–5: Effects of infusions of ghCRH44 into the lateral ventricle (Experiment 3), amygdala (Experiment 4) or BNST (Experiment 5) on morphine withdrawal.** Morphine pellets were implanted 5 days later because of stereotaxic surgery. Twice on each of days 3 and 4 post-pellet implantation rats were familiarized with the test apparatus. On day 5 rats were placed in the plastic buckets and 5 min later they received an infusion of either aCSF, 3 μg ghCRH44, or 5 μg ghCRH44, i.c.v. (Experiment 3); or aCSF, 1 μg ghCRH44, or 3 μg ghCRH44 in the CeA or BNST (Experiments 4 and 5). Ten minutes later they were injected s.c. with naloxone. These designs yield three groups in Experiment 3 (morphine–aCSF, morphine–3 μg ghCRH44, morphine–5 μg ghCRH44) and six groups in Experiments 4 and 5 (placebo–aCSF, placebo–1 μg ghCRH44, placebo–3 μg ghCRH44, morphine–aCSF, morphine–1 μg ghCRH44, morphine–3 μg ghCRH44). Each group was injected on test with naloxone. There were seven to nine rats per group on test. Behavior was recorded as described above. Rats were tested in the tail-flick test 15 min later. Blood was collected via nicking the tail vein 15 min later (30 min post-naloxone).

The single-factor, three-group design of Experiment 3 does not permit direct comparison with placebo-pelleted control animals. There have been two well controlled studies on the effects of i.c.v. microinjections of CRH antagonists on the somatic opiate withdrawal syndrome (Lu et al., 2000; Brugger et al., 1998), and these studies have indicated that the actions of CRH antagonists are selective to the opiate withdrawal. The goal of Experiment 3 was simply to confirm the important findings from these studies in the preparation used here. Because the results of Experiment 1 indicated that injecting placebo-pelleted rats with saline has minimal impact on behavioral, hormonal, and pain modulatory responses, and because such placebo-pelleted control rats are included in Experiments 4 and 5, we considered this condensed design to be useful and consistent with the goal of minimizing the suffering and number of animals used.

**In situ hybridization histochemistry**

Sections 10 μm thick were cut on a cryostat and thaw-mounted on polylysine=coated slides. Tissue was air-dried and stored at −80°C until processing for *in situ* hybridization. Sections were collected from the BNST to the ventral hippocampus. Every 100 μm a section was collected for Cresyl Violet staining.

**Before in situ hybridization histochemistry, brain sections were placed in 4% phosphate-buffered paraformaldehyde and fixed for 1 h at room temperature. Sections were rinsed three times in 2× saline sodium citrate (SSC; 1×SSC = 150 mM sodium chloride and 15 mM sodium citrate). Slides were then placed in a solution of 0.1 M triethanolamine with 0.25% acetic acid at room temperature for 10 min. The tissue was rinsed in water and dehydrated through a series of alcohols. After air drying, the sections were hybridized with 32P-labeled cRNA probe. The rat CRH probe was 770 nucleotides long directed against the rat CRH mRNA coding region (GenBank accession number 412043) and was cloned in our laboratory. The probe was labeled in a reaction mixture consisting of linearized plasmid (1 μg), 1×transcription buffer (Epicerentic Technologies, Madison, WI, USA), 125 μCi [35S]UTP (Amersham, Arlington Heights, IL, USA), 125 μCi [35S]CTP (Amersham), 150 μM ATP, 150 μM GTP, 10 mM dithiothreitol (DTT), 20 U RNAse inhibitor, and 0.6 U appropriate RNA polymerase. The reactions were incubated at 37°C for 2 h. The resulting probes were separated from free nucleotides on a Sephadex G50-50 column. The probes were diluted in hybridization buffer (50% formamide; 10% dextran sulfate; 3×SSC; 50 mM sodium phosphate buffer, pH 7.4; 1× Denhardt’s solution; 0.1 mg/ml yeast RNA; and 10 mM DTT) to yield 106 c.p.m./70 μl. Diluted probes (70 μl) were placed on each slide and the sections coveredslipped. Slides were placed in plastic trays lined with filter paper dampened with 50% formamidex50% water. Trays were sealed and incubated at 55°C for 16 h. Coverslips were then floated off in 2×SSC, and the slides rinsed an additional three times in 2×SSC. Sections were then incubated in RNase A (200 μg/ml) at 37°C for 1 h and rinsed in 2×SSC, 1×SSC, 0.5×SSC. Tissue was washed to a final stringency of 0.1×SSC at 65°C for 1 h. Slides were then washed in distilled water, dehydrated through a series of alcohol, and air-dried. Sections were then exposed to Kodak XAR film (Eastman-Kodak, Rochester, NY, USA) for 3 (hypothalamus) or 7 days (amygdala and BNST). Specificity of the cRNA probe was tested by pre-treating sections for 1 h with RNase (200 μg/ml) or attempting to hybridize a sense ribo-probe from the same plasmid insert.

Optical density measurements were quantified from X-ray film using NIH Image software, with specific location of signal confirmed by comparison with the Cresyl Violet stained sections 50 μm from the target section. Measurements were taken from a series of eight to 12 sections through the rostral-caudal extent of the structures of interest, except the hypothalamus where measurements were based on a series of five to seven sections. The optical density values were corrected for background (by defining signal as that activation which exceeded 3.5 standard deviation units above mean gray value of background), multiplied by the total area of the signal (number of pixels) above background in the sample, and then averaged to produce one data point for each brain region per animal.

**Corticosterone radioimmunoassay**

Blood was collected into capillary tubes containing EDTA. Blood samples were then centrifuged at 2000 r.p.m. for 10 min. The resulting plasma was pipetted into chilled Eppendorf microcentrifuge tubes and stored at −20°C until assayed.

The assay was conducted using a specific antibody raised in our laboratory with less than 3% cross-reactivity with other steroids (Dr. D. Helmreich, University of Michigan, personal communication). Plasma samples were diluted 1:100 in 0.05 M sodium phosphate buffer containing 0.25% bovine serum albumin (pH 7.4). Corticosterone was released from binding protein by heat (70°C; 30 min). Duplicate samples of 100 μl, to which were added 50 μl of tracer [3H]corticosterone (Amersham; 50 Ci/mmol, 10000 c.p.m. per tube), and 50 μl of antibody (final concentration 1:12800), were incubated at 4°C overnight. The addition of 0.5 μl of chilled 1% charcoal/0.1% dextran mixture in buffer for 10 min at 4°C served to separate bound from free corticosterone. Samples were then centrifuged for 8 min at 3000 r.p.m. The supernatant was poured into 4 ml of scintillation fluid, and bound [3H]corticosterone was counted on a Packard CA2000 liquid scintillation analyzer and compared with a standard curve (0–80 μg/dl). The intra- and inter-assay variability coefficients were approximately 10%.
Histology

Correct ventricular placement was verified by the angiotensin drinking test. In brief, rats received an infusion of 9.2 pmol angiotensin II (Sigma) and were allowed access to water for 10 min. Rats which consumed less than 5 ml of water were excluded from the analysis. Unfixed brains from remaining experiments were sectioned coronally at 40 μm and stained with Cresyl Violet. The location of cannula tips was determined at the microscope according to the boundaries described by Paxinos and Watson (1985).

Statistical analysis

Withdrawal signs were converted into a global withdrawal score (Gellert and Holtzmann, 1978; Maldonado et al., 1992). A score was allocated to each sign of withdrawal and a global withdrawal score was obtained by summing these scores. For checked signs, a score of 2 was allocated to the presence of diarrhea, facial fasciculations/teeth chattering, and ptosis; a score of 3 allocated to postural abnormalities, ejaculation or genital grooming, and irritability/vocalization on handling; a score of 5 allocated to chromodacryorrhea, and a score of 7 was allocated if there were one or two wet dog shakes and a score of 4 allocated if there were three or more wet dog shakes; a score of 3 allocated to postural abnormalities, ejaculation or genital grooming, and irritability/vocalization on handling; a score of 5 allocated to chromodacryorrhea, and a score of 7 was allocated if there were one or two wet dog shakes and a score of 4 allocated if there were three or more wet dog shakes; a score of 2 was allocated to the presence of diarrhea, facial fasciculations/teeth chattering, and ptosis; a score of 3 allocated to postural abnormalities, ejaculation or genital grooming, and irritability/vocalization on handling; a score of 5 allocated to chromodacryorrhea, and a score of 7 was allocated if there were one or two wet dog shakes and a score of 4 allocated if there were three or more wet dog shakes.

RESULTS

Experiment 1: Characterization of the behavioral, pain modulatory, and corticosterone responses during naloxone-precipitated morphine withdrawal

The mean and S.E.M. global withdrawal scores for rats in Experiment 1 are shown in the left panel, mean and S.E.M. tailflick latencies in the middle panel, and mean and S.E.M. plasma corticosterone levels in the right panel of Fig. 1. Inspection of Fig. 1 indicates the presence of withdrawal behavior among rats in the morphine-naloxone group, accompanied by a clear hyperalgesia persisting for 30 min, and a potent and prolonged increase in plasma corticosterone.

The 2×2 ANOVA of global withdrawal scores revealed a significant main effect for type of pellet pretreatment (F(1,30) = 231.9; P < 0.0001), a significant main effect for injection on test of naloxone-saline (F(1,30) = 239.6; P < 0.0001), and a significant 2×2 interaction (F(1,30) = 223.6; P < 0.0001), indicating a differential effect of naloxone among morphine-pelleted rats. Post-hoc comparisons revealed what is obvious from the figure: rats undergoing morphine withdrawal (group morphine-naloxone) displayed a significantly greater withdrawal syndrome than rats in the remaining three groups (P < 0.05), which did not differ from each other (P > 0.05).

The 2×2 ANOVA of tailflick latencies revealed significant main effects for pellet pre-treatment (F = 5.8 and 14.9; P = 0.02 and 0.001) and significant two-way interactions (F = 4.7 and 8.5; P = 0.04 and 0.007) at the 15- and 30-min time points, indicating a differential effect of naloxone among morphine-pelleted rats. Analyses at other time points were not significant (all F < 3.4, all P > 0.05). Post-hoc comparisons at the 15- and 30-min time points revealed a significant hyperalgesia among rats undergoing morphine withdrawal as compared to each of the remaining three groups (P < 0.05), which did not differ from each other (P > 0.05).

The 2×2 ANOVA of plasma corticosterone levels revealed significant main effects for pellet pre-treatment (F = 33.8, 59.2, and 24.3; P < 0.0001), injection of naloxone versus saline (F = 41.2, 47.6, and 26.7; P < 0.0001), and significant two-way interactions (F = 70.9, 78.5, and 35.8; P < 0.0001) at the 30-, 120- and 240-min time points, indicating a differential effect of naloxone among morphine-pelleted rats. Post-hoc comparisons at these time points again confirmed what is obvious from the figure: rats undergoing morphine withdrawal (group morphine-naloxone) had significantly higher levels of...
Fig. 2. Color autoradiographs showing CRH mRNA in representative sections from the CeA (top panels), BNST (middle panels), or hypothalamus (bottom panels) for rats in Experiment 2. Rats were pre-treated with either morphine or placebo pellets and injected on test with naloxone or not injected.
plasma corticosterone than rats in the remaining three groups \((P<0.05)\), which did not differ from each other \((P>0.05)\).

**Experiment 2: Changes in CRH mRNA associated with morphine withdrawal**

Figure 2 shows the distribution of CRH mRNA in representative sections through the CeA (panel A), BNST (panel B), and hypothalamus (panel C) for rats in each of the four groups. Figure 3 shows the mean and S.E.M. optical density measurements of CRH mRNA in these structures.

Inspection of Figs. 2 and 3 reveals that neither chronic morphine nor morphine withdrawal affected BNST CRH mRNA, but that these manipulations as well as a single injection of naloxone up-regulated CeA CRH mRNA, and finally that morphine withdrawal selectively up-regulated paraventricular nucleus of the hypothalamus (PVN) CRH mRNA. The \(2 \times 2\) ANOVA of CeA CRH mRNA optical densities revealed a significant main effect for type of pellet pre-treatment \((F(1,19)=6.5; P=0.01)\) but not injection of naloxone \((F(1,19)=1.7; P>0.05)\). Importantly, there was a significant \(2 \times 2\) interaction \((F(1,19)=10.2; P=0.005)\) which indicates a differential effect of naloxone and saline among placebo-pelleted rats. Post-hoc comparisons showed that rats in groups morphine alone, placebo–naloxone, and morphine–naloxone each had significantly higher levels of CRH mRNA in the CeA than rats in the group placebo alone \((P<0.05)\) but that these groups did not differ from each other \((P>0.05)\). The \(2 \times 2\) ANOVA of BNST CRH mRNA optical densities failed to detect a significant effect for pellet treatment \((F(1,19)=1.8; P>0.05)\), injection of naloxone \((F(1,19)<1; P>0.05)\), or a significant \(2 \times 2\) interaction \((F(1,19)=1.7; P>0.05)\). The \(2 \times 2\) ANOVA of PVN CRH mRNA optical densities revealed a significant main effect for type of pellet pre-treatment \((F(1,19)=11.4; P=0.004)\) but not injection of naloxone \((F(1,19)=2.1)\). Importantly, there was a significant \(2 \times 2\) interaction \((F(1,19)=9.2; P=0.005)\) indicating a differential effect of naloxone among morphine-pelleted rats. Post-hoc comparisons showed that rats undergoing morphine withdrawal had significantly higher levels of PVN CRH mRNA compared to rats in the remaining three groups \((P<0.05)\), which did not differ from each other \((P>0.05)\).

**Experiment 3: Effects of an infusion of \(\alpha hCRH_{9-41}\) into the lateral ventricle on morphine withdrawal**

The mean and S.E.M. global withdrawal scores, tail-flick latencies, and plasma corticosterone levels following i.c.v. infusions of aCSF or \(\alpha hCRH_{9-41}\) are shown in Fig. 4. Inspection of the figure indicates that i.c.v. infusions of \(\alpha hCRH_{9-41}\) reduced global withdrawal scores...
Fig. 5. Location of cannula microinjection tips in Experiment 4 (A) and Experiment 5 (B).
(left panel) and plasma corticosterone responses (right panel) but not hyperalgesia (middle panel) during morphine withdrawal.

The ANOVA revealed a significant main effect on global withdrawal scores of i.c.v. infusion \( (F(2,19) = 6.8; \ P = 0.001) \). Post-hoc comparisons showed that rats receiving an i.c.v. infusion of either 3 or 5 \( \mu \)g of \( \alpha \)CRH SDS had significantly lower global withdrawal scores than rats receiving an i.c.v. infusion of aCSF \( (P < 0.05) \), but there was no effect for dose of the antagonist \( (P > 0.05) \). Inspection of the data indicated that the i.c.v. infusions of \( \alpha \)CRH SDS reduced the number of escape jumps as well as the frequency of other signs of withdrawal (e.g., salivation, ptosis, irritability). Indeed, the ANOVA revealed a significant overall effect for infusion on the number of escape jumps \( (F(2,19) = 3.4; \ P = 0.05) \). The post-hoc analysis showed that rats infused with 5 \( \mu \)g of \( \alpha \)CRH SDS each had significantly fewer escape jumps than rats receiving an i.c.v. infusion of aCSF \( (P < 0.05) \) but there was no difference in escape jumps between the aCSF-infused rats and the rats infused with 3 \( \mu \)g of \( \alpha \)CRH SDS \( (P > 0.05) \). The \( \chi^2 \) analysis revealed a significant difference in the frequency of salivation (\% of subjects in group morphine-aCSF = 43; \% of subjects in group morphine-3 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS = 0; \% of subjects in group morphine-5 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS mean = 0) \( (P < 0.01) \), ptosis (\% of subjects in group morphine-aCSF = 86; \% of subjects in group morphine-3 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS = 14; \% of subjects in group morphine-5 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS mean = 13) \( (P < 0.01) \) and irritability (\% of subjects in group morphine-aCSF = 100; \% of subjects in group morphine-3 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS = 43; \% of subjects in group morphine-5 \( \mu \)g \( \alpha \)CRH SDS-\( \beta \)CRH SDS mean = 38) \( (P < 0.03) \) such that each of these signs of morphine withdrawal was reduced among rats receiving i.c.v. infusions of \( \alpha \)CRH SDS.

The ANOVA failed to demonstrate a significant effect for i.c.v. infusions on tail-flick latencies \( (F < 1; \ P > 0.05) \). However, it did reveal a significant effect for i.c.v. infusions on plasma corticosterone levels \( (F = 8.9; \ P < 0.001) \). The post-hoc analysis showed that rats infused with either 3 \( \mu \)g or 5 \( \mu \)g of \( \alpha \)CRH SDS each had significantly lower plasma corticosterone levels than rats receiving an i.c.v. infusion of aCSF \( (P < 0.05) \) but did not differ from each other \( (P > 0.05) \).
Experiment 4: Effects of an infusion of $\alpha$hCRH$_{9-41}$ into the amygdala on morphine withdrawal

Figure 5A depicts the location of the microinjection cannula tips for rats in Experiment 4. Three rats were excluded from the analysis, one from each of groups placebo–1 µg $\alpha$hCRH$_{9-41}$, morphine–1 µg $\alpha$hCRH$_{9-41}$, and morphine–3 µg $\alpha$hCRH$_{9-41}$, due to incorrect cannula placement.

The mean and S.E.M. global withdrawal scores, tail-flick latencies, and plasma corticosterone levels following amygdala infusions of aCSF or $\alpha$hCRH$_{9-41}$ are shown in the top three panels of Fig. 6. Inspection of the figure indicates that amygdala infusions of $\alpha$hCRH$_{9-41}$ reduced global withdrawal scores (left panel) as well as the withdrawal-associated hyperalgesia (middle panel) but failed to affect plasma corticosterone responses (right panel).

The 2×3 factorial ANOVA of global withdrawal scores revealed significant main effects for type of pellet pre-treatment (placebo–morphine) ($F_{(1,49)}=595.8; P<0.0001$) and intra-amygdala infusion ($F_{(2,49)}=3.9; P=0.02$), as well as a significant two-way interaction ($F_{(2,49)}=3.5; P=0.04$), indicating a differential effect of the CRH antagonist among morphine-pelleted rats. Post-hoc analysis indicated that each of the morphine-pelleted groups had significantly higher global withdrawal scores than placebo-pelleted groups ($P<0.05$).

Importantly, this analysis showed that rats infused with 1 or 3 µg of $\alpha$hCRH$_{9-41}$ had significantly lower global withdrawal scores than rats infused with aCSF ($P<0.05$), but that there was no effect of antagonist dose ($P>0.05$). This decrease in global withdrawal scores following amygdala infusions of $\alpha$hCRH$_{9-41}$ was most consistently reflected by a reduction in escape jumping (see inset in Fig. 6). Indeed, the 3×2 ANOVA of escape jumps revealed an overall significant main effect for escape jumps with morphine versus placebo ($F=32.7; P<0.01$), as well as an overall significant main effect for intra-amygdala infusions ($F=5.6; P=0.006$), and a significant two-way interaction ($F=5.9; P=0.005$). The post-hoc analysis showed rats pre-treated with morphine and infused with 1 or 3 µg of $\alpha$hCRH$_{9-41}$ each had significantly fewer escape jumps than rats pre-treated with morphine and infused with aCSF ($P<0.05$), but that there was no effect for antagonist dose ($P>0.05$). Although other signs of opiate withdrawal were reduced in some animals, the $\chi^2$ analysis failed to detect any significant differences in the frequency of any other signs of withdrawal (all $P>0.05$).

The 2×3 factorial ANOVA of tail-flick latencies revealed a significant main effect for type of intra-amygdala infusion ($F=4.4; P=0.01$) but not pellet pre-treatment ($F=1.7; P>0.05$). Importantly, there was a significant two-way interaction ($F=7.3; P=0.002$), indicating a differential effect of the CRH antagonist among morphine-pelleted rats. The post-hoc analysis showed that rats pre-treated with morphine and infused with aCSF were hyperalgesic relative to rats in each of the placebo-pelleted groups ($P<0.05$) and that this hyperalgesia was abolished by infusion of 3 µg $\alpha$hCRH$_{9-41}$ ($P<0.05$) returning tail-flick latencies to the level of placebo-pelleted rats ($P>0.05$).

The 2×3 factorial ANOVA of plasma corticosterone levels revealed a significant main effect for type of pellet pre-treatment ($F=23.9; P<0.0001$) but not intra-amygdala infusion ($F=1.0; P>0.05$) or a significant two-way interaction ($F<1; P>0.05$). Thus, although withdrawal from morphine was associated with elevated plasma corticosterone levels, intra-amygdala infusions of $\alpha$hCRH$_{9-41}$ were without effect on plasma corticosterone levels.

Experiment 5: Effects of an infusion of $\alpha$hCRH$_{9-41}$ into the BNST on morphine withdrawal

Figure 5B depicts the location of the microinjection cannula tips for rats in Experiment 5. Six rats were excluded from the analysis due to incorrect cannula placement, one from each of groups placebo–3 µg $\alpha$hCRH$_{9-41}$, morphine–aCSF, morphine–1 µg $\alpha$hCRH$_{9-41}$, and morphine–3 µg $\alpha$hCRH$_{9-41}$, and two rats from group placebo–1 µg $\alpha$hCRH$_{9-41}$.

The mean and S.E.M. global withdrawal scores, tail-flick latencies, and plasma corticosterone levels following BNST infusions of aCSF or $\alpha$hCRH$_{9-41}$ are shown in the bottom three panels in Fig. 6. Inspection of the figure indicates that BNST infusions of $\alpha$hCRH$_{9-41}$ were without effect on global withdrawal scores (left panel), withdrawal-associated hyperalgesia (middle panel), and plasma corticosterone responses (right panel).

There was a significant main effect for type of pellet pre-treatment (placebo vs. morphine) ($F_{(1,41)}=760.2; P<0.0001$) but not intra-BNST infusion ($F_{(2,41)}<1; P>0.05$). The two-way interaction was also not significant ($F_{(2,41)}<1; P>0.05$). Thus, although morphine-pre-treated rats displayed a withdrawal syndrome, the severity of this syndrome was not affected by intra-BNST infusions of $\alpha$hCRH$_{9-41}$.

The 2×3 factorial ANOVA of tail-flick latencies also revealed a significant main effect for type of pellet pre-treatment ($F=19.9; P<0.0001$) but not intra-BNST infusion ($F<1; P>0.05$). The two-way interaction was also non-significant ($F<1, P>0.05$). Thus, although morphine-pre-treated rats displayed a withdrawal-associated hyperalgesia, this hyperalgesia was not affected by intra-BNST infusions of $\alpha$hCRH$_{9-41}$.

The 2×3 factorial ANOVA of plasma corticosterone levels revealed a significant main effect for type of pellet pre-treatment ($F=31.6; P<0.001$) but not intra-BNST infusion ($F<1; P>0.05$). The two-way interaction was again non-significant ($F<1; P>0.05$). Thus, although withdrawal from morphine was associated with elevated plasma corticosterone levels, this was not affected by intra-BNST infusions of $\alpha$hCRH$_{9-41}$.

**DISCUSSION**

Injected with naloxone, morphine-dependent rats displayed a variety of withdrawal-related behaviors (e.g., escape jumping, wet dog shakes), as indexed by increased...
global withdrawal scores, which were accompanied by hyperalgesia as well as a potent and prolonged increase in plasma corticosterone levels. This withdrawal syndrome was accompanied by changes in CRH mRNA. Specifically, CRH mRNA was up-regulated in the central nucleus of the amygdala following opiate withdrawal, chronic morphine, or injection of naloxone. CRH mRNA was also selectively up-regulated in the PVN following morphine withdrawal. There was no change in BNST CRH mRNA in any of the groups. There was evidence that morphine withdrawal symptoms depended, at least in part, upon CRH actions in multiple brain regions. Specifically, an i.c.v. infusion of the CRH receptor antagonist 1,4,7-trisnor-endo-CRH9-41 reduced the severity of the global withdrawal syndrome, as indexed by reductions in escape jumping, multiple autonomic indices of withdrawal (e.g., salivation, ptosis), and levels of plasma corticosterone. These effects of the i.c.v. antagonist were mediated, at least in part, via actions in the amygdala because infusions of 1,4,7-trisnor-endo-CRH9-41 into the amygdala also reduced the severity of this withdrawal syndrome as indexed by reductions in withdrawal-associated escape jumping and hyperalgesia. By contrast, there was no evidence here that BNST CRH receptors contributed to either the behavioral, pain modulatory, or endocrine consequences of morphine withdrawal because infusions of 1,4,7-trisnor-endo-CRH9-41 into the BNST were without effect on each of these end points. Importantly, the actions of the 1,4,7-trisnor-endo-CRH9-41 in the amygdala were restricted to rats pre-treated with morphine and injected on test with naloxone, were dose-dependent on at least one measure, were not observed in rats with incorrect cannula placement, and did not include the effects on autonomic and endocrine function observed following i.c.v. infusions, indicating that they cannot be attributed to non-selective actions of the antagonist. Together, these results provide compelling evidence that CRH contributes to morphine withdrawal and that this contribution occurs in an anatomically specific fashion. These experiments confirm recent reports that CRH receptor antagonists reduce the severity of morphine withdrawal (Brugger et al., 1998; Iredale et al., 2000). Specifically, the effects of i.c.v. infusion of a CRH receptor antagonist on morphine withdrawal reported here were similar to those reported elsewhere. However, these experiments are the first to identify an important locus of action for these effects of i.c.v. infusions of CRH antagonists. Specifically, these experiments have indicated that the effects of i.c.v. CRH antagonists are mediated, at least in part, by their actions in the amygdala. Moreover, the present experiments are the first to document changes in the expression level of CRH mRNA during opiate withdrawal and to relate these changes to aspects of opiate dependence and the withdrawal syndrome. Thus, CRH mRNA was increased in the PVN during opiate withdrawal and consistent with this increase, an i.c.v. infusion of the CRH receptor antagonist reduced the withdrawal-associated rise in plasma corticosterone levels. CRH mRNA in the CeA was increased during opiate dependence and remained elevated during withdrawal. Indeed, it appears that a block-ade or down-regulation of the opioid receptor function sets the stage for an up-regulation of CRH expression in the CeA (see below). Importantly, experiments showed that CRH neurotransmission in the amygdala was related to the behavioral and pain modulatory consequences of morphine withdrawal because an infusion of the CRH receptor antagonist prevented withdrawal-associated hyperalgesia and escape jumping.

It could be suggested that it is more appropriate to compare the effects of CRH receptor antagonism during naloxone-precipitated morphine withdrawal with morphine-dependent rats not undergoing morphine withdrawal. That is, it could be suggested that the micro-injections of the CRH antagonist in Experiments 3–5 were effective in alleviating opiate withdrawal because they disrupted some process not critical for the generation of opiate withdrawal per se. We consider this possibility unlikely for four reasons. Firstly, previous experiments have shown that infusions of the CRH antagonist do not affect basal levels of activity or pain sensitivity in rats under conditions similar to those used here (Kalin et al., 1998). Thus, it is unlikely that the amygdala infusions of the antagonist affected the withdrawal-associated hyperalgesia or escape jumping through some non-specific mechanism. Secondly, Experiment 1 showed clearly that the behavioral, pain modulatory, and endocrine measures used were only sensitive to morphine withdrawal: neither chronic morphine nor injection of naloxone affected these end points. Thus, it is not clear how alterations in these end points by the CRH antagonist can be explained except with reference to an influence on morphine withdrawal. Thirdly, the effects of infusions of the CRH receptor antagonist into the amygdala during morphine withdrawal were similar to the effects of lesions of this structure during morphine withdrawal (Calvino et al., 1979). Finally, the effects of infusions of the CRH receptor antagonist into the amygdala during morphine withdrawal are consistent with the known role of this structure in the organization of defensive behavior and pain modulation (McNally, 1999). These findings confirm selectivity of action. When combined with previous reports that CeA CRH contributes to withdrawal-induced place aversion learning (Heinrichs et al., 1995), we can suggest a common role for CeA CRH in the behavioral, pain modulatory, and aversive motivational consequences of opiate dependence.

Interestingly, these experiments suggest that opioid activity in the amygdala controls CRH gene expression thereby setting the stage for increased CRH function during opiate dependence. Specifically, a single injection of naloxone in a placebo-treated animal selectively and rapidly increased CRH gene expression in the CeA. The absence of any effect of this injection of naloxone in placebo-treated animals in the other brain regions studied (e.g., PVN, BNST) indicates that this change in amygdala CRH gene expression cannot be explained by non-specific aspects of the experimental procedure (e.g., the stress of the injection ritual). Instead, this finding suggests that there is an endogenous opioid tone inhibiting the expression of CRH which is restricted to the
amarygdala and that this inhibition may be constitutively active because the effects of opioid receptor antagonism on CRH gene expression were apparent 4 h following injection. This is the first time that such a regulation of amygdala CRH gene expression by an opioid manipulation has been reported. In addition, a similar up-regulation of CRH gene expression was observed following chronic opioid agonist treatment (groups morphine alone and morphine-naloxone). It is well established that chronic morphine reduces opioid neurotransmission via adaptation at multiple levels of signal transduction (for review see Nestler, 1992). Indeed we have shown that under present conditions there is almost complete tolerance to an injection of morphine in morphine-pelleted rats as indexed by the tail-flick test (G.P. McNally and H. Akil, unpublished observations, see also Maldonado et al., 1996). Moreover, the lack of differences in tail-flick latencies in the absence of naloxone (Experiment 1), and the presence of hyperalgesia among rats undergoing morphine withdrawal, is consistent with the claim that the administration regime employed here results in almost complete adaptation in the opioid system. In other words, reductions in opioid neurotransmission, achieved either through antagonist administration or through chronic agonist-induced adaptation, may result in a subsequent increase in CRH neurotransmission. It is known that increases in cAMP and the cAMP-response element-binding protein (CREB) increase the transcriptional activation of CRH (Itoi et al., 1996). Thus, we hypothesize that endogenous opioids or acute injection of morphine, by activating the G_i/G_o-coupled μ-opioid receptor (MOR) and decreasing cAMP and CREB levels (for review see McNally and Akil, 2002), would lead to decreased CRH mRNA expression, whereas blockade or down-regulation of this signaling during chronic opiate administration would lead to an increased transcriptional activation of CRH. This hypothesis is consistent with the obvious functional opposition between anxiogenic actions of CRH and anxiolytic actions of opioids in the amygdala (Good and Westbrook, 1995; Swiergel et al., 1993; Takahashi et al., 1989).

Anatomical studies have begun to shed light on the relationship between the opioids and CRH in the amygdala. Specifically, we have recently shown that within the CeA pro-enkephalin and CRH mRNA are both co-expressed with GABA mRNA but in different GABAergic neurons (Day et al., 1999). Moreover, these two cell populations are differentially activated following immune stress (Day et al., 1999). By contrast to the peptide precursors, less is known about the receptors. In the amygdala the majority of cells expressing the CRH-R1 receptor, the critical receptor subtype mediating opiate withdrawal (Iredale et al., 2000), are located in the basolateral nucleus (De Souza and Grigoriadis, 1995). However, the MOR is expressed in the CeA (Mansour et al., 1995). Thus, it is possible that CeA MOR would be expressed in CRH-positive neurons, thereby mediating the effects of opioids in the CRH. It should be noted that the distinctive CRH-positive and enkephalin-positive GABAergic populations also exist in the BNST (Day et al., 1999). However, the pattern of expression of the relevant receptors remains unknown in that structure as well.

Two caveats bear on interpretation of the relationship of CRH mRNA levels to opiate dependence. First, opioid regulation of CRH activity and gene expression is likely to be a necessary but not sufficient condition for the generation of specific sequelae of dependence. In the present experiments CRH mRNA was up-regulated across each of the opiate manipulations (chronic agonist, acute antagonist, or chronic agonist and acute antagonist) yet signs of withdrawal attenuated by a CRH receptor antagonist were observed only in rats subjected to chronic morphine and acute injection of naloxone. This raises the interesting possibility that the actions of at least one other transmitter/peptide system in the amygdala subsequent to CRH activity may be necessary for production of the behavioral (e.g., escape jumping) and pain modulatory (e.g., hyperalgesia) responses indicative of opiate withdrawal. One candidate for this downstream signaling is noradrenaline because its actions in the amygdala contribute critically to the end points of opiate withdrawal measured here (Taylor et al., 1998). Second, this opioid regulation of CRH transcription may not occur with all opiate agonists or administration regimes. For example, Zhou et al. (1996) failed to detect changes in amygdala or hypothalamic CRH mRNA following chronic methadone treatment in rats. The procedural differences between these experiments as well as differences in the pharmacology of morphine and methadone temper speculation for the cause of this discrepancy. Clearly, further research into the factors governing alterations in the expression of non-opioid genes during opiate dependence, so-called between-systems adaptation, is required. The present experiments, as well as recent investigations (Bohn et al., 2000), suggest that these alterations may provide important insights into the nature of plasticity and causal mechanisms for opiate dependence.

The present experiments cannot exclude the involvement of BNST CRH in the aversive motivational properties of morphine withdrawal and this is worth further investigation. However, it is worth recalling that Milanes et al. (1998) failed to detect any change in BNST CRH peptide content during morphine withdrawal but did detect significant changes in PVN CRH peptide content. Similarly, the present experiments did not detect a significant alteration in CRH gene expression in the BNST during opiate withdrawal, despite such changes in gene expression being apparent in the amygdala and PVN. These findings argue against a role for BNST CRH in morphine withdrawal. Accordingly, these data bear directly on the roles accorded CRH in the BNST and CeA in responding to unpredictable (i.e., unconditioned) and predictable (i.e., conditioned) aversive stimuli or stressors. The evidence in the literature to date indicates that CRH actions in the BNST coordinate responding to unconditioned stimuli or stressors (e.g., footshock) whereas these actions in the CeA coordinate responding to learned or conditioned stimuli rendered dangerous or fearful by their association with such stressors (Erb and
Instead, it is worth considering whether the location of the stressor, interoceptive versus exteroceptive, may also determine BNST versus amygdala CRH activity. Interestingly, previous dissociations of the role of amygdala and BNST CRH have been based on the use of stressors in the animal’s external milieu, such as footshock, bright lights, or acoustic stimuli. Very little attention has been directed towards examining the generality of these results to stressors in the animal’s internal milieu, such as opiate withdrawal or illness and infection. In particular, the CeA may be especially sensitive to autonomic input via the nucleus tractus solitarius (Saper, 1995), and the enkephalin population may be uniquely responsive to such input. Thus, we suggest that chronic opiate dependence and withdrawal may trigger altered autonomic responses which access the CeA enkephalinergic population, down-regulate signaling via opioid receptors, thereby disinhibiting transcriptional activation of CRH in the CeA.

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