Pharmacology Biochemistry & Behavior, Vol. 32, pp. 919–926. Maxwell Pergamon Macmillan plc, 1989. Printed in the U.S.A. 0091-3057/89 $3.00 + .00

CRH and α-Helical-CRH Modulate Behavioral Measures of Arousal in Monkeys

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Received 27 June 1988

WINSLOW, J. T., J. D. NEWMAN AND T. R. INSEL. *CRH and α-helical-CRH modulate behavioral measures of arousal in monkeys*. PHARMACOL BIOCHEM BEHAV **32**(4) 919–926, 1989.—Several neuropeptides involved in the control of pituitary-adrenal activation have also been shown to have behavioral effects which may be mediated by actions on brain mechanisms independent of pituitary release. The behavioral effects of intraventricular administration of CRH and the synthetic peptide antagonist α-helical-CRH were assessed in socially separated squirrel monkeys. Treated monkeys were presented with a sequence of behavioral challenges including undisturbed social separation, presentation of a mirror image, and presentation of a "predator" stimulus. The test sequence was repeated at several time intervals after administration of the peptides. CRH produced dose-related increases in several species-typical measures of arousal including motor activity, vigilance-checking, and marking. Pretreatment with α-helical-CRH prevented the increased motor activity but not the marking behavior associated with CRH. When administered alone, α-helical-CRH increased vigilance-checking. In addition, α-helical-CRH increased aggressive behaviors exhibited at the mirror stimulus. The data provide further support for a central role for CRH in the mediation of both activational and inhibitory behavioral responses to stressful stimuli. These data also suggest both antagonistic and partial agonist effects for α-helical-CRH.

Corticotropin-releasing hormone Squirrel monkeys Stress Arousal Motor behavior Anxiety Aggression

INTEREST in the behavioral effects of neuropeptides has focused on differentiating between their effects on pituitary hormone release and their direct biochemical and electrophysiological actions in the CNS (34). Several peptides have well characterized modulatory influence on behavior independent of their hypothalamic effects on pituitary release (13,16). This may also be true for corticotropin-releasing hormone (CRH) (28). Immunoreactive CRH and CRH binding sites have been located in brain areas outside those associated with direct control of pituitary release in rat (12, 40, 43), cynamologous monkeys (32) and squirrel monkeys [(36), Winslow and Insel, in preparation]. Initial studies of CRH have demonstrated pronounced motor activation and "anxiogenic-like" effects which may represent central actions of this peptide (1, 3, 15, 24, 25). Intraventricular (ICV) administration of synthetic CRH to rats affected measures of emotionality and "anxiety" such as decreased open-field activity, prolonged habituation to a novel environment, and enhanced suppression of punished behavior (4,45). These effects are due to central as opposed to pituitary activation since the behavioral activation produced by ICV CRH was unaffected by pretreatment with dexamethasone (5), or by hypophysectomy (15), but was significantly reduced following administration of the anxiolytic chlordiazepoxide (3). A recently synthesized fragment of CRH, α-helical-CRH (9–41), effectively blocks CRH-induced pituitary release of ACTH in rats (39). ICV administration of α-helical-CRH has also been reported to attenuate stress-induced changes in the exploratory behavior of mice (2), and feeding (27), shock-induced fighting (46) and freezing (26) in rats, providing further evidence of a role for CRH in controlling the behavioral response to stress.

The characterization of the behavioral effects of ICV CRH in primates is more complex. Compared to rodents, primates show a substantially different distribution of brain CRH [(36), Winslow and Insel, in preparation] and the behavioral response to ICV CRH appears to depend on the test conditions (23,24). Rhesus monkeys exhibited increased struggling, visual checking, and vocalization following ICV administration of CRH when tested in a restraining chair. However, the same monkeys showed increased huddling and lying down when observed in the home cage. These latter behaviors were compared to the "despair" response exhibited by macaque monkeys following peer or mother-infant separations (23).

We investigated the effect of the central administration of CRH and a synthetic antagonist, α-helical-CRH, on the response of individual squirrel monkeys to provocative stimuli presented during brief separations from peer social housing. Studies in a similar setting have demonstrated significant behavioral agitation associated with increased pituitary adrenal activity (8). The vocal and motor behavior of monkeys in this setting have been shown to be differentially sensitive to the social status of the separated monkey (10), presentation of "predatory" stimuli (8), and pharmacological manipulations (18–20, 49). Three stimulus conditions were designed to evoke differential levels of arousal and stimulus-specific behaviors. The objective of these experiments was to assess the action of intraventricular CRH and α-helical-CRH in the presence of stimuli which evoke measurable, *species-typical* behaviors.

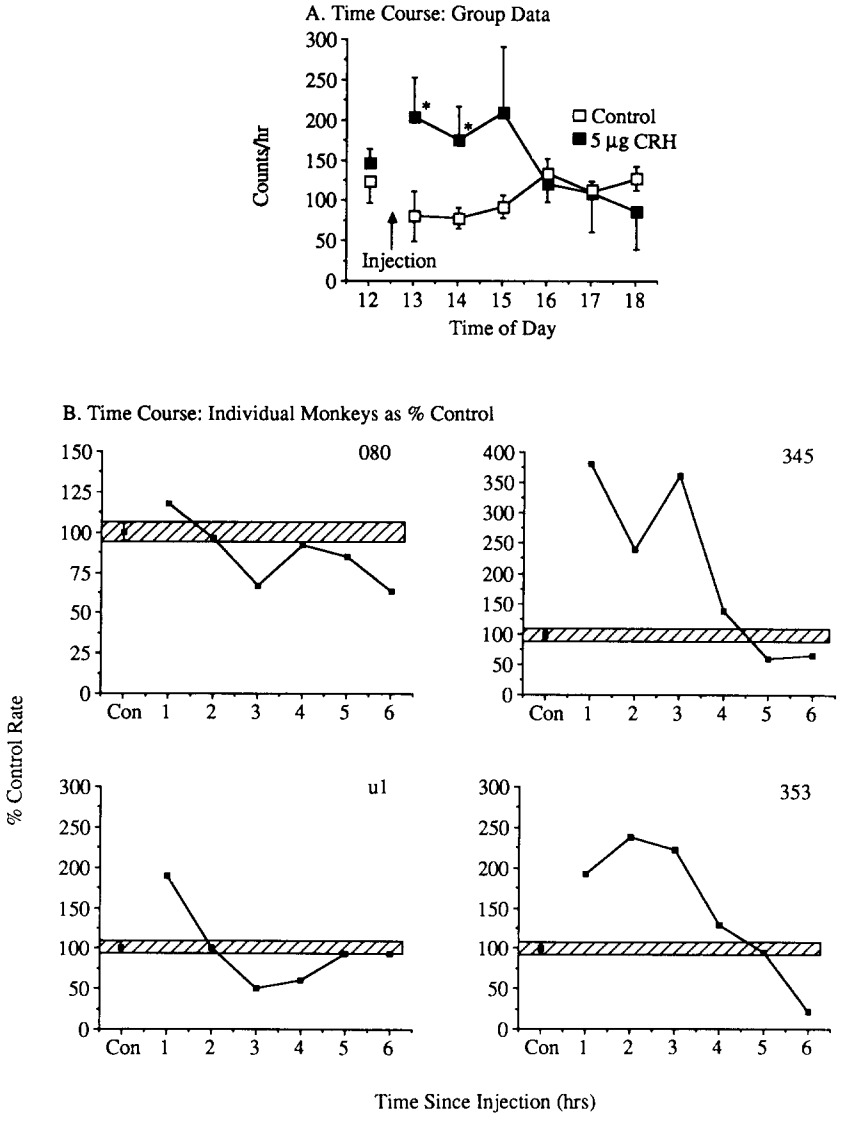

FIG. 1. (A) The total counts per hour reported by portable activity monitors mounted in vests worn by four individually housed squirrel monkeys in their home cages. Open data points represent the mean for four monkeys measured during the 5 days prior to administration of CRH. Filled data points represent the group means measured after administration of 5 μg CRH. Vertical lines represent ± 1 s.e.m. Asterisks refer to Dunnett's *t*-test comparisons of each time point with the pretreatment activity count, $p<0.05$. (B) Measures derived from individual monkeys treated with CRH. Values represent activity as a percent of average performance during previous baseline trials. Control values are represented by a horizontal bar and refer to the mean and ± 1 s.e.m. across six hours and are provided as a measure of within subject variability.

METHOD

Subjects

Four adult male squirrel monkeys (*Saimiri sciureus*) of the gothic arch variety and weighing 800–1200 g were used. Monkeys were individually housed in a primate vivarium with controlled temperature (28–30°C) and light cycle (lights on from 7:00–19:00 hr). Home cages were equipped with free access to food and water, and permitted full visual, auditory, and extensive tactile contact with other monkeys.

Surgery

Anesthetized (Somnifer, 20 mg/kg, IP) squirrel monkeys were placed in a Horsely-Clark stereotaxic instrument and secured with ear bars and eye pieces. Stainless steel guide cannulae (28 gauge, Plastic Products) were lowered unilaterally into the lateral ventricle [10.0 mm anterior to interaural zero; ±2.0 mm lateral; 20.0 mm below dura; (17)] and covered with a threaded stylette to prevent contamination.

Behavioral Testing

In a preliminary experiment to determine time course, monkeys were dressed in vests equipped with portable activity monitors with solid state memory. Activity monitors measured and recorded movement in three dimensions as function of rate of switch

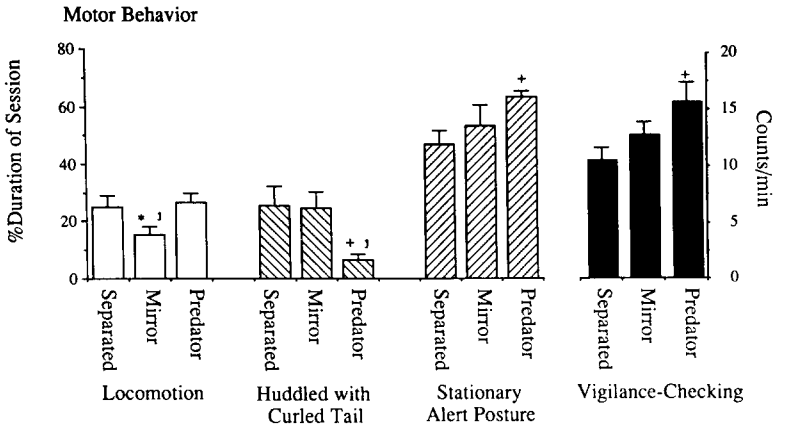

FIG. 2. The distribution of time spent in various motor activities during baseline trials. Columns represent the mean of four monkeys in two vehicle sessions expressed as % duration of a stimulus presentation spent in each behavioral category. Also represented is the rate of vigilance-checking measured during each stimulus condition. Vertical lines represent 1 s.e.m. Symbols refer to Newman-Keuls comparisons between each stimulus condition within a behavioral category: asterisk = Mirror vs. Separated; Dagger = Predator vs. Mirror; plus = Separated vs. Predator.

displacements per unit time (11). During a 2-week adaptation period, monkeys were captured, chair restrained for brief periods and returned to their home cages daily. Monitor memories were read every 3–4 days and when activity appeared to be relatively stable over a continuous 4-hour period after return to the home cage, a dose of 5.0 μg CRH was administered ICV the following day. Treated monkeys were returned to their home cages and monitors were read 24 hr later.

On the basis of these pilot studies in the home cage, subsequent behavioral testing progressed in two phases. In the first phase monkeys were adapted to the capture and chair restraint process in brief daily sessions. Once per week, 5 min after capture and restraint, individual monkeys were placed in transport cages and carried to a wire test cage (2 × 1 × 1 m) in an adjacent, sound-attenuating room. Behavior in the cage was recorded on videotape (Beta III, with high fidelity sound) for 15 min. When the behavior of these monkeys appeared stable in repeated weekly tests, peptide testing was begun.

During the second phase, monkeys were captured, restrained and administered selected doses of CRH (0.1, 1.0, 10.0 μg), α-helical-CRH (10.0 μg) or vehicle. At 5 min after peptide administration monkeys were transported to the test cage and presented with three successive stimulus conditions: 1) during the first 10 min of each test session monkeys were left isolated and undisturbed in the test cage; 2) after 10 min of undisturbed separation, a mirror mounted on the side of the cage was uncovered by remote control for 5 min; 3) finally, the mirror was covered and a "predator" stimulus [a large, fur-covered hand-puppet (18)] was presented at the front of the cage for 3–30-sec intervals alternated with 30-sec intervals with stimulus removed. At the end of the test, monkeys were returned to their home cages. Based on the results of pilot experiments, this 18-min sequence was repeated at three intervals: 5 min, 1 hr, and 3 hr after peptide administration.

Subsequently, videotapes were scored by a single observer for the frequency and duration of all occurrences of motor behavior including locomotion (walking, running, climbing and jumping), sitting with curled tail, stationary alert posture and grooming.

Additional behaviors were scored as potential species-typical indices of arousal including marking, vigilance-checking, vocalization and aggressive display. In novel and mildly stressful test conditions, squirrel monkeys exhibit several marking behaviors including urine washing, back rolling, sneezing and muzzle rubbing, and anogenital rubbing (41). Vigilance-checking (≥90° change in gaze direction) is sensitive to drug treatment (33,38) and may be related to an important survival strategy against predation in wild populations (7). Squirrel monkeys of the gothic-arch variety exhibit reliable threat displays when confronted with a mirror image in their home cage (30,37). The topography of these responses, including genital display, cage-shaking, and jumping at the image conform to the aggressive threat display exhibited by monkeys in seminatural conditions (33). Vocal behavior was recorded on the audio track of the videotape and assessed sonographically according to the criteria of Newman (35).

Peptide Administration

Doses of corticotropin-releasing hormone (0.01, 0.1, 1.0 μg/μl; Bachem Chemicals) were prepared in buffered, sterile saline. α-Helical-CRH (2.0 μg/μl) was prepared in slightly basic sterile distilled water (pH = 8.0; to facilitate solubilization) and frozen prior to testing. Immediately before testing the α-helical-CRH stock was diluted to 1.0 μg/μl with PBS. Five minutes before the first behavioral test, each monkey was rapidly removed from his home cage and placed in a primate restraint chair. The threaded stylette was removed from the guide cannula and replaced with a 30-gauge inner cannula. Using a Hamilton microsyringe, 10 μl of peptide solution was infused over a 60-sec interval.

Statistical Analysis

For comparisons of control performances in each of the behavioral test conditions, the duration of each behavior was expressed as percent of trial duration. The distribution of time spent in several behavioral categories was compared using a chi-squre test of independence. Repeated measures ANOVAs for test conditions were performed within each behavioral category and Newman-Keuls (q) test comparisons were calculated where appropriate (47).

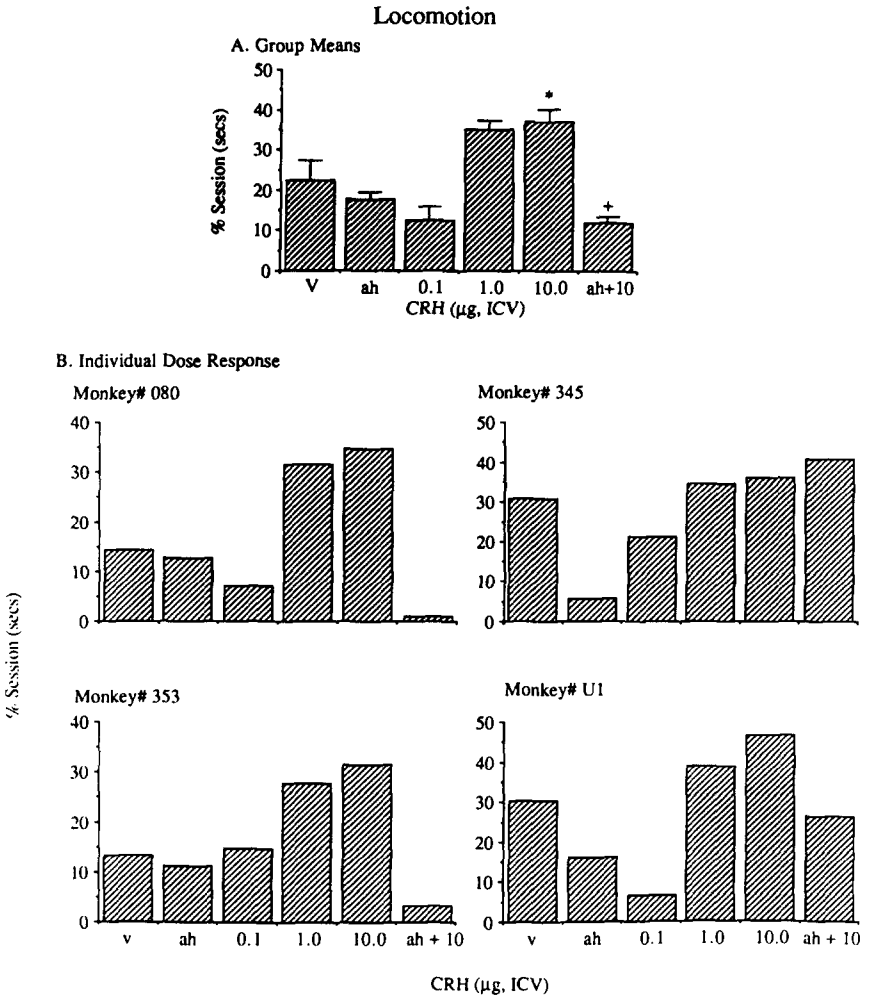

FIG. 3. (A) Locomotion after administration of doses of CRH and 10 μg α-helical-CRH (ah) alone and in combination. Since no interaction between dose, stimulus, or time since injection was detected, columns represent the mean of four monkeys as percent of session collapsed across time and stimulus conditions. Vertical lines represent 1 s.e.m. Asterisks refer to Dunnett's *t*-test comparisons of each peptide dose with the vehicle control, plus refers to Newman-Keuls comparison between 10.0 μg CRH and 10 μg α-helical-CRH plus 10 μg CRH, $p<0.05$. (B) Individual dose response to peptide administration for each of the four squirrel monkeys.

For analysis of peptide effects, the frequency and duration of each behavior was analyzed separately with a repeated measures ANOVA for dose, test condition, and time since injection. Dunnett's (t_D) or Newman-Keuls test comparisons (q_r) were calculated where appropriate (47). When the assumptions of the ANOVA could not be met, Mann-Whitney U comparisons were performed. A p level of 0.05 for two-tailed distributions was accepted as statistically significant.

RESULTS

A moderately high dose of CRH rapidly increased the motor activity of monkeys in their home cage for up to 3 hours after administration compared to untreated control measures, $F(1,5)=2.736$, $p=0.023$ (Fig. 1A). Inspection of individual records revealed variation in both the intensity and time course (Fig. 1B) for each monkey.

During the vehicle control trials, significant differences in the distribution of time allocated to individual behavioral categories were detected for each of the three stimulus conditions ($\chi^2=18.62$, $p=0.02$). Monkeys spent differing amounts of time locomoting, sitting with curled tail and in the stationary alert posture depending on the stimulus conditions. The "predator" stimulus elicited the highest amounts of time spent in the stationary alert posture, least amount of time huddled with curled tail, and the highest rate of vigilance-checking compared to the mirror or undisturbed separation tests (Fig. 2). Monkeys spent the least amount of time locomoting during the mirror stimulus associated with time spent inspecting the mirror and displaying at their reflection. Locomotion in the undisturbed separation and "predator" tests was expressed primarily as rapid pacing and was accompanied in one monkey by frequent isolation peep calls (Monkey #345). Measures of grooming and marking were relatively low and did not vary with test condition.

Administration of doses of CRH and α-helical-CRH significantly affected the amount of time spent locomoting, $F(5,15)=6.69$, $p<0.05$; this effect was not influenced by either stimulus condition, or time since injection. Post hoc comparisons revealed

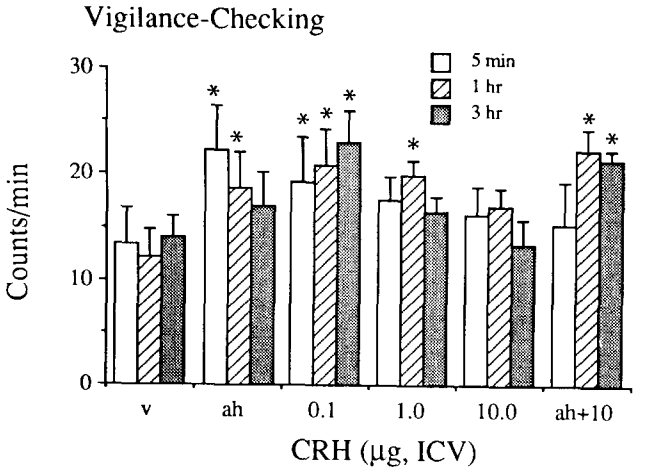

FIG. 4. The rate of vigilance-checking after administration of doses of CRH and 10 μg α-helical-CRH (ah) alone and in combination. Since no interaction between dose and stimulus condition was detected, columns represent the mean of four monkeys collapsed across stimulus conditions tested 5 min, 1 hr, and 3 hr after treatment. Vertical lines represent 1 s.e.m. Asterisks refer to Dunnett's *t*-test comparisons of each peptide dose with the vehicle control, $p<0.05$.

a significant increase in time spent locomoting following administration of the 10 μg dose of CRH ($t_D=2.41$, $p<0.05$). This increase was blocked by concurrent administration of α-helical-CRH ($q_5=5.83$, $p<0.05$; Fig. 3A). Examination of effects of CRH on individual monkeys also suggests a biphasic action: the low dose decreased and higher doses increased time spent locomoting (Fig. 3B).

The rate of vigilance-checking was increased following administration of CRH and α-helical-CRH, $F(5,15)=3.467$, $p<0.05$. This peptide effect was influenced by the time since injection, $F(10,30)=2.91$, $p<0.05$, but not test condition. Analysis of individual dose effects revealed that 0.1 μg CRH significantly increased the rate of checking when tested at 5 min ($t_D=2.64$, $p<0.05$), 1 hr ($t_D=3.77$, $p<0.05$) and 3 hr ($t_D=4.01$, $p<0.05$) after administration (Fig. 4). Checking was also increased by 1.0 μg CRH at 1 hr after administration ($t_D=3.36$, $p<0.05$). Ten μg α-helical-CRH increased checking at 5 min ($t_D=3.95$, $p<0.05$) and 1 hr ($t_D=2.86$, $p<0.05$) after administration. Although 10 μg CRH did not affect checking, combined administration of 10 μg CRH and 10 μg α-helical-CRH produced an increased rate of checking when tested at 1 hr ($t_D=4.43$, $p<0.05$) and 3 hr ($t_D=3.25$, $p<0.05$) after peptide administration.

Administration of CRH and α-helical-CRH significantly affected both the frequency of marking, $F(5,15)=4.902$, $p=0.007$, and aggressive display posture, $F(5,15)=4.229$, $p=0.013$, depending on time since injection and the test condition. The 1.0 and 10.0 μg doses of CRH significantly increased the frequency of marking during the undisturbed separation test at 5 min after administration (1.0: $t_D=2.53$; 10.0: $t_D=2.42$, $p<0.05$), but not 1 or 3 hr. Ten μg α-helical-CRH reduced the frequency of marking when administered alone ($t_D=2.74$, $p<0.05$), but did not block increased marking when combined with 10 μg CRH ($t_D=3.61$, $p<0.05$). Marking during the mirror and the "predator" tests was unaffected by either CRH or α-helical-CRH.

α-Helical-CRH produced a significant increase in the frequency of aggressive displays exhibited by treated monkeys during the mirror test at 5 min ($t_D=3.51$, $p<0.05$) and 1 hr ($t_D=2.68$, $p<0.05$). CRH did not affect or slightly reduced the frequency of aggressive display exhibited by treated monkeys, and partially blocked the increase produced by α-helical-CRH (Fig. 5A). Examination of individual dose responses to the low dose of CRH suggests that 3 of the 4 monkeys showed an increased likelihood of exhibiting display behavior (Fig. 5B).

Two monkeys exhibited high rates of vocal behavior associated with different stimulus conditions. Monkey #345 exhibited a moderate rate of isolation calling during social separation which was unaffected by α-helical-CRH or 0.1 μg CRH, and reduced following administration of 1.0 and 10.0 μg CRH (Fig. 6A). Monkey #080 exhibited a moderate rate of display calls (variable peep and harsh display calls) in the presence of the mirror stimulus, and consistent with the change in aggressive display, this vocalization was increased following administration of α-helical-CRH and reduced following CRH (Fig. 6B). Combined administration of α-helical-CRH and CRH did not restore either vocalization to vehicle levels.

DISCUSSION

A preliminary study of the time course of a moderate dose of CRH demonstrated a rapid onset, long-lasting increase in measures of activity recorded in the home cage. The finding of CRH-induced increased activity in the home cage of individually housed squirrel monkeys differs from Kalin *et al.* (24), who reported reduced activity and behavioral depression in the home cage of treated rhesus macaques. Differences may be related to dose, method of measurement, or species differences. For example, species differences in response to separation stress are indicated by the observation that adult rhesus monkeys exhibit a profound behavioral depression following prolonged separation from a peer group, while adult squirrel monkeys appear to remain agitated and hyperactive (44,48).

Baseline observations of the behavior of squirrel monkeys in the test cage measured reliable differences in response to the three stimulus conditions. In particular, monkeys spent the most time in the stationary alert posture and the least amount of time in the huddled sitting posture during presentation of the "predator" stimulus. Locomotion was comparable during the undisturbed separation and "predator" tests and reduced in the presence of the mirror. Previous experiments have demonstrated that similar test conditions elicit significant changes in cortisol and ACTH. Coe *et al.* (8) measured a significant increase in plasma cortisol following a 60-min separation and presentation of a predator stimulus. We have measured a significant increase in ACTH but not cortisol in samples collected after a 15-min separation (Winslow and Newman, unpublished observations). The frequency of several behaviors was also related to individual stimulus conditions: vigilance-checking was highest during the "predator" stimulus condition, consistent with the observation that vigilance-checking is an important defense strategy against predation in wild populations (7). Aggressive threats were most likely during the mirror test, while marking was frequent during all three conditions. These differences provided an opportunity to assess the effects of peptide administration on several unique classes of species-typical behavior.

The effects of intraventricular administration of CRH and α-helical-CRH on behavior depended on several factors including the stimulus conditions, time since injection, and class of behavior. The high dose of CRH produced a long-lasting increase in locomotor activity which was reduced by concurrent administration of α-helical-CRH. These results are consistent with previous findings of increased motor activation and exploration by rats (45), mice (1), and agitated behavior of chair restrained monkeys (24) treated with ICV CRH. α-Helical-CRH effectively blocked CRF-induced increased motor activation and response suppression (6), and the suppression of feeding behavior (27) when administered

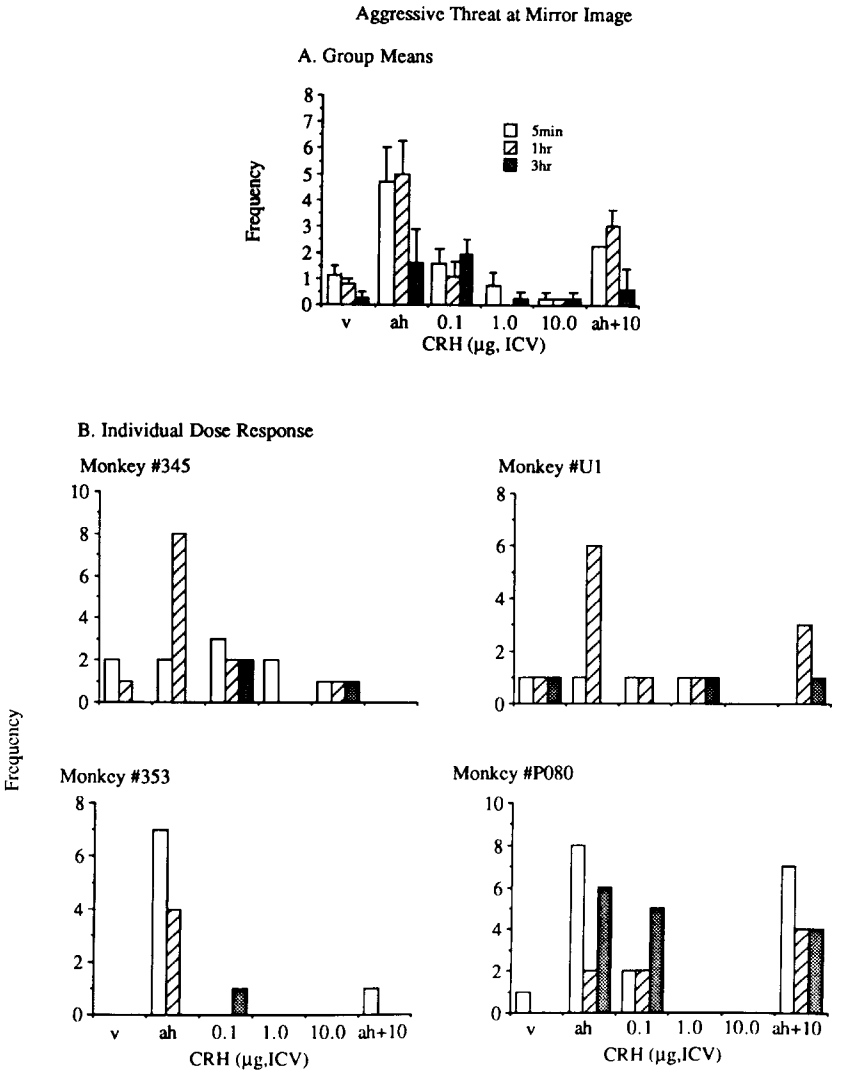

FIG. 5. (A) The frequency of aggressive threat (genital display, cage shaking, and jumping at reflection) directed at a mirror image after administration of doses of CRH and 10 µg α-helical-CRH (ah) alone and in combination. Vertical lines represent 1 s.e.m. Asterisks refer to Dunnett's *t*-test comparisons of each peptide dose with the vehicle control, $p<0.05$. (B) Individual dose response to peptide administration for each of the four squirrel monkeys.

ICV to rats. In contrast, the rate of vigilance-checking was increased by the low dose of CRH and by α-helical-CRH. α-Helical-CRH also increased vigilance-checking when administered in combination with the highest dose of CRH. These findings may indicate partial agonist properties of α-helical-CRH on measures of alertness.

The frequency of marking during the undisturbed separation condition was increased by CRH, but only during the first test after administration. This change was not antagonized by α-helical-CRH. Increases in the incidence of marking have previously been associated with the seasonal mating cycle (9), separation distress in juvenile squirrel monkeys, although only in a familiar environment (21), and with the presentation of novel stimuli and arousal in juveniles and adults (41). In addition, one monkey exhibited a high rate of isolation calling in this condition, and consistent with effects on isolation calling in rat pups (22), CRH produced a dose-related reduction in calling rate. The increased marking behavior and decreased vocalization following CRH administration may be related to increased arousal associated with apprehension and agitation (8).

CRH appears to be involved in the mediation of responses to social encounters. For example, intraventricular administration of CRH increased and α-helical-CRH decreased shock-induced defensive-aggression in rats (46). Under more natural testing conditions, ICV CRH decreased the sexual (42) and social behavior [(14); Mayor and Insel, unpublished observations] of rats, and offensive aggression in mice (31). Squirrel monkeys of the gothic-arch variety reliably exhibit aggressive displays to their mirror image when presented in their home cage (30). In the current experiments, the mirror test was presented in a novel cage and the rate of aggressive displays was low. Administration of CRH did not further reduce the low rate of aggressive display under these conditions, however the frequency of display was increased up to an hour after administration of α-helical-CRH.

In summary, the highest dose of CRH administered intraventricularly produced rapid, long-lasting changes in motor behavior independent of stimulus condition, consistent with a state of high arousal and agitation. Lower doses of CRH differentially modulated species-typical behavioral responses to provocative stimuli. The data suggest possible biphasic actions for CRH expressed as

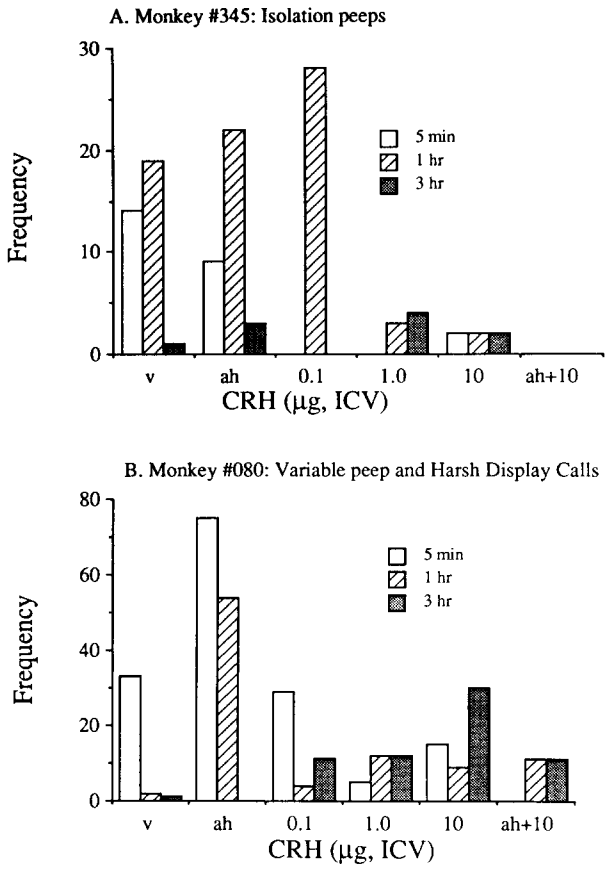

FIG. 6. (A) The frequency of isolation peeps expressed by monkey #345 during the separated undisturbed condition at 5 min, 1 hr, and 3 hr after administration of doses of CRH and 10 μg α-helical-CRH (ah). (B) The frequency of variable peep and harsh display calls expressed by monkey #080 in the presence of the mirror stimulus at 5 min, 1 hr, and 3 hr after administration of doses of CRH and 10 μg α-helical-CRH.

increased vigilance, approach and likelihood of aggressive behavior at low concentrations compared to increased likelihood of escape behavior (rapid pacing) and withdrawal at higher concentrations. Dose-related differences may reflect the central involvement of CRH in modulating different behavioral strategies for coping with "nonrhythmic" stimuli (29), such as exploration or withdrawal from novel stimuli, and attack or flight from provocative stimuli. Administration of α-helical-CRH antagonized the effects of the high dose of CRH on motor behavior and vigilance-checking, but not the effects of lower doses on vigilance and marking behavior. α-Helical-CRH administered alone increased vigilance in all test conditions and the frequency of aggressive displays exhibited by treated monkeys directed at their mirror image. Increases in stimulus-appropriate behavior may reflect the disinhibition of behavior partially suppressed in test environment as a consequence of reduced stress response. Alternatively, partial agonist properties of α-helical-CRH may activate behavior in a pattern similar to that recorded following administration of low doses of CRH.

ACKNOWLEDGEMENTS

Preparation of this contribution and research was supported by a Intramural Research Service Award from the National Institute of Child Health and Human Development to J.T.W.

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