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Effects of corticotropin-releasing hormone on distress vocalizations and locomotion in maternally separated mouse pups

Anneloes Dirks[a,*], Eric W. Fish[b], Takefumi Kikusui[b], Jan van der Gugten[a], Lucianne Groenink[a], Berend Olivier[a,c], Klaus A. Miczek[b,d]

[a]*Department of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands*
[b]*Department of Psychology, Tufts University, Medford, MA 02155, USA*
[c]*Department of Psychiatry, Yale University, New Haven, CT 06508, USA*
[d]*Departments of Psychiatry, Pharmacology and Experimental Therapeutics, and Neuroscience, Tufts University, Medford, MA 02155, USA*

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Abstract

The behavioral effects of corticotropin-releasing hormone (CRH) appear to depend on the baseline state of arousal of the animal. In this study, this hypothesis was tested using a 4-min maternal separation procedure in 7-day-old male and female mouse pups (outbred CFW strain). Two intensities of stress were used to assess the effects of intracerebroventricularly administered r/hCRH: a mild stress condition where the ambient temperature was close to nest temperature (30 °C) and rates of maternal separation-induced ultrasonic vocalizations (USVs) were relatively low (ca. 25/4 min), and a more stressful condition where the temperature was 19 °C and the rates of USVs were high (ca. 250/4 min). Differential effects of CRH on vocalization rate and locomotor behavior were observed to be dependent on the level of stress. In the more stressful 19 °C condition, r/hCRH dose-dependently reduced the number of USVs without affecting motor behavior, as indexed by grid crossings. In contrast, in the 30 °C condition, only the highest dose of r/hCRH reduced calling while r/hCRH activated motor behavior over a wider range of doses. These effects were independent of hypothalamus–pituitary–adrenal (HPA) axis activity, as measured by plasma corticosterone levels. The present study indicates that in mouse pups, the effects of CRH administration depend on baseline levels of arousal and that the behavioral effects of CRH administration can be dissociated under mild and more stressful conditions.

Keywords: Anxiety; Ultrasonic vocalization; Pups; Motor activity; CRH; CRF; Mice; Midazolam; Stress; Corticosterone

1. Introduction

Corticotropin-releasing hormone (CRH) plays a pivotal role in the response of an organism to various stressors by coordinating neuroendocrine, autonomic, behavioral and immunological responses to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Holsboer, 1999; Koob and Heinrichs, 1999; Koob et al., 1993). Indeed, intracerebroventricular (icv) infusion of CRH elicits a constellation of changes closely similar to those induced by many types of stress, including alterations in the autonomic nervous system, increases in hypothalamus–pituitary–adrenal (HPA) axis activity, and various stress- and anxiety-related behavioral changes (see Dunn and Berridge, 1990; Koob and Heinrichs, 1999; Koob et al., 1993). Furthermore, chronic CRH hyperactivity is implicated in human stress-related and affective disorders, including major depression and generalized anxiety disorder (see Arborelius et al., 1999; Heim and Nemeroff, 1999).

It has been suggested that the behavioral effects of CRH administration depend on the baseline state of arousal of the animal (e.g., Dunn and Berridge, 1990; Menzaghi et al., 1993; Koob and Heinrichs, 1999). When administered to nonstressed animals under low levels of arousal, CRH is behaviorally activating, while under more stressful conditions, administration of CRH results in enhanced behavioral responses to stress. For example, in rats tested in a familiar environment, icv CRH produces a dose-dependent activation of locomotor activity, while in a novel open field, CRH

* Corresponding author. Tel.: +31-30-253-3357; fax: +31-30-253-7387.
E-mail address: A.Dirks@pharm.uu.nl (A. Dirks).

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reduces locomotion and increases freezing behavior (Sutton et al., 1982). These findings would suggest that the inferred endogenous levels of CRH determine the effects of CRH administration. This study tested the hypothesis that the effects of CRH administration depend on the intensity of the stressor by using a maternal separation procedure in mouse pups.

Separation from dam and littermates induces distress-like reactions in infant rodents (e.g., Noirot, 1966, 1972). Distress in rat and mouse pups is signaled by the emission of 30–80 kHz ultrasonic vocalizations (USVs) that evoke search and retrieval of the pup by the dam (Allin and Banks, 1972; Smotherman et al., 1974). Vocalization rate is enhanced by various stressors, including exposure to low temperatures (Allin and Banks, 1971; Okon, 1972). In contrast, stimuli from the dam or littermates decrease the rate of vocalization (Hofer and Shair, 1987). Drugs with antidepressant, anxiolytic, and/or antistress effects, including compounds that act on 5-HT_{1A}, 5-HT_{1B}, the $GABA_A$ receptor complex, or opioid receptors, appear to be particularly effective in reducing the rate of vocalization (e.g., Benton and Nastiti, 1988; Nastiti et al., 1991; Winslow and Insel, 1991a; Vivian et al., 1997; Olivier et al., 1998a,b; Fish et al., 2000; Rowlett et al., 2001). Some putative anxiogenic drugs enhance calling, whereas a number of other psychotropic agents without anxiolytic-like effects are inactive (see Winslow and Insel, 1991b; Olivier et al., 1998b). The maternal separation procedure is relatively simple and useful to study stress- and anxiety-related processes.

Brain CRH receptors are functional early in postnatal life (Insel et al., 1988). In rat pups, centrally administered CRH is associated with a decreased rate of USVs, which appear not secondary to decreases in arousal, thermoregulatory capacity, or to peripheral actions of the peptide (Insel and Harbaugh, 1989; Harvey and Hennessy, 1995). Although the decrease in USVs is surprising, there are indications that CRH and USV emission rate are related in a curvilinear fashion: at resting levels of CRH, vocalizing is minimal; when a pup is isolated at ambient temperature, CRH levels begin to increase and the pup begins to vocalize; at yet higher CRH levels, the pup again becomes quiet (see Insel and Harbaugh, 1989; Harvey and Hennessy, 1995; Hennessy et al., 1999). Furthermore, CRH has been shown to be necessary but not sufficient to produce a high rate of vocalization when rat pups were maintained on warm home cage bedding (Harvey and Hennessy, 1995). Taken together, these data are consonant with the hypothesis that the inferred levels of endogenous CRH determine the effects of CRH administration.

In the present study, the effects of icv administered CRH in mouse pups were assessed in one condition where testing was close to nest temperature (i.e., 30 °C) and yielded low rates of USVs, and a condition with a temperature of 19 °C that yielded high rates of USVs (see also Olivier et al., 1998b). The number of grid crossings, an index for motor activation, was also examined to determine whether in infant mice CRH simply inhibits or stimulates all behaviors, or, whether analogous to adult rats, the effects of CRH on locomotor behavior are also dependent on dose and testing conditions. Since hypothermia may represent a confounding variable in the interpretation of the USVs (Allin and Banks, 1971; Blumberg and Alberts, 1990; Sokoloff and Blumberg, 1997), change in rectal temperature after icv CRH injections was also determined. Lastly, since CRH is the key regulator of the HPA axis, plasma corticosterone levels, as an index of HPA axis activity, were assessed.

2. Materials and methods

2.1. Animals

CFW mouse pups ($N = 208$) were bred on site from parents obtained from Charles River Breeding Laboratories (Wilmington, MA, USA) and housed with both parents in clear polycarbonate cages ($28 \times 17 \times 14$ cm) with pine shavings as floor covering. Purina rodent chow and tap water were freely available through the wire lid of the cage. The vivarium was maintained at constant temperature (21 ± 1 °C) and humidity (30–40%), and on a 12-h light/dark cycle (lights on at 0800 h).

The pups were taken from a total of 26 litters. Only litters with eight pups or more were used, and treatments were balanced across litters in such a way that each dose occurred at least once in every litter. The pups were counted at birth (Day 0) and not disturbed until the day of testing.

The mice were cared for according to the 'Guide for the Care and Use of Laboratory Animals' (1996). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Tufts University.

2.2. Apparatus and measurements

The testing apparatus was located in a procedure room that was separate from the animal housing colony. USVs were recorded in a sound-attenuated chamber ($49.5 \times 38 \times 34$ cm) that was illuminated by a 10-W red light and was fitted with a one-way mirror (19×16.5 cm) to allow behavioral observation. A square aluminum pan (23×23 cm) was suspended in a water bath to maintain surface temperature. The surface was divided into 2-cm squares that served as grids for the measurement of locomotor behavior. As described previously (Fish et al., 2000), ultrasounds were detected by a high-frequency condenser microphone (Bruel and Kjaer, Naerum, Denmark; Model No. 4135), preamplifier (Bruel and Kjaer; Model No. 2633), and amplifier (Bruel and Kjaer; Model No. 2610) in conjunction with a Krohn-Hite filter (Krohn-Hite, Cambridge, MA, USA; Model No. 3550R) that produced a flat frequency response between 30 and 100 kHz. The output was monitored on a 20-MHz oscilloscope (Goldstar 059020 A, Cerritos, CA, USA) and connected to a computer (Macintosh II) running customized software for signal detection.

The analog signals were converted to digital representations using a GWI-AMP analog-to-digital converter (GW Instruments, Somerville, MA, USA) that also enabled additional amplification of the signal. Audible sounds produced by the mouse pup, such as 'squeals' or scratches upon the surface, were eliminated using rejection parameters that accepted sounds between 30 and 100 kHz, longer than 0.01 s in duration, and that had an intersound interval of longer than 0.02 s. The program recorded, among others, the total number of USV during the 4-min test for each pup tested.

2.3. Icv injections

Icv injections were made using an injector and a 30-gauge beveled, hypodermic needle fitted into a 25-gauge cannula so that 2 mm protruded from the tip of the cannula. The injector was connected to a Hamilton syringe by polyethylene tubing (PE-20). Toluidine blue solution (0.4 μl) to mark the injection site, drug or saline (1.0 μl), and small air bubbles as dividers to prevent mixing were injected in unanesthesized pups. Pilot experiments demonstrated that 1% toluidine blue was a reliable dye for visualization of the injection site and did not show any adverse side effects. The Hamilton syringe was placed in a syringe pump (Sage Instruments, Cambridge, MA, USA; Model 341A) to provide constant infusion rate.

The conscious pup was gently restrained and the injector inserted 2 mm deep, ca. 2.5 mm posterior from bregma on midline through the scalp and skull, which can be easily penetrated at this age. The solution was infused over a period of about 60 s and the injector was left in place for an additional 60 s to allow diffusion. After the separation test, the pup was decapitated and its brain was inspected for injection localization. Only pups with clear diffusion of the dye in the ventricular system were included in the statistical analyses (approximately 82% of total).

2.4. Procedure

The procedure was based on a between-subject design. Seven-day-old pups were used since it has been demonstra-ted that the emission of maternal separation-induced USVs peaks around Day 7 in mouse pups and declines as the pups grow older and locomotor behavior increases (Elwood and Keeling, 1982; Fish et al., 2000). Each session began by removing an entire litter (8–12 pups) and a handful of bedding from the home cage and placing them in an incubator that maintained nest temperature (35 °C). After ca. 20 min, the pups were weighed, marked, and screened for the emission of USVs at temperature of 19 °C. Only pups that weighed between 3.5 and 5.5 g and that reached the criterion of six USVs during a 30-s separation were used as experimental animals. Pups were injected with the appropriate drug or vehicle (with the exception of pups of the no-injection group), counterbalanced across litters, and rectal temperatures were taken using a thermo-probe (Yellow Springs Instruments, Yellow Springs, OH, USA; Type YSI 555 N034, o.d. 0.7 mm) attached to a YSI-2111-Tele Thermometer (Yellow Springs Instruments). The probe was lubricated with mineral oil and inserted ca. 10 mm and kept in place until the temperature measurement was stable for at least 3 s. After recording rectal temperature, the pups were returned to the incubator until time of testing. After the appropriate injection interval (see Section 2.5), a second rectal temperature was taken immediately before the separation test. The pup was placed in the center of the surface and USVs and the number of grid crossings was recorded for 4 min. An experimenter counted a grid crossing when half of the pup's body crossed into the next grid. A typical experimental session lasted approximately 3 h and experimental sessions were conducted between 0800 and 2000 h. No differences in baseline rates of vocalization were observed according to the time of day, as also reported previously (Fish et al., 2000).

2.5. Experiments

For validation of the current icv injection procedure, the effects of the benzodiazepine midazolam were tested initially. Midazolam (Sigma, St. Louis, MO, USA) was dissolved in saline and injected 10 min before the separation test at temperature of 19 °C. After the test, pups ($n = 15$ per treatment) were decapitated and the injection site was verified. Two intensities of the same stressor (maternal separation) were used to assess the effects of CRH: a mild stress condition where the ambient temperature was close to nest temperature (30 °C), and a more stressful condition where the temperature was 19 °C. Rat/human CRH (kind gift from Dr. S.C. Heinrichs, Boston College) was dissolved in saline and was administered 30 min before the separation test. Groups consisted of 13–18 pups per treatment for the 30 °C condition (except no-injection group: $n = 6$) and 11–14 pups per dose for the 19 °C condition. After the test, the pup was decapitated, injection site was verified, and trunk blood was collected with pipette tips flushed with heparin for corticosterone determination. After the sessions, the Eppendorf vials containing the blood samples were centrifuged at 2800 rpm for 10 min at 4 °C, then plasma was collected and stored at −20 °C until assay. Plasma corticosterone levels were determined in duplicate using a commercially available radioimmunoassay (RIA) for rat corticosterone (ICN Biomedicals, Costa Mesa, CA, USA). Detection limit of this assay is 3.125 ng/ml.

2.6. Statistical analysis

For consistency reasons, especially with regard to the figures, data from all experiments were analyzed using nonparametric Kruskal–Wallis tests. When appropriate, posthoc Mann–Whitney tests were used to compare each treatment to vehicle. In all posthoc tests, Bonferroni correction of α for

Table 1
Effects of icv midazolam on USVs and locomotion

Midazolam icv	USVs	Grid crossings
Saline	206 (171–317)	19 (11–34)
1.0 μg	180 (113–213)	26 (21–39)
3.0 μg	88 (5–167)*	38 (30–51)*

$n = 15$ per dose; data expressed as median values with interquartile range (25th–75th percentile).
* $P < .05$ vs. saline control.

repeated between-subject comparisons was applied. Mann–Whitney tests were used to analyze differences between the noninjected and saline-treated pups.

3. Results

3.1. Icv midazolam

Icv administered midazolam dose-dependently reduced vocalization rate (Table 1; $\chi^2 = 14.10$, $df = 2$, $P < .005$). Post-hoc tests revealed that pups treated with 3.0 μg of midazolam emitted significantly fewer USVs than pups treated with saline. Midazolam also affected locomotor behavior (Table 1; $\chi^2 = 10.31$, $df = 2$, $P < .01$) by significantly increasing the number of grid crossings at the 3.0-μg dose. The pups showed clear signs of motor impairment and ataxia. Body temperature was not affected by midazolam (data not shown).

3.2. Icv r/hCRH

3.2.1. USVs

When tested at surface temperature of 19 °C, the icv injection procedure significantly reduced USVs as compared to the control mice that were tested through the entire procedure but did not receive an injection (Fig. 1A; no-injection vs. saline, Mann–Whitney test, $P < .05$). Administration of r/hCRH further reduced significantly the emission of USVs at the 0.3- and 1.0-μg doses (Fig. 1A; $\chi^2 = 36.98$, $df = 5$, $P < .001$). At the 30 °C temperature, there was no significant difference between the number of USVs emitted by pups from the no-injection and saline groups (Mann–Whitney test, n.s.]. Vocalization rates were reduced by icv r/hCRH (Fig. 1B; $\chi^2 = 9.36$, $df = 3$, $P < .05$), but only significantly at the 1.0-μg dose.

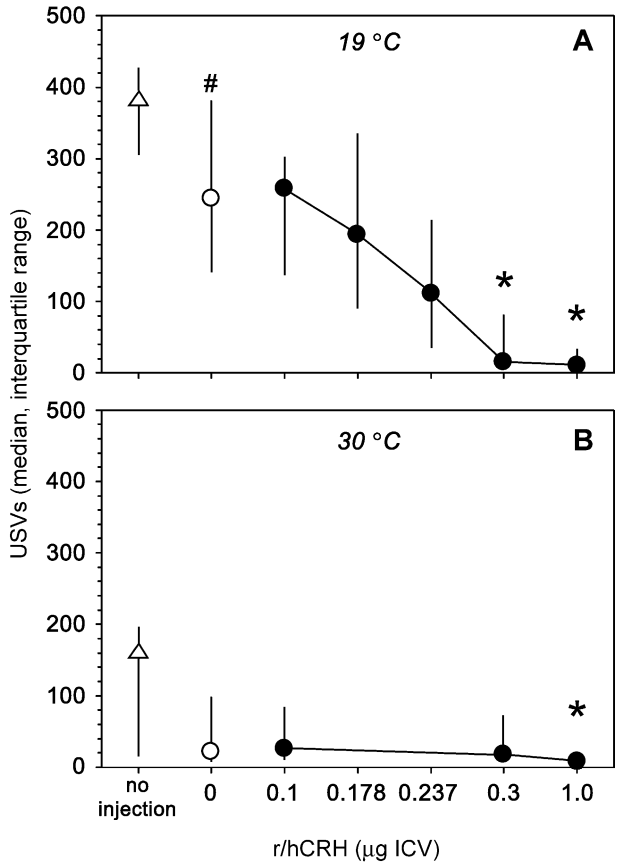

Fig. 1. The effects of icv r/hCRH on USV of mouse pups. Top (A): Test temperature of 19 °C ($n = 11–14$ per dose). Bottom (B): Test temperature of 30 °C ($n = 13–18$ per dose). Data are expressed as median values and interquartile range (25th–75th percentile). * $P < .05$ vs. saline control.

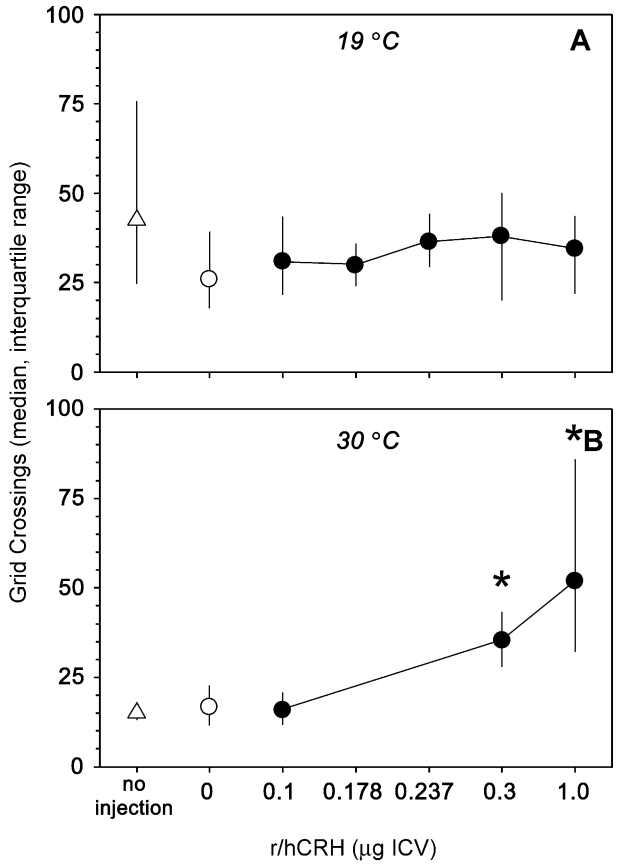

Fig. 2. The effects of icv r/hCRH on grid crossings of mouse pups. Top (A): Test temperature of 19 °C ($n = 11–14$ per dose). Bottom (B): Test temperature of 30 °C ($n = 13–18$ per dose). Data are expressed as median values and interquartile range (25th–75th percentile). * $P < .05$ vs. saline control.

3.2.2. Locomotor behavior

CRH had no significant effect on locomotion (Fig. 2A; $\chi^2 = 4.15$, $df = 5$, n.s.) in the 19 °C test condition, but increased grid crossings at the 30 °C test temperature (Fig. 2B; $\chi^2 = 22.42$, $df = 3$, $P < .001$). Pups treated with 0.3 and 1.0 μg of r/hCRH made significantly more grid crossings than saline-treated pups. In both conditions, the icv injection procedure itself did not affect locomotion (Fig. 2; Mann–Whitney test, n.s.).

3.2.3. Body temperature

Body temperature was not affected by icv injections or r/hCRH in either test condition (data not shown).

3.2.4. Plasma corticosterone levels

In both testing conditions, icv injections with saline significantly increased plasma corticosterone levels as compared to mice of the no-injection group (Fig. 3; Mann–Whitney test, $P < .05$). In the 19 °C condition, plasma corticosterone levels were increased after r/hCRH administration (Fig. 3A; $\chi^2 = 19.45$, $df = 5$, $P < .005$) at the 1.0-μg dose. In the 30 °C condition, r/hCRH did not have an additional effect on corticosterone levels, which were already increased by injection and handling (Fig. 3B; $\chi^2 = 5.65$, $df = 3$, n.s.).

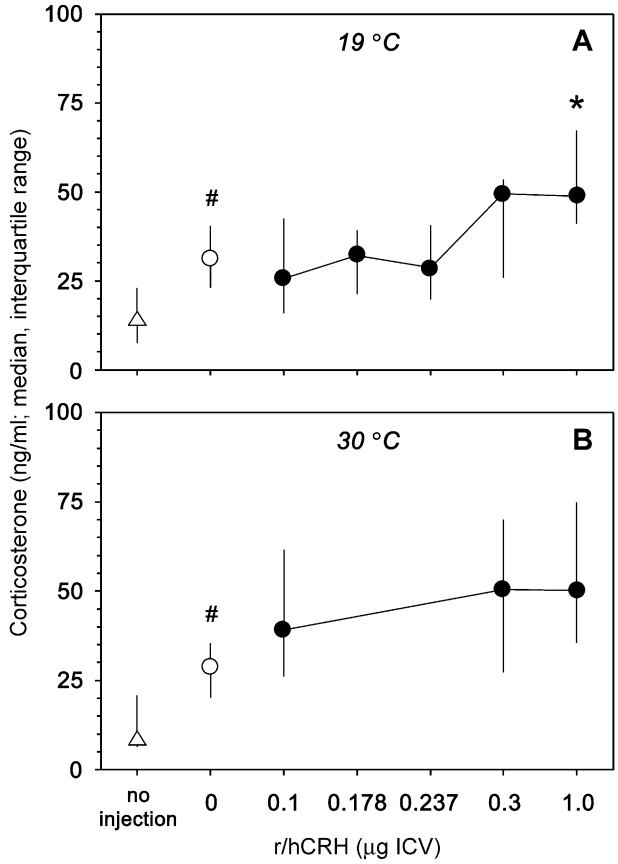

Fig. 3. The effects of icv r/hCRH on plasma corticosterone levels of mouse pups. Top (A): Test temperature of 19 °C ($n = 11–14$ per dose; no-injection group: $n = 12$). Bottom (B): Test temperature of 30 °C ($n = 11–17$ per dose; no-injection group: $n = 6$). Data are expressed as median values and interquartile range (25th–75th percentile). $^{\star}P < .05$ vs. saline control; $^{\#}P < .05$ vs. no-injection group.

4. Discussion

In this study, the hypothesis that the effects of CRH administration depend on the intensity of the stressor was tested using a maternal separation procedure in mouse pups. When tested at an ambient temperature of 19 °C, r/hCRH reduced the high rates of maternal separation-induced USVs without affecting motor activity. However, at the 30 °C temperature, with relatively low rates of USVs, only the highest dose of r/h CRH reduced the emission of USVs but increased motor activity across a wider range of doses. These results are consistent with previous demonstrations of CRH-induced decreases in USVs (Insel and Harbaugh, 1989; Harvey and Hennessy, 1995). These effects of r/hCRH at both test temperatures were independent of changes in body temperature and HPA axis activity as measured by plasma corticosterone levels. The results of saline-treated mouse pups and control mice that were tested through the entire procedure but did not receive an injection suggest that the icv injection procedure in unanesthesized pups and without guide cannulae did not induce severe additional stress.

For validation of the current icv injection procedure, the effects of the benzodiazepine midazolam were assessed. Midazolam given icv reduced the rate of USVs and increased motor activity, similar to other benzodiazepines in mouse (Benton and Nastiti, 1988; Nastiti et al., 1991; Cirulli et al., 1994; Fish et al., 2000; Rowlett et al., 2001) and rat pups (Vivian et al., 1997; Olivier et al., 1998b). The locomotor behavior of the pups was ataxic and occurred in a burst-like fashion rather than being suppressed as is typical in adult animals, resembling the increased activity seen in the excitation phase after anesthetics such as ether and pentobarbital, resulting in the paradoxical increase in grid crossings. Given the close similarities between centrally and peripherally administered midazolam, we concluded that the icv administration route could be used in combination with the maternal separation procedure in mouse pups.

In the present study, two levels of stress were used to assess stress-dependent effects of icv administered r/hCRH: a low-stress situation where the ambient temperature was kept close to nest temperature (30 °C), probably representing isolation distress only, and a more stressful situation where additional cold stress was imposed, with a temperature of 19 °C. R/hCRH reduced the high rates of maternal separation-induced USVs when tested at 19 °C, while only the highest dose of r/hCRH reduced the emission of USVs at 30 °C, consistent with earlier findings (Harvey and Hennessy, 1995). In rodent pups, decrease in body temperature is typically associated with an increase in USVs (Allin and Banks, 1971; Blumberg and Alberts, 1990; Sokoloff and Blumberg, 1997). As r/hCRH was without effect on body

temperature of the mouse pups in both stress conditions, hypothermia is an unlikely explanation for the reducing effects of CRH on vocalization rate. A more likely explanation for the decrease in USVs in the 19 °C condition might be that CRH specifically inhibits the USV response, making the pup behave as if in a prolonged isolation state (Insel and Harbaugh, 1989). Following several minutes of social isolation, the normal rate of USVs decreases to about the level observed following CRH (Insel and Harbaugh, 1989). The decreased USV response in more stressful conditions might be considered adaptive as a 'stressed' but quiet pup is less likely to be detected by a predator (Insel and Harbaugh, 1989). It has been demonstrated that footshocks (Takahashi et al., 1991) or cues from an unfamiliar adult male conspecific, which is a potential predator (Takahashi, 1999), reduce USVs in neonatal pups. Our data in mouse pups agree with these data in rat pups, and suggest that specific inhibition of the USV response may underlie the observed effects of CRH in the 19 °C condition. Results on centrally or peripherally administered CRH in guinea pig pups on audible distress vocalizations also agree with this hypothesis (reviewed in Hennessy et al., 1999). Furthermore, the results of the present study agree with the hypothesis that the arousal state determines the effects of administered CRH on vocalization rate. That is, when the inferred levels of endogenous CRH are low (i.e., in the nest and possibly at test temperature 30 °C), vocalizing is minimal and administration of CRH in doses up to 1.0 μg do not affect vocalization rates. In contrast, when endogenous levels of CRH are substantially increased (i.e., at test temperature 19 °C), vocalization rates are high and increasing CRH levels even further by CRH administration results in quieting of the pup. Additional support for this conclusion is derived from the finding that the number of USVs in the saline group is reduced when compared to the no-injection group, most likely due to the additional stress of the injection procedure.

Interestingly, the results of the present study indicate differential effects of CRH on different behavioral endpoints. In contrast to USVs, grid crossings were not affected by r/hCRH in the 19 °C condition, but increased dose-dependently in the 30 °C condition. The increased motor activity of pups at the 30 °C condition is consistent with the effects of CRH in adult rats tested in a familiar environment. Icv CRH dose-dependently activates locomotor activity in a familiar environment, while in a novel stressful environment CRH reduces locomotion and increases freezing behavior (Sutton et al., 1982), although in another study, locomotor behavior of rat pups was not affected by CRH (Harvey and Hennessy, 1995). Furthermore, our data suggest that the effects of CRH on USV rate in the 19 °C condition are not due to an overall inhibition of behavior, since grid crossings were unaffected in this condition.

In infant rodents, a 'stress hyporesponsive period' (SHRP) can be observed during the first two postnatal weeks (from Postnatal Days 3 to 14) that is characterized by an attenuated HPA axis response to stress (see Diez et al., 1976; Rosenfeld et al., 1992; Vazquez, 1998). However, prolonged maternal separation can produce HPA axis activation in rodent pups in the SHRP (e.g., Walker et al., 1991; Rosenfeld et al., 1992; Cirulli et al., 1994; Vazquez, 1998). In the present study, isolation from littermates and rectal temperature measurement induced a plasma corticosterone response exceeding the levels in nonhandled, nontested control pups that were below the detection limit of the RIA (data not shown). Icv injections of saline further increased corticosterone secretion in both stress conditions above the no-injection control group. Only the highest dose of r/hCRH (1.0 μg) significantly augmented corticosterone levels in the 19 °C condition when compared to saline controls, while in the 30 °C condition, CRH was without effect. In a previous study in rat pups used in vocalization studies, only peripheral CRH was found to be associated with an increase in plasma corticosterone (Insel and Harbaugh, 1989). Although in both test conditions plasma corticosterone levels were increased by icv injections with saline compared to no injection, marked stress-dependent differences in behavior between those two groups were evident. Furthermore, there was no correlation between USVs and plasma corticosterone levels after exogenous CRH (data not shown). Taken together, these results extend previous findings that the observed r/hCRH effects on USVs and locomotor behavior are independent of HPA axis activity (Cirulli et al., 1994).

In conclusion, the present study provides further evidence in favor of the hypothesis that, the baseline response to a stressor determines the behavioral effects of CRH administration. Furthermore, the present study indicates that CRH can affect behavioral endpoints differentially dependent on the intensity of the stressor, i.e., in a mild stress condition, preferentially locomotion; and in a more stressful condition, preferentially distress vocalizations.

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