Pharmacology Biochemistry & Behavior, Vol. 32, pp. 197–201. Pergamon Press plc, 1989. Printed in the U.S.A. 0091-3057/89 $3.00 + .00

Central Administration of Corticotropin Releasing Factor Alters Rat Pup Isolation Calls

THOMAS R. INSEL AND CARROLL R. HARBAUGH

Laboratory of Clinical Science, NIHAC/NIMH, Box 289, Poolesville, MD 20837

Received 2 May 1988

INSEL, T. R. AND C. R. HARBAUGH. *Central administration of corticotropin releasing factor alters rat pup isolation calls.* PHARMACOL BIOCHEM BEHAV 32(1) 197–201, 1989.—Rat pups, when socially isolated, emit ultrasonic vocalizations which are believed to indicate distress. This study investigated the effect of intracerebroventricular (ICV) administration of corticotropin releasing factor (CRF) on the production of ultrasonic isolation calls. Following a 2-minute baseline isolation test, rat pups (5–6 days old) were injected ICV with CRF or the CRF antagonist, α-helical CRF (9–41). Thirty minutes later, calls were significantly decreased following CRF (0.1 and 0.01 μg) and increased following the CRF antagonist (1.0 μg). These effects were not explained by changes in locomotor activity, thermoregulation, or plasma glucocorticoid levels following peptide administration. Peripheral administration of CRF (1.0 and 10.0 μg) did not alter the number of isolation calls.

Corticotropin releasing factor Ultrasonic calls Isolation distress CRF antagonist

CORTICOTROPIN releasing factor (CRF) is a 41 amino acid neuropeptide with endocrine, physiologic, and behavioral effects related to stress (16,29). The endocrine effects of CRF, including the release of corticotropin (ACTH) and β-endorphin, are mediated by selective membrane bound receptors in the pituitary (8). Central administration of the peptide has been associated with physiologic changes resembling a stress response, such as increases in heart rate, blood pressure, and plasma levels of catecholamines (5). In addition, following intracerebroventricular (ICV) injections of CRF, dose-dependent increases in behavioral activation (27), enhanced response to novelty (4), and decreased food ingestion (18) and reproductive behavior (24) have been reported. These various behavioral effects are believed to be mediated by selective brain receptors (9), although the regional localization of the effects of exogenous peptide remains unclear (6).

Recently, we reported a marked increase in brain CRF receptors in the developing rat (12). In the first week of postnatal life, CRF receptors were more than 300% of their adult level with an extremely dense distribution in striatum and cortex. In addition, many of these receptors were coupled to adenylate cyclase, suggesting that they were functional early in postnatal life. There is however, no available information about behavioral or physiologic responses to centrally administered CRF in development.

The studies described here investigate whether centrally administered CRF might have behavioral effects related to stress in infant rats. Although several of the measures of stress previously reported with CRF administration to adult rats, such as changes in exploratory behavior, food intake, or sexual behavior are not relevant to altricial infants, one reproducible behavior associated with the stress of isolation in the rat pup is a species-typical vocalization. Rat pup isolation calls are ultrasonic (35–45 kHz), monotonic sounds which are potent stimuli for maternal retrieval (2,25). They can be elicited within the first day of postnatal life and continue throughout the first 2 weeks whenever a pup is separated from its littermates and mother (22). Several observations link this behavior to stress or anxiety. Calls increase as environmental conditions become more harsh or novel compared to the maternal nest (11). Anxiolytic compounds, such as the benzodiazepines, decrease the rate of calling (10,15), while anxiogenic compounds, such as pentylenetetrazol, increase the rate of calling (15). Furthermore, rat pups from an emotionally reactive inbred strain call more than their nonreactive congeners (14). On the basis of these observations, we predicted that CRF would increase the rate of ultrasonic isolation calls in the rat pup.

METHOD

Offspring of our Sprague-Dawley breeders (original stock from Taconic Farms, Taconic, NY) were used when either 5 or 6 days old. Until the day of the experiment, pups were housed with both parents in a 55×31×21 cm cage kept in the colony room at 24°C with a 12-12 light-dark cycle. Parents were removed 45 minutes prior to testing.

For baseline tests, each pup was removed from its littermates, weighed, and then placed for 2 minutes in the recording chamber. This chamber is a Plexiglas container (46×29 cm) with a 5×5 cm grid drawn on the floor and a microphone

TABLE 1

MEANS (± SEM) VOCAL AND LOCOMOTOR SCORES BEFORE AND AFTER ICV TREATMENTS

Dose (μg)	n	Base Voc	Post Voc	Base Loc	Post Loc
Saline	13	134.6 ± 9.2	115.5 ± 11.1	10.6 ± 3.9	12.1 ± 3.9
CRF 0.1	9	120.5 ± 15.7	11.0 ± 3.8*	9.5 ± 3.2	4.4 ± 1.0
CRF 0.01	7	115.4 ± 8.7	50.3 ± 11.2*	13.3 ± 2.2	12.3 ± 2.6
CRF 0.001	8	117.7 ± 17.4	62.2 ± 12.2	10.2 ± 3.2	3.7 ± 1.6
CRF − A 1.0	10	135.7 ± 19.5	162.9 ± 27.7*	11.7 ± 4.2	13.8 ± 4.2
CRF 0.01 + A 1.0	9	123.7 ± 13.4	99.4 ± 14.5	3.1 ± 1.8	0.2 ± 0.1

*Signifies $p<0.05$ by post hoc comparison to saline.

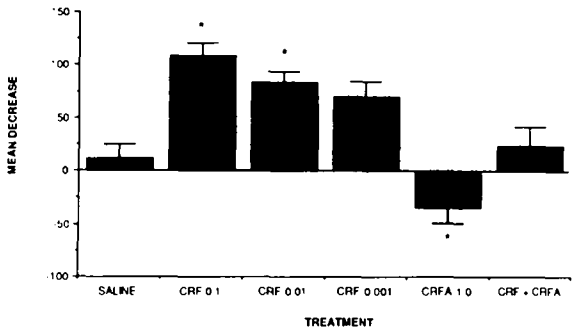

FIG. 1. Change in vocalizations within 2-minute test period, 30 minutes following ICV 1 μl injection with saline, CRF (0.1, 0.01, 0.001 μg), α-helical CRF (1.0 μg), or α-helical CRF (1.0 μg) + CRF (0.01 μg). Raw means are shown in Table 1. Note that upward deflection denotes decrease in number of calls. There is a significant overall effect for treatment, $F(5,46)=10.18$, $p=0.0001$, with * indicating significant ($p<0.05$) differences from saline.

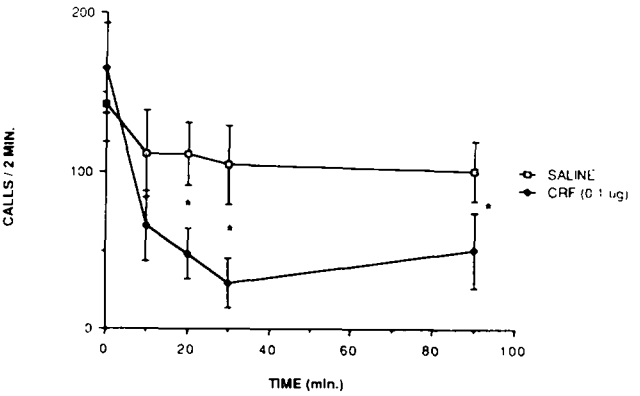

FIG. 2. Longitudinal recording of vocalizations after saline or CRF (0.1 μg) ICV injection shows a significant difference between treatments, $F(1,3)=7.034$, $p=0.02$, with CRF decreasing calls more than saline (* indicates $p<0.05$) at 20, 30, and 90 minutes.

(Bruel and Kjaer Model 4385, Copenhagen) suspended within a parabolic reflector 10 cm above the floor. At the end of the 2-minute baseline test, each pup was injected with peptide or saline, then returned to its litter (without parents). Thirty minutes later, pups were retested to assess treatment effects. In a subsequent longitudinal study, pups were injected in an identical fashion and then tested (for 2 minutes) at 10, 20, 30, and 90 minutes following treatment with intervals between tests spent with littermates.

Ultrasonic calls were transformed by a digital sound spectrum analysis system providing on-line the number of calls in each 2-minute session (7). Records were stored on a magnetic disk to permit subsequent analysis for the frequency range and relative power of each call. Locomotor activity was recorded by visual scoring of crossovers on the floor grid of the recording chamber. Room temperature in all studies was 24°C. Pup temperatures were recorded with both a skin probe (YSI-J-8443) and a rectal probe (YSI-K74367) at the end of the isolation test.

o-CRF (1–41) and α-helical CRF (9–41) were obtained from Bachem (Torrance, CA). CRF was dissolved in phosphate buffered saline (PBS); α-helical CRF was initially dissolved in deionized water at pH 8.0, then brought to a final concentration in PBS. Saline was given in the form of PBS. Peptides or PBS were injected in a volume of 1 μl using a 30-g needle in unanesthetized pups. At 6 days of age, the rat skull is not completely calcified and landmarks can be directly visualized through the skin for percutaneous injections. To ensure that injections were actually ICV, each injection included 20% India ink by volume. Immediately after the posttreatment recording session, each pup was decapitated and the brain was inspected for injection site. Only data from pups with filling of at least one lateral ventricle were included in the analysis. Subcutaneous injections were given at the nape of the neck in a volume of 100 μl using a 30-g needle.

Doses of CRF were 0.001, 0.01, and 0.10 μg/pup and for α-helical CRF was 1.0 μg/pup. Between 7 and 13 pups were used at each dose (total n=56). Each active treatment condition included pups from at least three or four litters. Saline controls, which were drawn from littermates of pups receiving active treatment, represent 8 litters. Pups calling less that 60 (n=14) or more than 260 (n=5) times within the 2-minute baseline were excluded to avoid floor or ceiling effects in the analysis.

Trunk blood was collected in heparinized tubes for corticosterone determination. After blood was spun for 10 minutes at 3,000 RPM, plasma was removed and frozen at −20°C

TABLE 2

CRF EFFECTS ON TEMPERATURE

	n	Baseline		Posttreatment	
		Skin	Rectal	Skin	Rectal
Saline	7	30.7 ± 0.47	30.6 ± 0.18	29.8 ± 0.67	30.5 ± 0.14
CRF (0.1 μg)	9	30.4 ± 0.43	30.2 ± 0.23	29.7 ± 0.41	30.1 ± 0.24

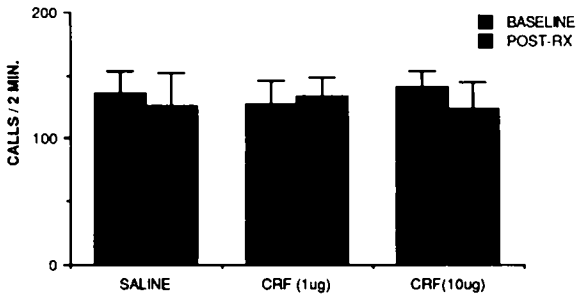

FIG. 3. Mean (±SEM) number of calls at baseline and 30 minutes following subcutaneous administration of saline, 1.0 μg CRF, or 10.0 μg CRF to 6-day-old pups. There are no significant differences in the rate of vocalization following peripheral administration of CRF.

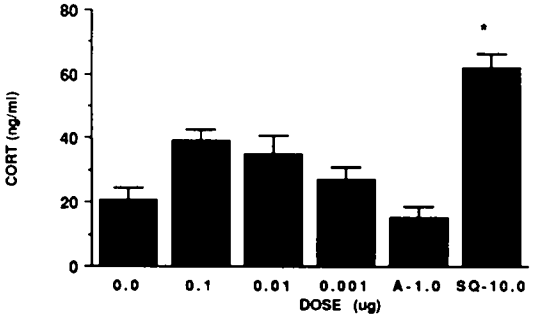

FIG. 4. Mean (±SEM) plasma concentration of corticosterone following either ICV or peripheral administration of CRF. Plasma levels of corticosterone are significantly increased (*signifies $p<0.05$) following peripheral but not central administration of CRF. These results, obtained from the same pups shown for vocalization data in Figs. 2 and 4, demonstrate a clear dissociation between calling rate and corticosterone changes following CRF administration.

until assay. Corticosterone was measured by radioimmunoassay using reagents provided by Radioassay Systems Laboratory (Carson, CA). Sensitivity of this assay is 3.0 ng/ml, with less than 1% cross reactivity with other steroids.

Data were analyzed with one-way ANOVA for both baseline values and for change from baseline. When significant group effects were present, group differences were analyzed by posthoc Duncan's Muliple Range test.

RESULTS

There were no significant group differences in baseline calling rate or locomotor scores (Table 1). Treatment was associated with significant group effects for vocalization, $F(5,49)=9.17$, $p=0.0001$, but not for locomotor scores, $F(5,49)=1.09$, $p=0.379$, assessed by one-way ANOVA on change scores from baseline.

Post hoc comparisons of the vocalization change scores disclosed significant differences ($p<0.05$) between saline and both the 0.1 and the 0.01 μg doses of CRF (Fig. 1). The rate of calling appeared to increase after administration of the 1.0 μg dose of the CRF antagonist; this change was significantly different from saline as well as all three doses of CRF. The CRF antagonist (1.0 μg) effectively blocked the decrease in vocalization observed after CRF (0.01 μg). Pups did not appear sedated nor did they show any evidence of convulsant activity after any of the treatments.

As the apparent decrease in vocalization following CRF might reflect fatigue following an initial increase in calling, an additional group of pups was recorded longitudinally following either ICV saline (n=6) or 0.1 μg CRF (n=6). As shown in Fig. 2, there is no evidence of an early burst of calling. The rate of calling decreased more with CRF than saline, $F(1,3)=7.034$, $p=0.02$, by repeated measures ANOVA examining changes from baseline. The rate of calling was significantly less (by post hoc test) in the CRF group at 20, 30, and 90 min following the ICV injection.

To determine if the change in vocalization rate following ICV CRF was secondary to thermoregulatory effects of the peptide, both skin and rectal temperature were monitored after ICV administration of either saline or 0.1 μg CRF. As shown in Table 2, there was no significant effect of centrally administered CRF on either skin or rectal temperature.

Finally, we wished to investigate if the CRF effects following ICV administration were due to a central target of action or secondary to transport of the peptide to a peripheral site. We administered either 1.0 μg CRF (n=8), 10.0 μg CRF (n=9), or saline (n=9) subcutaneously to 6-day-old pups. As shown in Fig. 3, peripheral administration of CRF did not significantly alter the rate of calling, $F(2,23)=0.377$, ns).

To further rule out a peripheral site of action, corticosterone was measured in the trunk blood of pups used in the ICV as well as subcutaneous CRF vocalization studies. As shown in Fig. 4, treatment effects were significant, $F(5,46)=15.2$, $p=0.0001$, but only peripheral CRF was associated with a significant ($p<0.05$ by post hoc test) increase in plasma corticosterone, although there was a trend for corticosterone to increase following the highest dose of CRF administered ICV.

DISCUSSION

On the basis of the substantial literature implicating CRF in the stress response, we predicted that centrally administered CRF would increase the rate of rat pup isolation calls. Contrary to this prediction, following ICV CRF, isolation calls were reduced in a dose-dependent fashion. As little as 0.01 μg CRF (roughly 1 μg/kg) decreased the rate of calling by 70%. This decrease was blocked by simultaneous treatment with the CRF antagonist, α-helical CRF (9–41). The decrease in calls following CRF was not due to sedation, an increase in body temperature, or a direct peripheral effect of the peptide.

The significant increase in calls after administration of the CRF antagonist is surprising. Most other recent behavioral studies with α-helical CRF (9–41) have not found intrinsic effects, although this analogue reverses stress-induced changes in aggression (29), feeding (17), and exploratory behavior (3). In the original description of the endocrine effects of α-helical CRF (9–41), Rivier and colleagues noted that the peptide decreased ACTH as well as blocking CRF-stimulated or stress-induced increases in ACTH. In the present study, this CRF antagonist was significantly different from both CRF and saline. In fact, 8 of 10 pups receiving the CRF antagonist increased their rate of calling from baseline compared to 5 of 13 receiving saline. Thus, it apears that on this behavioral measure, this CRF analogue has an intrinsic effect which is opposite to the native peptide.

During the first two postnatal weeks of the rat, plasma glucocorticoids are low and increase only slightly in response to several stressors (23). In spite of the very low levels of glucocorticoids, there is a modest but significant increase in response to social isolation throughout this relatively stress nonresponsive period (26). For this reason, one might presume that CRF in extra-hypothalamic sites would increase during isolation and that exogenous CRF would be associated with increased rates of ultrasonic isolation calls. How can the observed decrease in calls be explained? This decrease following CRF might be considered adaptive if a "stressed" quiet pup would be less likely to be detected by a predator. In fact, following several minutes of social isolation, the normal rate of calling decreases to about the level observed following CRF (author's unpublished data). One explanation for the paradoxical decrease in calling might be that CRF shuts down the behavioral response, shifting the pup prematurely into this prolonged isolation state. In other words, the rate of calling might vary as a U-shaped function with the level of distress—very low stress (following morphine or diazepam) and very high stress (following CRF) both associated with low rates of calling. One test of this hypothesis would be to administer CRF to "unstressed," nonisolated pups. If the peptide is, in fact, associated with increased distress, then vocalizations might be expected to increase with CRF while they are virtually absent with saline. In fact, Panksepp and co-workers have presented preliminary evidence of just this sort with young chicks (19). Chicks normally emit fewer distress calls if a mirror is present. Following CRF administration, the rate of distress calls does not change for chicks in isolation but increases for isolated chicks presented with a mirror.

Another possible explanation for the decrease in isolation calls following CRF is that the peptide has effects in development that are quite distinct from its role in the adult brain. Kalin has also found a decrease in distress calls following central administration of CRF to young rhesus monkeys (personal communication, 1988). Similarly, the alpha-2 adrenergic antagonist yohimbine, which appears to have clinical "anxiogenic" effects in adults, decreases locomotor behavior and isolation calls in rat pups (Kehoe, in press). Presumably these paradoxical effects in development reflect immature receptors or incomplete postsynaptic circuitry. With CRF, however, the available evidence suggests that the receptors appear very early in development although the ontogeny of the pathways in CRF receptor fields may lag behind (12).

The absence of significant effects following subcutaneous administration of CRF is somewhat surprising. In preliminary studies, we have noted relatively high penetration of CRF across the blood-brain barrier until postnatal day 28. If only 0.1% of the peripherally administered dose of 10 μg were to reach the central site of action, one would expect to observe a 70% reduction in the rate of calling. The failure to see any reduction in calling cannot be explained by a failure of absorption or rapid metabolism of the peptide, as corticosterone increases several fold in these animals, demonstrating activation of the pituitary. It is certainly possible that CRF has a rate-increasing peripheral effect (suggested at the 1.0 μg dose) which is counteracted by the central rate decreasing effects at the higher dose.

In summary, centrally administered CRF is associated with a decrease in the rate of isolation calls in 5–6-day-old-rat pups. This effect is not secondary to decreases in arousal or thermoregulatory capacity nor is it due to peripheral actions of the peptide. These results, along with the previous report of abundant CRF receptors in the rat pup brain, raise the possibility that CRF has an important physiologic role in development.

REFERENCES

1. Allin, J. T.; Banks, E. M. Effects of temperature on ultrasound production of infant albino rats. Dev. Psychobiol. 4:149–158; 1971.
2. Allin, J. T.; Banks, E. M. Functional aspects of ultrasound production by infant albino rats (rattus norvegicus). Anim. Behav. 20:175–185; 1972.
3. Berridge, C. W.; Dunn, A. A corticotropin-releasing factor antagonist reverses the stress-induced changes of exploratory behavior in mice. Horm. Behav. 21:393–401; 1987.
4. Britton, D. R.; Koob, G. F.; Rivier, J.; Vale, W. Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. Life Sci. 31:363–367; 1982.
5. Brown, M. R.; Fisher, L. A.; Rivier, J.; Spiess, J.; Rivier, C.; Vale, W. Corticotropin-releasing factor: Effects on the sympathetic nervous system and oxygen consumption. Life Sci. 30:207–210; 1982.
6. Brown, M. Corticotropin releasing factor: Central nervous system sites of action. Brain Res. 39:10–14; 1986.
7. Burkholder, J. H.; Hill, J. L.; Vaughan, W. J.; Cascio, H. E. A broadhand digitizing rat detector: Simultaneous recording from all sound frequencies in the range of rat ultrasonic vocalizations. Behav. Res. Methods Instrum. 14:511–518; 1982.

8. De Souza, E. B.; Perrin, M. H.; Rivier, J.; Vale, W.W.; Kuhar, M. J. Corticotropin-releasing factor receptors in rat pituitary gland: autoradiographic localization. Brain Res. 296:202–207; 1984.
9. De Souza, E. B.; Insel, T. R.; Perrin, M. H.; Vale, W. W.; Kuhar, M. J. Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: An autoradiographic study. J. Neurosci. 5:3189–3203: 1985.
10. Gardner, C. R. Inhibition of ultrasonic distress vocalications in rat pups by clordiazepoxide and diazepam. Drug Dev. Res. 5:185–193; 1985.
11. Hofer, M. A.; Shair, H. N. Isolation distress in two-week-old rats: Influence of home cage, social companions, and prior experience with littermates. Dev. Psychobiol. 20:465–476; 1987.
12. Insel, T. R. The ontogeny of brain receptors for corticotropin-releasing factor and the development of their functional association with adenylate cyclase. J. Neurosci. 8:4151–4158; 1988.
13. Insel, T. R.; Gelhard, R. E.; Miller, L. P. Rat pup isolation distress and the brain benzodiazepine receptor. Dev. Psychobiol.; in press.
14. Insel, T. R.; Hill, J. L. Infant separation distress in genetically fearful rats. Biol. Psychiatry 22:783–786; 1987.
15. Insel, T. R.; Hill, J. L.; Mayor, R. B. Rat pup ultrasonic isolation calls: Possible mediation by the benzodiazepine receptor complex. Pharmacol. Biochem. Behav. 24:1263–1267; 1986.
16. Koob, G. F.; Bloom, F. E. Corticotropin-releasing factor and behavior. Fed. Proc. 44:259–263; 1985.
17. Krahn, D. D.; Gosnell, B. A.; Grace, M.; Levine, A. S. CRF antagonist partially reverses CRF-and stress-induced effects on feeding. Brain Res. Bull. 17:285–289; 1986.
18. Morley, J. E.; Levine, A. S. Corticotropin releasing factor, grooming and ingestive behavior. Life Sci. 31:1459–1464; 1982.
19. Panksepp, J.; Normansell, L.; Herman, B.; Bishop, P.; Crepeau, L. Neural and neurochemical control of the separation distress call. In: Newman, J. D., ed. The physiology of mammalian vocalization. New York: Plenum Press; 1988:263–300.
20. Pappas, B. A.; Walsh, P. Behavioral comparison of pentylenetetrazol, clonidine, chlordiazepoxide and diazepam in infant rats. Pharmacol. Biochem. Behav. 19:957–961; 1983.
21. Rivier, J.; Rivier, C.; Vale, W. Synthetic competitive antagonists of corticotropin-releasing factor: Effect on ACTH secretion in the rat. Science 224:889–891; 1984.
22. Sales, G. D.; Pye, D. Ultrasonic communication by animals. London: Chapman and Hall; 1974:149–231.
23. Sapolsky, R. M.; Meaney, M. J. Maturation of the adrenocortical stress response: Neuroendocrine control mechanisms and the stress hypo-responsive period. Brain Res. Rev. 11:65–76; 1986.
24. Sirinathsinghji, D. J. S.; Rees, L. H.; Rivier, J.; Vale, W. Corticotropin-releasing factor is a potent inhibitor of sexual receptivity in the female rat. Nature 305:232–235; 1983.
25. Smotherman, W. P.; Bell, R. W.; Starzec, I.; Elias, J.; Zachman, T. A. Maternal responses to infant vocalizations and olfactory cues in rats and mice. Behav. Biol. 12:55–66; 1974.
26. Stanton, M. E.; Wallstrom, J.; Levine, S. Maternal contact inhibits pituitary-adrenal stress responses in preweanling rats. Dev. Psychiobiol. 20:131–145; 1987.
27. Sutton, R. E.; Koob, G. F.; Le Moal, M.; Rivier, J.; Vale, W. Corticotropin releasing factor produces behavioural activation in rats. Nature 297:331–333; 1982.
28. Tazi, A.; Dantzer, R.; Le Moal, M.; Rivier, J.; Vale, W.; Koob, G. F. Corticotropin-releasing factor antagonist blocks stress-induced fighting in rats. Regul. Pept. 18:37–42; 1987.
29. Vale, W.; Spiess, J.; Rivier, C.; Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394–1397; 1981.
30. Walker, C.-D.; Sapolsky, R. M.; Meaney, M. J.; Vale, W. W.; Rivier, C. Increased pituitary sensitive to glucocorticoid feedback during the stress nonresponsive period in the neonatal rat. Endocrinology 119:1816–1821; 1986.