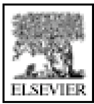

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Age-specific threats induce CRF expression in the paraventricular nucleus of the hypothalamus and hippocampus of young rats

Christoph P. Wiedenmayer[a, b], Ana M. Magariños[c], Bruce S. McEwen[c] and Gordon A. Barr[b, d]

[a]Department of Psychiatry, Columbia University, New York, NY 10032, USA
[b]Developmental Psychobiology, New York State Psychiatric Institute, New York, NY 10032, USA
[c]Laboratory of Neuroendocrinology, Rockefeller University, New York, NY 10021, USA
[d]Department of Psychology, Hunter College, New York, NY 10021, USA

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Abstract

Young animals respond to threatening stimuli in an age-specific way. Their endocrine and behavioral responses reflect the potential threat of the situation at a given age. The aim of the present study was to determine whether corticotropin-releasing factor (CRF) is involved in the endocrine and behavioral responses to threat and their developmental changes in young rats. Preweaning 14-day-old and postweaning 26-day-old rats were exposed to two age-specific threats, cat odor and an adult male rat. The acute behavioral response was determined during exposure. After exposure, the time courses of the corticosterone response and of CRF expression in the paraventricular nucleus of the hypothalamus (PVN) and in extrahypothalamic areas were assessed. Preweaning rats became immobile when exposed to cat odor or the male rat, whereas postweaning rats became immobile to cat odor only. Male exposure increased serum corticosterone levels in 14-day-old rats, but cat odor failed to increase levels at either age. Exposure induced elevation of CRF mRNA levels in the PVN that paralleled changes in corticosterone levels. CRF may thus play a role in endocrine regulation and its developmental changes during early life. Neither cat odor nor the adult male altered CRF mRNA levels in the bed nucleus of the stria terminalis (BNST) or the amygdala, but both stimuli increased levels in the hippocampus. Hippocampal CRF mRNA expression levels did not parallel cat odor or male-induced immobility, indicating that CRF is not involved in this response in young rats but may be involved in aspects of learning and

memory.

Keywords: Immobility; Fear; Predation; HPA axis; Glucocorticoid

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Introduction

Interest in the neural processes that govern behavioral changes in early life has increased considerably over the last decade. Basic as well as clinical research has demonstrated that the brain undergoes fundamental changes in early development and that such changes may contribute to age-specific alterations in behavior (Spear, 2000). The succession of changes in the neural organization allows the growing organism to adapt to rapidly changing

environments (Oppenheim, 1981 and Stamps, 2003). Ontogenetic plasticity, however, renders an individual vulnerable to aversive experience and increases the risk for psychopathologies later in life (Sánchez et al., 2001 and Steinberg and Avenevoli, 2000). For example, traumatic experience may shape the development of neural systems that mediate fear- and anxiety-related behaviors and is associated with the development of anxiety disorders such as posttraumatic stress disorder (Heim and Nemeroff, 2001). Knowledge of the neural processes underlying ontogenetic changes in behavior is crucial in our understanding of the extent and limits of developmental plasticity.

In young rats, behavioral responses to threatening stimuli that are ecologically relevant change during the first weeks of life. When exposed to an unfamiliar adult male rat, preweaning rats become immobile (freeze), whereas around weaning and after weaning, rats approach the male (Hepper, 1986, Takahashi, 1992 and Wiedenmayer and Barr, 1998). In contrast, rats become immobile throughout ontogeny when exposed to cat cues, although responsivity increases with age (Bronstein and Hirsch, 1976, Hubbard et al., 2004 and Wiedenmayer and Barr, 2001b). Endocrine responses to threat undergo changes in early life as well. Various stressors, such as exposure to cold, to ether vapor, or restraint, increase corticosterone levels in an age-specific way (Dent et al., 2000a, Walker et al., 1991 and Yi and Baram, 1994). For example, a saline injection increased corticosterone levels only in 18-day-old but not in younger rats (Dent et al., 2000b).

Behavioral and endocrine responsivity to threat and their ontogenetic changes may be mediated by the neuropeptide corticotropin-releasing factor (CRF). CRF plays a critical role as neuromodulator in both the fear and stress pathway. CRF was originally identified as a releasing factor that activates the hypothalamus–pituitary–adrenal (HPA) stress axis (Vale et al., 1981). Upon aversive stimulation, CRF is secreted from cells in the paraventricular nucleus of the hypothalamus (PVN) and induces, via a cascade of neuroendocrine events, the release of glucocorticoids from the adrenal glands (Herman and Cullinan, 1997). CRF acts also as a neurotransmitter in brain pathways outside the hypothalamus that mediate fear- and anxiety-related behaviors. Aversive stimulation induced the expression of CRF mRNA (Hsu et al., 1998, Kalin et al., 1994 and Makino et al., 1999) and the release of CRF (Cook, 2002, Merali et al., 1998 and Merlo Pich et al., 1995) in brain areas of the fear pathway (LeDoux, 2000) such as the amygdala and the bed nucleus of the stria terminalis (BNST). When CRF antagonists or CRF antisense were centrally infused either into the ventricular system or directly into the amygdala or locus ceruleus of rats submitted to aversive stimulation, responses such as freezing, anxiety-like behaviors in the elevated plus-maze, and withdrawal into a shelter were attenuated or blocked (Heinrichs et al., 1992, Kalin et al., 1988, Skutella et al., 1994, Smagin et al., 1996, Swiergiel et al., 1992, Swiergiel et al., 1993 and Takahashi et al., 1989).

Consistent with a dual role of CRF, aversive stimuli that activated CRF in the amygdala induced CRF expression in the PVN as well (Hsu et al., 1998 and Kalin et al., 1994). When CRF was blocked centrally, both HPA axis activity and fear-related behaviors were attenuated (Skutella et al., 1994). Mice that lack the CRF receptor 1 in the amygdala and other extrahypothalamic areas but not in the PVN showed reduced anxiety-like behaviors, but their basal HPA activity was unaffected (Müller et al., 2003).

In young animals, CRF has been implicated in developmental aspects of HPA axis regulation (for reviews, see Brunson et al., 2001, Dallman, 2000 and Levine, 2001). Only a few studies have demonstrated that CRF is involved in behavioral responses to aversive stimulation. CRF and CRF antagonists modulated ultrasonic vocalizations in preweaning rat pups that were separated from dam and nest (Harvey and Hennessy, 1995, Insel and Harbaugh, 1989 and Kehne et al., 2000). However, little is known about the role of CRF in other anxiety- and fear-like behaviors in the infant.

The aim of the present study was to examine if developmental changes of threat-induced CRF activation parallel changes in the endocrine and behavioral response in young rats. CRF expression levels were assessed in the PVN and in extrahypothalamic areas of the fear pathway in preweaning 14-day-old and postweaning 26-day-old rats exposed to cat odor or an adult male rat. We hypothesized that in preweaning rats, both male and cat cues induce immobility, corticosterone secretion, and CRF expression, whereas in postweaning rats, only cat cues are effective.

Materials and methods

Animals

Long-Evans hooded rats were housed under standard laboratory conditions in a colony room with a 12-h light–dark cycle with light onset at 6:00 AM. When the females were pregnant, the males were removed from the breeding cages. Cages were monitored daily in the morning and evening for the presence of newborn pups, and the date of birth was considered as day 0. On postnatal day 23, the mother was removed, and littermates were kept together in the same cage. Because standard laboratory cages are too small for litters of 10 animals kept together up to an age of 26 days, rats were housed in larger cages (55 × 38 × 21 cm). A sexually experienced unrelated adult male was housed in the same colony room. Treatments were according to the guidelines of the Institutional Animal Care and Use Committee.

Little is known about sex differences in endocrine and behavioral responses and in CRF expression in young rats. No gender effect was found in male-induced immobility in 14- and 21-day-old rats (Wiedenmayer and Barr, 1998). The studies quoted in this paper that assessed corticosterone and CRF mRNA levels used in most cases mixed litters. However, sex differences were never reported or discussed. In the present study, only male rats were used.

Testing procedures

On the day of testing, on postnatal days 14 and 26, rats of a litter were assigned to four groups: (1) unexposed control, (2) control-exposed, (3) cat odor-exposed, and (4) male-exposed. First, one rat was taken from the home cage and decapitated within a minute. This animal represented the unexposed control that provided basal measurements of corticosterone and CRF (see below). Then one of the groups 2–4 was removed from the home cage and placed in the testing cage. Each group consisted of a small huddle of three rats to decrease isolation-induced stress (Hennessy and Weinberg, 1990 and Hofer and Shair, 1980). The order of testing of these three groups, the exposed groups, was alternated between litters. The translucent testing cage (46 × 25 × 21 cm) was subdivided into two equal compartments by a wire-mesh partition positioned in the middle of the cage. The compartment into which the young rats were placed contained home cage bedding to simulate the nest area. The other compartment of the testing cage was empty. The rats were left to acclimate for 5 min. Afterwards, the stimulus was placed in the adjacent compartment for 5 min. For the group ‘cat odor-exposed,’ the stimulus consisted of a container (11 × 12 × 8 cm) with approximately 100 g of soiled cat bedding, which was taken out of litter boxes in our animal facility in the morning. For the group ‘male-exposed,’ the stimulus was the adult male rat. The ‘control-exposed’ animals were tested with the adjacent compartment empty. After exposure, the three rats of a group were not returned to the home cage because interactions with the dam may alter the consequences of exposure (Wiedenmayer et al., 2003). They were instead set aside for corticosterone and CRF mRNA assessment (see below). All tests were conducted between 1:00 and 5:00 PM during the light period.

Behavioral observations

During the 5 min of exposure, the behavior of the young rats was recorded. Every 15 s, it was noted whether the rats were immobile or not. Immobile was defined as any posture in which the animal did not exhibit any movement except for respiration, and was expressed as a percentage of the scans. Twenty-two litters of 14-day-old rats and 17 litters of 26-day-old rats were tested.

Corticosterone radioimmunoassay

As described above, one rat was decapitated within a minute for basal measurements (unexposed control, day 14: $N = 21$, day 26: $N = 17$). One rat from the three exposed groups was decapitated 15 min (day 14: $N = 7$, day 26: $N = 5$), 30 min (day 14: $N = 7$, day 26: $N = 7$), or 60 min (day 14: $N = 7$, day 26: $N = 5$) after the onset of exposure. Trunk blood was collected in chilled EDTA-coated tubes (Sherwood, St. Louis, MO) and centrifuged (4000 rpm) at 4°C for 15 min. Plasma was stored at −30°C until assayed. Corticosterone levels were determined by solid-phase radioimmunoassay (DPC, Los Angeles, CA). Assay sensitivity was 0.5 μg/dl. Intraassay and interassay variability were 12.2–4.3% and 14.9–5.8%, respectively.

In situ hybridization histochemistry

CRF expression was determined by quantifying CRF mRNA abundance with in situ hybridization. In adults, CRF mRNA levels are generally assessed at the peak of expression 2–4 h after stimulation (for example, Helmreich et al., 1999, Imaki et al., 1992, Kalin et al., 1994 and Ma et al., 1997). In young rats, elevation of CRF mRNA levels can already be detected after 15 and 30 min (Dent et al., 2000b). We therefore assessed CRF mRNA levels 30 min and 3 h after the onset of stimulation. Basal CRF mRNA levels were determined for the unexposed control rats ($N = 7$ at both ages). Thirty ($N = 7$) and 180 min ($N = 7$) after the onset of exposure, one rat from each of the three exposed groups at both ages was injected intraperitoneally with an overdose of nembutal (pentobarbital sodium, Abbott Laboratories, North Chicago, IL). The anesthetized rats were decapitated, and their brains were rapidly removed over powdered dry ice and stored at −80°C. The frozen brains were sectioned coronally (20 μm) in a cryostat at −20°C and thaw-mounted on Vectabond-coated slides (Vector Laboratories, Burlingame, CA). Three out of every 12 sections were collected per slide, and slides were stored at −80°C. Sections were fixed at 4°C in 2% paraformaldehyde and washed in 0.5 × SSC buffer (sodium chloride, sodium citrate buffer). Sections were air-dried at room temperature, rinsed for 1 min in acetylation buffer, and then acetylated for 10 min with 0.25% acetic anhydride dissolved in acetylation buffer. Slides were washed twice in 2 × SSC for 5 and 2 min and finally air-dried. Sections were hybridized overnight at 37°C in a humidified chamber with a solution consisting of 1 × Denhard's (1% bovine serum albumin, 1% polyvinylpyrrolidone, 1% Ficoll), 3 × SSC buffer, 50% formamide, 10% dextran sulfate, 10 mM DTT, 100 μg/ml of sheared and denaturated salmon sperm DNA, 400 μg/ml of tRNA, 1 mM EDTA, 4 μg/ml heparin, and the oligonucleotide probe (see below). The hybridization solution was applied in a volume of 300 μl. The next day, the sections were washed two times in 1 × SSC buffer/1 mM DTT followed by one wash at 45°C in 1 × SSC/1 mM DDT and one final wash at 45°C in 1 × SSC (20 min each). Sections were dried and washed in 70%, 85%, and 100% ethanol. Hybridized sections were apposed to film (Hyperfilm MP, Amersham Pharmacio Biotech, Piscataway, NJ) for 1–4 days. Sections of the four groups were assayed together. After the films were developed, sections were stained with cresyl violet for orientation purposes.

Radiolabeling of the oligonucleotide probes

The precursor for CRF, which consists of 187 amino acids (Jingami et al., 1985), was detected with an antisense synthetic oligonucleotide probe that was labeled with ^{35}S-dATP. The following probe (Oligos Etc., Wilsonville, OR) was used: 5′CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC3′.

The 48-base probe corresponded to amino acids 22–37, and its specificity was determined previously (Young et al., 1986). The probe was labeled at the 3′ end by terminal transferase (Roche, Indianapolis, IN). The reaction consisted of 200 mM potassium cacodylate buffer, 25 mM cobalt chloride, ^{35}S-dATP (Amersham Pharmacia Biotech), and deoxynucleotidylexotransferase (total reaction volume of 11 μl). The reaction mixture was incubated at 37°C for 1 h, and the oligonucleotide probe was separated from unincorporated isotope by chromatography on a G-25 Sephadex Quick Spin column (Boehringer Mannheim, Roche) at room temperature. The column buffer consisted of 10 mM Tris-Cl buffer and 1 mM EDTA. Activity of the probe was 8×10^6 cpm/ml.

Quantification of CRF mRNA levels

CRF mRNA abundance was determined by quantifying gray levels on X-ray autoradiograms in regions corresponding to the PVN, subdivisions of the BNST, amygdala, and hippocampus (Paxinos and Watson, 1998). The BNST was subdivided into medial, lateral, and ventral nuclei; the amygdala was subdivided into the basolateral complex [consisting of the lateral and basolateral nuclei (Maren, 1999), called in the following basolateral amygdala], central and medial amygdala; the hippocampus was subdivided into dentate gyrus, CA3, and CA1. Gray levels were quantified with an image analysis system utilizing the NIH Image 1.49 software by a person without knowledge of the treatment of the groups. Film background values were subtracted from all sections. The following number of sections per animal was quantified bilaterally: PVN 3–4, BNST 3, amygdala 3–4, and hippocampus 4–5 sections. The values were averaged for each animal to generate an optical density value that corresponded to CRF mRNA abundance per animal per brain structure. Sections of animals from the four groups were matched for corresponding neuroanatomical levels.

Data analysis

Immobility, corticosterone, and CRF mRNA levels in each brain area at each age were analyzed by separate factorial analyses of variance (ANOVA), and Newman-Keuls tests were used for post hoc comparisons. For immobility, the three exposed groups (control-exposed, cat odor-exposed, male-exposed) were within litter variables and were treated as repeated measures. For corticosterone and CRF mRNA data, all four groups were the within litter variables.

Results

Immobility

In 14-day-old rats, immobility during exposure differed significantly across the three exposed groups [$F(2,42) = 126.6$, $P < 0.001$]. Male-exposed rats were more immobile than control-exposed and cat odor-exposed rats ($P < 0.001$), and cat odor-exposed rats were more immobile than controls ($P < 0.01$, Fig. 1). On day 26, immobility during exposure differed significantly across groups [$F(2,32) = 30.2$, $P < 0.001$]. Cat odor-exposed rats were significantly more immobile than control-exposed and male-exposed rats ($P < 0.001$), which did not differ from each other (Fig. 1).

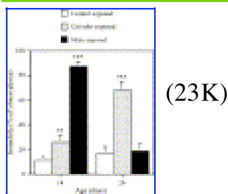

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Fig. 1. Immobility (means ± SE) of preweaning 14-day-old rats and postweaning 26-day-old rats ($N = 22$ and 17, respectively) during 5 min of exposure to cat odor or an adult male rat. Control-exposed rats were tested with the cage empty. ** $P < 0.01$, *** $P < 0.001$.

Corticosterone

In 14-day-old rats, there was a main effect for groups 15 min [$F(3,18) = 13.9$, $P < 0.001$], 30 min [$F(3,18) = 12.6$, $P < 0.001$], and 60 min [$F(3,18) = 3.6$, $P < 0.05$] after the onset of exposure. Corticosterone levels in the exposed groups were higher than in unexposed controls (baseline) at all three time points ($P < 0.05$), with the exception of control-exposed rats that did not differ from unexposed rats after 60 min. Thirty minutes after exposure, male-exposed rats had higher corticosterone levels than control-exposed and cat odor-exposed rats ($P < 0.05$, Fig. 2). The three exposed groups did not differ from each other 15 and 60 min after exposure.

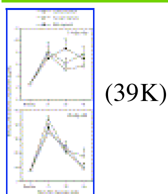

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Fig. 2. Time course of plasma corticosterone (means ± SE) in preweaning 14-day-old and postweaning 26-day-old rats before and after exposure to cat odor or an adult male rat. Control-exposed rats were tested with the cage empty. Baseline: day 14 $N = 21$; day 26 $N = 17$; 15 min: day 14 $N = 7$; day 26 $N = 5$; 30 min: day 14 $N = 7$; day 26 $N = 7$; 60 min: day 14 $N = 7$; day 26 $N = 5$. * $P < 0.05$.

Levels in 26-day-old rats differed significantly across groups 15 min [$F(3,12) = 26.6$, $P < 0.001$] and 30 min [$F(3,18) = 6.5$, $P < 0.01$] but not 60 min after exposure. Levels of the three exposed groups were higher than baseline levels from unexposed controls ($P < 0.01$) 15 and 30 min after exposure, but the three exposed groups did not differ from each other (Fig. 2).

CRF expression

PVN

In 14-day-old rats, CRF mRNA levels in the PVN differed significantly across the four groups 30 min after exposure [$F(3,18) = 5.9$, $P < 0.01$]. Levels did not differ between unexposed and control-exposed rats. Levels were higher in male-exposed rats compared to unexposed and exposed controls ($P < 0.05$) and were higher in cat odor-exposed compared to unexposed rats [$P < 0.05$, Fig. 3]. Fig. 4 depicts CRF mRNA abundance in the exposed groups. After 180 min, CRF mRNA levels still differed across groups [$F(3,18) = 8.5$, $P < 0.01$]. Levels of the three exposed groups were significantly higher than those of the unexposed animals ($P < 0.01$), but exposed groups did not differ from each other (Fig. 3).

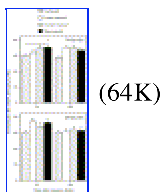 (64K)

Fig. 3. Time course of CRF mRNA expression (means ± SE) in the paraventricular nucleus of the hypothalamus (PVN) of preweaning 14-day-old rats and postweaning 26-day-old rats after exposure to cat odor or to an adult male rat. Control exposed rats were tested with the cage empty. $N = 7$ for each age and each time point. $*P < 0.05$.

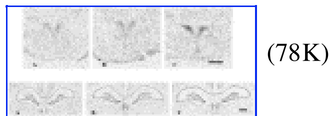 (78K)

Fig. 4. Photomicrographs showing CRF mRNA expression 30 min after exposure to (B) cat odor and (C) an adult male rat. (A) Represents a control exposed animal that was tested with the cage empty. (Top) The paraventricular nucleus of the hypothalamus of 14-day-old rats. Scale bar = 0.5 mm. (Bottom) The dentate gyrus of 26-day-old rats. Scale bar = 1 mm. Film images are representative for group means. They were cropped without any image alteration.

In 26-day-old rats, CRF mRNA levels in the PVN differed across groups 30 min after exposure [$F(3,18) = 7.5$, $P < 0.01$]. Levels of exposed animals were higher than unexposed ($P < 0.05$) but did not differ from each other (Fig. 3). There was no difference across the four groups 180 min after exposure.

BNST, amygdala

CRF mRNA levels in the medial, lateral, and ventral nucleus of the BNST and in the basolateral, central, and medial nucleus of the amygdala did not differ across groups 30 and 180 min after exposure in 14- and 26-day-old animals.

Hippocampus

In 14-day-old rats, CRF mRNA levels in the dentate gyrus and CA1 differed significantly across groups 180 min after exposure [$F(3,18) = 5.6$, $P < 0.01$, $F(3,18) = 4.2$, $P < 0.05$, respectively]. In the three exposed groups, CRF mRNA levels were higher in both hippocampal layers compared to unexposed controls ($P < 0.01$, $P < 0.05$, respectively, Fig. 5).

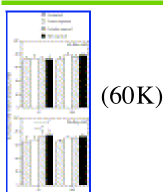 (60K)

Fig. 5. Time course of CRF mRNA expression (means ± SE) in the dentate gyrus of preweaning 14-day-old and postweaning 26-day-old rats after exposure to cat odor or an adult male rat. Control-exposed rats were tested with the cage empty. $N = 7$ for each age and each time point. $*P < 0.05$.

In 26-day-old rats, CRF mRNA levels in the dentate gyrus differed significantly across groups 30 min [$F(3,18) = 5.1$, $P < 0.01$] and 180 min [$F(3,18) = 6.7$, $P < 0.01$] after exposure. After 30 min, cat odor- and male-exposed rats had higher levels than unexposed and control-exposed rats ($P < 0.05$, Fig. 5). The microphotograph in Fig. 4 shows CRF mRNA abundance in the three exposed groups. After 180 min, all three exposed groups had significantly higher levels in the dentate gyrus than unexposed controls ($P < 0.05$) but did not differ from each other (Fig. 5). CRF mRNA levels in CA1 showed a tendency to be different across groups after 180 min [$F(3,18) = 2.7$, $P < 0.07$], with exposed groups being elevated compared to baseline ($P < 0.09$).

CRF mRNA levels in CA3 did not differ from baseline at both ages.

Discussion

Acute exposure to ecologically salient threats induced behavioral and endocrine responses and increased CRF expression in an age-specific way in young rats. Changes in CRF mRNA levels in the PVN paralleled changes in corticosterone levels, indicating a role of CRF in endocrine regulation. In contrast, exposure to threatening stimuli did not increase CRF mRNA levels in the amygdala or BNST, and levels in the hippocampus did not parallel the immobility response. Therefore, CRF seems to contribute to the regulation of the endocrine response and its developmental changes but not to the immediate behavioral response to threat in the infant rat.

Behavioral response

Young rats exposed to cat odor or the male rat responded in a way that reflected the potential threat of the situation. They became immobile when exposed to cat odor or the adult male rat before weaning and to cat odor after weaning. The postweaning rats were not immobile in the presence of the adult male rat that, at that age, does not represent an infanticidal threat (Paul and Kupferschmidt, 1975 and Takushi et al., 1983). Responsivity to cat odor increased with age, which may reflect increased predation threat in older rats (Wiedenmayer and Barr, 2001b). Such differential responsivity seems to be adaptive because it provides the animal with fitness benefits. Immobility in the presence of the adult male before weaning may prevent infanticide, whereas after weaning, when infanticide stops (Paul and Kupferschmidt, 1975), approaching the male may help familiarize the young rat with group members (Takahashi, 1992). On the other hand, immobility in the presence of cat cues seems to represent an antipredator strategy effective throughout life (Fanselow and Lester, 1988). Immobility seems to be the most effective response in young rats because it reduces the likelihood to be detected by an intruding adult male rat or a hunting cat (Thor et al., 1981).

Endocrine response

Acute exposure also led to rapid elevation of glucocorticoid levels at both ages in all exposed groups. Within 15 min after the onset of exposure, young rats had increased blood serum concentration of corticosterone. The time course and magnitude of the endocrine response depended on the age of the rats and to a lesser degree on the type of stimulus.

On day 14, exposure increased corticosterone levels in all exposed animals, but levels were significantly higher in male-exposed pups. It could be argued that exposure to a behaving male rat provides much stronger stimulation than cat odor. However, behaviorally, the pups did respond to cat odor. Also, male odor alone is effective in inducing a glucocorticoid response. Placing 5-day-old pups on soiled bedding from an adult male rat increased their corticosterone levels (Tanapat et al., 1998). Elevated corticosterone levels to the male but

not to cat odor indicate differential endocrine responsivity in 14-day-old rats, although it has been postulated that the activation of the HPA axis is unspecific and general (for review, see Pacák and Palkovits, 2001). These findings also indicate that an ecologically relevant stimulus can induce a rapid and distinct endocrine response in 14-day-old rats despite their age.

It has been argued that the presence of the mother inhibits HPA axis activation, and separation from her disinhibits the HPA axis in preweaning rats (Levine, 2001). Separation from the mother may have affected two aspects of the endocrine response in the 14-day-old rats. First, the testing procedure, which induced corticosterone secretion in the control-exposed animals as well, included maternal separation. The removal of maternal cues can be sufficient to elevate glucocorticoid levels. In 10- and 15-day-old rats separated as a group from their mother, corticosterone levels increased gradually and were significantly elevated after 2 h of separation (Kuhn et al., 1990). Our findings in the control-exposed group indicate that separation from the mother rapidly increases glucocorticoid levels and therefore is a potent stressor in preweaning rats. Second, the mother appears to regulate the temporal course of the corticosterone response. When a stressful stimulation is terminated, corticosterone levels may remain elevated in the absence of the mother. For example, corticosterone levels remained elevated for several hours after restraint or cold stress in 10-, 12-, and 13-day-old rats kept separated from their mothers (Walker et al., 1991 and Yi and Baram, 1994). Contact with a lactating dam in a novel environment inhibited isolation-induced elevation of corticosterone in 12-, 16-, and 20-day-old rats (Stanton et al., 1987), and contact with the mother decreased male-induced corticosterone levels in 14-day-old rats (Wiedenmayer et al., 2003). In the present study, the 14-day-old rats remained separated from their mother after exposure, and their corticosterone levels were still elevated 60 min after the termination of exposure.

The HPA axis undergoes profound developmental changes in early life. Between postnatal days 2 and 14, basal corticosterone levels are low, and the stimulus-induced activation of the HPA axis is diminished (Levine, 2001 and Sapolsky and Meaney, 1986). A reduction of corticosterone secretion seems to be required for normal development of the nervous system (Levine, 2001 and Sapolsky and Meaney, 1986). After this stress hyporesponsive period, the magnitude of the corticosterone response increases gradually with age (Walker et al., 1991). In the present study, basal and stimulus-induced corticosterone levels in 26-day-old rats were higher than the levels in preweaning 14-day-old rats. On day 26, basal levels and the magnitude of the stimulus-induced glucocorticoid response resembled the levels of adult rats. Acute exposure to a compound of fox odor increased corticosterone from 8 μg/dl baseline levels to stimulus-induced levels of 37 μg/dl 20 min later in adult rats (Morrow et al., 2000). A similar response was induced when a cat odor-impregnated cloth was placed for 5 min outside the home cage of adult rats. Adult corticosterone levels increased from approximately 12 to 52 μg/dl within 30 min (File et al., 1993). The time course of the glucocorticoid response in adult rats after short, acute stimulation includes an increase of corticosterone levels within 15 min and a return to baseline levels within 45–60 min (Kabbaj et al., 2000). A similar decline was found in the 26-day-old rats, which indicates a fast recovery from exposure and contrasts the prolonged elevated levels on day 14.

On day 26, contrary to our prediction, corticosterone levels did not differ between the three different exposed conditions, indicating that exposure increased corticosterone levels in an unspecific way. Although cat odor induced high levels of immobility, it was not effective in inducing a stronger endocrine response compared to the male. Alternatively, the experimental procedure may have elevated corticosterone to maximum levels, which prevented an additional increase induced by cat odor. Procedures included exposure to a novel environment that may have been sufficiently aversive to activate the HPA axis. Rats were tested in groups of three to decrease isolation-induced stress (Hennessy and Weinberg, 1990 and Hofer and Shair, 1980) and in a compartment that contained soiled home cage

bedding to mimic the nest area. Nevertheless, manipulations such as picking the rats up and novel features of the testing cage such as the wire mesh screen could have produced elevated corticosterone levels. It remains to be investigated if corticosterone increases in control-exposed animals could be prevented, for example, by habituating the rats to the testing procedure, and if in that case, cat odor-exposed rats would display an increased glucocorticoid response.

The profile of the immobility response was not fully matched by the profile of the glucocorticoid response. For example, cat odor induced freezing but did not elevate corticosterone levels in 14-day-old rats. Such dissociation supports the findings that different neural systems underlie the behavioral and endocrine responses to threat (de Kloet et al., 1988).

CRF expression

After exposure to cat odor and the adult male rat, CRF mRNA levels were elevated in components of the stress and fear pathway. The increase of CRF mRNA abundance may indicate that the exposure induced CRF biosynthesis. Synthesis may lead to refilling of stores in axon terminals and replenish previously released CRF (Dallman, 2000). Increased synthesis may also up-regulate CRF and affect reactivity to subsequent stimulation by supporting a higher rate of utilization in a later encounter with the threat (Aubry et al., 1999, Avishai-Eliner et al., 2002 and Brunson et al., 2001). In the following, changes of CRF expression in the four investigated brain areas and their relation to endocrine and behavioral responses are discussed.

PVN

Acute exposure to an aversive situation such as restraint, immobilization, and tail shock increases CRF mRNA levels in the PVN of adult rats (Aubry et al., 1999, Helmreich et al., 1999, Hsu et al., 1998, Kalin et al., 1994 and Ma et al., 1997). Similar situations increase CRF mRNA abundance in the PVN of young rats. Restraint rapidly elevated CRF mRNA levels in 6-, 12-, and 18-day-old rats (Dent et al., 2000a). Forty to 60 min of exposure to cold increased CRF mRNA levels in 9-, 10-, and 16-day-old rats (Hatalski et al., 1998, Hatalski et al., 2000 and Yi and Baram, 1994). We extend these findings and demonstrate that brief exposure to an ecologically relevant threat, an adult male rat, rapidly increased CRF expression in an age-specific way in the PVN of preweaning rats. Cat odor was less effective and only slightly increased CRF mRNA levels in preweaning rats 30 min after the onset of exposure. Such rapid expression seems to depend on the type of stimulus. The mild stress of a saline injection led to CRF expression within 15 min in 6-, 12-, and 18-day-old rats (Dent et al., 2000b), whereas cold stress increased CRF mRNA levels only 4 h after stress termination in 16-day-old rats (Yi and Baram, 1994).

CRF mRNA levels were also increased 3 h after exposure in 14-day-old rats and 30 min after exposure in 26-day-old rats. The elevation of CRF mRNA levels in all exposed groups at both ages can be linked to the endocrine response. Stimulus-induced elevation of CRF mRNA abundance in the PVN indicates the activation of the HPA axis. In adults, elevated CRF mRNA levels were accompanied by increased corticosterone concentrations (Helmreich et al., 1999 and Ma et al., 1997), and intraventricular CRF antisense injection blocked CRF expression in the PVN and ACTH release (Skutella et al., 1994). In preweaning rats, stimulus-induced CRF expression and corticosterone concentrations were concurrently elevated after restraint stress (Dent et al., 2000a) and cold stress (Hatalski et al., 1998 and Hatalski et al., 2000). When preweaning rats were immunized against CRF, increases in corticosterone after cold stress were abolished (Yi and Baram, 1994). In the present study, the time course of CRF mRNA expression in the PVN matched the time

course of the glucocorticoid response. On day 14, exposure, particularly to the male, induced a rapid increase in both CRF mRNA and corticosterone concentrations. Levels of CRF mRNA and corticosterone remained elevated for an extended period of time. It has to be further investigated whether corticosterone levels remain elevated for 3 h, thus paralleling CRF levels. On day 26, both central CRF mRNA and peripheral corticosterone levels were elevated rapidly after exposure and were back to pretest levels within 180 and 60 min, respectively.

Taken together, stimulation-induced CRF expression paralleled the endocrine response in an age-specific way and may therefore have contributed to the regulation of the HPA axis and its developmental changes.

BNST, amygdala

The BNST and the amygdala are components of the fear pathway and mediate responses to aversive stimuli (Davis, 2000 and LeDoux, 2000). In adult rats, both structures express CRF (Beyer et al., 1988). Aversive stimulation such as restraint or social stress induced the release of CRF at the amygdala (Merali et al., 1998), increased levels of CRF mRNA in the amygdala and BNST (Hsu et al., 1998 and Makino et al., 1999), and CRF antagonists infused into the amygdala decreased fear-like behavior (Swiergiel et al., 1993). Findings in young rats are however not as conclusive. Acute cold stress did not elevate CRF mRNA in the central amygdala in 9- and 10-day-old pups (Hatalski et al., 1998). In the present study, neither cat odor nor the adult male rat induced CRF expression in the BNST or amygdala of preweaning and postweaning rats. Because brain areas involved in responses to threat undergo maturational changes during early life (Sullivan et al., 2000), the CRF system in the BNST and amygdala may not be functionally mature in young rats. However, CRF receptor expression in the amygdala increases after birth and peaks on postnatal day 9 (Avishai-Eliner et al., 1996). The amygdala of preweaning rats is also responsive to male threat as male exposure induced the expression of the immediate early gene c-*fos* and of preproenkephalin, the precursor of the opioid peptide enkephalin, in the amygdala of preweaning rats (Wiedenmayer and Barr, 2001a and Wiedenmayer et al., 2002). CRF in the amygdala and BNST does therefore not seem to underlie immobility to cat odor and the male nor developmental changes in immobility.

Hippocampus

The hippocampus of young rats contains more cells that produce CRF than the adult hippocampus; thus it is highly sensitive to stimulation (Avishai-Eliner et al., 2002). In 10-day-old rats, hyperthermia increased CRF mRNA expression in the dentate gyrus, CA3, and CA1 (Hatalski et al., 2000). We extend these findings and demonstrate that cat odor and male exposure induced a rapid increase in CRF mRNA levels in the dentate gyrus in 26-day-old rats. Three hours after exposure, CRF mRNA levels were elevated in all exposed conditions compared to unexposed controls in the dentate gyrus of 26-day-old rats and in the dentate gyrus and CA1 of 14-day-old rats. Such increased CRF expression in the hippocampus could be involved in the modulation of the behavioral and endocrine responses.

The rat hippocampus is activated by odor cues from predators or conspecifics (Heale et al., 1994 and Vanderwolf, 1992) and mediates responses to threat such as freezing (Blanchard and Blanchard, 1972 and Kjelstrup et al., 2002). In preweaning rats, hippocampal lesions reduced male-induced immobility (Takahashi, 1995). In the present study, however, levels of stimulus-induced CRF expression did not parallel the changes in immobility and therefore do not appear to have contributed to the acute behavioral response. But because the hippocampus is critically involved in learning (Sanders et al., 2003), CRF expression

could play a role in the consequences of exposure. In young rats, hippocampal CRF is mainly found in GABAergic interneurons (Chen et al., 2001 and Yan et al., 1998) and has modulatory function by amplifying excitability of pyramidal cells (Hollrigel et al., 1998). In adult rats, CRF modulates glutamatergic transmission and increases synaptic efficacy (Wang et al., 2000). Injection of CRF into the hippocampus increased memory retention (Lee et al., 1992), and blocking of hippocampal CRF mRNA with antisense oligonucleotides impaired memory retention in an inhibitory avoidance task (Wu et al., 1997). Whether CRF in the hippocampus modulates memory function in young rats by facilitating learning about the adult male and cat odor encounter remains to be investigated.

Three hours after exposure, hippocampal CRF levels were elevated in all exposed groups at both ages. Again, expression levels did not parallel patterns of immobility. Rather, increased CRF expression could indicate a role in HPA axis regulation. Circulating glucocorticoids exert feedback inhibition on the endocrine stress response in several time domains (Campeau et al., 1998 and Herman and Cullinan, 1997). In delayed feedback, glucocorticoids bind to mineralocorticoid and glucocorticoid receptors in the hippocampus. Through genomic mechanisms, in a time frame of hours, the hippocampus exerts inhibitory feedback on PVN activity (Young and Vazquez, 1996). Although it has been demonstrated that glucocorticoids increase CRF expression in the amygdala (Schulkin et al., 1998) and BNST (Makino et al., 1994), the effect of corticosterone on hippocampal CRF expression and the exact role of CRF in negative feedback inhibition of the HPA axis remain unknown. A recent study has for the first time demonstrated that CRF receptors in the hippocampus are required for the inhibition of the HPA axis (Müller et al., 2003). It can be speculated that delayed elevation of hippocampal CRF levels in the present study may have contributed to the regulation of the HPA axis.

Conclusions

The major findings of this study are that exposure to acute age-specific threats induced an immobility response followed by rapid secretion of corticosterone. Aversive stimulation induced the expression of CRF in brain areas involved in the activation and regulation of the HPA axis. Such CRF expression was age-dependent, indicating a role in the developmental changes of the endocrine response. CRF was expressed only in one of the areas that mediate fear-like behaviors in adult rats, the hippocampus. The expression pattern in the hippocampus did, however, not parallel the developmental changes in immobility, indicating that CRF is not involved in this behavioral response in young rats. The results thus demonstrate that ecologically relevant stimuli induce rapid gene expression in brain areas underlying neuroendocrine regulation and that this expression pattern changes during early ontogeny.

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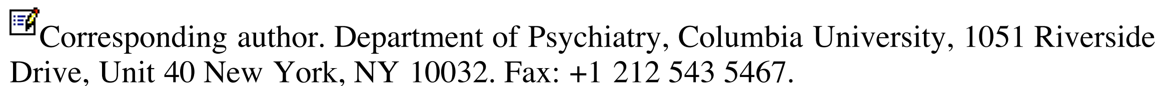

Corresponding author. Department of Psychiatry, Columbia University, 1051 Riverside Drive, Unit 40 New York, NY 10032. Fax: +1 212 543 5467.

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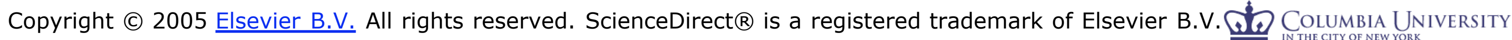

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