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Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups

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Abstract

In many altricial species, fear responses such as freezing do not emerge until sometime later in development. In infant rats, fear to natural predator odors emerges around postnatal day (PN) 10 when infant rats begin walking. The behavioral emergence of fear is correlated with two physiological events: functional emergence of the amygdala and increasing corticosterone (CORT) levels. Here, we hypothesize that increasing corticosterone levels influence amygdala activity to permit the emergence of fear expression. We assessed the relationship between fear expression (immobility similar to freezing), amygdala function (c-fos) and the level of corticosterone in pups in response to presentation of novel male odor (predator), littermate odor and no odor. CORT levels were increased in PN8 pups (no fear, normally low CORT) by exogenous CORT (3 mg/kg) and decreased in PN12 pups (express fear, CORT levels higher) through adrenalectomy and CORT replacement. Results showed that PN8 expression of fear to a predator odor and basolateral/lateral amygdala activity could be prematurely evoked with exogenous CORT, while adrenalectomy in PN12 pups prevented both fear expression and amygdala activation. These results suggest that low neonatal CORT level serves to protect pups from responding to fear inducing stimuli and attenuate amygdala activation. This suggests that alteration of the neonatal CORT system by environmental insults such as alcohol, stress and illegal drugs, may also alter the neonatal fear system and its underlying neural control.

Keywords

Corticosterone; Predator odor; Development; Infant rat; Fear; Amygdala; Hippocampus; Development

1. Introduction

Early adverse experience has been identified as a significant cause of adult behavioral problems, although very little is known about how these early experiences affect the infant and subsequent brain development (Glaser, 2000; Grossman et al., 2003; Gunnar, 2001; Sanchez et al., 2001; Teicher et al., 2003). It is well known that long-term adverse experience induces long-lasting changes in behavior and brain development, but a single exposure to a severe threat (such as taste aversion conditioning, predator presence) can also influence long-term changes in behavior and brain development (review in Wiedenmayer, 2004). Clinically, abused children show heightened fear and stress responses (Gunnar et al., 2001), although attenuated fear responses have also been documented (Gunnar, 2001; King et al., 2001). The cause of this clinical variability is unknown, although modification of the stress systems, the hypothalamus-pituitary-adrenal system that releases the stress hormone cortisol, and the other component of the stress axis composed of the locus coeruleus-amygdala has been implicated (Anand and Shekhar, 2003; Gunnar and Donzella, 2002; Kalin, 2003; Sanchez et al., 2001; Shekhar et al.,

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2003). Using an animal model, we began to assess the effects of early adverse experiences by presenting a naturally fearful odor (novel male odor) while manipulating the stress hormone corticosterone (CORT; homologous to primate cortisol) and assessed the early development of both the fear response and the neural structure implicated in fear responsiveness, the amygdala. We suggest that understanding the developmental neurobiology of infant fear responses will aid us in understanding how early adverse experiences alter brain development.

In altricial species, fear responses emerge later in development. For example, a delayed defensive response is seen in the young rabbit's response to hawks (Pongrácz and Altbäcker, 2000) and stranger anxiety emerges around 9 months in children (Joseph, 1999). In rats, the novel adult male is a predator, but a defensive response to male odor does not emerge until approximately postnatal day (PN) 10, when pups begin to walk and sometimes leave the nest (Bolles and Woods, 1964; Bronstein and Hirsch, 1976; Brown, 1986; Mennella and Moltz, 1988; Paul and Kupferschmidt, 1975; Takahashi et al., 1991; Takahashi, 1992; Wiedenmayer and Barr, 2001). This PN10 early defensive response is characterized by an immature version of freezing sometimes referred to as immobility (Takahashi, 1994b), and corresponds with an increasing level of the stress hormone corticosterone, with later inclusion of fleeing (Wiedenmayer et al., 2003) and an analgesic response (Wiedenmayer and Barr, 1998) at weaning when the stress response to predator odor becomes more similar to the adult's response (Dielenberg and McGregor, 2001; Perrot-Sinal et al., 1999).

The development of infant rat freezing to novel male odor and its associated neuroendocrine response has been characterized by Takahashi and his colleagues. They manipulated the age freezing emerged by increasing neonatal CORT levels (PN8). Specifically, premature freezing was elicited by increasing CORT levels, and freezing was eliminated by removing the primary source of CORT (adrenalectomy, ADX) in older pups (PN12; Takahashi and Rubin, 1993; Takahashi, 1994a,b). The ability of CORT to alter pups' fear system is related to the stress hyporesponsive period (SHRP), when neonatal rats have very low basal levels of CORT and the CORT response to stressors (such as shock) is blunted (Grino et al., 1994; Levine, 1962, 2001; Walker et al., 1986). The SHRP blunted response to stress is believed to be due to tactile stimulation (licking and nursing) and/or feeding received during maternal care (Levine, 1962; Suchecki et al., 1993; Van Oers et al., 1998b).

As the SHRP begins to end, the immobility component of the PN10 fear response emerges along with amygdala's participation in the fear response (Wiedenmayer and Barr, 2001). Specifically, during the SHRP, presentation of male odor does not elicit freezing and does not activate the amygdala, whereas a few days later the novel male odor elicits both freezing and amygdala participation (Wiedenmayer and Barr, 2001). Additional evidence suggests the hippocampus may also have a role in the development of freezing. CORT introduced directly into the hippocampus over 5 days accelerates cholinergic hippocampal development and the development of freezing (Takahashi, 1995; Takahashi and Goh, 1998). Others have found CORT (5 mg/kg from PN2 to PN6) to decrease hippocampus neurogenesis (Gould et al., 1991b,c; Gould and Cameron, 1997) suggesting CORT doses and regimen are important for hippocampal development during both infancy and adulthood (Cameron and Gould, 1994; Gould and Tanapat, 1999).

In the present study, we hypothesized that CORT is involved in the emergence of the fear response through the participation of the amygdala. It should be noted that CORT can be manipulated within the nest through the mother. First, mothers' CORT levels are transmitted to pups through her milk, including the high CORT levels induced by stress (Yeh, 1984). Second, the amount of maternal sensory stimulation provided alters pups' endogenous CORT levels, with high levels of stimulation maintaining low CORT levels and maternal deprivation

increasing CORT levels (Dent et al., 2000,2001;Levine et al., 1992;Suchecki et al., 1993). Third, pharmacological insults also alter pups' CORT levels. Maternal ingestion of alcohol appears to prematurely end the SHRP (Weinberg, 1994) and opiates dampen infants' CORT levels (Lesage et al., 1996,1998). Therefore, our direct manipulations of pup CORT levels on fear expression and the amygdala may be related to brain and behavior alterations that occur normally during development.

2. Methods

2.1. Subjects

The subjects were 108 male and female PN8 and PN12 Long Evans rat pups born and bred at the University of Oklahoma's vivarium (Harlan, Indianapolis, IN). The pups and their mothers were housed in polypropylene cages (34 cm × 29 cm × 17 cm) lined with aspen shavings and cages were kept in a temperature (23 °C) and light (07:00–19:00 h) controlled room. Food and water were available at all times. The day of birth was designated PN0, and litters were culled to 10 on PN1. Each experimental condition did not use more than one male and one female from each litter.

2.2. Corticosterone manipulation

For PN8 or PN12 pups with CORT replacement, pups were injected with either CORT (3.0 mg/kg, i.p.) or saline 30 min prior to odor presentations (Moriceau and Sullivan, 2004;Takahashi, 1994a). For PN12 pups, endogenous CORT was eliminated by ADX at PN8. Dorsal incisions to extract the adrenal glands were performed on anesthetized pups (isoflurane). SHAM-operated controls received dorsal incisions, but the adrenal glands were left intact. Following recovery from surgery (approximately 1 h), pups were returned to the mother until testing.

2.3. Odor presentations

Odors were presented to pups on either PN8 or PN12. Pups were placed in individual 600 ml glass beakers and given a 5 min adaptation period to recover from experimenter handling. They were then presented with the odor for 5 min. Either no odor, littermate, or adult male rat odor (rat pups had no prior experience with male odor) was delivered by a flow dilution olfactometer. Littermate and adult male odors were generated by placing each into separate round, airtight glass enclosures (width, 20.32 cm; height, 20.96 cm) connected to the olfactometer for odor delivery. Odor timing was controlled with a Chronotrol (Chronotrol Corporation, San Diego, CA).

2.4. The total time immobile/freezing was recorded

It should be noted that pups do not show the entire spectrum of behaviors associated with freezing in the adult rat. For example, there is no piloerection and crouching position in PN12–14 pups, and immobile/freezing was defined as the cessation of body movement (Takahashi, 1994a,b;Wiedenmayer and Barr, 2001).

2.5. Immunohistochemistry

Ninety min following odor delivery, PN8 and PN12 pups were decapitated and their brains were quickly removed, frozen in 2-methylbutane at –45 °C and stored in a –70 °C freezer. For analysis, brains were sectioned in a cryostat (20 µm; Minotome Plus, TBS, Durham, NC) at –20 °C. Two out of every four sections were collected. Each third section was used for immunohistochemical processing (Fisher colorfrost/plus) and each fourth section was placed on a microscope slide for cresyl violet staining to permit localization of the amygdala and hippocampus. Sections of adult male-exposed and control animals were post-fixed and

processed together. Sections were preincubated in hydrogen peroxide for 5 min. The sections were processed for 20 h at 4 °C in the primary antibody, rabbit anti-Fos (Santa Cruz Biotechnology, sc-52) diluted to 1:500 in phosphate buffer saline (pH 7.2). They were then rinsed and incubated in the secondary antibody, goat anti-rabbit (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Finally, the slides were processed using the ABC kit (Vectastain Elite, Vector Laboratories, Burlingame, CA). Stained sections were dehydrated in ethanol and coverslipped.

2.6. RIA

The levels of circulating CORT were determined from heart blood of PN8 and PN12 pups after 5 min exposure to littermate or male odor. Duplicate plasma samples were analyzed for CORT using the Rat corticosterone Coat-a-Countkit (Radioassay Systems Labs, Inc., Carson, CA). The sensitivity of the assay was 5 ng/ml. The intra-assay coefficient of variation was 1–9%.

2.7. Data analysis

Fos-positive cells were visualized using a microscope (Olympus with a 10× objective) equipped with a drawing tube. Using a rat brain atlas (Paxinos et al., 1991), cresyl violet sections were used to outline the basolateral/lateral complex, the medial nucleus and the central nucleus of the amygdala, as well as CA1, CA3 and the dentate gyrus of the hippocampus. Fos labeled cells were unilaterally counted by an experimenter blind to the training conditions. A Fos positive cell had to have a labeled nucleus to be considered distinct from the background. For each animal, the mean number of cell counts was calculated by averaging the counts from two sections. Comparisons of the number of Fos-positive cells were made with ANOVA and post hoc Fisher tests for each brain area. Freezing behavior was also analyzed with ANOVA followed by post hoc Fisher tests.

3. Results

3.1. PN8 behavior

As illustrated in Fig. 1A, saline treated PN8 pups did not exhibit immobility/freezing to any odor presentations, whereas CORT (3 mg/kg) injected pups showed a significant increase in immobility/freezing time to the adult male rat odor. ANOVA analysis revealed a significant interaction between odor presentation and drug treatment $F_{(2,36)} = 56.067$, $P < 0.0001$; post hoc Fisher tests revealed that the CORT-male odor group was significantly different from each of the other PN8 groups at the $P < 0.05$ level. Minimal or no immobility/freezing was detected in response to either peer odor or the control no odor presentation. These results replicate previous work from other laboratories (Takahashi, 1994; Wiedenmayer and Barr, 2001).

3.2. PN8 hippocampus

No difference was found between groups in the CA1, CA3 or dentate gyrus of the hippocampus.

3.3. PN8 amygdala

A significant increase in Fos-positive cells was found in the basolateral/lateral complex of the amygdala of pups that received both the adult male rat odor and a CORT injection as compared to each of the other groups (Fig. 2A; ANOVA—odor presentation and drug, $F_{(2,17)} = 11.979$, $P < 0.001$; post hoc Fisher tests revealed that the CORT-adult male odor group was significantly different from each of the other PN8 groups at the $P < 0.05$ level.). Minimal Fos-positive cells were found in the medial and central amygdala (nonsignificant ANOVA). This suggests that CORT either directly or indirectly permitted basolateral/lateral amygdala activation during the adult male odor presentation.

3.4. PN8 and PN12 RIA

A significant increase in CORT level was found (ANOVA, $F_{(3,8)} = 6.345$, $P < 0.05$) and post hoc Fisher tests ($P < 0.05$ level) revealed that PN12 pups exposed to male odor (56.67 ± 6.67 ng/ml) had significantly higher CORT levels compared to all other groups at both ages. PN12 pups exposed to littermate odor had a RIA value of 37.33 ± 6.67 ng/ml and PN8 RIA values for male odor and littermate odor were 27.33 ± 3.33 and 34.00 ± 0 ng/ml respectively.

3.5. PN12 behavior

As indicated in Fig. 1B, saline injected PN12 rats showed normal immobility/freezing to the adult male odor but immobility/freezing was eliminated in pups without CORT (ADX). However, replacement CORT in ADX pups (ADX/CORT) reinstated freezing in the presence of an adult male. These data replicate those of Takahashi (1994a,b) and suggest that CORT is required for the emergence of immobility/freezing during ontogeny. ANOVA analysis revealed a significant interaction between training condition and drug treatment $F_{(4,45)} = 10.330$, $P < 0.0001$; post hoc Fisher tests ($P < 0.05$ level) revealed that the SHAM and ADX/CORT pups showed significantly more freezing to male odor compared to each of the other groups. Minimal or no freezing was detected in response to either littermate odor or the control no odor presentation.

3.6. PN12 hippocampus

No difference was found between groups in the CA1, CA3 or dentate gyrus of the hippocampus.

3.7. PN12 amygdala

As illustrated in Fig. 2B, both PN12 groups that showed freezing to male odor had a significant increase in Fos-positive cells in the basolateral/lateral complex of the amygdala in response to adult male odor than to no odor. It should be noted that the SHAM-male odor condition Fos expression was significantly higher than the ADX/CORT-male odor condition group. The Fos response was minimal in all other PN12 pups. ANOVA analysis revealed a significant interaction between training condition and drug treatment $F_{(4,27)} = 6.292$, $P < 0.05$; post hoc Fisher tests revealed that the SHAM-male odor pups were significantly different from each of the other groups. The ADX/CORT male odor group was significantly different from each of the other groups, except the ADX-littermate group at the $P < 0.05$ level.

No group differences were found in the central nucleus of the amygdala or the medial nucleus of the amygdala.

4. Discussion

Our results suggest that the emergence of the stress CORT system permits the expression of fear (freezing) mediated by direct or indirect activation of the basolateral/lateral amygdala. Specifically, we could prematurely activate the expression of fear and the basolateral/lateral amygdala in pups during SHRP through exogenous CORT. We were also able to retard the developmental expression of fear and basolateral/lateral amygdala activation by preventing the end of the SHRP through ADX. These results replicate those of Wiedenmayer and Barr (2001) and Takahashi (1994a,b) but also extend their results to suggest that CORT may be implicated in the activation of the amygdala, either directly or indirectly, to permit the expression of infant fear. However, it should be noted that a longer exposure (30 min) to predator odor can increase CORT levels in younger pups (Tanapat et al., 1998) and could possibly activate the amygdala even in younger pups.

Our amygdala data, while consistent with other developmental analysis by Wiedenmayer and Barr (2001), are not consistent with the adult literature that suggests the medial nucleus of the amygdala, not the basolateral/lateral complex, is important in the response to predator odor (Dielenberg et al., 2001; Dielenberg and McGregor, 2001; Li et al., 2004). Our failure to show hippocampal participation in the predator odor response is also consistent with the work of Wiedenmayer and Barr (2001) but is also in sharp contrast to the adult literature (Heale et al., 1994; Mesches et al., 1999). It should be noted that Wiedenmayer and Barr (2001) found the inclusion of the hippocampus in the predator odor fear circuit at PN21 (weaning) and based on the learning literature, the hippocampus may not be functionally mature until weaning (Fanselow and Rudy, 1998; Green and Stanton, 1989; Rudy and Morledge, 1994). The reasons for developmental change in the predator odor circuitry is unclear and suggests the circuit may change with maturation and the inclusion of other fear related behaviors. For example, Wiedenmayer and Barr (2001) have shown that the medial nucleus of the amygdala does not become incorporated in the predator odor neural circuitry until around PN21 (weaning) when the fleeing response emerges.

While our data show a correlation between amygdala function and the expression of fear associated with systemic CORT manipulations, it is possible that CORT is working indirectly on the amygdala. Although the amygdala does contain CORT receptors during the SHRP and amygdala plasticity may be dependent upon CORT, other brain areas contain CORT receptors, connect with the amygdala, and are involved in fear such as the hippocampus, bed nucleus of the stria terminalis (BNST) and paraventricular nucleus of the hypothalamus (Cintra et al., 1993; Crain et al., 1979; Dielenberg and McGregor, 2001; Meaney et al., 1985; Nair and Gonzalez-Lima, 1999; Rosenfeld et al., 1988a,b,1993; Sarrieau et al., 1988; Stutzmann et al., 1998). For example, prolonged CORT treatment over 4–5 days has been shown to accelerate the development of freezing and influence cholinergic hippocampal dentate granule cells' development (Takahashi, 1995; Takahashi and Goh, 1998). However, due to the rapid action of CORT on the unlearned fear system in the present experiment (30 min), it is unlikely that neurogenesis could account for the precocial emergence of freezing in our PN8 pups. Perhaps the most cohesive assessment of neural development of pup freezing in response to predator odor was done by Wiedenmayer and Barr (2001), who showed that the basolateral amygdala, the locus coeruleus, the periaqueductal gray and the paraventricular nucleus of the hypothalamus each begin to participate in the response to a naturally fearful odor as freezing emerges. They also assessed weanling pups (PN21) and found the recruitment of the hippocampus, the BNST and the medial amygdala as the fleeing response emerges as a response to predator odor. Overall, the unlearned fear circuit appears complex and the data suggest that more than one circuit may be used for freezing and change over development.

We have found similar effects of CORT manipulation on the development of learned fear and participation of the amygdala in fear conditioning. Specifically, fear conditioning (odor 0.5 mA) in neonatal rat pups actually results in a preference for that odor (Camp and Rudy, 1988; Sullivan et al., 1986,2000), although pups feel pain (Barr, 1995). In striking similarity to the unlearned fear system documented here, fear conditioning evoked odor aversion is not learned until around PN10, when the amygdala participates in odor-shock (0.5 mA) conditioning (Sullivan et al., 2000). Furthermore, we were able to alter the developmental expression of fear conditioning through manipulations of the CORT system similar to those described in the present experiments (Moriceau and Sullivan, 2004). Thus, both learned fear and unlearned fear appear to emerge at the same age, with the amygdala and CORT system implicated in both fear responses. Similarly to differences in amygdala participation found for predator odor, a more global response is found in the adult amygdala compared to the infant amygdala. Specifically, while our amygdala response was limited to the basolateral/lateral complex, the adult literature shows the basolateral/lateral complex, medial and central nuclei

of the amygdala are activated by adult fear conditioning (Beane et al., 2002; Davis, 1997; Fanselow and LeDoux, 1999; Fanselow and Gale, 2003; Fendt and Fanselow, 1999; Goldstein et al., 1996; Johnson et al., 1992; Lee et al., 1994; Maren, 1999; Morrow et al., 2000; Roozendaal et al., 1991; Vazdarjanova et al., 2001; Walker and Davis, 1997). These differences may reflect the developmental changes in the fear circuitry.

These data suggest that modification of pups' CORT system may modify the maturation of the fear system to predator odors. There are myriad ways pup CORT levels can be modified including through the mother's milk (Yeh, 1984), the amount of maternal sensory stimulation (Dent et al., 2000, 2001; Levine et al., 1992; Suchecki et al., 1993) and maternal ingestion of alcohol (Weinberg, 1994) or opiates (Lesage et al., 1996, 1998). Indeed, the effects of stress and maternal behaviors that manipulate the CORT system appear to alter the trajectory of brain development (Brunson et al., 2001; Dent et al., 2000, 2001; Eghbal-Ahmadi et al., 1997; Francis et al., 2002; Huot et al., 2002; Smith et al., 1997; Zhang et al., 2002). Enhancing maternal behavior that maintains low CORT levels in pups reduces emotionality and enhances adult maternal behavior, while environmental stressors such as deprivation increase emotionality and produce a malfunctioning stress system. These early developmental manipulations of the infant's CORT system produce long-term changes in baseline CORT levels, hormones and neurotransmitters related with the stress system and associated receptors in a myriad of brain structures (Avishai-Eliner et al., 1999; Caldji et al., 1998; Kent et al., 1997; Liu et al., 1997; Meaney et al., 1988, 1989, 1996; Okimoto et al., 2002; Rosenfeld et al., 1991; Rots et al., 1996; Stanton et al., 1988; Van Oers et al., 1998a; Vasquez et al., 1996; Yi et al., 1994).

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Fig 1.

Mean ($\pm$ S.E.M.) number of immobility/freezing responses for (A) PN8 and (B) PN12 pups to novel male odor, littermate odor or no odor. Some groups had values at or near zero and are not detectable on this graph. Asterisks represent significant differences from each of the other groups ($P < 0.05$).

Fig 2. Mean ($\pm$ S.E.M.) number of Fos-positive cells in the basolateral/lateral complex of the amygdala of rats exposed to an adult male odor, littermate odor or no odor for (A) PN8 and (B) PN12 pups. Some groups had values at or near zero and are not detectable on this graph. Asterisks represent significant differences from each of the other groups ($P < 0.05$).