Early-Life Stress Disrupts Attachment Learning: The Role of Amygdala Corticosterone, Locus Ceruleus Corticotropin Releasing Hormone, and Olfactory Bulb Norepinephrine

Stephanie Moriceau,^{1,2,4} Kiseko Shionoya,^{4,5} Katherine Jakubs,^{4,6} and Regina M. Sullivan^{1,2,3,4}

¹Emotional Brain Institute, Nathan Kline Institute, Orangeburg, New York 10962, ²Child and Adolescent Psychiatry, Child Study Center, New York University Langone Medical Center, New York, New York 10016, ³New York University Center for Neural Science, New York, New York 10003, ⁴Department of Zoology, University of Oklahoma, Norman, Oklahoma 73019, ⁵Laboratory Neurosciences Sensorielles, Centre National de la Recherche Scientifique, Université Lyon, 69007 Lyon, France, and ⁶National Institutes of Health, Bethesda, Maryland 20892

Infant rats require maternal odor learning to guide pups' proximity-seeking of the mother and nursing. Maternal odor learning occurs using a simple learning circuit including robust olfactory bulb norepinephrine (NE), release from the locus ceruleus (LC), and amygdala suppression by low corticosterone (CORT). Early-life stress increases NE but also CORT, and we questioned whether early-life stress disrupted attachment learning and its neural correlates [2-deoxyglucose (2-DG) autoradiography]. Neonatal rats were normally reared or stressed-reared during the first 6 d of life by providing the mother with insufficient bedding for nest building and were odor–0.5 mA shock conditioned at 7 d old. Normally reared paired pups exhibited typical odor approach learning and associated olfactory bulb enhanced 2-DG uptake. However, stressed-reared pups showed odor avoidance learning and both olfactory bulb and amygdala 2-DG uptake enhancement. Furthermore, stressed-reared pups had elevated CORT levels, and systemic CORT antagonist injection reestablished the age-appropriate odor-preference learning, enhanced olfactory bulb, and attenuated amygdala 2-DG. We also assessed the neural mechanism for stressed-reared pups' abnormal behavior in a more controlled environment by injecting normally reared pups with CORT. This was sufficient to produce odor aversion, as well as dual amygdala and olfactory bulb enhanced 2-DG uptake. Moreover, we assessed a unique cascade of neural events for the aberrant effects of stress rearing: the amygdala–LC–olfactory bulb pathway. Intra-amygdala CORT or intra-LC corticotropin releasing hormone (CRH) infusion supported aversion learning with intra-LC CRH infusion associated with increased olfactory bulb NE (microdialysis). These results suggest that early-life stress disturbs attachment behavior via a unique cascade of events (amygdala–LC–olfactory bulb).

Introduction

Early-life stress appears to disrupt the programming of the hypothalamus–pituitary–adrenal axis, the limbic system (hippocampus, amygdala, prefrontal cortex, etc.), and the locus ceruleus (LC), all of which are considered critical mediators of early-life trauma on later life compromised mental health in humans and animal models (Caldji et al., 1998; Glaser, 2000; Dent et al., 2001; Sanchez et al., 2001; Grossman et al., 2003; Teicher et al., 2003; De Bellis, 2005; Plotsky et al., 2005; Gunnar and Quevedo, 2007; Champagne et al., 2008; McEwen, 2008; Cirulli et al., 2009; Gunnar et al., 2009; Lupien et al., 2009). Furthermore, early-life prolonged stress produces an exaggerated corticosterone (CORT) response, increased corticotropin releasing hormone (CRH) expression, and heightened norepinephrine (NE) levels (Smith et al., 1997; Hatalski et al., 1998; Koob,

1999; Koob and Heinrichs, 1999; Vazquez et al., 2006; Korosi and Baram, 2008).

These neural targets of early stress may have particular importance because they overlap with the infant's unique attachment learning neural circuit, which is used to support attachment to the mother via learning the maternal odor (Brunjes and Alberts, 1979; Galef and Kaner, 1980; Campbell, 1984; Sullivan et al., 1986, 1990, 2000a; Moriceau and Sullivan, 2004b; Roth and Sullivan, 2005; Moriceau et al., 2006). This circuit also enables myriad stimuli (milk, stroking, 0.5 mA shock) to support this odor learning required for pup survival and is characterized by robust odor approach learning and attenuated aversion learning even when the reinforcer is painful, such as 0.5 mA shock, tail pinch (Haroutunian and Campbell, 1979; Sullivan et al., 1986; Camp and Rudy, 1988; Sullivan et al., 2000a), or abusive mother (Roth and Sullivan, 2005). The enhanced learned odor approach responses is supported by a unique infant hyperfunctioning LC that releases copious amounts of NE into the olfactory bulb to produce the learning-induced plasticity of the olfactory bulb (Shipley et al., 1985; McLean and Shipley, 1991; Sullivan and Wilson, 1991, 1995; Wilson and Sullivan, 1991; Rangel and Leon, 1995; Langdon et al., 1997; Moriceau and Sullivan, 2004b). It is

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Correspondence should be addressed to Stephanie Moriceau, The Nathan Kline Institute, Emotional Brain Institute, 140 Old Orangeburg Road, Orangeburg, NY 10962. E-mail: SMoriceau@nki.rfmh.org.

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important to mention that moderate levels of olfactory bulb NE support preference learning, whereas higher NE levels support aversion learning in rat pups (Sullivan et al., 1989, 1991; Yuan et al., 2000; Harley et al., 2006; Christie-Fougere et al., 2009). The attenuated odor avoidance learning is supported by suppression of amygdala attributable to pups' naturally low CORT, and CORT is critical for early-life amygdala plasticity (Sullivan et al., 2000a; Moriceau and Sullivan, 2006; Moriceau et al., 2006). Indeed, in older animals, CORT increase produces a cascade of events beginning with CORT activation of the amygdala increasing the expression of CRH mRNA into the central amygdala (Makino et al., 1994; Hatafski et al., 1998; Hsu et al., 1998; Van Bockstaele et al., 1998; Korosi and Baram, 2008) and then CRH release exciting the LC to increase the release of NE (Butler et al., 1990; Makino et al., 1994; Curtis et al., 1997; Hatafski et al., 1998; Hsu et al., 1998; Lehnert et al., 1998; Van Bockstaele et al., 1998, 2001; Page and Abercrombie, 1999; Bouret et al., 2003; Dunn et al., 2004; Jedema and Grace, 2004; Korosi and Baram, 2008).

Thus, there is overlap in the attachment learning circuit and the targets of early-life prolonged stress, and here we test whether this is important for the unique effects of stress in infancy. Specifically, we describe a functionally compromised attachment neural circuit produced by early-life stress that may contribute to the enduring effects of early-life stress via disrupted social behavior of pups to the mother.

Materials and Methods

Subjects

Subjects were both male and female Long–Evans rat pups born and bred in our colony (originally from Harlan Lab Animals). Mothers and pups were housed in polypropylene cages (34 cm width × 29 cm length × 17 cm height) lined with pine shavings and were kept in a temperature-controlled (23°C) and light-controlled (7:00 A.M. to 7:00 P.M.) room. Food and water were available *ad libitum*. The day of parturition was considered postnatal day 0 (P0), and litters were culled to 12 on P1. No more than one male and one female from a litter were used in each experimental condition, and no difference in behaviors or neural activity between male and female was found. All procedures were approved by the Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Stressed rearing

From P1 to P7, the dam and her pups were housed in a cage with limited nesting/bedding material, a modified version of the Baram procedure [1000 ml, 0.5 inch layer compared with the normal 4500 ml, 3 inch layer (Gilles et al., 1996; Brunson et al., 2005)]. This limited bedding environment decreased the dams' ability to construct a nest, which results in frequent nest building, transporting/rough handling of pups, and less licking and nursing (Table 1). No significant difference in weight between conditioning group and rearing condition was found ($F_{(3,46)} = 0.236$, $p < 0.8707$), which replicates previous research (Gilles et al., 1996; Avishai-Eliner et al., 2001).

Maternal and pup behaviors were observed two times a day (day and night), with each session lasting 30 min. The maternal behaviors observed were as follows: (1) stepping/jumping on, in which the mother steps or jumps on the pup; (2) rough handling, in which the mother aggressively grooms a pup or transports a pup by a limb; (3) chase tail, in which the mother was running after its own tail; (4) nest building, in which the mother was building a nest; (5) licking, in which the mother was licking its pups; and (6) nursing, in which the mother was nursing the pups.

Systemic drug injection

P7 pups were injected with either CORT (Corticosterone HBC complex, 3.0 mg/kg, i.p., dissolved in saline; Sigma), CORT antagonist (mifepristone, RU 38486, 5.0 mg/kg, i.p., dissolved in saline; Sigma), or saline

Table 1. Frequency of maternal behaviors observed during mother–infant interactions within the normally reared and stressed-reared paradigm

	Percentage of observation periods in which behaviors occurred	
	Stressed-reared paradigm	Normally reared paradigm
Abnormal behaviors		
Steps or jump on	41.0%	2.0%
Rough handling	9.0%	0%
Chase tail	5.0%	0%
Nest building	19.0%	6.0%
Normal behaviors		
Lick	12.0%	33.0%
Nurse	45.0%	75.0%
Weight of pups	15.01 g	14.22 g

(Takahashi, 1994; Moriceau and Sullivan, 2004a) 24 h and 30 min before conditioning.

Radioimmunoassay

The levels of circulating CORT were determined from heart blood of P7 pups after normal rearing and stressed-rearing. Pups were anesthetized with pentobarbital and blood taken from the heart's ventricle via a thoracotomy through the diaphragm using a 23 gauge needle. All blood was collected between 12 and 2pm immediately after conditioning. Blood samples were centrifuged at 14,000 rpm for 6 min. Plasma was stored at -70°C until radioimmunoassay was performed. Duplicate plasma samples were analyzed for CORT using the Rat Corticosterone Coat-a-Countkit (Radioassay Systems Labs). The sensitivity of the assay was 5 ng/ml. The intraassay coefficient of variation was 1–9%.

Surgery

On P5, pups were anesthetized by inhalation (with isoflurane) and placed in an adult stereotaxic apparatus modified for use with infants. Stainless steel cannulas (30 gauge tubing) were implanted bilaterally in the LC or the amygdaloid complex through holes drilled in the overlying skull. Stereotaxic coordinates derived from the atlas of Paxinos were used as reference and adapted through pilot work (Paxinos et al., 1991; Sullivan et al., 2000b) for implanting cannulas into the LC (caudal, -1.40 mm; lateral, ± 0.60 mm from lambda) or into the amygdaloid complex (caudal, -0.80 mm; lateral, ± 3.00 mm from bregma). The cannulas were lowered 5.5 mm (LC) or 5.0 mm (amygdala) from the surface of the skull. The cannulas were fixed to the skull with dental cement. To ensure patency of the cannulas, guide wires were placed in the lumen of the tubing until conditioning. After recovery from surgery (generally within 30 min), pups were returned to the dam and littermates for a 2 d recovery period until conditioning. The survival rate of the surgery was 91%, including recovery from anesthesia and cannibalism from the mother.

Pharmacological treatment

On the day of the conditioning, bilateral cannulas were attached with polyethylene PE10 tubing to a Harvard syringe pump driving two Hamilton microliter syringes. The cannulas were filled (10 s for amygdala cannulas and 12 s for LC cannulas at rate of $0.5 \mu\text{l}/\text{min}$) with either drug (described below) or control. During the first 20 min conditioning period, for both pups with amygdaloid cannulas or LC cannulas, drug or control was infused at $0.1 \mu\text{l}/\text{min}$, for a total infusion volume of $2.0 \mu\text{l}$ as described previously (Sullivan et al., 1992, 2000b; Moriceau and Sullivan, 2004b). After conditioning, pups were disconnected from the syringe pump and returned to the nest until testing the following day.

Amygdala infusions. Pups with bilateral cannulas into the amygdala received either CORT (50 or 100 ng; Sigma) or cholesterol (Sigma).

LC infusions. Pups with bilateral cannulas implanted into the LC received either CRH (50, 100, or 200 ng; Sigma) or saline.

Odor–0.5 mA shock conditioning

On P7, pups were randomly assigned to one of the three following conditioning groups: (1) paired odor–shock, (2) unpaired odor–shock, or (3) odor only. Pups were placed in individual 600 ml clear plastic beakers and given a 10 min adaptation period to recover from experimental

handling. During a 45 min conditioning session, pups received 11 presentations of a 30 s peppermint odor [conditioned stimulus (CS)] and a 1 s, 0.5 mA footshock (unconditioned stimulus; Lafayette scrambled shock generator), with an intertrial interval of 4 min. Peppermint odor was delivered by a flow dilution olfactometer (2 L/min flow rate) at concentration of 1:10 peppermint vapor. Paired odor–shock pups received a shock overlapping with the last second of the 30 s odor presentation. Unpaired odor–shock pups received the shock and odor presentation pseudorandomly. Odor and shock were never coincident in this group. Odor-only pups received only the peppermint odor presentation.

During conditioning, the number of limbs moving was recorded (0 indicated no movement of the extremities; 5 indicated movement of all five extremities, including head) 20 s before presentation as well as the first 20 s of the odor presentation and the shock delivery (Hall, 1979).

Behavioral testing: Y-maze

The day after conditioning, pups were given a Y-maze test consisting of a start box ($8.5 \times 10 \times 8$ cm) and two arms ($8.5 \times 24 \times 8$ cm) separated via two sliding doors. This test required pups to choose between two arms of a Plexiglas Y-maze, one containing the peppermint odor CS (25 μ l of peppermint odor placed on a Kim Wipe) and the other containing the familiar odor of pine shavings (20 ml of clean shaving in a Petri dish, same bedding used in home cage). For 5 s before the door to each arm was opened, a pup was placed in the start box. Each pup was given 60 s to choose an arm. A response was considered a choice when a pup's entire body was past the entrance to the alley. Pups received five trials with 30 s between trials, and the floor was wiped clean between each trial (Sullivan and Wilson, 1991). The testing was done blind to the conditioning groups, and no drugs were infused during testing.

Behavioral testing: immobility/freezing

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 P7–P8 pups, and immobile/freezing was defined as the cessation of body movement (Takahashi, 1994; Hunt and Campbell, 1999; Richardson et al., 2000; Wiedenmayer and Barr, 2001; Moriceau et al., 2004). Odors were presented to pups the day after conditioning. Pups were placed in individual 600 ml plastic beakers and given 5 min adaptation period to recover from experimenter handling. They were then given five odor presentations (2 L/min, 1:10 odor/air ratio, intertrial interval of 4 min). The odor was presented with an olfactometer as described above (same intensity and flow rate).

Microdialysis procedure

P5 rat pups were anesthetized by isoflurane and placed in a stereotaxic frame adapted for rat pup surgery as described previously. A cannula was placed into the right LC (used to infuse CRH), and a microdialysis guide cannula (8 mm long, 500 μ m diameter acrylic resin; EICOM) was positioned into the right olfactory bulb; two additional holes were drilled for two skull screws (EICOM), and both were secured to the skull with dental cement. After surgery, pups were returned to the dam and littermates for a 1 d recovery period before experimentation.

On the day of the experiment, pups were placed in a 27-cm-diameter acrylic circular cage (EICOM) and were able to move freely and kept at 27°C. The guide cannula was used for insertion of the microdialysis probe (A-I-8-02, 8 mm length, 2 mm membrane, 220 μ m diameter; EICOM). The probes were perfused with artificial CSF (in mM: 147 NaCl, 2.7 KCl, 1.2 CaCl₂, and 0.85 MgCl₂) at a flow rate of 1.5 μ l/min. The dead volume of the collection apparatus is 4 μ l. Dialysate was collected automatically in a refrigerated (4°C) microfraction collector (EFC-82; EICOM) in which every vial contained 2 μ l of 12.5 mM perchloric acid/250 μ M EDTA. After completion of the experiment, dialysate samples were immediately stored at –80°C until HPLC analysis.

NE was assessed by HPLC with electrochemical detection (HPLC-EC). HPLC-EC consisted of a 150 $\times$ 2.1 mm SC-50DS, 5 μ m particle column (EICOM). Mobile phase (0.1 M citric acid, 0.25 mM octyl sulfate sodium salt, 0.5 mM EDTA, 0.085 mM triethylamine, and 6% acetonitrile, pH 2.4) was delivered at 0.23 ml/min by a EICOM EP-300 pump. Neurotransmitters were detected with a graphite carbon detector electrode maintained at +0.75 V relative to an Ag/AgCl reference

Figure 1. P7 pups stressed-reared versus normally reared and odor–0.5 mA shock conditioning. **A**, Number of choices toward the CS odor during the Y-maze test (5 trials). **B**, Freezing to the conditioned odor presentations (cue test, 5 odor presentations). **C**, CORT levels immediately after odor–0.5 mA shock conditioning. **D–G**, ¹⁴C 2-DG autoradiography during odor–0.5 mA shock conditioning in the olfactory bulb odor-specific loci of the glomerular layer (**D**), lateral nucleus of the amygdala (**E**), basolateral nucleus of the amygdala (**F**), and central nucleus of the amygdala (**G**). * $p < 0.05$, significantly different from the other groups ($n = 5$ –8 per group).

electrode. Neurochemical concentrations were estimated using chromatographic peak areas and calibration curves obtained with standard mixtures of known monoamine compounds. During the course of dialysate autoinjection fractions, a standard mixture was injected every fifth sample to monitor and correct calibration curves.

Assessing neural correlates within the olfactory bulb and the amygdala

P7 pups were injected with ¹⁴C 2-deoxyglucose (2-DG) (20 μ Ci/100 g, s.c.) 5 min before the 45 min odor–shock conditioning. Immediately after conditioning, pups were decapitated, and their brains were quickly removed, frozen in 2-methylbutane (–45°C), and stored in a –70°C freezer. Then, brains were sectioned (20 μ m) in a –20°C cryostat, and every other section was saved to be placed on a coverslip and exposed for 5 d along with standards (¹⁴C standards 10 $\times$ 0.02 mCi; American Radiolabeled Chemicals) to x-ray film (Coopersmith and Leon, 1986; Sullivan and Wilson, 1995).

Olfactory bulb. The olfactory bulb does not require staining because anatomical landmarks are clearly visible with 2-DG, with odors producing an odor-specific pattern of 2-DG uptake within the glomerular layer of the olfactory bulb, which was expressed relative to the periventricular core of the bulb (Greer et al., 1982). These odor-specific loci, along with the periventricular core, were measured using quantitative optical densitometry with NIH Image software (Coopersmith and Leon, 1986; Sullivan and Leon, 1986). To quantify 2-DG uptake, the computer constructed a calibration curve that related the gray value of ^{14}C standards that were exposed with the brain sections to that of determined value. The autoradiographs were observed for the presence of odor-specific glomerular layer foci, which are several times above the background uptake (see Fig. 2F). Five readings were taken from the periventricular core and the odor-specific loci. Data were analyzed as the uptake within the odor-specific loci relative to the uptake in the periventricular core (Sullivan and Wilson, 1995). Analyses were made blind to conditioning groups.

Amygdala. Specific amygdala nuclei (central, basolateral, and lateral nuclei) were identified by counterstaining sections with cresyl violet and by making a template of that brain area for use with the autoradiographs. The 2-DG uptake was expressed relative to 2-DG uptake in the corpus callosum (which did not vary among conditioning groups) to control for differences in section thickness and exposure (see Fig. 2G) (Sullivan et al., 2000a,b).

Three readings were taken from the central, lateral, and basolateral nuclei of the amygdala. Data were analyzed as the uptake within the central, lateral, and basolateral nuclei of the amygdala relative to the uptake in the corpus callosum (Sullivan and Wilson, 1995). Analyses were made blind to conditioning groups.

Histology and drugs spread

After behavioral testing, brains were removed and frozen in 2-methylbutane (-45°C) and stored in a -70°C freezer. For analysis, brains were sectioned ($20\ \mu\text{m}$) in a -20°C cryostat, and cresyl violet staining was used to verify LC and amygdaloid complex cannulas placements. Cannula tracks are shown in Figures 3D (amygdala) and 4D (LC).

To characterize the extent of drug diffusion within and outside of the LC and the amygdaloid complex, additional pups were used. On P7, pups were anesthetized by urethane and placed in a stereotaxic apparatus. For the LC, holes were drilled through the skull at 1.4 mm posterior to lambda and ± 0.60 mm from the midline. A $10\ \mu\text{l}$ Hamilton syringe was lowered 5.5 mm from the surface of the skull, which placed the tip near the LC. The pups were infused with $2\ \mu\text{l}$ of a saline solution of [^3H]CRH ($0.37\ \mu\text{Ci}/\mu\text{l}$; PerkinElmer Life and Analytical Sciences). For the amygdaloid complex, holes were drilled through the skull at 0.80 mm posterior to bregma and ± 3.00 mm from the midline. A $10\ \mu\text{l}$ Hamilton syringe was lowered 5.0 mm from the surface of the skull, which placed the tip near the amygdaloid complex. The pups were infused with $2\ \mu\text{l}$ of a saline solution of [^3H]CORT ($1\ \mu\text{Ci}/\mu\text{l}$; PerkinElmer Life and Analytical Sciences). Twenty minutes after infusion, the brains were quickly removed and frozen in methylbutane at -45°C . Brains were sliced in $20\ \mu\text{m}$ coronal sections. The slides were apposed to a tritium storage phosphor screen (GE Healthcare). After 14 d exposure, the screen was scanned at a pixel density of $50\ \mu\text{m}$ ($5000\ \text{dots}/\text{cm}^2$) with a STORM 820 Phosphor Imager (Molecular Dynamics). Phosphorimaging of the slides results in a TIFF image file (Tucker et al., 2002; Moriceau and Sullivan, 2004b; Moriceau et al., 2006).

Corticosterone Injection to Mimic Stressed-Rearing

Figure 2. Odor–0.5 mA shock conditioning in P7 pups with CORT injection versus saline injection. **A**, Number of choices toward the CS odor during the Y-maze test (5 trials). **B–E**, ^{14}C 2-DG autoradiography during odor–0.5 mA shock conditioning in the olfactory bulb odor-specific loci of the glomerular layer (**B**), lateral nucleus of the amygdala (**C**), basolateral nucleus of the amygdala (**D**), and central nucleus of the amygdala (**E**). **F**, Pseudocolor 2-DG autoradiography of the olfactory bulb. The odor-specific glomerular layer foci are indicated by arrows and are shown for a control animal on the left and an experimental animal on the right. **G**, Pseudocolor 2-DG autoradiography of the lateral, basolateral, and central nuclei of the amygdala. * $p < 0.05$, significantly different from the other groups ($n = 5–7$ per groups).

Statistical analysis

Comparisons were made between groups using the ANOVA test, followed by *post hoc* Fisher's tests. In all cases, the level of significance was set at $p < 0.05$ level.

Results

Early-life stress effect on learning and neural circuit

As shown in Figure 1A, at P7, normally reared paired odor–0.5 mA shock pups learned an odor preference, whereas stressed-reared paired pups learned an avoidance. However, CORT antagonist injection in stressed-reared paired pups reinstated the learned odor preference. The Y-maze ANOVA revealed a main effect of rearing condition ($F_{(1,46)} = 24.939$, $p < 0.0005$), a significant effect of conditioning group ($F_{(3,46)} = 36.157$, $p < 0.0001$), and a significant interaction between conditioning group and rearing condition ($F_{(3,46)} = 24.939$, $p < 0.0001$); *post hoc* Fisher's tests revealed that the stressed-reared paired groups differed significantly from the normally reared paired group and the stressed-reared paired pups receiving a CORT antagonist injection. Also, the normally reared paired group, the stressed-reared paired pups receiving a CORT antagonist injection, and the stressed-reared paired groups each differed significantly from each of the control groups at the $p < 0.05$ level.

Furthermore, as illustrated in Figure 1B, stressed-reared paired pups showed freezing compared with normally reared paired pups or stressed-reared paired pups receiving a CORT antagonist injection, which do not show freezing behavior. The freezing behavior ANOVA revealed a main effect of rearing condition ($F_{(1,32)} = 66.016$, $p < 0.0001$), a significant effect of conditioning group ($F_{(3,32)} = 81.926$, $p < 0.0001$), and a significant

interaction between conditioning group and rearing condition ($F_{(3,32)} = 66.016, p < 0.0001$); *post hoc* Fisher's tests revealed that the stressed-reared paired groups differed significantly from each of the control groups at the $p < 0.05$ level.

Also, Figure 1C shows that paired, unpaired, and odor-only pups exposed to stressed-rearing had increased levels of CORT compared with normally reared pups. ANOVA for the CORT levels revealed a significant main effect of rearing condition ($F_{(1,22)} = 54.060, p < 0.0005$), a main effect of conditioning group ($F_{(1,22)} = 4.298, p < 0.05$), and a significant interaction between conditioning group and rearing condition ($F_{(2,22)} = 6.873, p < 0.005$); *post hoc* Fisher's tests revealed that the paired, unpaired, and odor-only stressed-reared pups were significantly different from the normally reared groups, although the paired, unpaired, and odor-only stressed-reared groups were also significantly different from one another at the $p < 0.05$ level.

Figure 1D illustrates an increased 2-DG uptake within the glomerular layer of the olfactory bulb of pups learning either an odor aversion (stressed-reared paired pups) or an odor preference (normally reared paired pups and stressed-reared paired pups receiving a CORT antagonist injection). No changes were observed in control groups. ANOVA revealed a main effect of conditioning group ($F_{(3,32)} = 22.171, p < 0.0001$); *post hoc* Fisher's tests revealed that each of the paired groups were significantly different from each of the control groups.

As illustrated in Figure 1E–G, stressed rearing, which results in pups learning an odor aversion rather than the age-typical odor preference, produced odor–shock-induced enhanced 2-DG uptake in the lateral, basolateral, and central amygdala nuclei. CORT antagonist injection prevented the enhance amygdala 2-DG uptake. Specifically, central amygdala nucleus ANOVA revealed a significant main effect of conditioning group ($F_{(3,32)} = 11.156, p < 0.0001$), a main effect of rearing condition ($F_{(1,32)} = 6.243, p < 0.05$), and a significant interaction between conditioning group and rearing condition ($F_{(3,32)} = 8.547, p < 0.0005$). The basolateral amygdala nucleus ANOVA revealed a significant main effect of conditioning group ($F_{(3,32)} = 7.975, p < 0.0005$), a main effect of rearing condition ($F_{(1,32)} = 10.215, p < 0.005$), and a significant interaction between conditioning group and rearing condition ($F_{(3,32)} = 8.358, p < 0.0005$). The lateral amygdala nucleus ANOVA revealed a significant main effect of conditioning group ($F_{(3,32)} = 8.827, p < 0.0005$), a main effect of rearing condition ($F_{(1,32)} = 9.375, p < 0.005$), and a significant interaction between conditioning group and rearing condition ($F_{(3,32)} = 9.484, p < 0.0001$); *post hoc* Fisher's tests revealed that basolateral, central, and lateral nucleus of the amygdala of stressed-reared paired pups differed from each of the other groups at the $p < 0.05$ level.

Mimicking stressed rearing by systemic CORT injection switches odor preference to odor aversion learning and alters the neural circuitry

We have demonstrated previously that systemic CORT injection during odor–0.5 mA shock conditioning switches odor-preference learning to odor aversion learning (Moriceau et al., 2006). Our goal here was to assess the effect of systemic CORT injection on the olfactory bulb and the participation of the amygdala (basolateral, lateral, and central nuclei) in learning and verify whether CORT injection was able to mimic the effect of early-life stress.

At P7, paired odor–shock pups injected with saline learned an odor preference (same as normally reared pups, experiment 1).

PN8 Amygdala Corticosterone Infusion

Figure 3. Odor–0.5 mA shock conditioning in P7 pups with intra-amygdala CORT infusion. **A**, Number of choices toward the CS odor in a Y-maze test. **B**, Locations of cannula tips (solid circles) for rats used for CORT infusion into the amygdala (Paxinos et al., 1991). **C**, Brain section counterstained with cresyl violet illustrating the extent of [^3H]CORT diffusion within the amygdala. **D**, Representative location of cannula tip near the basolateral complex of the amygdala. The cannula tip placement is marked by an arrow. The location of the basolateral complex of the amygdala is illustrated in gray ($n = 7$ –12 per group). * $p < 0.05$, significantly different from the other groups.

However, systemic CORT injection permitted pups to learn odor avoidance instead (similarly to stressed-reared pups, experiment 1) (Fig. 2A). The Y-maze ANOVA revealed a main effect of drug treatment ($F_{(1,34)} = 22.514, p < 0.0001$) and a significant interaction between conditioning group and drug treatment ($F_{(2,34)} = 23.849, p < 0.0001$); *post hoc* Fisher's tests revealed that the saline-

paired group and the CORT-paired groups each differed significantly from each of the control groups and also from one another at the $p < 0.05$ level.

Figure 2B depicted an increased 2-DG uptake within the glomerular layer of the olfactory bulb of pups learning either an odor aversion (CORT-injected pups) or an odor preference (saline-injected pups). No changes were observed in control groups. ANOVA revealed a main effect of conditioning group ($F_{(5,22)} = 23.507$, $p < 0.0001$); *post hoc* Fisher's tests revealed that each of the paired groups were significantly different from each control group.

As illustrated in Figure 2, C–E, CORT injection, which causes pups to learn an odor aversion rather than the age-typical odor preference, produced odor-shock-induced enhancement in basolateral and lateral amygdala nuclei 2-DG uptake. Specifically, basolateral nucleus ANOVA revealed a significant main effect of conditioning group ($F_{(2,23)} = 12.597$, $p = 0.0005$), a main effect of drug treatment ($F_{(1,23)} = 9.086$, $p < 0.05$), and a significant interaction between conditioning group and drug treatment ($F_{(2,23)} = 6.287$, $p < 0.05$). The lateral nucleus ANOVA revealed a significant main effect of conditioning group ($F_{(2,23)} = 12.304$, $p = 0.0005$), a main effect of drug treatment ($F_{(1,23)} = 7.039$, $p < 0.05$), and a significant interaction between conditioning group and drug treatment ($F_{(2,23)} = 3.927$, $p < 0.05$). The central nucleus ANOVA revealed a significant interaction between conditioning group and drug treatment ($F_{(2,21)} = 6.144$, $p < 0.05$); *post hoc* Fisher's tests revealed that basolateral, lateral, and central nuclei of the amygdala of paired CORT-injected pups differed from each of the other groups at $p < 0.05$ level.

CORT infusion into the amygdaloid complex and CRH infusion into the LC permits odor aversion learning

Here we assess CORT action on the amygdala–LC axis during odor aversion learning with odor-shock conditioning. Specifically, we mimic the activation of the amygdala by CORT and the subsequent activation of the LC by central amygdala CRH afferents to permit the release of NE into the olfactory bulb.

As shown in Figure 3A, paired pups that received amygdala CORT infusions (50 or 100 ng) during odor-shock conditioning exhibited a subsequent odor aversion. ANOVA revealed a significant main effect of conditioning group ($F_{(2,67)} = 10.525$, $p = 0.0001$), a main effect of drug treatment ($F_{(2,67)} = 24.899$, $p < 0.0001$), and a significant interaction between conditioning group and drug treatment ($F_{(4,67)} = 17.966$, $p < 0.0001$); *post hoc* Fisher's tests revealed that paired pups infused with 50 or 100 ng CORT, and paired pups infused with cholesterol into the amygdala each differed significantly from each of the control groups at the $p < 0.05$ level. Amygdala cannula tip placements are shown in Figure 3B. All tip placements were < 1 mm from the basolateral complex of the amygdala. As demonstrated in Figure 3C, most of the drug diffusion was limited to the amygdala, most notably the

Figure 4. Odor-shock conditioning in P7 pups with intra-LC CRH infusion. **A**, Number of choices toward the CS odor in a Y-maze test. **B**, Locations of cannula tips (solid circles) for rats used for CRH infusion into the LC. **C**, Brain section counterstained with cresyl violet illustrating the extent of [3 H]CRH diffusion within the LC. **D**, Representative location of cannula tip near the LC. The cannula tip placement is marked by an arrow. The location of the LC is illustrated in red ($n = 6$ –10 per group). * $p < 0.05$, significantly different from the other groups.

Figure 5. Effect of CRH infusion into the LC on the level of NE measured by microdialysis and HPLC in the olfactory bulb of sensitive-period pups (P7). * $p < 0.05$, significantly different from the other groups.

lateral and central nuclei. However, some drug spread was found ~ 1 mm outside the amygdala.

As shown in Figure 4A, paired pups that received 100 or 200 ng CRH infused into the LC during odor-shock conditioning exhibited an odor aversion, whereas 50 ng CRH infusion prevented the odor-preference learning in paired pups. ANOVA revealed a significant effect of drug treatment ($F_{(3,76)} = 6.182$, $p < 0.001$) and a significant interaction between conditioning group and drug treatment ($F_{(6,76)} = 10.537$, $p < 0.001$); *post hoc* Fisher's tests revealed that LC CRH 100 and 200 ng paired pups and LC paired saline pups each differed significantly from each of the control groups at the $p < 0.05$ level. Cannula tip placements directed at the LC, which were all < 1 mm from the LC, are shown in Figure 4B. Among our cannulated animals, two paired pups that received 100 ng of CRH had misplaced cannula, and they did

shock) and non-noxious (stroking) stimulus-evoked NE release in the olfactory bulb, with neonatal pups releasing significantly more NE than older pups/adult (Kimura and Nakamura, 1987; Nakamura et al., 1987; Nakamura and Sakaguchi, 1990; McLean and Shipley, 1991; Sullivan et al., 1992, 2000b; Sullivan and Wilson, 1994; Langdon et al., 1997; Yuan et al., 2000). The mechanism for the prolonged neonatal LC response appears because of the lack of functional $\alpha 2$ inhibitory noradrenergic autoreceptors that terminate the LC response in older pups/adult (Nakamura et al., 1987; Pieribone et al., 1994; Scheinin et al., 1994; Winzer-Serhan et al., 1999).

In contrast, in older pups and adults, the LC has different characteristics limiting NE release that is associated with $\alpha 2$ inhibitory autoreceptor functional emergence (Nakamura et al., 1987; Nakamura and Sakaguchi, 1990; Scheinin et al., 1994). This NE level decrease is responsible for the termination of the sensitive period in pups' NE-dependent rapid odor-preference learning (Moriceau and Sullivan, 2004b). Furthermore, as described above, the LC is activated via CRH afferent from the amygdala during stress in adults. Indeed, CRH antagonist application into the LC prevents fear conditioning (Bouret et al., 2003), whereas CRH administered locally into the adult LC is able to increase the fear response (Valentino et al., 1983; Dunn and Everitt, 1987; Butler et al., 1990; Emoto et al., 1993; Borsody and Weiss, 1996; Curtis et al., 1997; Page and Abercrombie, 1999; Bouret et al., 2003; Dunn et al., 2004; Jedema and Grace, 2004; Reyes et al., 2006; Dunn and Swiergiel, 2008). This increase in fear response is associated with a firing rate increase of the LC NE neurons and application of CRH antagonist into the LC blocked this effect. Here, we showed that, during the sensitive period, early-life stress (stressed-reared or CORT-injected paired pups) increases olfactory bulb 2-DG uptake. Furthermore, CRH infusion directly into the LC permits odor aversion learning associated with a large NE increase into the olfactory bulb—probably the consequence of too much NE into the bulb attributable to the unique neonatal LC response characteristics. Therefore, our data suggest that the 2-DG increase is not attributable to the activation of the attachment circuit per se but to the infant's unique hyperfunctioning LC being stimulated by CRH. However, because of the incompletely defined NE pathway between the LC, amygdala, and A_1/A_2 noradrenergic nuclei, other potential pathways cannot be eliminated, including NE feedback to the basolateral nucleus of the amygdala.

Pup behavior and neural changes: early-life stress and elevated CORT

The present results, together with results from the Baram laboratory (Gilles et al., 1996; Avishai-Eliner et al., 2001), suggest that elevation of CORT on pups' behavior leads to disruption of social interactions with the mother, as well as learning about the mother. These disruptions in behavior are presumably because of neural changes within the amygdala and LC (Avishai-Eliner et al., 2001; Brunson et al., 2005; Levine, 2005; Guijarro et al., 2007; Champagne et al., 2008; McEwen, 2008; Cirulli et al., 2009; Lupien et al., 2009). The present data suggest that the effects of early stress on the infants' LC and amygdala have immediate consequences for pups' interactions with the mother via disruption in attachment.

As demonstrated previously, early-life stress alters the developmental trajectory of myriad brain areas, including the amygdala and LC, with elevated CORT implicated as a causal factor (Caldji et al., 1998; Dent et al., 2001; Sanchez et al., 2001; Plotsky et al., 2005; Champagne et al., 2008; McEwen, 2008; Cirulli et al.,

2009; Lupien et al., 2009). The maternal stress procedure used here, which is based on the insufficient nest/bedding paradigm developed in the Baram laboratory, also produces an increase in pups' CORT through maternal rough handling of pups (Gilles et al., 1996; Avishai-Eliner et al., 2001). This precocious increase in CORT is significant because it indicates that early-life stress prematurely ends the stress hyporesponsive period, which is characterized by pups' low basal CORT levels and the failure to mount the stress-induced CORT release (Rosenfeld et al., 1992; Grino et al., 1994; Levine, 2001).

Implications

Early-life stress seems to manipulate the precise timing for the participation of specific brain regions in learning. Specifically, these data suggest the fear circuit (amygdala–CORT LC–CRH) is coactivated with the attachment circuit (LC–NE–olfactory bulb). Because early-life activation of LC CRH has been linked with later-life behavioral and neural deficits, these data may suggest a very specific route of activation during early-life pain, which is blocked without stress. Furthermore, this attachment/fear circuit overlaps with neural correlates of compromised mental health, including depression and anxiety in humans (Heim et al., 2001; Nestler et al., 2002; Teicher et al., 2003; Gunnar et al., 2009; Lupien et al., 2009). Overall, there is remarkable convergence between this new early-life stress paradigm and other paradigms, as well as the clinical literature (Kaufman, 1991; Kaufman et al., 1997; Caldji et al., 1998; Lehnert et al., 1998; Glaser, 2000; Dent et al., 2001; Kaufman and Charney, 2001; Sanchez et al., 2001; Grossman et al., 2003; Teicher et al., 2003; Plotsky et al., 2005; Gunnar and Quevedo, 2007; Cirulli et al., 2009).

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