

Corticosterone Influences on Mammalian Neonatal Sensitive-Period Learning

Stephanie Moriceau and Regina M. Sullivan
University of Oklahoma

Infant rats exhibit sensitive-period odor learning characterized by olfactory bulb neural changes and odor preference acquisitions critical for survival. This sensitive period is coincident with low endogenous corticosterone (CORT) levels and stress hyporesponsivity. The authors hypothesized that low corticosterone levels modulate sensitive-period learning. They assessed the effects of manipulating CORT levels by increasing and removing CORT during (Postnatal Day 8) and after (Postnatal Day 12) the sensitive period. Results show that (a) exogenous CORT prematurely ends sensitive-period odor–shock-induced preferences; (b) adrenalectomy developmentally extends the sensitive period as indicated by odor–shock-induced odor-preference learning in older pups, whereas CORT replacement can reinstate fear learning; and (c) CORT manipulation modulates olfactory bulb correlates of sensitive-period odor learning in a manner consistent with behavior.

Sensitive periods for enhanced learning have been documented in a wide range of species. For example, language acquisition in humans is more easily learned prior to puberty, the ewe has a few hours postpartum when she may bond with her lamb, and imprinting in avian species occurs during the first few hours posthatching. However, sensitive periods can also involve suppression of other types of learning that could interfere with attachment and learning about the mother, such as fear conditioning, inhibitory conditioning, and passive avoidance (Blozovski & Cudennec, 1980; Camp & Rudy, 1988; Collier, Mast, Meyer, & Jacobs, 1979; Emerich, Scalzo, Enters, Spear, & Spear, 1985; Haroutunian & Campbell, 1979; Myslivecek, 1997; Sullivan, Hofer, & Brake, 1986; Sullivan, Landers, Yeaman, & Wilson, 2000). At least in rats, learning during the preweaning period results in long-lasting behavioral and neural effects that later influence sexual performance, mate choice, and maternal behavior (Coopersmith & Leon, 1986; Fillion & Blass, 1986; Moore, Jordan & Wong, 1996; Pager, 1974; Shah, Oxley, Lovic, & Fleming, 2002; Woo & Leon, 1988).

Neonatal rats rapidly learn an odor preference following odor–milk pairings, but also after pairing odor with 0.5-mA shock (Camp & Rudy, 1988; Sullivan & Hall, 1988). This learning is not due to an altered pain threshold (Barr, 1995; Emerich et al., 1985; Stehouwer & Campbell, 1978), but appears to be due to the failure of the amygdala to participate in neonatal fear conditioning (Sullivan et al., 2000). Because pups are limited to olfactory, gustatory, and somatosensory system functioning, the learned odor preference for maternal odor is critical for survival. A similar pain-attachment learning system has been demonstrated in avian im-

printing, young dogs, nonhuman primates, and, perhaps, in abused children (Harlow & Harlow, 1965; Helfer, Kempe, & Krugman, 1997; Hess, 1962; Rajecki, Lamb, & Obmascher, 1978; Sanchez, Ladd, & Plotsky, 2001). This suggests that evolution has produced an attachment system to ensure that young approach their caregiver, regardless of the quality of care (Hofer & Sullivan, 2001).

Corticosterone (CORT) is implicated in sensitive-period termination in rats. First, the sensitive period coincides with the stress hyporesponsive period when tactile stimulation received during maternal care maintains low CORT levels and stress (including shock) does not normally produce adrenal gland CORT release (Levine, 1962; Van Oers, de Kloet, Whelan, & Levine, 1998). Second, if the ontogenetic increase in CORT is prevented by Postnatal Day (PN) 8 adrenalectomy (ADX), the normal emergence of inhibitory conditioning and the developmental emergence of fear to natural odors in PN10 pups are prevented, whereas injecting a PN8 pup with CORT causes the premature expression of fear to natural odors (Bialik, Pappas, & Roberts, 1984; Takahashi, 1994; Takahashi & Rubin, 1993).

The neonatal olfactory-based attachment system is associated with acquisition of olfactory bulb neural enhancement of responses to both natural and learned attachment odors, such as enhanced immediate-early gene activity (*c-fos*); enhanced ¹⁴C 2-deoxyglucose (2-DG) uptake in focal, odor-specific glomerular regions; modified single-unit response patterns of mitral/tufted cells; and olfactory bulb anatomical changes (Johnson, Woo, Duong, Nguyen, & Leon, 1995; Sullivan & Leon, 1986; Sullivan, Wilson, & Leon, 1989; Wilson & Leon, 1988; Wilson, Sullivan, & Leon, 1987; Woo, Coopersmith, & Leon, 1987). As with attachment behavioral changes, these neural changes are retained into adulthood, but their acquisition is dependent on infant experiences (Coopersmith & Leon, 1986; Pager, 1974; Woo & Leon, 1988).

To assess the role of CORT in the neonatal sensitive period, we used our mammalian attachment model with odor–shock-induced odor preference. We manipulated CORT levels before (PN8) and after (PN12) the sensitive period and assessed learned odor preference/aversion and learning-induced olfactory bulb changes.

Stephanie Moriceau and Regina M. Sullivan, Department of Zoology, University of Oklahoma.

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Correspondence concerning this article should be addressed to Stephanie Moriceau, Department of Zoology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019. E-mail: smoriceau@ou.edu

Method

Subjects

The subjects were 198 male and female PN8 and PN12 Long-Evans rat pups born and bred in our colony (originally from Harlan Lab Animals). They were housed in polypropylene cages (34 cm × 29 cm × 17 cm) lined with pine shavings and were kept in a temperature (23 °C)- and light (0700–1900)-controlled room. Food and water were available ad libitum. The day of birth was considered PN0, and litters were culled to 10 on PN0–1. No more than 1 male and 1 female from a litter were used in each experimental condition.

Odor–Shock Conditioning

Pups were conditioned during the sensitive period (PN8) or after the sensitive period (PN12) in one of the following 45-min classical conditioning groups: (a) paired odor–shock, (b) unpaired odor–shock, and (c) odor only. Pups were placed in individual 600-ml glass beakers and were given a 10-min adaptation period to recover from handling. During 45 min of conditioning stimuli, pups received 11 presentations of a 30-s citral odor (conditioned stimulus [CS]) and a 1-s 0.5-mA tail shock (unconditioned stimulus; Lafayette Instruments, Lafayette, IN), with an intertrial interval of 4 min. Citral odor was delivered by a flow dilution olfactometer (2 L/min flow rate) at a concentration of 1:10 citral vapor. Paired odor–shock pups received the 30-s odor, with shock overlapping with the last second of the odor presentation. Unpaired odor–shock pups received the shock 2 min after each odor presentation. Odor-only pups received only the citral odor presentation.

During conditioning, the number of limbs moving (0 = *no movement of the extremities*; 5 = *movement of all five extremities*) was recorded 20 s before presentation as well as for 20 s during the odor presentation (Hall, 1979). These measures permit a general assessment of behavioral activity during training in motorically immature rats.

Manipulating CORT

Thirty minutes prior to training, pups were injected with either CORT (1.5 and 3.0 mg/kg, intraperitoneally) or saline (Takahashi, 1994). Injected CORT leaves the system after 8–12 hr, indicating that testing was performed on drug-free rats (Goodman & Gilman, 1985). On the basis of radioimmunoassay done in our laboratory, the 3.0 mg/kg dose CORT produces CORT levels similar to those found in nonmaternally deprived and stressed (endotoxin injection) pups at PN6 (Dent, Smith, & Levine, 1999).

Endogenous CORT was eliminated by ADX at PN8 for training of PN12 pups. Pups were anesthetized (Isoflurane), and dorsal incisions were made to extract the adrenal glands. Sham-operated controls received dorsal incisions, but the adrenal glands were left intact. Following recovery from surgery (approximately 1 hr), pups were returned to the mother until training. CORT receptors are present and functional throughout neonatal development (Alexis, Kittraki, Spanou, Stylianopoulou, & Sekeris, 1990; Chao, Choo, & McEwen, 1989; Kittraki, Alexis, Papalopoulou, & Stylianopoulou, 1996; Yi, Masters, & Baram, 1994).

ADX was verified at PN13 in the late afternoon with a commercially available kit (¹²⁵I CORT, sensitivity of 5.7 ng/ml; Radioimmunoassay Systems Lab, Inc., Carson, CA). Heart blood samples were taken and centrifuged at 10,000 cpm for 3 min; the plasma was then divided into aliquots and stored at –70 °C for later analysis.

Assessing Learning: Y Maze

The day after conditioning, pups were given a Y-maze test when all CORT had been eliminated (Goodman & Gilman, 1985). This test required pups to choose between two arms of a Plexiglas Y maze (start box: 8.5

cm × 10 cm × 8 cm; choice arms: 8.5 cm × 24 cm × 8 cm): one containing the citral odor CS (20 μl of citral odor placed on a KimWipe), and the other containing the familiar odor of pine shavings (20 ml of clean shaving in a petri dish). A pup was placed in the start box (habituation chamber) during the 5 s before the door to each alley was opened. 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 & Wilson, 1991). Testing was done blind to the training condition.

Assessing Neural Correlates Within the Olfactory Bulb

PN 8 and PN 12 pups were injected with 2-DG (20 μCi/100 g, subcutaneously) 5 min prior to the 45-min odor–shock conditioning. Immediately following conditioning, pups were decapitated and their brains quickly removed, frozen in 2-methylbutane (–45 °C), and stored in a –70 °C freezer. For analysis, brains were sectioned (20 μm) in a –20 °C cryostat, and every other section was saved to be placed on a cover slip and exposed for 5 days along with standards (Carbon 14 standard 10 × .02 mCi; American Radiolabeled Chemicals Inc., St. Louis, MO) to X-ray film (Coopersmith & Leon, 1986; Sullivan & Wilson, 1995).

Odors produce an odor-specific pattern of 2-DG uptake within the glomerular layer of the olfactory bulb that is enhanced with neonatal odor conditioning (Coopersmith & Leon, 1986; Sullivan & Leon, 1986). These odor-specific loci, along with the periventricular core, were measured using quantitative optical densitometry with National Institutes of Health image software. To quantify 2-DG uptake, the computer constructed a calibration curve that related the gray value of ¹⁴C 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. Five readings were taken from the periventricular core and the center of the odor-specific loci. Data were analyzed as the uptake within the odor-specific loci relative to the uptake in the periventricular core (Sullivan & Wilson, 1995). Readings were made blind to the training condition.

Statistical Analysis

Comparisons were made between groups using the analysis of variance (ANOVA) test followed by post hoc Fisher's tests (Winkler & Hays, 1975).

Results

Early Termination of the Sensitive Period Through Exogenous CORT (PN8 Pups)

As illustrated in Figure 1A, following odor–shock conditioning, sensitive-period pups injected with saline learned an odor preference, whereas CORT (3.0 mg/kg) injection prevented the odor preference learning. An ANOVA revealed a significant effect of training condition, $F(2, 71) = 10.44, p = .01$; a main effect of drug treatment, $F(2, 71) = 12.59, p < .01$; and a significant interaction between training condition and drug treatment, $F(4, 71) = 6.75, p < .01$; post hoc Fisher's tests revealed that both the saline and the 1.5 mg/kg CORT-paired groups differed significantly from each of the control groups at the $p < .05$ level ($n = 7–10$ pups per group). While the 3.0 mg/kg CORT-paired group was significantly different from the unpaired saline group, this difference was not found for the unpaired CORT groups.

Analysis of behavior during odor–shock conditioning demonstrated that the acquisition curves were significantly different for

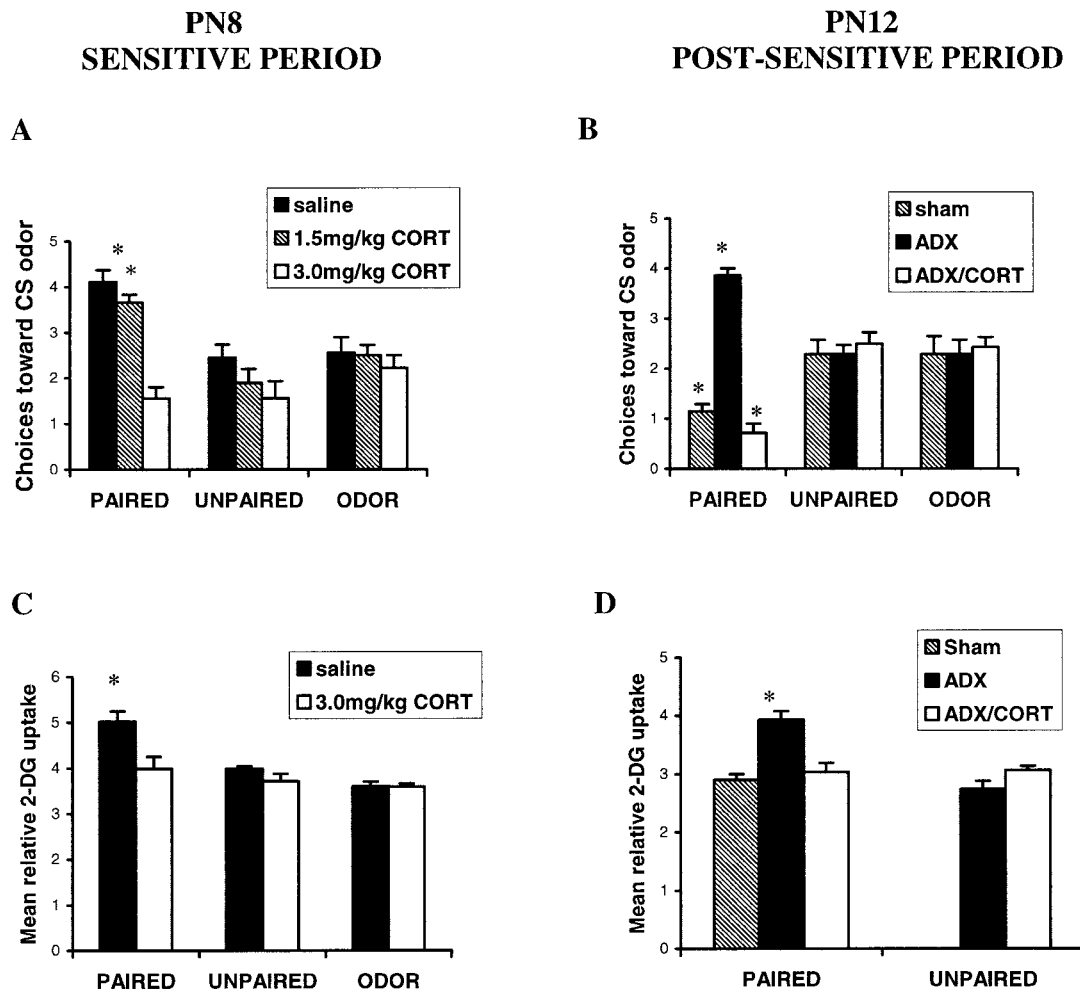


Figure 1. Mean (\pm SEM) number of conditioned odor choices in a Y-maze test (total of five trials) for (A) sensitive-period Postnatal Day (PN) 8 pups and (B) post-sensitive-period PN12 pups. Mean (\pm SEM) level of odor-induced olfactory bulb focal ^{14}C 2-deoxyglucose (2-DG) uptake in (C) sensitive-period PN8 pups and (D) post-sensitive-period PN12 pups. Asterisks represent significant differences from all others groups ($p < .05$). CS = conditioned stimulus; CORT = corticosterone; ADX = adrenalectomy.

the Trial \times Condition (see Figure 2A) repeated measures ANOVA, $F(20, 700) = 28.81$, $p < .01$, but not for the Trial \times Drug interaction, $F(20, 700) = 0.65$, $p = .87$.

Extending the Sensitive Period by Eliminating CORT (PN12 Pups)

As indicated in Figure 1B, ADX PN12 pups demonstrated a shock-induced odor preference, whereas sham pups exhibited the age-appropriate odor aversion. The dependence of aversion learning on CORT was supported by the aversion learning seen in ADX pups given CORT replacement. An ANOVA revealed a significant effect of training condition, $F(2, 53) = 3.56$, $p < .05$; a main effect of drug treatment, $F(2, 53) = 15.39$, $p < .01$; and a significant interaction between training condition and drug treatment, $F(4, 53) = 19.13$, $p < .01$; post hoc Fisher's tests revealed that the sham and the ADX-plus-CORT groups were significantly lower, whereas the ADX group was significantly higher than each of the

control groups at the $p < .05$ level ($n = 7$ pups per group). ADX significantly reduced CORT levels (sham 15.4 ± 3.9 ng/ml vs. ADX 1.8 ± 0.9 ng/ml), $t(25) = 5.13$, $p < .01$.

Analysis of behavior during odor-shock conditioning demonstrated that the acquisition curves were significantly different for the Trial \times Condition (Figure 2B) repeated-measure ANOVA, $F(20, 740) = 21.29$, $p < .01$, but not for the Trial \times Drug interaction, $F(40, 740) = 1.20$, $p = .19$.

CORT Modification of Sensitive-Period Olfactory Bulb Neural Correlates of Imprinting Consistent With the Behavioral Effects

The odor-specific pattern of 2-DG uptake within the glomerular layer of the olfactory bulb was measured for 2-DG labeling density. As illustrated by the PN8 (sensitive period) pup data in Figure 1C, the olfactory bulb showed enhanced 2-DG uptake following odor-shock conditioning and replicated previous results (Sullivan

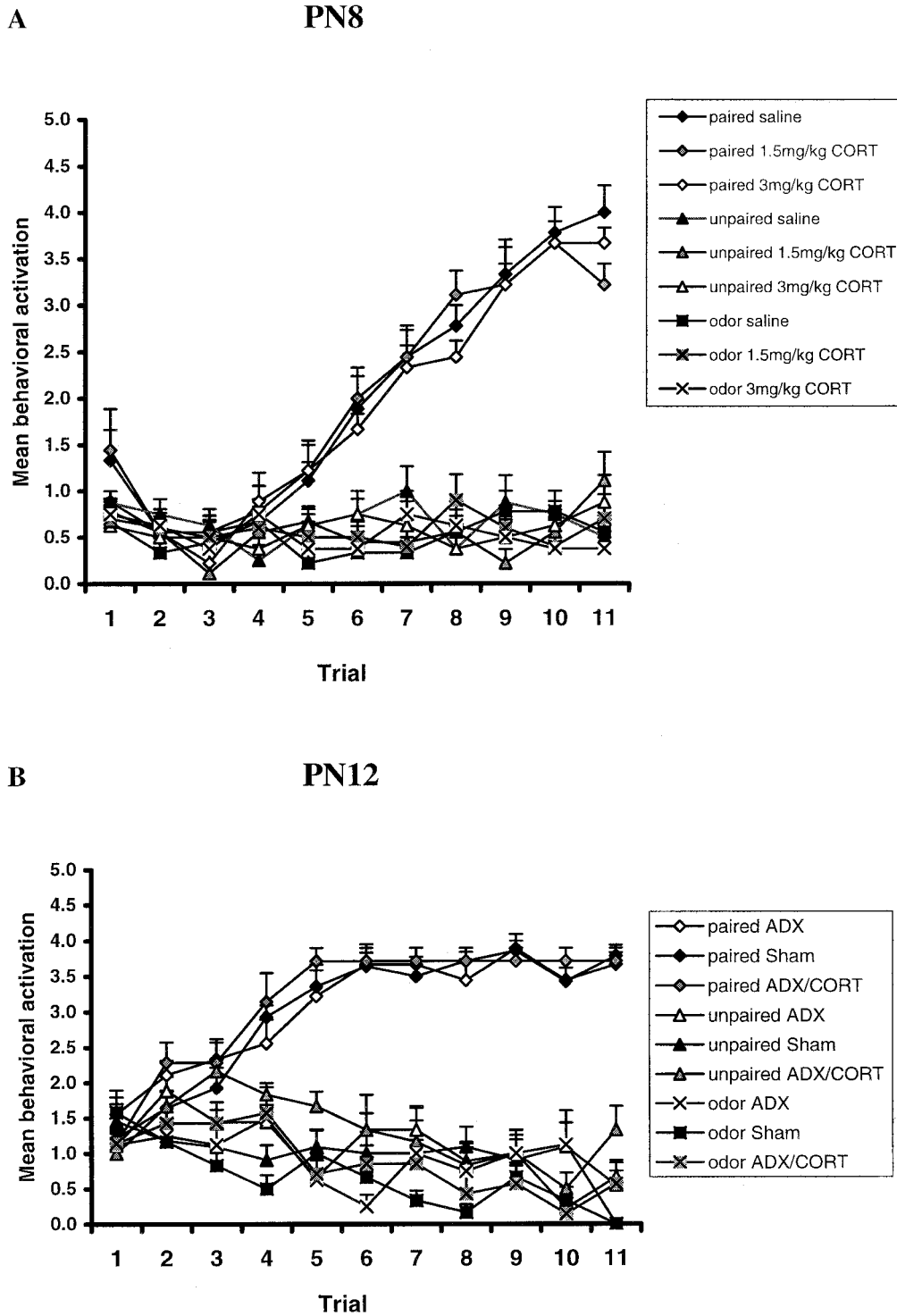


Figure 2. Mean (\pm SEM) number of responses (behavioral activation) to the odor conditioned stimulus during odor-shock acquisition for (A) sensitive period Postnatal Day (PN) 8 pups and (B) post-sensitive-period PN12 pups. CORT = corticosterone; ADX = adrenalectomy.

& Wilson, 1995). However, odor-shock CORT pups showed levels of uptake similar to pups in the control learning conditioning, $F(5, 25) = 10.91, p < .01$; post hoc Fisher's tests revealed that the odor-shock conditioning, CORT-injected groups were signifi-

cantly different from each of the control groups at the $p < .01$ level ($n = 5-6$ pups per group).

Older post-sensitive-period pups (PN12) do not normally show enhanced olfactory bulb uptake, which was replicated in our

present results (Sullivan & Wilson, 1995). However, eliminating CORT through ADX appeared to maintain the olfactory bulb's ability to produce the neonatal learning-induced plasticity characteristic of the sensitive period (Figure 1D). This plasticity was eliminated in ADX pups given CORT replacement, $F(4, 20) = 13.85, p < .01$; post hoc Fisher's tests revealed that the odor-shock conditioning combined with the ADX group was significantly different from each of the control groups at the $p < .01$ level ($n = 5$ pups per group).

Discussion

Neonatal rat pups have unique learning abilities to ensure that the olfactory-based attachment to the mother is quickly learned. The low CORT levels characteristic of the postnatal sensitive period for attachment learning appear to be critical in permitting these unique learning abilities. Specifically, we found that (a) CORT will prematurely end the sensitive period as indicated by preventing odor-shock-induced preference learning, (b) an ADX, which prevents the natural production of CORT, will extend the sensitive period to PN12 as indicated by odor-shock conditioning producing an odor preference, while CORT replacement can reinstate pups' ability to learn fear conditioning, and (c) manipulation of CORT modulates the neural correlates of sensitive-period odor learning in a manner consistent with behavior. Specifically, preventing the endogenous increase of CORT (ADX) extends the temporal parameters in which early learning can modify olfactory bulb glomerular layer 2-DG uptake. Moreover, the injection of CORT during the sensitive period (PN8) prevents the learning-induced olfactory bulb neural changes characteristic of sensitive-period learning. The effects of CORT are limited to acquisition, because CORT levels return to baseline at approximately 10 hr prior to testing (Goodman & Gilman, 1985). However, the CORT dose (3mg/kg) used in this study, demonstrated by radioimmunoassay done in our laboratory (data not shown), generated CORT levels similar to those found in stressed rats at PN12 after an endotoxin injection (potent stimulant of the hypothalamic-pituitary-adrenal axis) and in maternally deprived rat pups at PN6 (Dent, Smith, & Levine, 1999, 2000). In a previous study, a higher dose was used (6 mg/kg) and eliminated because it produced a hypoactivity in the behavior of rat pups. The role of CORT in developmental emergence of other behaviors supports the notion that low CORT levels during the sensitive period prevent pups from learning conditioned inhibition and passive avoidance (Bialik et al., 1984; Collier et al., 1979).

The low level of CORT during the neonatal period is referred to as the stress hyporesponsive period (Grino, Paulmyer-Lacroix, Faudon, Renard, & Anglade, 1994; Levine, 1962, 2001; Rosenfeld, Suchecki, & Levine, 1992; Walker et al., 1986). The exact neural mechanisms for the stress hyporesponsive period remain elusive; however, two factors influence pups' CORT level. First, sensory stimuli from the mother during normal mother-infant interactions maintain pups' CORT at low levels; without maternal stimulation (deprivation) for a few hours, CORT levels become elevated (Levine, 2001). Second, stress-induced elevated maternal CORT levels are delivered to pups through milk (Yeh, 1984). Thus, maternal behavior modulates pup CORT levels and may influence temporal aspects of the sensitive period.

Stress and maternal behavior have profound short- and long-term effects on the development of brain areas mediating stress: the amygdala-locus coeruleus and the hypothalamus-pituitary-adrenal axis (Dent, Smith, & Levine, 2001; Eghbal-Ahmadi, Hatalski, Avishai-Eliner, & Baram, 1997; Francis, Young, Meaney, & Insel, 2002; Huot, Plotsky, Lenox, & McNamara, 2002; Zhang et al., 2002). Additionally, hormones and neurotransmitters associated with the stress system exhibit both short- and long-term effects, including modification of baseline CORT levels, enhanced stress response, and CORT receptor modifications (Avishai-Eliner, Hatalski, Tabachnik, Eghbal-Ahmadi, & Baram, 1999; Kent, Tom, & Levine, 1997; Lightman & Harbuz, 1993; Liu et al., 1997; Meaney et al., 1996; Okimoto et al., 2002; Suchecki, Mozaffarian, Gross, Rosenfeld, & Levine, 1993; Vasquez, Van Oers, Levine, & Akil, 1996; Yi et al., 1994). Also, in humans, the effects of early stress events are implicated in the emergence of psychiatric disorders during adult life (review Teicher et al., 2003).

CORT retains an important role in adult fear conditioning and is associated with acquisition deficits. A systemic injection of CORT increases freezing in response to a CS paired with footshock, and decreasing CORT levels cause reduced contextual fear and memory retrieval (Corodimas, LeDoux, Gold, & Schulkin, 1994; Pugh, Tremblay, Fleshner, & Rudy, 1997; Roozendaal, 2002; Roozendaal, Bohus, & McGaugh, 1996). However, opposed to the results observed here in pups, adult manipulation of CORT during acquisition does not alter whether odor-shock conditioning produces an odor preference or aversion, thus indicating unique effects of CORT during early development.

Although the site of CORT action has not been directly assessed in the present experiments, work on adult fear conditioning suggests that the amygdala may be important. The amygdala is involved in both learned and unlearned fear (Amaral et al., 2003; Cahill et al., 1999; Doron & LeDoux, 1999; Fanselow & LeDoux, 1999; Otto, Couzens, & Herzog, 2000; Otto et al., 1997; Schettino & Otto, 2001). CORT facilitates amygdala plasticity in a fear-conditioning paradigm (Stutzman, McEwen, & LeDoux, 1998). Until the emergence of amygdala function at PN10, neonatal pups do not express both learned and unlearned fear (Sullivan et al., 2000; Takahashi, Turner, & Kalin, 1991; Wiedenmayer & Barr, 2001). It should be noted that very high-intensity shock (1.0–1.5 mA) does produce an odor aversion in neonatal pups via odor-illness conditioning (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Kucharski & Spear, 1984; Rudy & Cheatle, 1977; Sullivan & Wilson, 1995), although this conditioning has been shown to occur without an amygdala (Bermudez-Rattoni & McGaugh, 1991; Schafe, Thiele, & Bernstein, 1998). Interestingly, while odor illness is easily learned by pups away from the mother, this learning is attenuated if conditioning is done while pups are suckling, indicating another constraint on pups' learning (Thiels & Alberts, 1991).

Glucocorticoid receptors are present in the neonatal olfactory bulb as well as in other brain areas, and CORT effects on adult learning have also been localized to the hippocampus and prefrontal cortex (Alexis et al., 1990; Diorio, Viau, & Meaney, 1993; Jacobson & Sapolsky, 1991; Kitraki et al., 1996). While the hippocampus and prefrontal cortex do not appear to be functional neonatally, the olfactory bulb has a uniquely important role in neonatal learning, and CORT may have direct effects on olfactory bulb odor processing (Crain, Cotman, Taylor, & Lynch, 1973;

Fanselow & Rudy, 1998; Sananes & Campbell, 1989; Stanton, 2000; Sullivan et al., 2000; reviews Hofer & Sullivan, 2001; Sullivan, in press). Specifically, during neonatal learning, olfactory bulb norepinephrine (NE) from the locus coeruleus (LC) is necessary for learning. During the sensitive period, the reinforcer increases LC firing and dramatically increases olfactory bulb NE (Nakamura & Sakaguchi, 1990; Rangel & Leon, 1995). The NE then prevents the primary output neurons of the bulb from habituating to the odor (Sullivan et al., 1989). While there is direct excitatory effect of NE on mitral cells (Yuan, Harley, McLean, & Knopfel, 2002), NE can also increase mitral-cell excitation by inhibiting the granule cells that normally inhibit the mitral cells (Aroniadou-Anderjaska, Zhou, Priest, Ennis, & Shipley, 2000; Nickell, Behbehni, & Shipley, 1994; Okutani, Yagi, & Kaba, 1999; Sullivan et al., 1989; Trombley, Hill, & Horning, 1999; Zhang, Okutani, Inoue, & Kaba, 2003; see Brennan & Keverne, 1997; Insel & Young, 2001, for similar odor learning during reproductive behavior). While CORT action in the bulb has not been assessed, hippocampal and amygdala studies suggest that CORT may cause the enhanced release of GABA, thereby preventing the NE excitatory effects on mitral cells (Minor & Hunter, 2002; Stutzmann et al., 1998). There is evidence that serotonin may modulate this cell interaction in both pup olfactory bulbs (Yuan et al., 2002) and adult hippocampus and amygdala (Minor & Hunter, 2002; Stutzmann et al., 1998).

In conclusion, our results suggest that neonatal odor-attachment learning and its neural correlates are temporally modified by CORT. Specifically, low levels of endogenous CORT maintain pups' unique ability to learn predominantly approach responses to their mother. This unique neonatal learning produces neural changes in the olfactory bulb that are still present in adulthood and that may underlie the neonatal attachment odor's ability to enhance mate choice and sexual and maternal behavior (Fillion & Blass, 1986; Fleming, O'Day, Kraemer, 1999; Moore et al., 1996).

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