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ONTOGENY OF FEAR: INFLUENCE OF CORTICOSTERONE

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By

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ONTOGENY OF FEAR: INFLUENCE OF CORTICOSTERONE

A Dissertation APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

BY

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Chapter 3 – Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups

Preface

I, Stephanie Moriceau, hold a principal author status for all manuscript chapters in this dissertation. However, Chapters 1-2 and 4-5 are co-authored by my supervisor. Chapter 3 is co-authored by my supervisor, Tania Roth and Terri Okotoghaide. Chapter 6 is co-authored by my supervisor and Kavita Trivedi.

A large part of Chapter 1 consists of a review chapter co-authored by Regina M. Sullivan. The review chapter is an article in Developmental Psychobiology and it is currently *in press.*

Chapter 2 is co-authored by Regina M. Sullivan. This chapter was published in *Journal of Neuroscience* (vol 24: 1182-1189) in 2004.

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Chapter 5 is co-authored by Regina M. Sullivan. This chapter will be divided in two articles: *Learning and Memory* and *Chemical senses.*

Chapter 6 is co-authored by Regina M. Sullivan and Kavita Trivedi. This chapter has been formatted for *Journal of Neuroscience*. Additional experiments will be added to this chapter before submission.

Chapter 1

Neurobiology of Infant Attachment

Modified version of *Developmental Psychobiology* (in press)

Abstract

A strong attachment to the caregiver is critical for survival in altricial species, including humans. While some behavioral aspects of attachment have been characterized, its neurobiology has only recently received attention. Using a mammalian imprinting model, we are assessing the neural circuitry that enables infant rats to attach quickly to a caregiver, thus enhancing survival in the nest. Specifically, the hyper-functioning noradrenergic locus coeruleus (LC) enables pups to learn rapid, robust preference for the caregiver. Conversely, a hypo-functional amygdala appears to prevent the infant from learning aversions to the caregiver. Adult LC and amygdala functional emergence correlates with sensitive period termination. This work suggests the neonatal brain is not an immature version of the adult brain but is uniquely intended to optimize attachment to the caregiver. Although human attachment may not rely on identical circuitry, the work reviewed here suggests a new conceptual framework in which to explore human attachments, particularly attachments to abusive caregivers.

The powerful influences of infant experiences on adult life are well established with strong support from both clinical and basic research, beginning with Freud. More recently, the psychiatrist John Bowlby proposed that infant relationships define future relationships and stressed the importance of understanding early attachment to the mother (Bowlby, 1965). He characterized human infant attachment in a specific, defined framework that permitted testing in an experimentally refined protocol easily applied to humans. Beyond that, his characterization of attachment is relevant throughout the animal kingdom. First, Bowlby noted that infants rapidly form an attachment to the caregiver. The classic example is imprinting in chicks, although human infants can also rapidly learn about the mother during the hours following birth (DeCasper & Fifer, 1980). Second, Bowlby noted infants undergo considerable abuse while remaining attached to the caretaker. In the avian model of imprinting, chicks will continue to follow their mother during the imprinting period even while being shocked (Hess, 1962; Salzen, 1970). Naïve post-critical period chicks (only hours older) are quickly able to learn an aversion to a surrogate mother when given similar shock presentations. A similar experiment in young dogs showed that puppies will learn a strong attachment to a handler providing shock or rough treatment (Fisher, 1955 cited in Rajecki, Lamb & Obmascher, 1978). This phenomenon extends to primates. The Harlows (1965) showed that nonhuman primate infants of abusive mothers still exhibited strong attachment, and recent work on a colony of abusive nonhuman primates shows similar results (Maestripieri, Tomaszycki &

Carroll, 1999; Sanchez, Ladd & Plotsky, 2001). Moreover, human children, even those abused by their caregiver, generally exhibit a strong attachment to that caregiver (review – Helfer, Kempe & Krugman, 1997). We have hypothesized that this attachment system may have evolved to ensure that altricial animals easily form a repertoire of proximity-seeking behaviors to the primary caregiver, regardless of the quality of the care they receive (Hofer & Sullivan, 2001).

In general, altricial species rely, at least to some extent, on learning about the mother to form attachment. This is exemplified in the avian imprinting model with its temporally defined sensitive period when the learning process is rapid and robust, although sensitive periods can be found in many species during developmental stages critical for survival. For example, postpartum animals quickly learn about their offspring; animals learn to identify their mate and, and as described here, infants learn about their caregiver (Brennen & Keverne, 1997; Insel & Young, 2001; Marlier, Schaal & Soussignan, 1998; Moffat, Suh & Fleming, 1993; Okere & Kaba, 2000).

Mammalian Imprinting Model

To assess the neurobiology of infant attachment, we have developed an infant rat model that conforms to the characteristics of attachment initially described by Bowlby. First, Bowlby stated that the infant rapidly forms an attachment to the caregiver. As illustrated in Figure 1 (top), neonatal rats very rapidly and easily learn an odor preference, although preference learning

becomes more adult-like after PN10 (bottom Figure 1). We modeled this rapid odor learning outside the nest using a classical conditioning paradigm in which a novel odor was paired with a positive stimulus such as stroking (left Figure 1; Sullivan, Brake, Hofer & Williams, 1986a; Sullivan, Hofer & Brake, 1986b, Pedersen, Williams & Blass, 1982). This learning occurs naturally in the nest to the maternal odor, although the preference can also be acquired to a novel odor applied to the mother (Galef & Kaner, 1980; Roth & Sullivan, submitted; Sullivan, Wilson, Wong, Correa & Leon, 1990; Terry & Johanson, 1996). Rapid odor learning may be a critical component of the altricial rat's survival because a newborn rat has limited sensory input (olfactory, somatosensory) and depends on learning its mother's odor for approach to the mother and nipple attachment (Polan & Hofer, 1999; Shair, Masmela, Brunelli & Hofer, 1997). This period of unique odor learning ends at postnatal day (PN) 10 and is called the sensitive period (Sullivan, Landers, Yeaman & Wilson, 2000a; see bottom of Figure 1 where learning is more adult-like). The second attachment characteristic defined by Bowlby is that infants can undergo considerable abuse while remaining attached to the caretaker. As is illustrated in Figure 1 (right, top), neonatal (PN6) rat pups learn to approach an odor even after that odor is paired with a painful stimulus (0.5mA shock), although older (PN12; right bottom) pups easily learn to avoid an odor paired with shock on the previous day (Sullivan et al., 2000a). Specifically, using a classical conditioning paradigm, pups exposed to an odor while receiving either a shock (0.5mA) or tail pinch subsequently express a preference for that odor (Camp & Rudy, 1988; Sullivan et al., 1986a; Sullivan et

al., 1986b; Sullivan et al., 2000a; Moriceau & Sullivan, 2004b). This shock-induced learning and preference acquisition is not due to the pups' inability to feel pain, since shock threshold varies little during this period of development (Barr, 1995; Emerich, Scalzo, Enters, Spear & Spear, 1985; Stehouwer & Campbell, 1978; Sullivan et al., 2000a).

While shock-induced preference acquisition may appear paradoxical, it may have developed to prevent pups from learning an aversion to the mother when being handled roughly in the nest. Indeed, rough treatment of pups by the mother is common in the nest. Mothers frequently step on pups when entering and leaving the nest or retrieve pups by a leg rather than at the nape of the neck. During these painful interactions, pups emit vocalizations associated with pain (Hofer, 1996). The benefits of a system preventing pups from learning an aversion to the mother are obvious since pups need to exhibit approach behaviors to procure the mother's milk, warmth and protection. Thus, in the altricial rat pup, the neonatal learning system seems specifically designed for attachment and is expressed behaviorally as an enhanced ability to acquire learned odor preferences and a decreased ability to acquire learned odor aversions (reviews – Hofer & Sullivan, 2001; Sullivan, 2001, 2003).

It should be noted that neonatal rats are able to learn aversive conditionings if an odor is paired with malaise (> 1.0 mA - strong shock or LiCl), since pups easily learn about interoceptive but not exteroceptive cues (Campbell,

1984; Haroutunian & Campbell, 1979; Miller, Molina & Spear, 1990; Rudy & Cheatle, 1977, 1978; Spear, 1978; Spear & Rudy, 1991). However, while odor illness associations are easily learned by pups away from the mother, this learning is diminished if LiCl conditioning is done while pups are suckling (Martin & Alberts, 1979; Melcer, Alberts & Gubernick, 1985).

During the sensitive period (PN1-9, the age when pups show enhanced preference learning and attenuated aversion learning), neonatal rats are confined to the nest. It is appropriate to learn only preferences, not aversions, in a situation where only the mother and other pups are encountered. However, as the sensitive period terminates around PN10, walking develops and the probability of leaving the nest greatly increases (Bolles & Woods, 1965). At this stage of development, pups require a more complex learning system more suited to the extra-nest environment. As illustrated in Figure 1 (bottom, PN12), the more mobile pup is more adult-like, with discriminating learning system to deal with the increasingly complex environment. Specifically, aversions are more easily learned and odor preferences are less easily learned, enabling pups to deal more appropriately with stimuli outside the nest. As is reviewed below, the pup's learning circuitry appears to show remarkable correspondence to its changing needs as its mobility increases.

Long-term Importance of Odors Learned in Infancy

In rats, early attachment-related odors appear to retain value into adulthood, although the role of the odor in modifying behavior changes from that used during infancy (attachment to the mother) to that used in adulthood (reproduction). Work done independently in the labs of Celia Moore (Moore, Jordan & Wong, 1996) and Elliot Blass (Fillion & Blass, 1986) demonstrated that adult male rats exhibited enhanced sexual performance when exposed to the odors experienced in infancy. These results are consistent with observations in other species on the influence of early experience on adult mate preference, such as avian imprinting (Slagsvold, Hansen, Johannessen & Lifjeld, 2002; Ten Cate & Vos, 1999).

Neural Circuitry Underlying Neonatal Attachment Learning

It is curious that neonatal rats can be classically conditioned, although brain areas known to be important in adult learning may not yet be functional (e.g. amygdala, hippocampus, frontal cortex; Fanselow & Rudy, 1998; Nair & Gonzalez-Lima,1999; Rudy & Morledge, 1994; Sananes & Campbell, 1989; Stanton, 2000; Sullivan et al., 2000a; Verwer, Van Vulpen & Van Uum, 1996). Thus, the infant rat must use a different learning circuit from adults, presumably one designed through evolution to provide rat pups with the neural circuitry required to survive and optimize attachment to a caregiver (Hofer & Sullivan, 2001). Three brain structures have been shown to have a role in the neonatal rat's sensitive period for heightened odor learning: the olfactory bulb, the noradrenergic locus coeruleus (LC) and the amygdala. The adult circuit for odor

learning appears more complex and includes the olfactory bulb, piriform cortex, hippocampus, amygdala, and orbitofrontal cortex (Hess, Gall, Granger & Lynch, 1997; Ramus & Eichenbaum, 2000; Roullet, Datiche, Lienard & Cattarelli, 2004; Schettino & Otto, 2001; Schoenbaum, Chiba & Gallagher, 1999; Sevelinges, Gervais, Messaoudi, Granjon & Mouly, 2004; Tronel & Sara 2002).

Olfactory Bulb. In contrast to learning in adult rats, neonatal odor learning produces changes in the olfactory bulb. The bulb is a simple structure with functional cell groupings called glomeruli that are intermediary between the input from the receptors on the olfactory nerve and the output via mitral cell dendrites. The glomerulus response in neonatal rats to an odor is modified after learning, with a corresponding change in the output signal of the olfactory bulb via the mitral cells. Importantly, this learning-induced olfactory bulb change occurs both naturally in the nest and in controlled learning experiments (McLean, Harley, Darby-King & Yuan, 1999; Yuan, Harley & McLean, 2003; Zhang, Okutani, Inoue & Kaba, 2003; Moriceau & Sullivan, 2004b; Sullivan & Leon, 1986; Sullivan et al., 1990; Wilson, Sullivan & Leon, 1987; Yuan, Harley, McLean & Knöpfel, 2002; Yuan, Mutoh, Debardieux & Knöpfel, 2004). As with the behavioral changes in attachment, the olfactory bulb neural changes described here are retained into adulthood but their acquisition is dependent upon experiences during infancy (Pager, 1974; Woo & Leon, 1988).

Recordings of mitral cells during learning indicate that the excitatory response of mitral cells to the CS odor continues throughout learning in the paired group (odor-reward), but habituation occurs in the control groups (Wilson & Sullivan, 1992). Molecular events within mitral cells during learning may provide insight into how the olfactory bulb response to the learned odor is permanently changed (McLean et al., 1999; Yuan et al., 2003; Zhang et al., 2003). Within minutes of acquisition, cAMP levels, induced by neurotransmitters binding, increase CREB phosphorylation (pCREB) and lead to changes in protein synthesis that allow a long-term CS-UCS association trace to form in mitral cells (Figure 2). Research by the McLean and Harley group shows that manipulation of CREB directly alters learning induced molecular events; mutant CREB mice (too little CREB) fail to learn. This learning-induced cascade of molecular events has been identified in a wide variety of species across development, suggesting that the molecular biology underlying memory storage is highly conserved across both development and species (Carew, 1996; Carew & Sutton, 2001; Kandel, 2001; Rankin, 2002). However, while learning-induced intracellular events appear unchanged with development, as outlined here, the neural circuitry involved in olfactory memory shows marked changes with development.

Locus Coeruleus (LC). The LC is a pontine nucleus and the sole source of norepinephrine (NE) for the olfactory bulb (McLean & Shipley, 1991; Shipley, Halloran & De la Torre, 1985). In sharp contrast to the role of NE in neonatal learning, the LC is not necessary for adult learning, although NE enhances or attenuates memories during consolidation in adults (Roozendaal, Nguyen, Power

& McGaugh, 1999). In the neonate, the NE from the LC is both necessary and sufficient for neonatal learning. Related experiments found that an odor preference can be rapidly acquired by activation of olfactory bulb NE ß-receptors with isoproterenol paired with odor stimulation (Langdon, Harley & McLean, 1997; Sullivan, Zyzak, Skierkowski & Wilson, 1992) or by direct stimulation of the LC, the source of olfactory bulb NE (Sullivan, Stackenwalt, Nasr, Lemon & Wilson, 2000b; Sullivan, Wilson, Lemon & Gerhardt, 1994). Moreover, destroying the LC or preventing olfactory bulb NE receptor binding prevents neonatal odor learning (Sullivan et al., 1992, 2000b). While many other neurotransmitters have a role in neonatal rat learning (dopamine – Weldon, Travis & Kennedy, 1991; Zhang, Okutani, Yagi, Inoue & Kaba, 2000; serotonin – McLean, Darby-King, Sullivan & King, 1993; McLean et al. 1999; Yuan et al., 2003; GABA – Okutani, Zhang, Yagi & Kaba, 2002; Okutani, Zhang, Otsuka, Yagi & Kaba, 2003; and opiates – Barr & Rossi, 1992; Kehoe & Blass, 1986; Roth & Sullivan, 2001, 2003), NE appears particularly important in learning-induced neural plasticity in development. For example, within the olfactory bulb, NE is required for the maintenance of the prolonged mitral cell response necessary for acquisition of an odor preference and olfactory bulb learning-induced changes (Wilson & Sullivan, 1991). A similar role for NE appears to reemerge in adult olfactory learning critical for survival, such as mating and infant care (Brennen & Keverne, 1997; Fleming, O'Day & Kraemer, 1999; Moffat et al., 1993; Okere & Kaba, 2000).

The LC's changing role in learning appears to be caused by developmental changes in the LC. Neonates show prolonged excitation of the LC and it releases enormous amounts of NE compared to the amount released after the sensitive period (Rangel & Leon, 1995). This decrease in NE release is controlled by functional changes in the maturing LC: 1) inhibitory $\alpha 2$ noradrenergic autoreceptors become functional and quickly terminate the LC's excitatory responses to stimuli; 2) LC excitatory $\alpha 1$ autoreceptor function becomes limited and no longer temporally extends the LC's response to sensory stimuli; and 3) decreases in electronic coupling of LC neurons limit the coordination of LC neuron firing (Marshall, Christi, Finlayson & Williams, 1991; Nakamura, Kimura, & Sakaguchi, 1987; Nakamura & Sakaguchi, 1990; Winzer-Serhan & Leslie, 1999). Given these observations, we hypothesize that the hyperactivation of the LC before PN10 is responsible for enhanced odor preference learning, and that maturation of the LC signals the termination of the sensitive period for learning in rat pups.

Amygdala. In the adult rat, the amygdala is important for the acquisition of the odor-shock induced odor aversion called conditioned fear (Cahill, McGaugh & Weinberger, 2001; Fanselow & Gale, 2003; Fanselow & LeDoux, 1999; Fendt & Fanselow, 1999; Maren, 2003; McGaugh, Cahill & Roozendaal, 1999; Pape & Stork, 2003; Pare, Quirk & LeDoux, 2004). Evidence suggests that the lack of a functional amygdala during neonatal odor-shock conditioning may underlie pups' difficulty in learning fear. First, behaviors associated with

amygdala function emerge around PN10: inhibitory conditioning, passive avoidance and olfactory-conditioned aversions (Blozovski & Cudennec, 1980; Collier, Mast, Meyer & Jacobs, 1979; Myslivecek, 1997; Sullivan et al., 2000b). Second, amygdala lesions during the neonatal sensitive period (PN1-9) do not prevent the acquisition of an odor preference, although slightly longer training is required (Sullivan & Wilson, 1993). A similar lesion in the adult greatly retards fear conditioning, and the unique traits of a neonatal amygdala cannot account for the dramatic differences in neonatal and adult amygdala lesions (Higley, Hermer-Vazquez, Levitsky & Strupp 2001; Maren, 1999). Third, the amygdala does not appear to participate in acquisition of odor-shock induced odor preference during the sensitive period (Figure 3; Sullivan et al., 2000b, Sullivan, 2001). However, following the termination of the sensitive period, when odor-shock conditioning produces an odor aversion, the amygdala is involved in learning. Fourth, similarly to conditioned fear, unconditioned fear of natural odors does not emerge until PN10 when the amygdala begins to participate in the odor response (Takahashi 1994; Wiedenmayer & Barr, 2001).

Immaturity of the amygdala may account for its lack of participation in neonatal sensitive period learning. Amygdala neurogenesis continues until PN14, although major nuclei subdivision occurs around PN7 (Bayer, 1980; Berdel, Morys & Maciejewska, 1997; Berdel & Morys, 2000; Morys, Berdel, Jagalska-Majewska & Luczynska, 1999). Synaptic development begins to appear around PN5 with a dramatic increase between PN10 and PN20, reaching

adult levels by PN30 (Mizukawa, Tseng & Otsuka, 1989). Behavioral data on the development of amygdala-dependent behaviors suggest that sequential maturation of specific amygdala microcircuits may be important (Hunt & Campbell, 1999; Richardson, Paxinos & Lee, 2002; Sananes, Gaddy & Campbell, 1988). Specifically, freezing first emerges in the olfactory, auditory and visual systems at PN10, 16 and 18, respectively. Learning ability for specific fear-related behaviors evoked by a sensory system also emerges sequentially. In odor-fear conditioning, pups learn freezing, heart rate and startle at PN10, 15 and 21, respectively, whereas in visual fear conditioning, pups exhibit learned freezing, heart rate and startle at PN18, 23 and 30, respectively. Ontogenetic connectivity of the amygdala with motor-related neural areas may also play a role in the ontogenetic emergence of these learned behaviors.

The attenuation of odor aversion conditioning during the sensitive period may also be due to immature major neural connections between the amygdala and other brain areas important in conditioning. For example, amygdala-hippocampus connections are still undeveloped, and the primary cortical input to the hippocampus from the entorhinal cortex is still developing (Crain, Cotman, Taylor & Lynch, 1973; Fanselow & Rudy, 1998; Nair & Gonzalez-Lima, 1999; Rudy & Morledge, 1994; Stanton, 2000). Furthermore, neonatal learning may not involve the cortex, and the frontal cortex is still undeveloped during this early neonatal period (Landers & Sullivan, 1999a,b; Verwer et al., 1996).

Sensitive Period Learning and the Hypothalamic-Pituitary-Adrenal Axis (HPA).

During adult stress, the adrenal gland can release corticosterone (CORT), while during the neonatal sensitive period the response of the adrenal is reduced. However, the neonatal adrenal gland is capable of releasing CORT when exposed to specific stressors such as cold and maternal separation (Gilles, Schultz & Baram, 1996; Levine, 2001). Pups' low CORT levels appear to be controlled by certain aspects of the maternal behavior, such as feeding and grooming (Sucheki, Rosenfeld & Levine, 1993; Van Oers, De Kloet & Levine, 1998). Thus, this early HPA system is limited in function and results in attenuated CORT release in response to shock during the neonatal sensitive period (Levine, 1962a). For example, while the adult rat responds to shock with a robust CORT release, the neonatal rat does not (Levine, 1962a, 2001; Van Oers et al., 1998). The attenuated neonatal CORT response appears to limit pups' ability to express unlearned fear (of predator odor), learned odor aversions (also called conditioned fear), passive avoidance and inhibitory conditioning. These behaviors normally emerge at PN10-11 (the end of the sensitive period) but can be delayed or advanced ontogenetically simply by removing the source of CORT or by prematurely elevating CORT levels (Bialik, Pappas & Roberts, 1984; Blozovski & Cudennec, 1980; Collier et al., 1979; review – Myslivecek, 1997; Takahashi, 1994; Takahashi & Rubin, 1993; Takahashi, Turner & Kalin, 1991). Previous work has shown potent CORT effects on the neonatal LC, amygdala, hippocampus, frontal cortex and HPA axis that last until adulthood

using the maternal deprivation paradigm (Dent, Smith & Levine, 2001; Eghbal-Ahmadi, Avishai-Eliner, Hatalski & Baram, 1999; Francis, Caldji, Champagne, Plotsky & Meaney, 1999; Swiergiel, Takahashi & Kalin, 1993). While CORT has strong effects on adult memory formation, its role in adult learning appears to be modulatory (McGaugh & Roozendaal 2002). These data suggest that stress during early infancy may be capable of modifying the neural systems underlying attachment and hence the adult functioning of these brain areas.

Consequences for Adult Behavior.

Early life experiences, including early attachment experiences, have an enormous impact on adult life in rodents, nonhuman primates and humans (Denenberg, 1963; Harlow & Harlow, 1965; Levine, 1962b; Rosenzweig, Bennett, Diamond, Wu, Slagle & Saffran, 1969; Schore, 2001). The documented overlap in brain areas associated with our attachment model, general early experiences, and later psychiatric problems strongly suggests that the neonatal effects involved the LC, amygdala, cerebellum and HPA axis, as well as presumably nonfunctional neonatal rat brain areas such as the hippocampus and frontal cortex (Dent et al., 2001; Francis et al., 1999; Gutman & Nemeroff, 2002; Heim & Nemeroff, 2001; Kaufman, Plotsky, Nemeroff & Charney, 2000; Levine, 2001; Perry, Pollard, Blakely, Baker & Vigilante, 1995; Teicher, Ito, Gold, Andersen, Dumont & Ackerman, 1997). Together, these data suggest a potential mechanism for the enduring effects of early attachment on adult psychiatric wellness.

Conclusions.

In summary, the present review outlines unique characteristics of neonatal learning that facilitate the infant rat's attachment to the mother. Specifically, pups exhibit enhanced preference learning and attenuated aversion learning. Considering the necessity of infant maternal odor preference learning for survival (nipple attachment, huddling, orientation), it is beneficial for pups to quickly learn a preference for the maternal odor and block aversion learning that would interfere with pups' attachment to the mother.

This review also suggests that pups' unique neural circuitry underlying infant learning may have evolved to ensure infants rapid attachment to the mother. This circuitry is not simply due to the absence or immaturity of brain structures but rather to the brain having unique characteristics: the olfactory bulb encodes learning, the noradrenergic LC is both necessary and sufficient for the preference learning, and the lack of amygdala participation underlies pups' attenuated aversion learning. This NE-dependent learning is similar to the neural basis of other survival-dependent behaviors in reproduction across species.

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Figure Captions

Figure 1: Mean number of CS odor choices (±S.E.M.) in an olfactory Y-maze test. Pups were trained during the sensitive period (PN6) with pleasant odor-stroke conditioning (upper left) or aversive odor-shock (0.5mA) conditioning (upper right), although pairings of either reward produced a subsequent odor preference at this early age. Older pups (lower), after the sensitive period (PN12), show more discriminating conditioning characteristic of adult animals; odor-stroke conditioning (lower left) was ineffective at producing an odor preference and odor-shock conditioning (lower right) produced a subsequent odor aversion.

Figure 2: Schematic representation of olfactory bulb input from the noradrenergic locus coeruleus, which is important in inducing early olfactory learning. If the odor is paired with a reward, activation of NE ß-receptors increases cAMP levels, which combined with the high levels of Ca^{++}, activate a cascade resulting in pCREB-mediated changes in gene transcription. These changes could result in odor-specific changes in mitral cell odor coding that would reflect the learned significance of the odor to the animal (Sullivan et al., 2000b; Yuan et al., 2003).

Figure 3: Amygdala activity, as measured by 14C autoradiography, of sensitive period pups (PN8) does not appear to participate in odor-shock conditioning and may underlie pups' difficulty in learning odor aversions. Older pups, past the

sensitive period, have an amygdala that participates in learning and easily form odor aversions (Sullivan et al., 2000a).

Figure 1

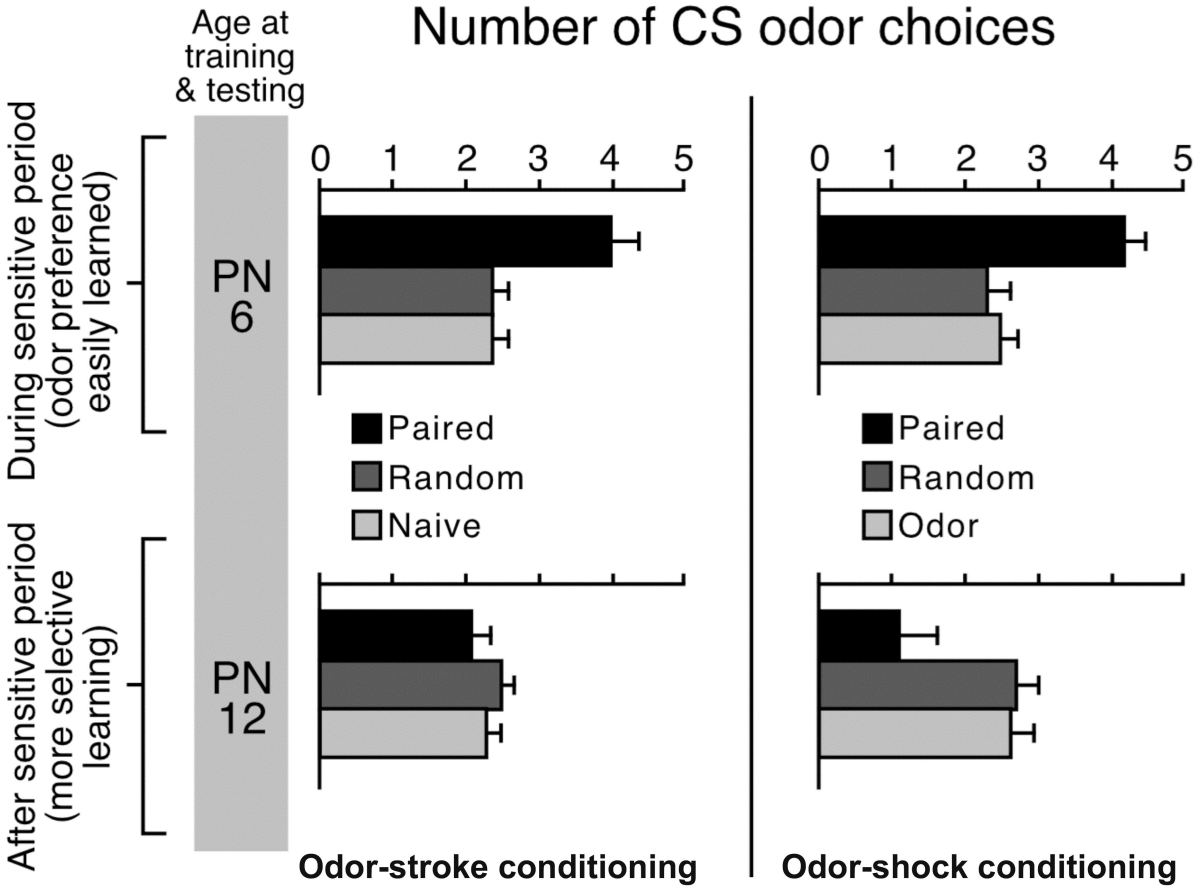

Age at training & testing
Number of CS odor choices
During sensitive period (odor preference easily learned)
After sensitive period (more selective learning)
PN 6
PN 12
0 1 2 3 4 5
Paired
Random
Naive
Odor
Odor-stroke conditioning
Odor-shock conditioning

Figure 2

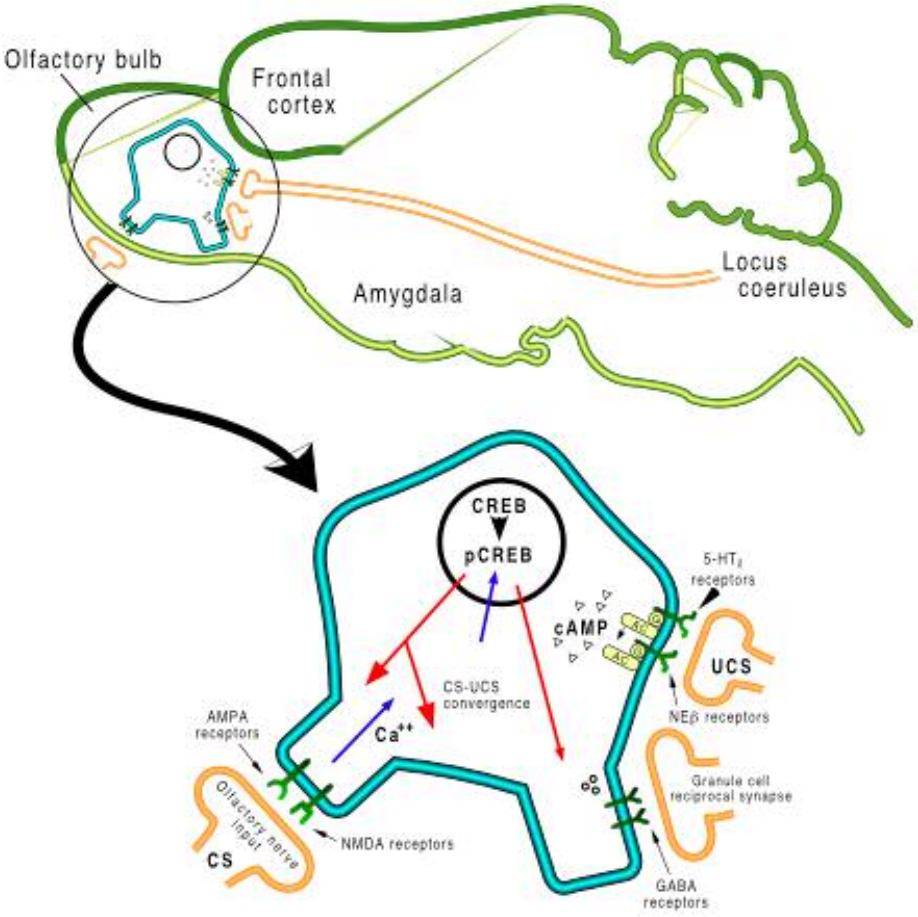
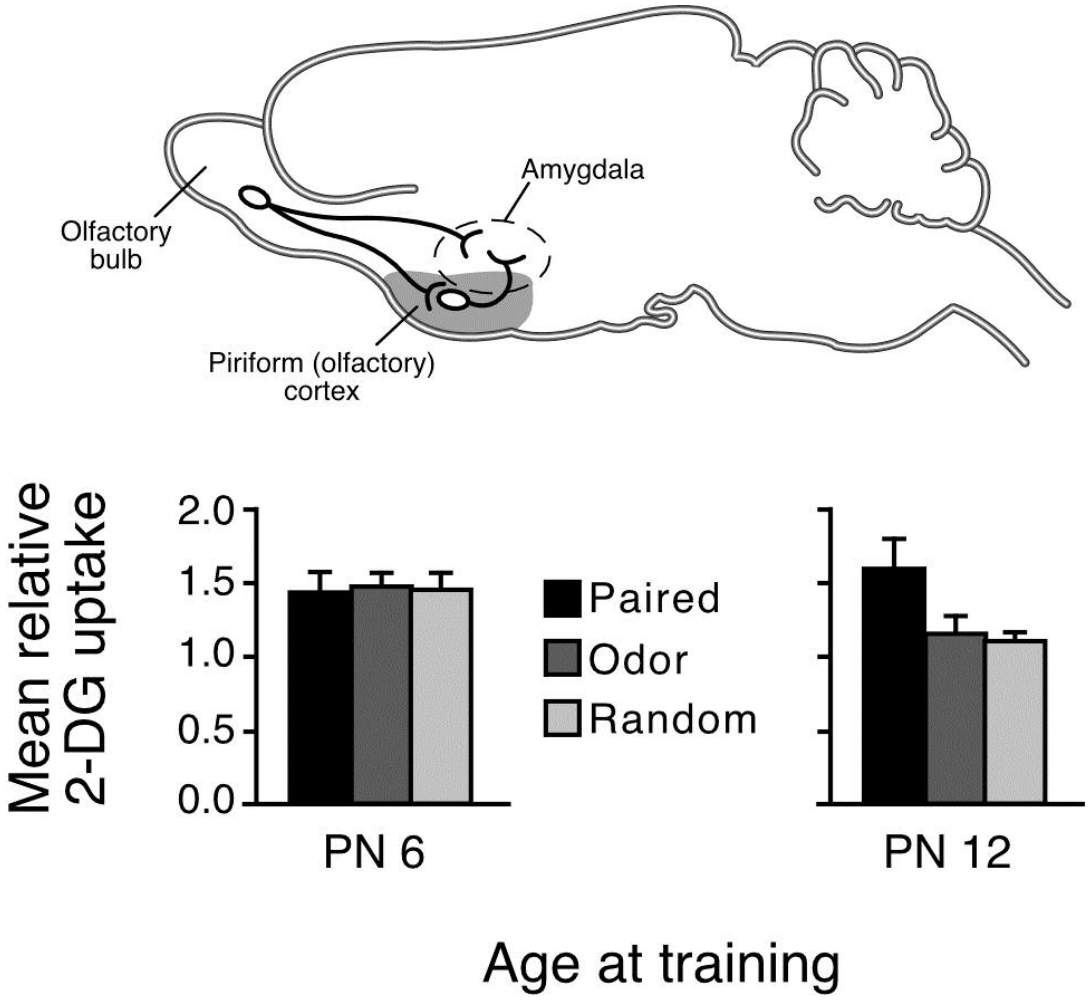

Olfactory bulb
Frontal cortex
Amygdala
Locus coeruleus
CREB
pCREB
cAMP
5-HT2 receptors
UCS
NEβ receptors
CS-UCS convergence
Ca++
AMPA receptors
Olfactory nerve input
CS
NMDA receptors
Granule cell reciprocal synapse
GABA receptors

Figure 3


Amygdala
Olfactory bulb
Piriform (olfactory) cortex
Mean relative 2-DG uptake
2.0
1.5
1.0
0.5
0.0
Paired
Odor
Random
PN 6
PN 12
Age at training

Chapter 2

Unique Neural Circuitry for Neonatal Olfactory Learning

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Abstract

Imprinting ensures that the infant forms the caregiver attachment necessary for altricial species survival. In our mammalian model of imprinting, neonatal rats rapidly learn the odor-based maternal attachment. This rapid learning requires reward-evoked locus coeruleus (LC) release of copious amounts of norepinephrine (NE) into the olfactory bulb. This imprinting ends at (postnatal day) P10, and is associated with a dramatic reduction in reward-evoked LC NE release. Here we assess whether the functional emergence of LC $\propto$2 inhibitory autoreceptors and the downregulation of LC $\propto$1 excitatory autoreceptors underlie the dramatic reduction in NE release associated with termination of the sensitive period. Post-sensitive period pups (P12) were implanted with either LC or olfactory bulb cannulas, classically conditioned with intracranial drug infusions (P14) and tested for an odor preference (P15). During conditioning, a novel odor was paired with either olfactory bulb infusion of a β-receptor agonist (isoproterenol) to assess the target effects of NE, or direct LC cholinergic stimulation combined with $\propto$2 antagonists and $\propto$1 agonists in a mixture to reinstate neonatal levels of LC autoreceptor activity to assess the source of NE. Pups learned an odor preference when the odor was paired with either olfactory bulb isoproterenol infusion or reinstatement of neonatal LC receptor activity. These results suggest LC autoreceptor functional changes, rather than olfactory bulb changes, underlie sensitive period termination.

Introduction

The strong influences of infant experiences on adult life will remain elusive until we understand the unique functions of the neonatal brain that lay the foundation for future brain processing. Childhood experiences, especially within the context of attachment, have a strong impact on emergence of adult mental health and character traits (Bowlby, 1969; Glaser, 2000; Schore, 2001; Teicher et al., 2003). Our mammalian imprinting model explores this issue and has shown that the neonatal rat uses a unique, simplistic learning circuit to acquire the life-sustaining odor attachment to the caregiver. Specifically, without brain areas normally involved in adult learning (nonfunctional hippocampus, amygdala, frontal cortex), the infant must rely on a unique learning circuit involving the olfactory bulb and locus coeruleus (LC) (Rudy and Morledge, 1994; Wilson and Sullivan, 1994; Vermer et al., 1996; Sullivan et al., 2000b; Stanton, 2000; Sullivan, 2003).

In our rat mammalian imprinting model using odor-reward pairings, neonatal rats can rapidly learn an odor-based attachment to their mother. This sensitive period learning is dependent on a simplistic learning circuit involving the olfactory bulb and LC and results in a rapid strong attachment to the mother corresponding with olfactory bulb metabolic and anatomical changes (Wilson et al., 1987; Woo et al., 1987; Wilson and Leon, 1988; Wilson and Sullivan, 1990; McLean et al., 1999; Sullivan, 2001). These learning-induced behavioral and neural changes require norepinephrine (NE) (LC lesion or blocking olfactory bulb

NE prevents learning), and an odor preference is learned from odor-NE pairings (LC stimulation or olfactory bulb NE infusions: Sullivan et al., 1992, 1994, 2000b; Yuan et al., 2003). While the behavioral and neural changes are dependent upon acquisition during the sensitive period, the odor is later important for adult mate choice, sex and maternal behavior (Pager, 1974; Coopersmith and Leon, 1986; Fillion and Blass, 1986; Woo and Leon, 1987; Moore et al., 1996; Fleming et al., 1999; Shah et al., 2002).

At P10, as the sensitive period ends, pups develop the motor abilities to leave the nest (Bolles and Woods, 1965) and their learning abilities become more adultlike. First, the infant's learning abilities expand to permit passive avoidance, active avoidance and inhibitory conditioning (Collier et al., 1979; Blozovski and Cudennec, 1980; Camp and Rudy, 1988; Myslivecek, 1997; Sullivan et al., 2000a). The developmental emergence of amygdala functioning seems to underlie these new learning abilities (Wilson and Sullivan, 1993; Sullivan et al., 2000a). Second, learning diminishes in concert with the loss of NE release to odor and reward (Rangel and Leon, 1995). Because the LC is the sole source of NE for the olfactory bulb, we assess here whether developmental changes in the LC may underlie pups' loss of rapid, robust odor preference learning. The LC contains recurrent collaterals along with corresponding LC NE autoreceptors to regulate LC function (Berridge and Waterhouse, 2003). While the LC $\propto$2 autoreceptors are present in the neonate (receptor autoradiography and mRNA: Winzer-Serhan et al., 1999), they do not appear to function until

approximately P10, at least in whole animals receiving sensory stimulation during extracellular single-cell LC recordings (Kimura and Nakamura, 1987; Nakamura et al., 1987; Nakamura and Sakaguchi, 1990). Specifically, extracellular recording of single LC neurons in response to sensory stimuli (1 sec air puff or electric shock) elicits prolonged excitation (20-30 sec) in neonates, whereas the same stimulation produces only a response duration measured in milliseconds in older pups. This developmental response difference appears to be attributable to the functional emergence of $\propto 2$ inhibitory noradrenergic autoreceptors, resulting in the LC autoinhibition within milliseconds of activation, and the functional attenuation of LC $\alpha 1$ excitatory noradrenergic autoreceptors which prolong LC neonatal responses (Nakamura et al., 1987; Pieribone et al., 1994; Scheinin et al., 1994). Based on these data, we hypothesized that developmental LC autoreceptor changes may be responsible for the failure of older pups to learn the rapid, robust odor preference simply because the LC releases insufficient amounts of NE to support learning. To test this hypothesis, we attempted to reinstate neonatal LC autoreceptor activity in post-sensitive period pups through infusions of an excitatory autoreceptor agonist-inhibitory autoreceptor antagonist mixture and assessed whether imprinting-like odor preference learning could be reinstated. Moreover, since the olfactory bulb is the site of the learning-induced changes, we assessed the role of NE within the olfactory bulb by intrabulbar infusions of a NE β-agonist. Our results suggest that the developmental change in LC autoreceptors, rather than the olfactory bulb, may underlie, at least in part, sensitive period termination.

Methods

Subjects. The subjects were male and female Long Evans rat pups born and bred in our colony at the University of Oklahoma (originally from Harlan Farms). Animals were housed in polypropylene cages (34 × 29 × 17 cm) lined with wood chips, and were kept in a temperature (23°C) and light (7:00 A.M.-7:00 P.M.) controlled room. Food and water were available ad libitum. The day of birth was considered P0 and litters were culled to 10 on P1. No more than one male and one female from a litter were used in each experimental condition. All procedures followed University of Oklahoma and NIH standards for animal treatment.

Cannula implantation. On P12, pups were anesthetized by inhalation (metofane - until tailpinch reflex eliminated) and placed in an adult stereotaxic apparatus adapted for use with infants. Stainless steel cannulas (30-gauge tubing) were implanted bilaterally in the olfactory bulb or LC through holes drilled in the overlying skull. Stereotaxic coordinates derived from the atlas of Paxinos et al., 1991 were used as a reference and adapted through pilot work (Sullivan et al 2000b) for implanting cannulas into the LC (caudal – 1.40 mm; lateral ±0.60 mm from lambda). The cannulas were lowered 5.5 mm from the surface of the skull, which placed the tip near the LC. The cannula assembly was fixed to the skull with dental cement and anchor wires (one for olfactory bulb, two for LC) were placed approximately 3 mm from the cannula holes (for more details see

Gilbert and Cain, 1980). To ensure patency of the cannulas, guide wires were placed in the lumen of the tubing until training. Olfactory bulb cannulas were implanted under visual guidance. After recovery from surgery (generally within 30 min), pups were returned to the litter and dam until training two days later.

Drug infusion protocol and conditioning. On P14, pups were placed in training chambers and bilateral cannulas were connected via polyethylene 10 tubing to a Harvard syringe pump driving two Hamilton microliter syringes. The cannulas were filled (6 sec for olfactory bulb cannulas and 12 sec for LC cannulas at 0.5 µl/min) with either drug (described below) or vehicle. During both the 10-min behavioral adaptation period and the 10-min training period, drug or vehicle was infused at 0.1 µl/min, for a total infusion volume of 2.0 µl as previously described (Sullivan et al., 1992; 2000b). Pups were given a 10-min adaptation period to permit acclimation to the training chamber and to allow drug distribution within the targeted brain area. During the subsequent 10 min, brain infusions continued and pups were presented with citral odor (0.25 µl, Sigma, St. Louis, MO), which served as the conditioned stimulus (CS). After training, pups were disconnected from the syringe pump and returned to the nest until testing the next day. Throughout adaptation and training, pups were continuously observed to ensure that all pups were healthy and responding normally to experimental stimuli. Pups exhibiting abnormally high (i.e. aversive) or low (i.e. lethargic) behavioral responsiveness were eliminated from the experiment during adaptation or conditioning.

Olfactory bulb infusions. Pups with bilateral cannulas in the olfactory bulbs received either isoproterenol (50 μM, Sigma), an NE ß-receptor agonist or vehicle (saline) (Sullivan et al., 1989).

LC infusions. Pups with cannulas implanted into the LC received one or more of the following compounds: acetylcholine (2 mM, Sigma, acetylcholine chloride; Sigma) which activates the LC (Adams and Foote, 1988); phenylephrine (1 mM L-phenylephrine; Sigma), which is an α1 agonist that stimulates the excitatory LC autoreceptors (Mouly et al., 1995); or idazoxan (2 μM; Sigma), which is an α2 antagonist that blocks the inhibitory LC autoreceptors (Devauges and Sara, 1990; Ivanov and Aston-Jones, 1995; Sullivan et al., 2000b).

Systemic injections. To confirm the noradrenergic basis of LC manipulation effects on odor learning, a separate group of LC-infused pups was injected systemically with the β-receptor antagonist propranolol (20 mg/kg, i.p.) 30 min before training (Sullivan et al., 1989).

Behavior Tests. On P15, pups were given one of two tests, depending on the experiments. Both tests, which have been widely used and dependably assess infant learning, are described below. No drugs were infused during testing and drugs present during training had left the system the previous day (Goodman and Gilman, 1985).

Two-odor choice test. This test measures the amount of time pups spent over a familiar odor (clean pine nest shavings) vs. the CS citral odor used during

conditioning. The test apparatus consisted of a Plexiglas arena (24 cm long × 14 cm wide) with a wire mesh floor that enabled pups to smell the odor beneath them. The floor was divided into two parts, separated by a 2 cm midline; one side contained the odor CS (0.20 μl citral placed on a 5 X 5 cm Kimwipe) and the other side contained familiar pine odor (160 ml wood chips). Pups were placed on the midline between the two odors (direction counterbalanced) and the amount of time each pup spent over each odor was monitored for three 60-sec trials, with 10 sec between each trial. Between each trial, the floor was cleaned with distilled water and dried (Sullivan et al., 1989; Cornwell-Jones, 1981).

Y-maze. This test requires pups to choose between two arms of a Plexiglas Y-maze (start box: 8.5 cm width, 10 cm length, 8 cm height; choice arms: 8.5 × 24 × 8 cm), one containing the citral odor CS and the other containing the familiar odor of pine shavings. Pups were placed in the start box (direction counterbalanced) and after 5 sec the door to each alley was opened, at which time pups were given 60 sec to choose an arm. A response was considered a choice when a pup's entire body was past the entrance to the alley. Occasionally (3 pups, only 1 trial each), pups did not make a choice within 60 sec, and in these cases the pups were removed and returned to the maze to repeat the trial. Pups received 5 trials with 30 sec between trials (Sullivan and Wilson, 1991).

Drug diffusion. In order to characterize the extent of drug diffusion within and outside of the LC, additional pups were used. On P12, pups were anesthetized by urethane and placed in a stereotaxic apparatus. Holes were

drilled through the skull at 1.4 mm posterior to lambda, and ±0.60 mm from the midline. A 10 μl 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 μl of a saline solution of [^{3}H]NE (56.9 Ci . mmol^{-1}. μm^{-1}, 0.37 Ci . mmol^{-1}. μl^{-1}; NEN Research Products), and 20 min after infusion, the brains were quickly removed and frozen in methyl butane at -45°C. Brains were sliced in 20 μm coronal sections. The slides were apposed to a tritium storage phosphor screen (Amersham Biosciences, USA). After 14 days exposure, the screen was scanned at a pixel density of 50 μm (5000 dots per cm^2) with a STORM 820 PhosphorImager (Molecular Dynamics, Sunnyvale, Calif). Phosphorimaging of the slides results in a tagged image file format (Tucker et al., 2002).

Histology. After behavioral testing, pups were overdosed with urethane and perfused with saline and 4% formalin. Brains were removed and postfixed in 4% formalin and 30% sucrose. Sections (40μm) were cut and cresyl violet staining was used to verify LC cannula placements.

Results

Behavioral observations of pups during training. Behavior was observed during training to ensure that the experimental manipulations did not adversely affect pups. Two pups were excluded from the study for exhibiting hyperactivity during training, and four other pups were excluded for weight loss between surgery and training. Pups gained weight over the course of the experiment, with initial weight of 28.67 g (s.e. ± 1.62) and 27.96 g (s.e. ± 0.94) for olfactory bulb and LC cannula pups, respectively. After testing, pups weighed 33.71 g (s.e. ± 1.18) and 32.45 g (s.e. ± 1.02) for olfactory bulb and LC cannula pups, respectively.

There were no statistical weight differences at either training or testing between groups for each cannula placement. Pup treatment did not change weight gain over the course of the experiment, although pups that did not gain weight after surgery were discontinued from the experiment (saline vs. drug treatment at surgery, training and testing (ANOVA, $F_{(3,76)}$= 0.198, NS)).

Post-sensitive period LC activation does not support odor learning. In contrast to neonatal rat pups during the sensitive period (Sullivan et al., 2000b), preweanling (P14) rat pups cannot learn an odor preference by simple activation of the LC during an odor presentation. As shown in Figure 1, only pups that received isoproterenol infused into the olfactory bulb exhibited a preference for the citral odor CS. ACh infusion into the LC, which produces an

odor preference in younger neonatal pups, was insufficient to produce an odor preference in these preweanling pups (ANOVA, $F_{(3,26)}$=6.834, p< 0.005). Post-hoc Fisher tests revealed that olfactory bulb isoproterenol pups spent significantly more time over the CS odor than each of the other groups (p< 0.05). It should be noted that there was a difference between the ACh LC group and the vehicle olfactory bulb groups (p< 0.05), but neither of these two groups differed from the vehicle LC group. Preweanling rat pups learn an NE-induced odor preference by reinstating the neonatal LC. As shown in Figure 2, only those pups that received activation of the LC by ACh concurrently with the blockade of LC inhibitory autoreceptors (α2 antagonist, idazoxan) and activation of the LC excitatory autoreceptors (α1 agonist, phenylephrine) during a citral odor presentation exhibited a subsequent preference for the citral odor ($F_{(5,24)}$=14.332, p<0.0001). Pups that received any combination of less than three of these drugs or vehicle did not exhibit learning as determined by post-hoc Fisher tests (each group spent significantly less time over the CS, p< 0.001). As illustrated in Figure 3, a systemic blockade of NE receptors during the training prevented the reinstatement of the NE-dependent odor learning seen during the neonatal sensitive period (ANOVA, conditioning group $\times$ odor, $F_{(2,17)}$= 18.338, p< 0.0001; post-hoc Fisher tests indicate that the pups injected with propranolol and infused concurrently with acetylcholine, idazoxan and phenylephrine are significantly different from each of the other groups, p< 0.03).

Drug diffusion. As demonstrated in Figure 4, the volume of drugs infused into the LC for data illustrated in Figure 2 diffused less than 1 mm from the LC.

Histology. Cannula tip placements for cannulas directed at the LC are shown in Figure 5. All tip placements were less than 1 mm from the LC except for one pup, which was excluded from the experiment. This pup, which received an infusion of ACh concurrently with phenylephrine and idazoxan, did not exhibit a subsequent preference for the citral odor, unlike pups receiving the same infusion into the LC.

Discussion

These results suggest that the developmental emergence of autoinhibition and attenuation of autoexcitation in the LC may be responsible, at least in part, for the termination of the infant rat's sensitive period for attachment learning. Our results showed that preweanling (P14) pharmacological LC manipulations intended to reinstate neonatal sensitive period LC function (attenuating LC autoinhibition and amplifying LC autoexcitation) are sufficient to reinstate the neonatal NE-dependent odor learning. Specifically, in postsensitive period P14 pups, we reversed the LC characteristics that emerge at P10 by blocking the LC $\propto$2 inhibitory autoreceptors and activating the LC $\propto$1 excitatory autoreceptors. The pharmacologically neotonized LC, combined with LC cholinergic stimulation paired with a novel odor, was sufficient to produce an odor preference that was blocked by systemic blockade of NE β receptors.

Our results also suggest the olfactory bulb remains plastic even after the sensitive period has terminated, since simply increasing olfactory bulb NE is sufficient to produce the rapid, NE-dependent odor preference. Indeed, increasing NE via LC stimulation or direct olfactory bulb infusions showed that the olfactory bulb maintains its ability to produce rapid acquisition of an odor preference. These results complement previous results on the important role of NE in developmental plasticity other than learning, such as occurs in the

developing visual system (Kasamatsu and Pettigrew, 1979, Kasamatsu et al., 1979; Bear and Singer, 1986; Shirokawa et al.,1989).

These results are in sharp contrast to the effects of NE on learning in adults, where NE appears to have a modulatory effect on acquisition and on attention consolidation (Gold and Van Buskirk, 1975; Liang et al., 1990; Selden et al., 1990; McGaugh et al., 1996; Roozendaal et al., 1999; Feenstra et al., 2001). Specifically, similarly to neonates, neural correlates of learning could be achieved by large NE infusions directly into the adult rat olfactory bulb, hippocampus and auditory cortex, suggesting the mature brain retains at least some potential for NE-dependent plasticity, provided sufficient levels of NE are available (Gray et al., 1986; Harley et al., 1996; Harley, 1998; Chaulk and Harley, 1998; Edeline, 1999).

The differences in the role of NE in neonatal and adult learning appear to be due to developmental differences in the LC's response to sensory stimuli. The infant and adult LC are both activated by sensory stimuli, but the adult LC is less likely than the infant's LC to respond to non-noxious stimuli (Foote et al., 1980; Kimura & Nakamura, 1985; Nakamura and Sakaguchi, 1990; Selden et al., 1990; Harris and Fitzgerald, 1991; Harley and Sara, 1992; Aston-Jones et al., 1994; Sara et al., 1995; Vankov et al., 1995; Mansour et al., 2003). Additionally, the adult LC habituates after repeated (or even single) stimulus presentations, whereas the infant LC fails to habituate (Nakamura et al., 1987; Nakamura and

Sakaguchi, 1990; Vankov et al., 1995). Even more dramatic is the response duration of the neonatal LC as compared to the preweanling/adult LC, with a 1-sec presentation of tactile stimulation causing a few milliseconds response in the adult LC, but a 20-30 sec response in the infant LC (Nakamura et al., 1987). These developmental differences in LC activity are reflected in dramatic age differences in stimulus-evoked NE release in the olfactory bulb, with neonatal pups (sensitive period age pups) releasing significantly more NE than slightly older pups (postsensitive period pups: Rangel and Leon, 1995). This developmental decrease in sensory-evoked release of NE in the olfactory bulb occurs despite the extensive increase in NE centrifugal fibers innervating the bulb at this developmental stage (McLean and Shipley, 1991). Together with the present results, these findings suggest that developmental changes in the LC may be responsible for the increasingly subtle role of NE in learning and memory as rats mature.

Throughout the lifespan, attachments continue to be formed within the context of mate selection, mating and care of the young. While the neural circuitry learning for these behaviors is more complex than that seen in the neonate, the importance of NE in learning reemerges (Levy et al., 1990; Brennan and Keverne, 1997; Insel and Young, 2001). There is some evidence to suggest the LC changes to accommodate the rapid learning required in these reproduction-related learning situations. Specifically, similarly to the neonatal rat

pup LC, spontaneous activity is greatly diminished during pregnancy, although the amount of NE released is greatly enhanced (Nakamura et al., 1988).

The large release of NE during neonatal rat learning appears to underlie the neural changes uniquely associated with learning during the sensitive period. During acquisition, the NE from the LC maintains the responsiveness of mitral cells (primary output neurons of the olfactory bulb), while the mitral cells of control animals (backward, odor only) quickly habituate (Wilson et al., 1987). During consolidation, a cascade of molecular events is implicated in odor-preference learning in neonatal rats. An important interaction between serotonin and NE permits increasing mitral cell cAMP and cAMP response element binding protein phosphorylation (Yuan et al., 2003; Zhang et al., 2003). This causes modifications in the olfactory bulb that last into adulthood, including enhanced odor-evoked glomerular layer 2-DG uptake and fos-like immunoreactivity (Coopersmith and Leon, 1984; Sullivan and Leon, 1986; Johnson et al., 1995), modified odor response patterns of mitral/tufted cells (Wilson et al., 1987) and morphological changes in the glomerular layer (Woo et al., 1987). The acquisition of these olfactory bulb learning-induced changes is limited to the sensitive period (Woo et al., 1987; Sullivan and Wilson, 1991).

As described above, as the pup's sensitive period ends, rapid odor preference conditioning ends. However, other types of learning emerge, such as fear conditioning, inhibitory conditioning and passive avoidance (Collier et al.,

1979; Blozovski and Cudennec, 1980; Camp and Rudy, 1988; Myslivecek, 1997; Sullivan et al., 2000a). The functional emergence of the amygdala seems to be responsible for the emergence of the learned fear system (odor – 0.5 mA shock: Sullivan et al., 2000a). Moreover, the functional emergence of the pup's ability to release corticosterone in response to shock and other stressors may be related to the ability of the amygdala to function in the fear conditioning situation (odor – 0.5 mA shock: Moriceau and Sullivan, in press; Moriceau and Sullivan, 2003). Thus, developmental changes in pups' learning abilities likely reflect the myriad of developmental brain changes occurring during early development. We have hypothesized that the changing learning system reflects the changes in developmental demands placed on pups as new behaviors such as walking emerge. Other developmental changes in learning have been documented to coincide with weaning and with the emergence of hippocampal-dependent spatial learning and potentiated startle (Hunt and Campbell, 1999; Richardson et al., 2000; Stanton, 2000).

Although some prenatal olfactory experience may contribute to the pup's olfactory attachment to the mother, postnatal experience in the form of classical conditioning appears necessary for attachment (Thoman et al., 1968; Galef and Sherry, 1973; Johanson and Hall, 1982; Pederson et al., 1982; Alberts and May, 1984; Sullivan et al., 1986a,b). A similar role for somatosensory learning, particularly the facial whiskers, may also be important in early attachment (Polan and Hofer, 1998; Landers and Sullivan, 1999a). Both somatosensory and

olfactory learning are NE-dependent (Landers and Sullivan, 1999b). Due to the late maturation of the auditory and visual systems in the rat, dependence on odor and somatosensory learning may be critical for survival (Moye and Rudy, 1985, 1987; Hunt and Campbell, 1999; Richardson et al., 2000). Learning in the olfactory system seems to be particularly important in several behaviors critical for survival (i.e. nipple attachment, huddling, orientation to the dam), and damage to the olfactory system or the source of the odor stimuli greatly reduces survival (Singh and Tobach, 1975; Hofer, 1976). Together, these results suggest that a unique learning circuitry in the neonate has evolved to ensure infants form a rapid, robust attachment to the mother (Sullivan et al., 2000b; Hofer and Sullivan, 2001). This unique neonatal learning circuit may be particularly important in the infant rat, where structures important to adult learning have not yet developed (amygdala: Sullivan et al., 2000a; hippocampus: Rudy and Morledge, 1994; cerebellum: Freeman and Nicholson, 2000). The present results are additional support that the LC is a fundamental component of this unique learning circuit, and that the special balance of NE autoreceptors within the developing LC helps define a sensitive period that is critical for infant learning and survival.

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Figure captions

Figure 1: Mean (± SEM) time spent over CS odor during the two-odor choice test. Training infusion into the olfactory bulb of the NE receptor agonist isoproterenol (Iso) significantly increased the learned relative odor preference compared with vehicle group. Training infusion into the locus coeruleus of acetylcholine (ACh), an LC stimulant, did not produce a significant change in odor preference compared with vehicle groups (n= 7-8 per group). Asterisk represents significant differences from all other groups ($p<0.05$).

Figure 2: Mean (± SEM) number of choices toward the conditioned stimulus (CS) odor during the Y-Maze test. Training infusion of acetylcholine (ACh) and α1 agonist, phenylephrine (α1 ago) and α2 antagonist, idazoxan (α2 antago) increased the learned relative odor preference compared with the other groups (n= 3-7 per group). Asterisk represents significant differences from all other groups ($p<0.001$).

Figure 3: Mean (± SEM) number of choices toward the conditioned stimulus (CS) odor during the Y-Maze test. Pretraining injection of propranolol (20 mg/kg) significantly reduced the learned relative odor preference expressed by pups receiving infusion of acetylcholine (ACh) and α1 agonist, phenylephrine (α1 ago) and α2 antagonist, idazoxan (α2 antago) compared with the control groups (n= 8-10 per groups). Asterisk represents significant differences from all other groups ($p< 0.03$).

Figure 4: A: Section from a P14 pup counterstained with cresyl violet. White arrows mark the locus coeruleus bilaterally. Actual cannula tip placement is outside the plane of this section. B: Same section as in A at the same magnification and orientation, characterizing the extent of an unilateral H^3 NE drug diffusion within the LC. A+B: Color overlay of H^3 NE diffusion on the histological section showing drug diffusion over the region of the locus coeruleus.

Figure 5: A. Representative location of cannula tip near the locus coeruleus. The cannula tip placement is marked by arrow. The location of the locus coeruleus is illustrated by an asterisk. B. Locations of cannula tips (solid circles) in rats used for the experiment shown in Figure 2. The locus coeruleus is illustrated in gray and marked by the horizontal arrows. Corresponding sections from the adult stereotaxic atlas of Paxinos et al. (1991) were determined, and relative distance from bregma for each coronal section based on the adult atlas is noted on the right.

Figure 1

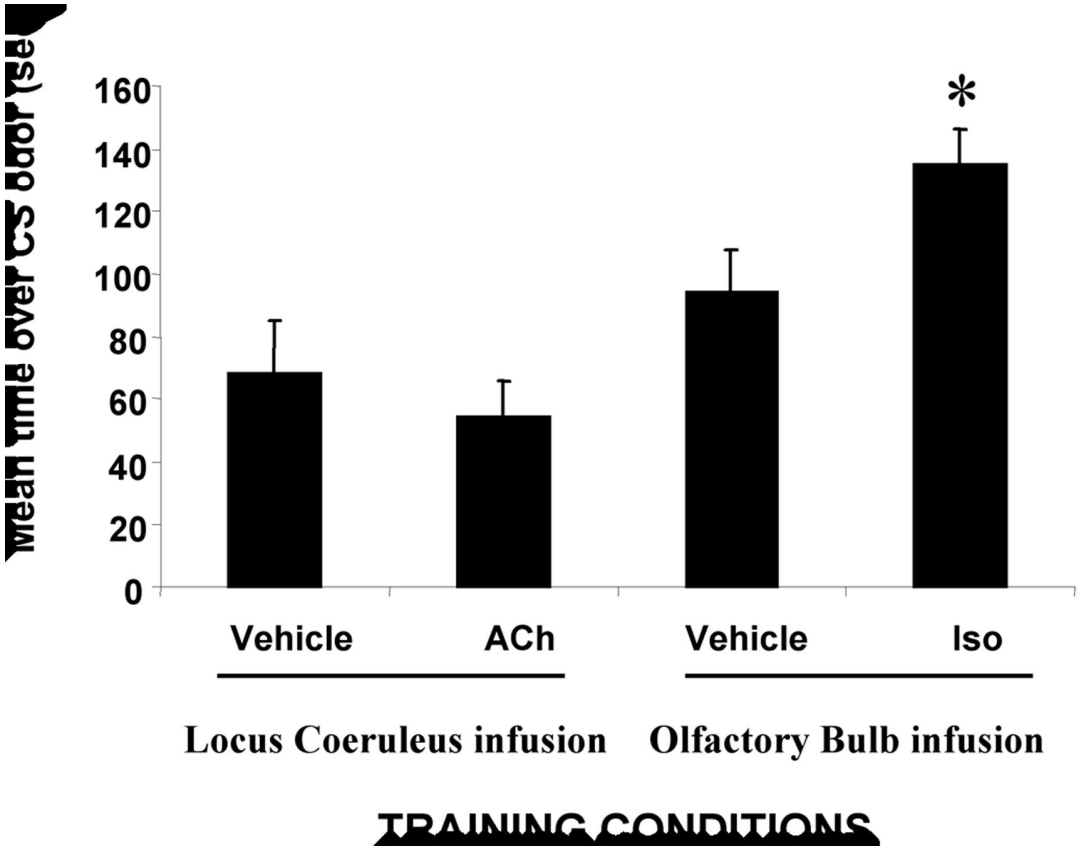
160
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Figure 2

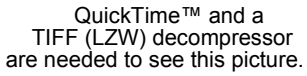
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Figure 3

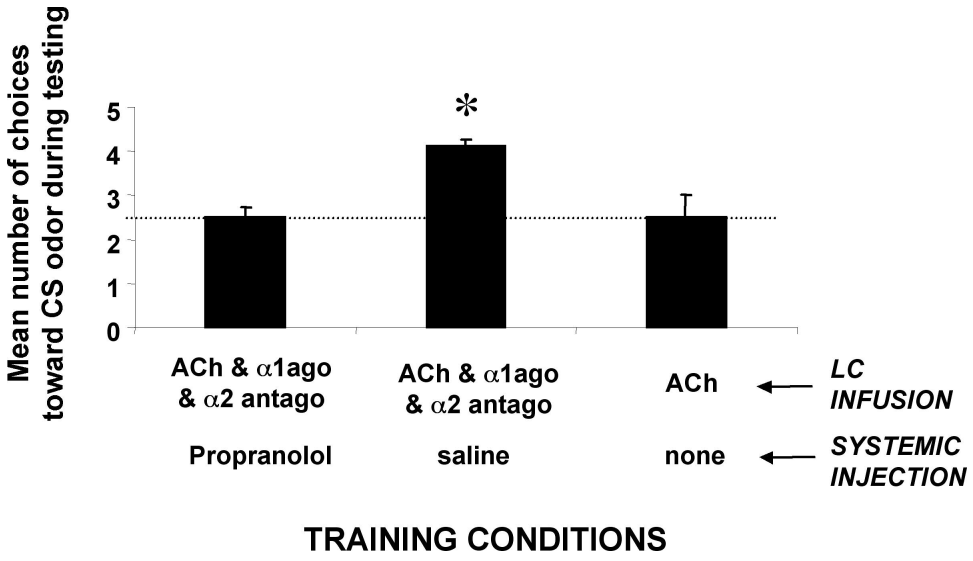
Mean number of choices toward CS odor during testing
5
4
3
2
1
0
*
ACh & α1ago & α2 antago
ACh & α1ago & α2 antago
ACh
LC INFUSION
Propranolol
saline
none
SYSTEMIC INJECTION
TRAINING CONDITIONS

Figure 4

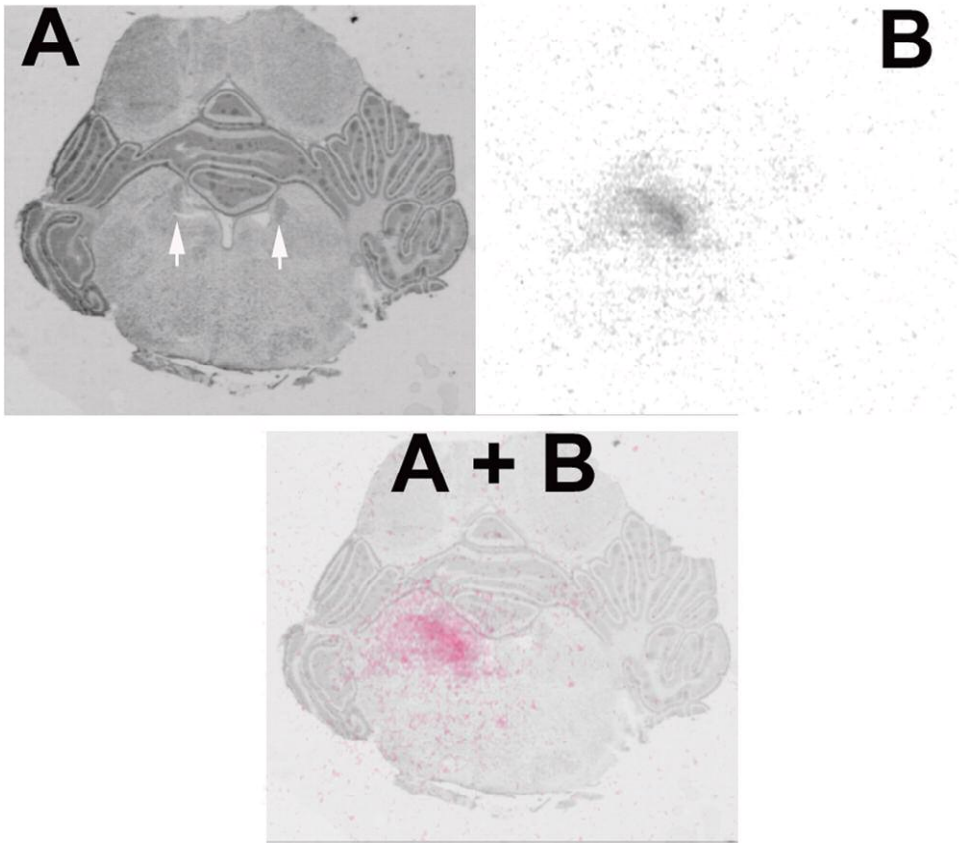

Figure 5


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Chapter 3

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 (3mg/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 amygdala activity could be premature0ly 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 drugs, may also alter the neonatal fear system and its underlying neural control.

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., 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 (Pongracz and Altbacker, 2000) and stranger anxiety emerges around 9 mths 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; Weidenmayer 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 CORT, 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 the 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 (5mg/kg from PN2 to PN6) to decrease hippocampal 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 mostly 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 to the mother also alter pups' CORT levels. Maternal ingestion of alcohol appears to prematurely end the SHRP (Weinberg, 1994) and maternal 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.

Methods

Subjects. The subjects were 108 male and female PN8 and PN12 Long Evans rat pups born and bred in our colony at the University of Oklahoma's vivarium (originally from Harlan, Indianapolis, IN). The pups and their mothers were housed in polypropylene cages (34 x 29 x 17 cm) lined with aspen shavings and cages were kept in a temperature (23°C) and light (0700-1900 hr) 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 used no more than one male and one female from each litter.

Corticosterone manipulation. For PN8 pups or PN12 pups with CORT replacement, pups were injected with either CORT (3.0 mg/kg, ip) 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 (approx 1 hr), pups were returned to the mother until testing.

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 minutes. 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 Chrontrol (Chrontol Corp., San Diego, CA).

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, 1994; Wiedenmayer and Barr, 2001).

Immunohistochemistry. Ninety min following odor delivery, PN8 and PN12 pups were decapitated and their brains were quickly removed, frozen in 2-methylbutane at -45^{o}C and stored in a -70^{o}C freezer. For analysis, brains were sectioned in a cryostat (20 μm; Minotome Plus, TBS, Durham, NC) at –20^{o}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^{o}C in the primary antibody, rabbit anti-Fos (Santa Cruz Biotechnology, sc-52) diluted to

1:500 in phosphate buffered saline (pH= 7.2). They were then rinsed and incubated in the secondary antibody, goat anti-rabbit (Vector Laboratories, Burlingame, CA) for 2 hours 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.

RIA. The levels of circulating CORT were determined from heart blood of PN8 and PN12 pups after 5 minutes exposure with 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 5ng/ml. The intraassay coefficient of variation was 1-9%.

Data analysis. Fos-positive cells were visualized using a microscope (Olympus with a 10x objective) equipped with a drawing tube. Using a rat brain atlas (Paxinos et al., 1991), cresyl violet sections were used to outline the basolateral 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 labeled cells was calculated by averaging the counts from 4 sections per animals. 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.

Results

PN8 behavior. As illustrated in Figure 1A, saline treated PN8 pups did not exhibit immobility/ freezing to any odor presentations, whereas CORT (3mg/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 littermate odor or the control no odor presentation. These results replicate previous work from other laboratories (Takahashi, 1994; Wiedenmayer and Barr, 2001).

PN8 hippocampus. No difference was found between groups in the CA1, CA3 or dentate gyrus of the hippocampus.

PN8 amygdala. A significant increase in Fos-positive cells was found in the basolateral 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 (Figure 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.). Few Fos-positive cells were found in the medial and central amygdala (nonsignificant ANOVA). This suggests that CORT either directly or indirectly

permitted basolateral amygdala activation during the adult male odor presentation.

PN8 and PN12 RIA. A significant increase in CORT level was found in PN12 pups exposed to male odor (ANOVA, $F_{(3,8)} = 6.345$, $p < 0.05$) and post hoc Fisher tests ($p < 0.05$ level) revealed that these pups (56.67± 6.67ng/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± 6.67ng/ml and PN8 RIA values for male odor and littermate odor were 27.33± 3.33 and 34.00± 0 ng/ml, respectively.

PN12 behavior. As indicated in Figure 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 (1994) 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.

PN12 hippocampus. No difference was found between groups in CA1, CA3 or dentate gyrus of the hippocampus.

PN12 amygdala. As illustrated in Figure 2B, both PN12 groups that showed freezing to male odor had a significantly greater increase in Fos-positive cells in the basolateral 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.

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 amygdala. Specifically, we could prematurely activate the expression of fear and the basolateral amygdala in pups during SHRP through exogenous CORT. We were also able to retard the developmental expression of fear and basolateral amygdala activation by preventing the end of the SHRP through ADX. These results replicate those of Weidenmayer and Barr (2001) and Takahashi (1994) 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 during the SHRP (Tanapat et al., 1998) and thus could possibly activate the amygdala even in younger pups.

Our amygdala data, while consistent with other developmental analyses by Wiedenmayer and Barr (2001), are not consistent with the adult literature that suggests the medial nucleus of the amygdala, not the basolateral 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 are unclear and suggest 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 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 Weidenmayer 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-0.5mA) 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 (0.5mA) 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 complex, the adult literature shows the basolateral complex, medial nucleus and central nucleus 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, 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 to 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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Figure captions

Figure 1. Mean (± sem) 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 ($p<0.05$).

Figure 2. Mean (± sem) number of Fos-positive cells in the basolateral 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 ($p<0.05$).

Figure 1

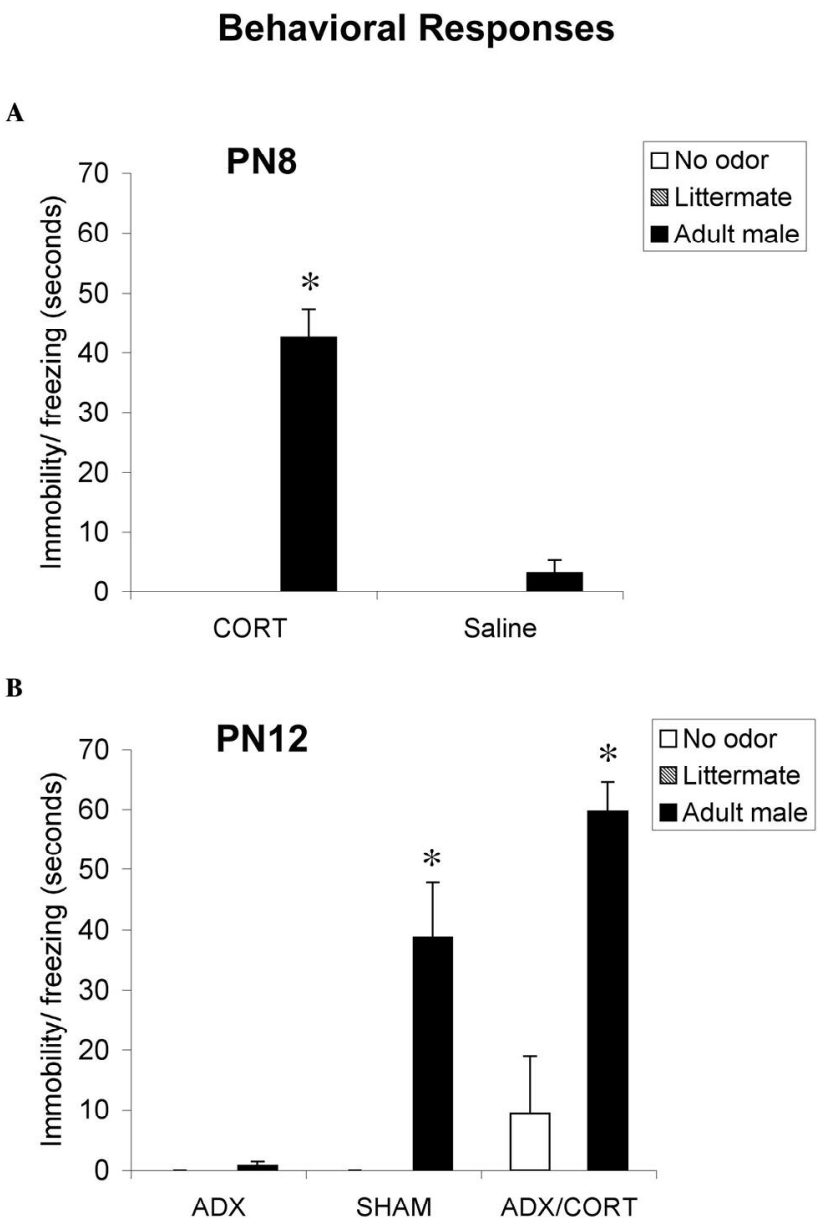
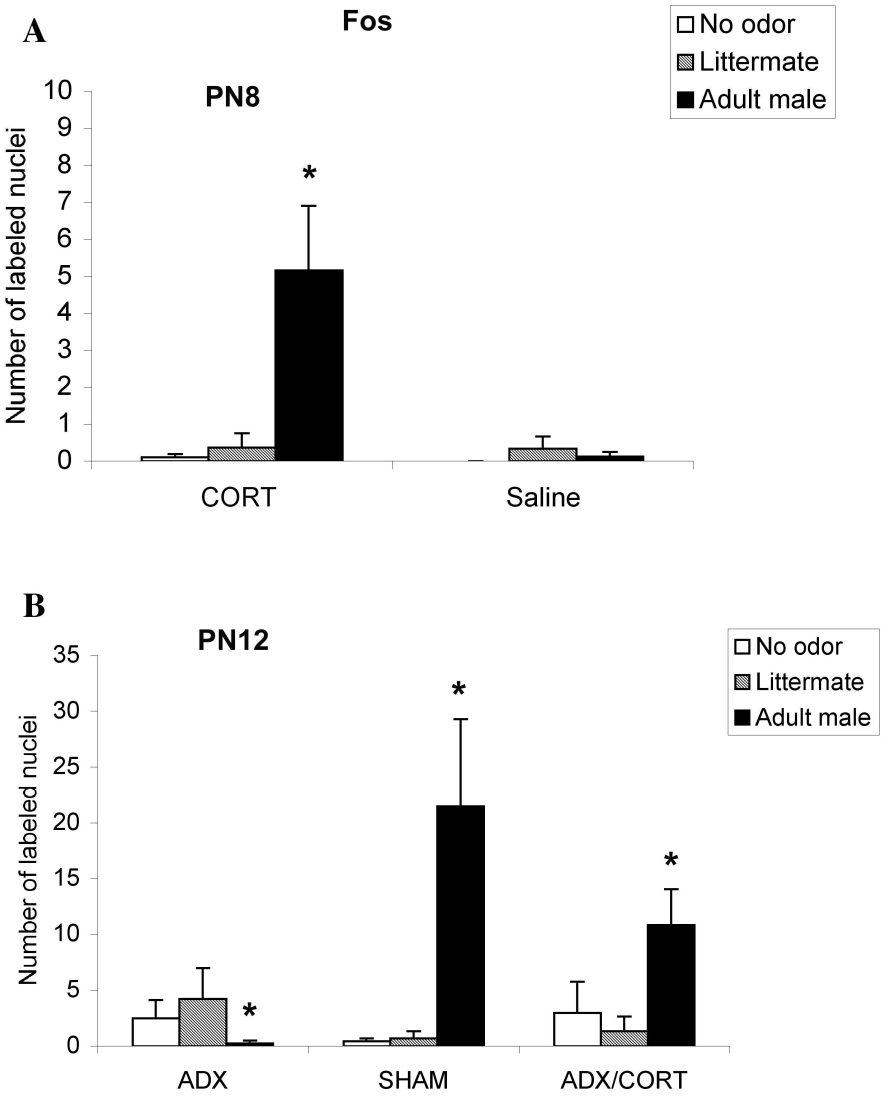

Behavioral Responses
A
PN8
No odor
Littermate
Adult male
Immobility/ freezing (seconds)
70
60
50
40
30
20
10
0
*
CORT
Saline
B
PN12
No odor
Littermate
Adult male
Immobility/ freezing (seconds)
70
60
50
40
30
20
10
0
*
*
ADX
SHAM
ADX/CORT

Figure 2


A
Fos
No odor
Littermate
Adult male
PN8
Number of labeled nuclei
10
9
8
7
6
5
4
3
2
1
0
*
CORT
Saline
B
PN12
No odor
Littermate
Adult male
Number of labeled nuclei
35
30
25
20
15
10
5
0
*
*
*
ADX
SHAM
ADX/CORT

Chapter 4

Corticosterone Influences on Mammalian Neonatal Sensitive Period Learning

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Abstract

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, while CORT replacement can reinstate fear learning, and (c) CORT manipulation modulates neural correlates of sensitive-period odor learning in a manner consistent with behavior.

Introduction

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 post-hatching. 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 et al., 1979; Emerich et al., 1985; Haroutunian & Campbell, 1979; Myslivecek, 1997; Sullivan et al., 1986: Sullivan et al., 2000). At least in rats, learning during the preweanling 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 et al., 1996; Pager, 1974; Shah et al., 2002; Woo & Leon, 1988). The odor enhancement of sexual behavior has also been documented following only a few days of odor-shock conditioning during the sensitive period (Sullivan et al., 2003).

Neonatal rats rapidly learn an odor preference following odor-milk pairings, but also after pairing odor with 0.5 mA shock (Sullivan & Hall, 1988, Camp & Rudy, 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 has been demonstrated in avian imprinting, young dogs, nonhuman primates and perhaps in abused children (Harlow & Harlow, 1965; Helfer et al., 1997; Hess, 1962; Rajecki et al., 1978; Sánchez et al., 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 et al., 1998b). 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 et al., 1984; Takahashi & Rubin, 1993; Takahashi, 1994).

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 ^{14}C 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 et al., 1995; Sullivan & Leon, 1986; Sullivan et al., 1989; Wilson & Leon, 1988b; Wilson et al., 1987; Woo et al., 1987). As with attachment behavioral changes, these neural changes are retained into adulthood, but their acquisition is dependent upon 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.

Methods

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: 1) paired odor-shock, 2) unpaired odor-shock, and 3) 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-sec citral odor (Conditioned stimulus [CS]) and a 1-s 0.5-mA tail shock (unconditioned stimulus [US]; Lafayette Instruments, Lafayette, IN), with an intertrial interval of 4 min. Citral odor was delivered by a flow dilution olfactometer (2L/min flow rate) and 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 odor presentation as well as 20 s during the odor presentation and the shock delivery (Hall, 1979). These measures permit a general assessment of behavioral activity during training in motorically immature animals.

Manipulating CORT. Thirty min 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 and Gilman, 1985). Based on RIA done in our laboratory, the 3.0 mg/kg dose of CORT produces CORT levels similar to those found in nonmaternally deprived, 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 et al., 1990; Chao et al., 1989; Kitraki et al., 1996; Yi et al., 1994).

ADX was verified at PN13 in the late afternoon with a commercially available kit (^{125}I CORT, sensitivity of 5.7ng/ml; Radioimmunoassay Systems

Lab, Inc., Carson, CA). Heart blood samples were taken, centrifuged at 10,000 cpm for 3 minutes, the plasma aliquoted 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 ^{14}C standards that were exposed with the brain sections. 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 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 (3mg/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 tests revealed that both the saline and the 1.5 mg/kg CORT-paired groups differed significantly from each of the control groups at 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 the Trial X Condition (Figure 2A) repeated-measure ANOVA, $F_{(20,700)}$ = 28.81, p < .01) but not for the Trial X Drug ($F_{(20,700)}$ = 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 ($F_{(4,53)}$ = 19.128, p< .01); post hoc Fisher 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 < .0001).

Analysis of behavior during odor-shock conditioning demonstrated that the acquisition curves were significantly different for the Trial X Condition (Figure 2B; repeated-measure ANOVA, $F_{(20,740)}$ = 21.29, p < .01, but not by trial X drug ($F_{(40.740)}$ = 1.197, p = .1918).

CORT modification of sensitive period olfactory bulb neural correlates of imprinting consistent with the behavioral effects. The odor-specific density of 2-DG uptake within the glomerular layer of the olfactory bulb was measured. As illustrated by the PN8 (sensitive period) pup data in Figure 1C, the olfactory bulb showed enhanced 2-DG uptake following odor-shock conditioning, replicating previous results (Sullivan & Wilson, 1995). However, odor-shock CORT pups showed levels of uptake similar to pups in the control groups ($F_{(5,25)}$ =10.91, p< .01; post hoc Fisher tests revealed that the odor-shock conditioning, saline-injected groups were significantly different from each of the control groups at the p < 0.0002 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 tests revealed that the odor-shock conditioning combined with ADX group was significantly different from each of the control groups at the $p < 0.01$ 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 2DG 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 (6mg/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 et al., 1994; Levine, 1962, 2001; Rosenfeld et al., 1992; Walker et al., 1986). The exact neural mechanisms for the 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-term and long-term effects on the development of brain areas mediating stress: the amygdala-locus coeruleus and the hypothalamus-pituitary-adrenal axis (Dent et al., 2001; Eghbal-Ahmadi et al., 1997; Francis et al., 2002; Huot et al., 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 et al., 1999; Kent et al., 1997; Lightman & Harbuz, 1993; Liu et al., 1997; Meaney et al., 1996; Okimoto et al., 2002; Sucheki et al., 1993; Vasquez et al., 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 foot-shock, and decreasing CORT levels causes reduced contextual fear and memory retrieval (Corodimas et al., 1994; Pugh et al., 1997; Roozendaal et al., 1996; Roozendaal, 2002). 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.

While 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 et al., 1997, 2000; Schettino & Otto, 2001). CORT facilitates amygdala plasticity in a fear-conditioning paradigm (Stutzman et al., 1998). Until

the emergence of amygdala function at PN10, neonatal pups do not express either learned or unlearned fear (Sullivan et al., 2000; Takahashi et al., 1991; Wiedenmayer & Barr, 2001). It should be noted that very high-intensity shock (1.0 -1.5mA) 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 et al., 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 et al., 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 et al., 1973; Fanselow & Rudy, 1998; Sananes & Campbell, 1989; Stanton, 2000; Sullivan et al., 2000; reviews: Hofer & Sullivan 2001; Sullivan 2003). 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 et al., 2002), NE can also increase mitral-cell excitation by inhibiting the granule cells that normally inhibit the mitral cells (Aroniadou-Anderjaska et al., 2000; Nickell et al., 1994; Okutani et al., 1999; Sullivan et al., 1989; Trombley et al., 1999; Zhang et al., 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 suggests 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 bulb (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 may underlie the neonatal attachment odor's ability to later enhance mate choice, sex

and maternal behavior (Fillion & Blass, 1986; Fleming et al., 1999; Moore et al., 1996).

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Figure Captions

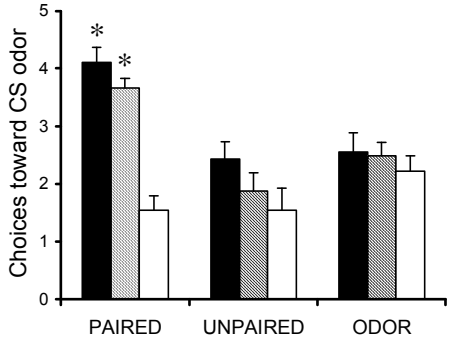
Figure 1. Mean (± SEM) number of conditioned odor choices in a Y-maze test (total of five trials) for (**A**) sensitive-period Postnatal Day (PN) 8 pups and (**B**) postsensitive-period Postnatal Day (PN) 12 pups. Mean (±SEM) level of odor-induced olfactory bulb focal ^{14}C 2-deoxyglucose uptake in (**C**) sensitive-period PN8 pups and (**D**) postsensitive-period PN12 pups. Asterisks represents significant differences from all others groups ($p<.05$). CS = conditioned stimulus; CORT = corticosterone; ADX = adrenalectomy. Error bars represent standard error of the mean.

Figure 2. Mean (± SEM) number of responses (behavioral activity) to the odor conditioned stimulus during odor-shock acquisition for (**A**) sensitive period Postnatal Day (PN8) pups and (**B**) post sensitive-period PN12 pups. CS = conditioned stimulus; CORT = corticosterone; ADX = adrenalectomy. Error bars represent standard error of the mean.

Figure 1

PN8
SENSITIVE PERIOD

A

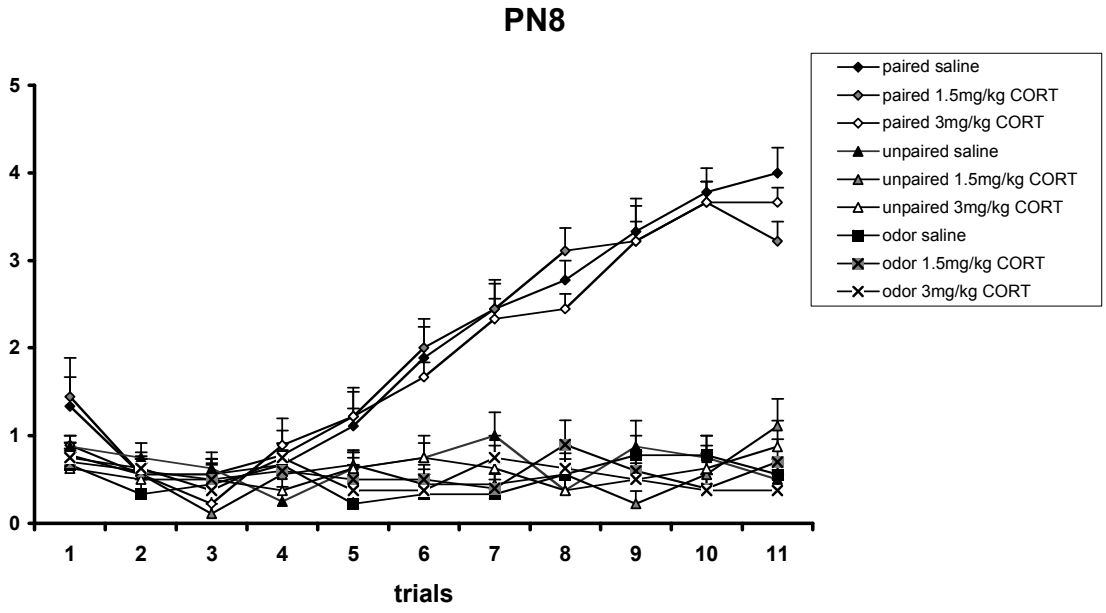


PN12
POST-SENSITIVE PERIOD

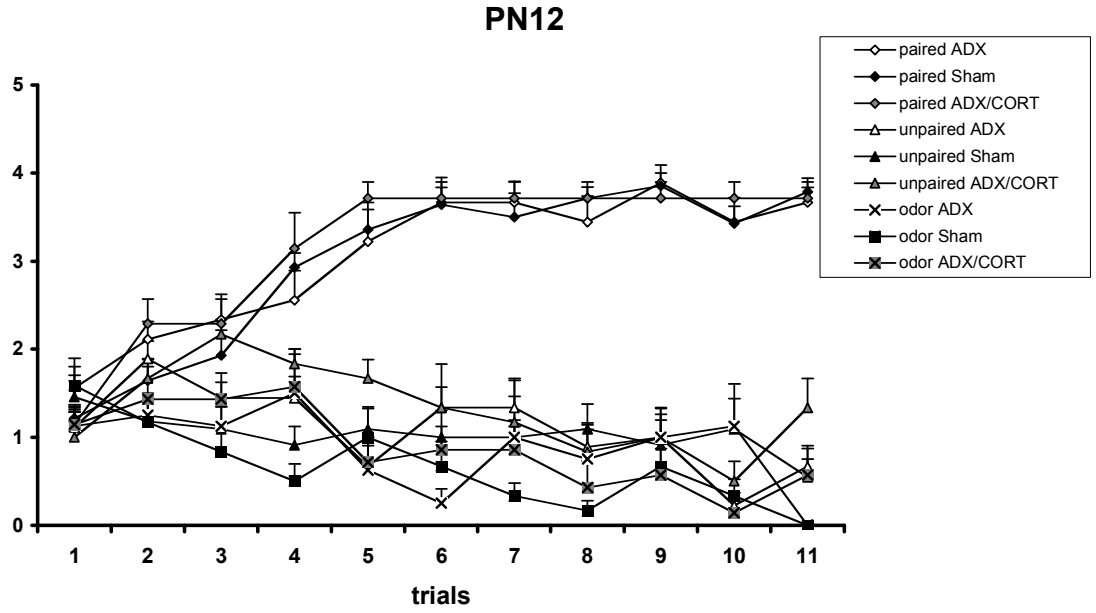
B

Figure 2

A


PN8
paired saline
paired 1.5mg/kg CORT
paired 3mg/kg CORT
unpaired saline
unpaired 1.5mg/kg CORT
unpaired 3mg/kg CORT
odor saline
odor 1.5mg/kg CORT
odor 3mg/kg CORT
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
trials

B


PN12
paired ADX
paired Sham
paired ADX/CORT
unpaired ADX
unpaired Sham
unpaired ADX/CORT
odor ADX
odor Sham
odor ADX/CORT
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
trials

Chapter 5

Permissive role of corticosterone on the ontogeny of fear conditioning and its neural circuitry

Abstract

Neonatal rats learn to prefer odors paired with pain, as is illustrated by the delayed ontogenetic emergence of fear conditioning until postnatal day 10 when the amygdala becomes incorporated into the learning circuit. This early neonatal period is referred to as the sensitive period when learning to prefer the mother's odor is required for attachment and survival. Here we show that low corticosterone (CORT) levels during the first 9 days of life appears to prevent pups from learning fear (avoidance) from odor-shock (0.5mA) pairings and underlies the unique attenuation of avoidance learning found during the sensitive period.

We paired an odor with 0.5 mA shock in either sensitive period (PN8) or postsensitive period (PN12) pups while manipulating CORT levels. We then assessed preference/aversion learning and the olfactory neural circuitry involved during acquisition (2-DG autoradiography). While sensitive period saline Paired odor-shock pups continued to learn an odor preference, PN8 Paired odor-shock CORT (3mg/kg, ip) pups showed a precocious odor aversion and neural activity within the odor learning circuit similar to that expressed by postsensitive period pups (increased activity in posterior piriform cortex and the amygdala's cortical, basolateral/lateral and medial nuclei, but decreased activity in the olfactory bulb and anterior piriform cortex). In postsensitive period pups (PN12), control Paired odor-shock pups showed an odor aversion, although CORT-depleted adrenalectomized (ADX) paired odor-shock pups showed odor preference learning and an odor learning circuit characteristic of the sensitive period

(nonparticipation of the amygdala, decreased posterior piriform cortex activity and increased olfactory bulb and anterior piriform cortex activity).

These results suggest that CORT has an unique role in the developmental emergence of olfactory fear conditioning, changing preference conditioning into aversive conditioning. This sensitive period CORT action is in sharp contrast to its role in the adult where it either strengthens or weakens learning. Since stimulation of pups by the mother modulates endogenous CORT level in pups, these data suggest that the quality of maternal care may alter the duration of the sensitive period.

Introduction

Fear conditioning in adult animals has been instrumental to our understanding of the neurobiology of learning and memory. Here we assess the development of fear conditioning to provide another perspective on the neurobiology of learning and memory that relies on the absence of brains areas considered critical in adult fear conditioning (immature prefrontal cortex, hippocampus and amygdala; Crain et al., 1979; Nair and Gonzalez-Lima, 1999; Sullivan et al., 2000a; Verwer et al., 1996; Waters et al., 1997) suggesting pups' fear conditioning can be assessed in a relatively simplified neural circuit.

Adult-like olfactory fear conditioning emerges around postnatal day (PN) 10, when the amygdala is recruited into the pup's learning neural circuit and the pup's sensitive period for attachment learning ends (Sullivan et al., 2000a). Indeed, pups learn a preference for an odor paired with pain prior to PN10 (tail or foot shock, tailpinch; Camp and Rudy, 1988; Moriceau and Sullivan, 2004b; Sullivan et al., 1986a,b,2000a). It should be noted that this paradoxical pain-induced preference learning is not due to an altered pain threshold and pups will vocalize and escape from a 0.5 mA shock (Barr, 1995; Emerich et al., 1985; Stehouwer and Campbell, 1978; Sullivan et al., 2000a). This suggests that, similar to the adult, the amygdala is required for fear conditioning in neonates (Fanselow and Gale, 2003; Maren, 2003; McIntyre et al., 2003; Sullivan et al., 2000a; Walker and Davis, 2002). Some areas important in adult fear

conditioning, such as the piriform cortex, have been shown to be important in pup conditioning, while other areas, such as the hippocampus, do not appear to participate in pup fear conditioning either before or soon after the sensitive period (Nair and Gonzalez-Lima, 1999; Roth and Sullivan, 2005; Rudy and Morledge, 1994).

Here we assess the role of corticosterone (CORT) on the emergence of olfactory fear conditioning and its corresponding neural circuit (anterior olfactory cortex (AOC), piriform cortex and amygdala). In adults, CORT appears to modulate learning by either strengthening or weakening adult fear conditioning (Hui et al., 2004; review in Roozendaal, 2000; Roozendaal et al., 1999b; Thompson et al., 2004) and appears to work directly on the amygdala and hippocampus (Jacobson and Sapolsky, 1991; Makino et al., 1995). However, in neonates, CORT has a more permissive role on fear learning with CORT dramatically altering what pups learn, although its site of action has yet to be determined. Specifically, removing the source of CORT (by adrenalectomy, ADX) prevents the end of the sensitive period shock-induced odor preference learning and its neural correlate at least until PN13 (Moriceau and Sullivan, 2004b). Other developmental studies also suggest that CORT is required for fear to a predator odor (novel adult male), passive avoidance conditioning and inhibitory conditioning (Bialik et al., 1984; Moriceau et al., 2004; Takahashi, 1994; Takahashi and Rubin, 1993). Furthermore, simply raising CORT levels in sensitive period pups is sufficient to prevent odor-shock attachment learning

(Moriceau and Sullivan, 2004b). Together, these results suggest that low CORT levels attenuate aversion learning, which prevents pups from avoiding the caregiver. Also, a low level of CORT during the sensitive period appears to enable the odor-shock induced odor preference and learning-associated enhanced olfactory bulb functioning. Since maternal odor controls mother-infant attachment and suckling, low CORT levels may have a critical role in attachment.

To further explore the development of fear conditioning in rat pups, we increased or decreased rat pups' CORT levels during and after the sensitive period respectively and assessed its effects on the developmental expression of fear conditioning and the olfactory neural circuit (via 2-DG) associated with this conditioning.

Methods

Subjects. The subjects were PN8 and PN12 male and female Long Evans rat pups born and bred in our colony (originally from Harlan Lab Animals). Dams were housed in polypropylene cages (34 × 29 × 17 cm) lined with pine shavings, and were kept in a room controlled for temperature (23°C) and light (0700-1900 hr). Food and water were available ad libitum. The day of parturition was considered PN0 and litters were culled to 12 on PN0-1. No more than one male and one female from a litter were used in each experimental condition. All procedures were approved by the University of Oklahoma Institutional Animal Care and Use Committee and follow NIH guidelines.

Odor-Shock Conditioning. At PN 8 or PN12, pups were assigned to one of the following 45 min classical conditioning groups: 1) paired odor-shock, 2) unpaired odor-shock, and 3) odor only. Pups were trained in individual 600 ml plastic beakers, and were given a 10 min adaptation period to recover from experimental handling. During a 45 min training session, pups received 11 presentations of a 30-sec peppermint odor (CS) and a 1 sec 0.5 mA tail shock (US; Lafayette), with an intertrial interval of 4 min. Peppermint odor was delivered by a flow dilution olfactometer (2 L/min flow rate) at a concentration of 1:10 peppermint vapor. Paired odor-shock pups received 11 pairings of the 30 sec odor with shock overlapping during the last 1 sec of the odor presentation. Unpaired odor-shock pups received the shock 2 min after each odor presentation. Odor only pups received only the peppermint odor presentation.

During conditioning, a general assessment of behavioral activity during training in motorically immature animals was done by recording the number of limbs moving (0= no movement of the extremities; 5= movement of all 5 extremities) 20 sec before the odor presentation as well as 20 sec during the odor presentation and the shock delivery (Hall, 1979; Moriceau and Sullivan, 2004b; Sullivan et al., 1994).

Manipulating Corticosterone (CORT). Pups were injected with either CORT (3.0 mg/kg, ip) or saline (Takahashi, 1994; Moriceau and Sullivan, 2004b) either 24 hrs and 30 min before training (2 injections) or 30 min before training (1 injection). Endogenous CORT was removed by adrenalectomy (ADX) at PN8 for training of PN12 pups. Pups were anesthetized using 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 (approx 1 hr), pups were returned to the mother and left undisturbed until training (Moriceau and Sullivan, 2004b).

Assessing learning – Y-maze. The day following conditioning, pups were given a behavioral Y-maze test when all exogenous CORT had left the system (Goodman and Gilman, 1985). This test required pups to choose between two arms of a Plexiglas Y-maze (start box: 8.5 cm width, 10 cm length, 8 cm height; choice arms: $8.5 \times 24 \times 8$ cm), one containing the peppermint odor CS (20 μl of peppermint odor on a KimWipe placed at the end of the alley) and the other

containing the familiar odor of pine shavings (20 ml of clean shavings in a petri dish placed at the end of the alley). The start box was separated from the alleys by two doors. A pup was placed in the start box (habituation chamber) for 5 sec before the door to each alley was opened. Each pup was given 60 sec 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 5 sequential trials with 30 sec between trials and the floor was cleaned between each trial (Sullivan and Wilson, 1991). Observation during the testing was done blind to the training condition.

Assessing neural correlates within the neural circuitry involved in olfactory learning. PN 8 and PN 12 pups were injected with ^{14}C 2-deoxyglucose (2-DG; 20 μCi/100g, sc) 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 standards 10×.02 mCi, American Radiolabeled Chemicals, Inc., St. Louis, MO) to x-ray film (Coopersmith and Leon, 1986; DiRocco and Hall, 1981; Nudo and Masterton, 2004; Sullivan and Wilson, 1995).

The brain areas examined 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 with conditioning group) to control for

differences in section thickness and exposure (Sullivan et al., 2000a). Brain areas examined were the anterior olfactory cortex (AOC; medial, lateral, ventral and dorsal nucleus of AOC), the anterior and posterior piriform cortex, and the basolateral/lateral (BLA), cortical (CoA), medial (AM) and central amygdaloid nuclei (AC).

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

Results

Increasing CORT during the sensitive period alters learning.

At PN8, paired odor-shock pups injected with saline learned an odor preference, whether given 1 or 2 injections of saline (see Figure 1A and 1B). Paired pups that received one injection of CORT 30 min before odor-shock conditioning (Figure 1A) did not learn either an odor preference or aversion, suggesting CORT disrupted learning and replicating our previous results (Moriceau and Sullivan, 2004b). *However, double injections of CORT (24 hrs & 30 min prior to paired odor-shock conditioning; Figure 1B) prematurely terminated the sensitive period for odor preference learning and permitted pups to learn odor avoidance instead.*

The single CORT injection ANOVA analysis revealed a main effect of drug treatment ($F_{(1.39)}$ = 22.570, $p < 0.0001$), and a significant interaction between training condition and drug treatment ($F_{(2.39)}$ = 9.237, $p < 0.001$); post hoc Fisher tests revealed that the saline-paired groups differed significantly from each of the control groups at the $p < 0.05$ level, while the 3.0mg/kg CORT-paired group was not significantly different from the control groups. The double CORT injection ANOVA analysis revealed a main effect of drug treatment ($F_{(1.41)}$ = 11.467, $p < 0.01$), and a significant interaction between training condition and drug treatment ($F_{(2.41)}$ = 13.099, $p < 0.0001$); post hoc Fisher tests revealed that the saline-paired group and the double CORT-paired groups each differed significantly from each of the control groups at the $p < 0.05$ level.

Increasing CORT during the sensitive period alters the neural circuitry of olfactory fear conditioning.

The olfactory pathway to the amygdala is illustrated in Figure 2. As illustrated in Figure 3 and 4, saline treated paired odor-shock pups had learning-induced changes in the anterior piriform cortex, which replicates previous results (Roth and Sullivan, 2005). As illustrated in Figure 3, at PN8, one injection of CORT 30 min before odor-shock conditioning, which only disrupts learning, prevented the learning changes observed in the anterior piriform cortex. As illustrated in Figure 4, double CORT injection (24 hrs & 30 min prior to conditioning), which causes pups to learn an odor aversion rather than the age-typical odor preference, produced changes in the posterior piriform cortex and the amygdala (cortical nucleus, basolateral/ lateral nucleus and medial nucleus). Thus, it appears that double CORT injection during the sensitive period (PN8) was sufficient to activate the neural circuit underlying odor aversion learning normally expressed in postsensitive period pups.

As illustrated in Figure 3B, one CORT injection caused a significant difference in the anterior piriform cortex ($F_{(5,22)}$ = 11.664, $p < 0.0001$). Post hoc Fisher tests indicated that the saline-paired group differed significantly from each of the other paired groups and each control group at $p < 0.05$ levels for the anterior piriform cortex. No statistical differences were found for the AOC, posterior piriform cortex, basolateral/lateral amygdala, medial amygdala, central amygdala or cortical amygdala.

As illustrated in Figure 4C, the double CORT injection caused a significant difference in the anterior piriform cortex ($F_{(5,23)}$ = 4.153, p< 0.01), posterior piriform cortex ($F_{(5,21)}$ = 7.297, p< 0.0005), basolateral/lateral amygdala ($F_{(5,23)}$ = 12.004, p< 0.0001), cortical amygdala ($F_{(5,19)}$ = 4.186, p< 0.01), and medial amygdala ($F_{(5,20)}$ = 5.221, p< 0.005). Post hoc Fisher tests revealed that the anterior piriform cortex of paired saline pups differed from each of the other groups (similar to saline pups in the CORT 1 injection experiment), while the paired double CORT group differed from the other groups for the posterior piriform, basolateral/lateral amygdala, cortical amygdala and medial amygdala at the p < 0.05 level. No statistically significant difference was found for the AOC.

Decreasing CORT after the sensitive period extends the sensitive period learning.

At PN12, sham paired odor-shock pups learned an odor aversion (see Figure 5A-B). Pups that received adrenalectomy (removal of endogenous CORT) before paired odor-shock conditioning (Figure 5A-B) showed an extension of the sensitive period and permit pups to learn to approach odors paired with 0.5 mA shock rather than the age-typical odor avoidance (Moriceau and Sullivan, 2004b). ADX pups that received CORT replacement with one injection of CORT 30 min (Figure 5A) or with two injections of CORT (24 hrs & 30 min; Figure 5B) before paired odor-shock conditioning learned to avoid odors paired with 0.5 mA shock.

The single CORT injection ANOVA analysis showed a significant effect of drug treatment ($F_{(2.61)}$ = 10.231, $p < 0.0005$) and a significant interaction between training condition and drug treatment ($F_{(4,61)}$ = 8.724, $p< 0.0001$); post hoc Fisher tests revealed that the sham, the ADX+CORT and the ADX-Paired groups were significantly different from each of the control groups at the $p < 0.05$ level.

The double CORT injections ANOVA analysis revealed a significant effect of drug treatment ($F_{(2,28)}$ = 4.334, $p < 0.05$) and a significant interaction between training condition and drug treatment ($F_{(4,28)}$ = 6.807, $p< 0.001$); post hoc Fisher tests revealed that the sham, the ADX+ double CORT and the ADX-Paired groups were each significantly different from each of the control groups at the $p < 0.05$ level.

Decreasing CORT at the end of the sensitive period alters the neural circuitry of olfactory fear conditioning.

The olfactory pathway to the amygdala is illustrated in Figure 2. As illustrated in Figures 6 and 7, sham Paired odor-shock pups had learning-induced changes in the posterior piriform cortex and the amygdala (cortical nucleus, basolateral/ lateral nucleus and medial nucleus) which replicates previous findings on the amygdala as a whole (Sullivan et al., 2000a) and provides information on specific subdivision of the amygdala. As illustrated in Figures 6 and 7, at PN12, adrenalectomy before odor-shock conditioning, which permitted the learning of an odor preference rather than the age-typical odor aversion, induced changes in the anterior piriform cortex and blocked the

appropriate age-changes seen in the posterior piriform cortex and the amygdala (cortical nucleus, basolateral/ lateral nucleus and medial nucleus). As illustrated in Figure 6B, CORT replacement with one injection of CORT 30 min before odor-shock conditioning, which permitted odor avoidance learning, prevented the learning changes in the anterior piriform cortex seen after adrenalectomy. As illustrated in Figure 7, CORT replacement with double CORT injections (24 hrs & 30 min prior to conditioning), which also causes pups to learn an odor aversion, reinstated neural changes in the posterior piriform cortex and the amygdala (cortical nucleus, basolateral/ lateral nucleus and medial nucleus). *As illustrated in Figures 6 and 7, adrenalectomy during the postsensitive period was sufficient to activate the neural circuit underlying odor preference learning in sensitive period pups.*

As illustrated in Figures 6 and 7, adrenalectomy caused a significant difference in the anterior piriform cortex ($F_{(5,18)}$ = 7.856, $p < 0.001$), the posterior piriform cortex ($F_{(5,18)}$ = 12.976, $p < 0.0001$), the basolateral/lateral amygdala ($F_{(5,18)}$ = 16.132, $p < 0.0001$), the medial amygdala ($F_{(5,16)}$ = 3.776, $p < 0.05$) and the cortical amygdala ($F_{(5,15)}$ = 6.425, $p < 0.05$). Post hoc Fisher tests indicated that the ADX-Paired groups differed significantly from each of the other Paired groups and each control group at the $p < 0.05$ level for the anterior piriform cortex. Additionally, the posterior piriform cortex, basolateral/lateral amygdala, medial amygdala and cortical amygdala of Sham-Paired groups differed from the ADX-Paired groups, the ADX-one CORT injection replacement-Paired pups and

the control groups at the $p < 0.05$ level. No statistical differences were found for the AOC.

As illustrated in Figure 6, one CORT injection caused a significant difference in the anterior piriform cortex ($F_{(4,23)} = 7.055$, $p< 0.001$). Post hoc Fisher tests indicated that the ADX-Paired groups differed significantly from each of the other Paired groups and each control group at the $p < 0.05$ level for the anterior piriform cortex. No statistical differences were found for the AOC.

As illustrated in Figure 7, double CORT injection caused a significant difference in the anterior piriform cortex ($F_{(5,18)} = 7.856$, $p< 0.001$), the posterior piriform cortex ($F_{(5,18)} = 12.976$, $p< 0.0001$), the basolateral/lateral amygdala ($F_{(5,18)} = 16.132$, $p< 0.0001$), the medial amygdala ($F_{(5,16)} = 3.776$, $p< 0.05$) and the cortical amygdala ($F_{(5,15)} = 6.425$, $p< 0.05$). Post hoc Fisher tests revealed that the anterior piriform cortex of ADX-Paired pups differed from each of the other groups while the Sham-Paired pups and ADX-double CORT injection replacement-Paired pups differed from each of the other groups for the posterior piriform cortex, basolateral/lateral amygdala, cortical amygdala and medial amygdala at the $p < 0.05$ level. No effects of training or drug condition were found within the AOC.

Discussion

These data suggest that during the sensitive period, the naturally low levels of CORT maintain pups' unique abilities to ensure a rapid learning of olfactory-based attachment to the mother even when interactions with the mother are painful. Specifically, we found that (a) increasing CORT over 24 hours permits the precocious ontogenetic emergence of fear conditioning and induces participation of brain areas implicated in adult fear learning (posterior piriform cortex and cortical, basolateral/lateral and medial nuclei of the amygdala), (b) the sensitive period can be prolonged by experimentally maintaining low level of CORT (ADX) in older pups, permitting odor-shock conditioning to continue to induce an odor preference and maintain the unique neural circuitry involved in sensitive period preference learning (increased olfactory bulb activity; Moriceau and Sullivan, 2004b) and anterior piriform cortex neural activity, and (c) CORT replacement in older ADX pups reinstates odor-shock-induced odor aversions and its underlying neural circuit (posterior piriform cortex and cortical, basolateral/lateral and medial nuclei of the amygdala).

The single CORT injection, in PN8 pups, just prior to conditioning blocked learning and all neural correlates associated with either preference or avoidance learning from odor-shock conditioning. The ability of a double CORT injection to permit odor-aversion learning in sensitive period pups suggests that CORT may be altering neural plasticity and/or gene expression, although further work is

required to explore the mechanism (Borski, 2000; De Kloet, 2004; Orchinik et al., 2002).

The ability of CORT manipulation to alter the termination of the pups' sensitive period is related to the 'Stress Hyporesponsive Period' when low CORT levels in pups are not raised by most stressful stimuli (i.e., restraint, shock; Grino et al., 1994; Levine, 1962, 2001; Rosenfeld et al., 1992). Sensory stimulation provided by the mother during nursing and grooming seems to control the pups' low CORT levels (Levine, 1962; Van Oers et al., 1998). In effect, prolonged maternal separation (~24 hrs) deprives pups of maternal sensory stimulation and permits an increase of pups' CORT level (Levine, 2001), while maternal sensory stimulation replacement reinstates the low level of CORT (Stanton and Levine, 1990; Suchecki et al., 1993).

Pups also have limited ability to learn inhibitory conditioning paradigms, suggesting pups have evolved a learning system thar prevents them from learning to avoid or inhibit responses to the mother. Specifically, during the sensitive period, an odor preference is learned following odor-pain (i.e. shock, tailpinch) conditioning (Sullivan et al., 1986; 2000a; Moriceau and Sullivan, 2004b; Camp and Rudy, 1988) and pups also fail to learn inhibitory conditioning and passive avoidance (Bialik et al., 1984; Blozovski and Cudennec, 1980; Collier et al., 1979; Myslivecek, 1997). Behavioral studies demonstrate that a low neonatal CORT level also appears to limit pups' ability to learn inhibitory

conditioning. Specifically, inhibitory conditioning emerges as the sensitive period ends and inhibitory conditioning can be delayed ontogenetically simply by removing the source of CORT (Bialik et al., 1984). It should also be noted that the expression of unlearned fear (predator odor) also emerges as the sensitive period ends around PN10 (Moriceau et al., 2004; Takahashi et al., 1991; Takahashi, 1992; Weidenmayer and Barr 2001) and its emergence is correlated with the participation of the amygdala in the response to predator odor (Moriceau et al., 2004; Weidenmayer and Barr 2001). Furthermore, increasing and decreasing pups' CORT permitted or prevented freezing, respectively (Takahashi, 1994; Takahashi and Rubin, 1993) and amygdala participation or nonparticipation, respectively (Moriceau et al., 2004). Together, these data suggest that CORT has an important role in terminating the sensitive period and facilitates the expression of both learned and unlearned fear.

During the neonatal sensitive period, CORT seems to have a unique effect, by changing the effect of odor-shock conditioning from preference learning to aversion learning. These data are in sharp contrast to the effects of CORT in the adult where CORT either enhances or attenuates learning and consolidation (Corodimas et al., 1994; Pugh et al., 1997; Roozendaal et al., 1996, 2002). Specifically, CORT injection permitted the expression of more fear-conditioned freezing in adults and removal of the source of CORT reduced fear conditioning (Hui et al., 2004; Pugh et al., 1997; Thompson et al., 2004). Also, CORT has a facilitative role in the acquisition of a spatial learning task (Akirav et al., 2004).

The unique neural circuitry used by pups during sensitive period learning suggests that the neonatal brain is specialized for optimizing attachment to the caregiver, regardless of the quality of care and expressed through heightened approach learning and attenuated avoidance learning. This heightened approach learning is supported by a unique neural circuit including the hyper-functioning locus coeruleus, the olfactory bulb and the anterior piriform cortex (Moriceau and Sullivan, 2004b; McLean et al., 1999; Rangel and Leon, 1995; Roth and Sullivan, 2005; Sullivan et al., 1992, 1994, 2000b; Wilson and Sullivan, 1990; Wilson et al., 1987; Wilson and Leon, 1988; Woo et al., 1987; Yuan et al., 2003) while attenuated avoidance learning is characterized by an lack of participation of the posterior piriform cortex and the amygdala, which is a brain area known to be important in adult fear conditioning (Cousens and Otto, 1998; Fanselow and Gale, 2003; Litaudon et al., 1997; Maren, 2003; McIntyre et al., 2003; Mouly et al., 2001; Roth and Sullivan, 2005; Sevelinges et al., 2004; Rosenkranz and Grace, 2002; Sullivan et al., 2000; Walker et Davis, 2002).

Thus, pups appear to have two sequentially-developing neural circuits associated with early learning; the first is specialized for the rapid preference conditioning associated with the sensitive period and the second, more “adult-like” circuit permits pups to easily learn aversions and is more selective in supporting odor preferences. The relevant brain areas are discussed below in terms of their roles in infant sensitive period learning, infant’s postsensitive period learning and adult learning.

Olfactory bulb. In neonate pups, olfactory classical conditioning produces cellular and physiological changes within the olfactory bulb, only acquired during the sensitive period and last into adulthood (Fillion and Blass, 1986; Fleming et al., 1999; Johnson et al., 1995; Moore et al., 1996; Sullivan and Wilson, 1991; Wilson et al., 1987; Woo et al., 1987; Yuan et al., 2003; Zhang et al., 2003). These learning-induced olfactory bulb changes depend upon norepinephrine (NE) release from the locus coeruleus, which prevents the mitral cells of the olfactory bulb from habituating to the odor (Sullivan et al., 1989; 1992; 2000b, Wilson et al., 1987). The unique response characteristics of the sensitive-period locus coeruleus permit a wide range of sensory stimuli to cause an abundant release of NE (Nakamura and Sakaguchi, 1990; Rangel and Leon, 1995). Recently, we were able to reinstate the sensitive-period heightened preference learning by reinstating the locus coeruleus autoreceptors that cause the abundant release of NE (Moriceau and Sullivan, 2004a). We have also shown that CORT modulates learning-induced neural correlates in the olfactory bulb (Moriceau and Sullivan, 2004b). Specifically, one systemic CORT injection prevents the olfactory bulb neural changes characteristic of the sensitive-period learning and depletion of CORT extends these typical neural changes beyond the sensitive period (Moriceau and Sullivan, 2004b). It is unclear if CORT directly affects the olfactory bulb, which contains CORT receptors (Kitraki et al., 1996) or acts indirectly, through modulation of NE levels, since the locus coeruleus also contains CORT receptors that inhibit LC responses. There may

also be other indirect routes for CORT to modify the olfactory bulb function, such as CORT in the amygdala causing release of corticotropin releasing factor (CRF) to activate NE release by the locus coeruleus (Curtis et al., 2002; Page and Abercrombie, 1999), resulting in too much NE. Specifically, there is an inverted U dose response curve for NE levels during conditioning, with too much NE producing an odor aversion (Sullivan et al., 1989).

Anterior Olfactory Cortex. The olfactory bulb output mitral cells' axons form the olfactory tract and relay information to the anterior olfactory cortex (AOC, Haberly, 2001). This anatomical pathway is illustrated in Figure 2. Also, the AOC is a major relay between the two olfactory bulbs via the anterior commisure, which becomes functional between PN6 and PN12 (King and Hall, 1990; reviewed in Shipley and Ennis, 1996; Wilson and Sullivan, 2003). The AOC, by projecting to both the ipsilateral and contralateral olfactory bulbs may ensure similar odor-evoked activity in the two bulbs and permit each bulb access to information.

While our results suggest that during both the sensitive period and the postsensitive period, the AOC is not involved in acquisition learning of odor-shock conditioning, it is important to note that previous studies showed an involvement of the AOC in memory. Specifically, olfactory stimulation through novel odor presentation increased the AOC activity of pups and adult rats (Astic and Saucier, 1982; Hall, 1987). Also in adults, presentation of a conditioned odor increased neural activity in the AOC (Funk and Amir, 2000; Hamrick et al., 1993).

Additionally, following an appetitive learning paradigm involving bar pressing for milk, the AOC of a conditioned neonatal rat (PN6) showed a small metabolic activity in response to odor stimulation (Hall, 1987). Although CORT receptors are present in the AOC (Kitraki et al., 1996), our present results suggest that during olfactory fear conditioning, CORT does not appear to be affecting the AOC.

Piriform Cortex. The piriform cortex is typically divided into anterior and posterior areas and each area has unique olfactory bulb connections. Mitral cells from the olfactory bulb project massively to the anterior piriform cortex, while the posterior piriform cortex receives projections from the anterior piriform cortex but a more limited input from mitral cells (anatomical pathway is illustrated in Figure 2; Johnson et al., 2000; Santiago and Shammah-Lagnado, 2004; Schwob and Price, 1984; review in Wilson and Sullivan, 2003). In adults, both the anterior and posterior piriform cortex appear to be involved in learning, but the posterior piriform cortex is more implicated in synaptic plasticity and memory processes (Barkai and Sahar, 2001; Haberly, 2001; Hasselmo and Barkai, 1995; Litaudon et al, 1997b; Mouly et al., 2001; Sevelinges et al., 2004; Tronel and Sara, 2002; Wilson et al., 2004). Our present results show that sensitive-period-preference learning involves the anterior piriform cortex, while postsensitive period aversion learning seems to involve the posterior piriform cortex. This suggests that the role of the anterior and posterior piriform cortices may differ during and after the

sensitive period perhaps indicative of the more complex plasticity associated with the posterior piriform (Best et al., 2004).

Since CORT receptors are localized within the neonate and adult piriform cortex (Ahima et al., 1991), it is possible that CORT acted directly on the piriform to alter its neural activity and hence alter learning. Research in the adult is consistent with this interpretation since stress and CORT seem to induce neuronal structural changes within the piriform cortex and this mechanism could modulate olfactory learning (Nacher et al., 2004). However, CORT may also work indirectly on the piriform through its action on the olfactory bulb (see above) or other structures that provide input to the piriform, such as the amygdala and the locus coeruleus (Datiche and Cattarelli, 1996; Luskin and Price, 1983). However, regardless of the mechanism used to alter the relative inputs to the anterior and posterior piriform cortex during infant learning, our results suggests that output from the piriform to the amygdala may differ during and after the sensitive period.

Amygdala. The anatomical input to the amygdala is illustrated in figure 2. The amygdala receives olfactory input (conditioned stimulus; CS) from a myriad of different pathways. First, olfactory information reaches the amygdala through direct input from the olfactory bulb to the cortical amygdala (McDonald, 2003; reviewed in Wilson and Sullivan, 2003). Second, the olfactory bulb also sends its output directly to the medial amygdala (Aggleton, 2001; Ferguson et al., 2001;

McDonald, 2003; Meredith, 1991). Third, there is an indirect input to the cortical amygdala via both the anterior and posterior piriform cortex. Finally, there is also indirect input to the basolateral/lateral amygdala via the posterior piriform cortex (Haberly, 2001; McDonald, 2003; Pitkanen, 2003; Price, 1973; Shipley et al., 1995; Schwob and Price, 1984; reviewed in Wilson and Sullivan, 2003). Lastly, there is thalamic inpt, although it is unclear if ths pathway is functional in the neonate (Bouwmeester et al., 2002; Eichenbaum et al., 1980; McBride and Slotnick, 1997).

The amygdala is thought to receive shock-related input (unconditioned stimulus, US) through the lateral amygdala from two parallel pathways either cortical (insula-amygdala including the parabriachal nucleus) or subcortical (thalamoamygdala including the posterior intralaminar nucleus) pathway, both of which receive shock-related input from the spinal cord (Blair et al., 2005; LeDoux, 2000; Shi and Davis, 1999).

The US and the olfactory CS converge in the basolateral nucleus of the amygdala (Otto et al., 2000). However, there is also some evidence for convergence of CS-US in the medial amygdala (Schettino and Otto, 2001). The CS in other sensory modalities (auditory, visual) appears to have an overlapping but distinct input to the amygdala through the lateral nucleus (McDonald, 1998; Rosenkrantz and Grace, 2002; Romanski and LeDoux, 1993; Swanson and Petrovitch, 1998), although convergence also appears in the basolateral nucleus.

This suggests that the neural pathway for learning may differ between sensory modalities.

There are interconnections, illustrated in figure 8, between almost all of the amygdala nuclei. The major flow of information involves convergence of input from the lateral, medial and cortical nucleus into the basolateral. The basolateral nucleus then projects to the central nucleus, which provides the major output to the thalamus (for attention and vigilance), the hypothalamus (for corticosterone release, increased blood pressure), the basal forebrain (for increased vigilance), the medulla (for increased attention and vigilance), the pons (for increased attention and vigilance, fear potentiation, increased respiration) and the midbrain (for freezing, ultravocalization, hypoalgesia; Fendt and Fanselow, 1999; Jolkonen and Pitkanen, 1998; Rosen, 2004).

The intra-amygdaloid connections shown for adults in Figure 8, could be different during development due to the immaturity of the infant amygdala. The basolateral/lateral amygdala begin to be distinct from other structures on the 17th day of embryonic life (Morys et al., 1999) and most of the neurons of the basolateral/lateral amygdala are generated before PN7 (Berdel et al., 1997). During the first days of postnatal life, the neurons of the basolateral and lateral amygdala begin to differentiate. Neurons of other nuclei of the amygdala such as the central and medial are generated a few days later (Morys et al., 1999), although all amygdala nuclei are still developing until adolescence (Berdel and

Morys, 2000; Berdel et al., 1997, Morys et al., 1999). Our results showed that the amygdala participates in olfactory fear conditioning around PN10, although CORT injection advanced this at least to PN8.

The inability of the amygdala to take part in the learning circuit of sensitive period pups suggests the amygdala is required for fear conditioning even in infancy (Sullivan et al., 2000, Moriceau and Sullivan, 2004, Roth and Sullivan, 2005; Wilson and Sullivan, 2003). We suggest that CORT induces amygdala activity either directly or indirectly, and could explain the emergence of fear conditioning in neonatal rats. In older pups and adults, fear conditioning is associated with amygdala activity, as illustrated by the disruption of fear conditioning due to the inactivation of the amygdala, and CORT is known to affect fear learning through the amygdala (Cousens and Otto, 1998; Fanselow and Gale, 2003; Maren, 2003; McIntyre et al., 2003; Packard and Cahill, 2001; Schafe et al., 2001; Sevelinges et al., 2005; Sullivan et al., 2000; Roth and Sullivan, 2005; Wallace and Rosen, 2001; Walker and Davis, 2002). Specifically, direct CORT infusion into the amygdala enhanced performance after an inhibitory conditioning (Roozendaal and McGaugh, 1997) and this effect was blocked by lesion of the amygdala (Roozendaal and McGaugh, 1996). CORT, acting through amygdala receptors, produced an increase in the expression of corticotropin-releasing-factor (CRF) mRNA in the central nucleus of the amygdala and increased the fear conditioned response (Makino et al., 1999; Shepard et al., 2000; Thompson et al., 2004).

Since NE is such a critical neurotransmitter for pup learning, a potential interaction between CORT and NE in pups should be explored. Since CORT has such distinct effects on neonatal and adult fear conditioning, comparisons of infant and adult data should be done with caution, although some insights may be gained. The adult amygdala is the locus of complex interaction between CORT and norepinephrine (NE) in modulating learning during consolidation (Quirarte et al., 1997; Roozendaal et al., 2002, 2004). Activation of the nucleus of the solitary tract permit the release of NE in the amygdala either via a direct projection or indirectly through the locus coeruleus, and stimulates cAMP levels within the amygdala, which permit fear learning (Daly et al., 1991; Fallon and Ciofi, 1992; Introini-Collison et al., 1991; Liang et al., 1995; Roozendaal et al., 1999; Roozendaal, 2002). CORT, by interacting with the NE response, interferes with the cAMP-PKA cellular cascade and potentiated fear learning (Roozendaal et al., 2002). Overall, our results suggest that CORT, through its action on the amygdala, has a critical role in the emergence of olfactory fear conditioning. However, in neonates, the exact cellular mechanism of CORT action within the amygdala is still undetermined.

In conclusion, our results show that, in the neonate, CORT has the unique role of switching preference conditioning to aversive conditioning. They also demonstrates that CORT appears to have an important role in the developmental emergence of brain areas in the olfactory neural pathway correlated with fear

conditioning, particularly the amygdala and piriform cortex. Since low levels of CORT are notable during the sensitive period and maintained by the mother's stimulation of pups, the quality of caregiving may be important in controlling sensitive period termination.

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Figure captions

Figure 1: Mean (± sem) number of choices toward the conditioned stimulus (CS) odor during the Y-maze test (total of 5 trials) for (**A**) sensitive period PN8 pups receiving a single CORT injection and (**B**) sensitive period PN8 pups receiving a double CORT injection. Asterisk represents a significant difference from each of the other groups ($p<0.05$).

Figure 2: Schematic diagram of the circuitry underlying olfactory fear conditioning in adult. Adapted from Best et al., 2005.

Figure 3: Effect of a single CORT injection on the 2-DG relative uptake in neonate (PN8) olfactory circuitry. Bars represent the Mean (±sem) level of 2-DG uptake in (**A**) the medial, lateral, dorsal and ventral nuclei of the AOC, (**B**) anterior and posterior piriform cortex and (**C**) basolateral/ lateral, medial, central and cortical nuclei of the amygdala. Asterisk represents a significant difference from all other groups ($p<0.05$).

Figure 4: Effect of a double CORT injection on the 2-DG relative uptake in neonate (PN8) olfactory circuitry. Bars represent the Mean (±sem) level of 2-DG uptake in (**A**) the medial, lateral, dorsal and ventral nuclei of the AOC, (**B**) anterior and posterior piriform cortex and (**C**) basolateral/ lateral, medial, central and cortical nuclei of the amygdala. Asterisk represents a significant difference from all other groups ($p<0.05$).

Figure 5: Mean (± sem) number of choices toward the conditioned stimulus (CS) odor during the Y-maze test (total of 5 trials) for (**A**) postsensitive period PN12 pups receiving a single CORT injection and (**B**) postsensitive period PN12 pups receiving a double CORT injection. Asterisk represents a significant difference from all other groups ($p<0.05$).

Figure 6: Effect of a single CORT injection on the 2-DG relative uptake in olfactory circuitry in the postsensitive period (PN12). Bars represent the Mean (±sem) level of 2-DG uptake in (**A**) the medial, lateral, dorsal and ventral nuclei of the AOC, (**B**) anterior and posterior piriform cortex and (**C**) basolateral/ lateral, medial, central and cortical nuclei of the amygdala. Asterisk represents a significant difference from all other groups ($p<0.05$).

Figure 7: Effect of a double CORT injection on the 2-DG relative uptake in olfactory circuitry in the postsensitive period (PN12). Bars represent the Mean (±sem) level of 2-DG uptake in (**A**) the medial, lateral, dorsal and ventral nuclei of the AOC, (**B**) anterior and posterior piriform cortex and (**C**) basolateral/ lateral, medial, central and cortical nuclei of the amygdala. Asterisk represents a significant difference from all other groups ($p<0.05$).

Figure 8: Schematic diagram of the interconnections between the amygdala nuclei in adults.

Figure 1

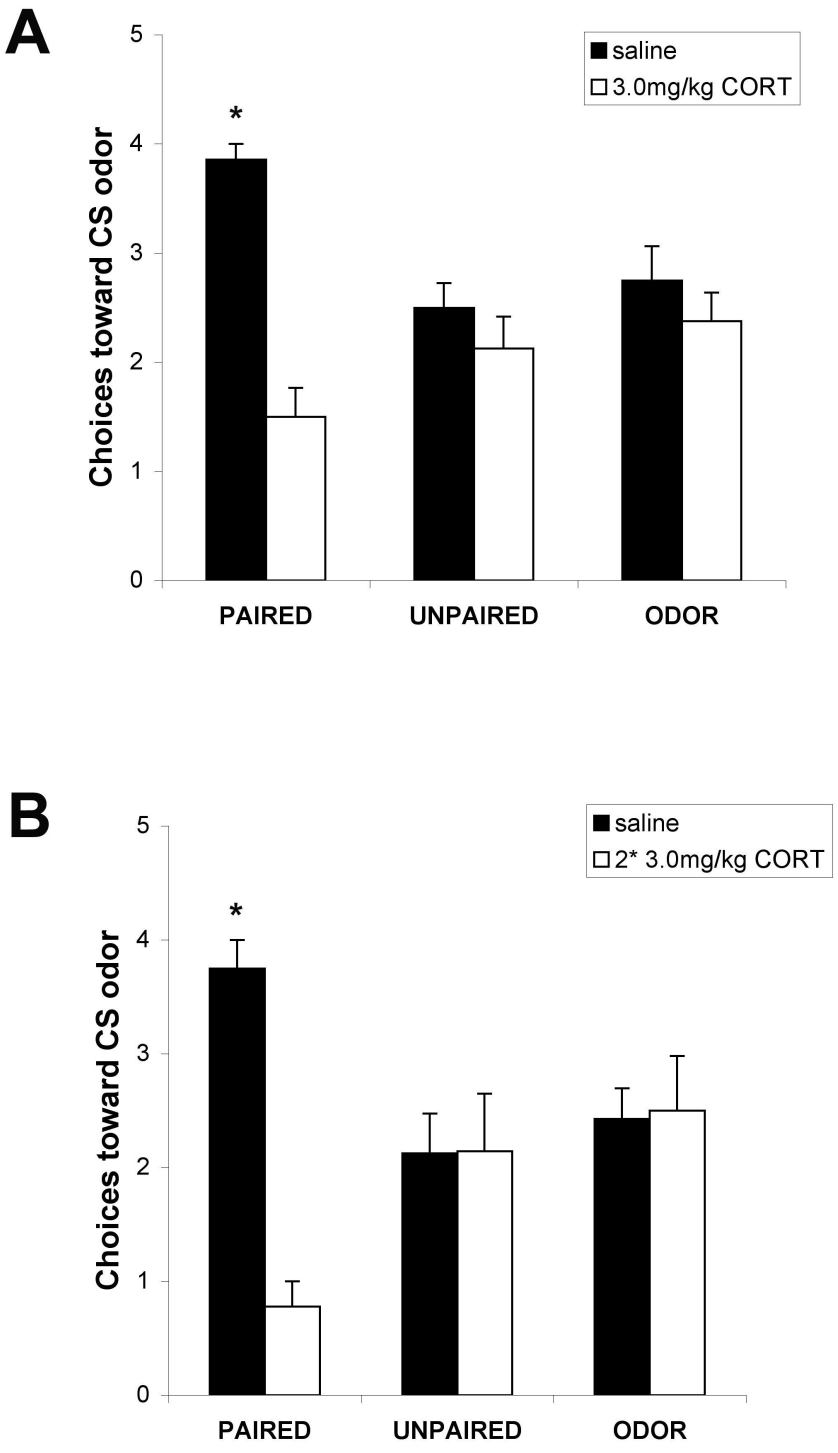

A
saline
3.0mg/kg CORT
Choices toward CS odor
0
1
2
3
4
5
*
PAIRED
UNPAIRED
ODOR
B
saline
2* 3.0mg/kg CORT
Choices toward CS odor
0
1
2
3
4
5
*
PAIRED
UNPAIRED
ODOR

Figure 2

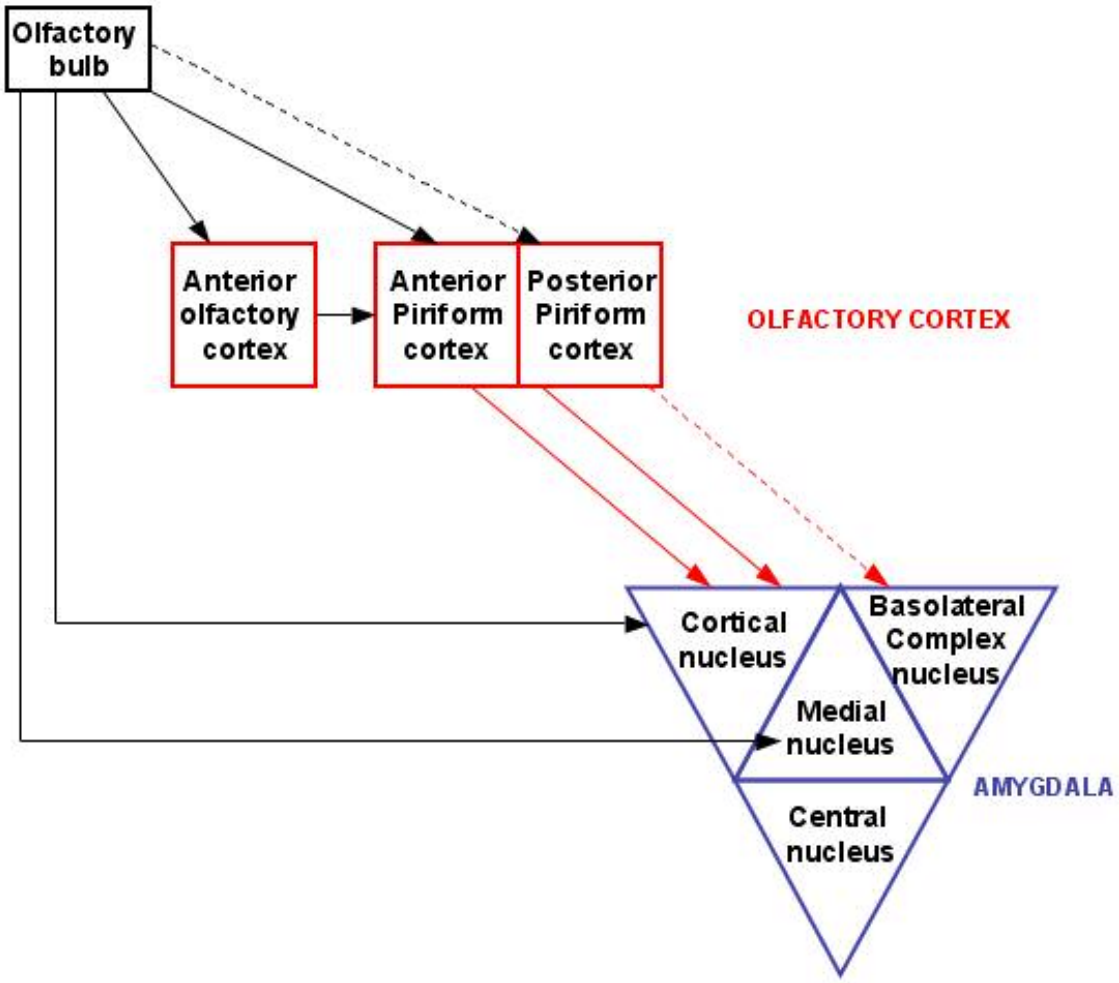

Olfactory bulb
Anterior olfactory cortex
Anterior Piriform cortex
Posterior Piriform cortex
OLFACTORY CORTEX
Cortical nucleus
Basolateral Complex nucleus
Medial nucleus
Central nucleus
AMYGDALA

Figure 3

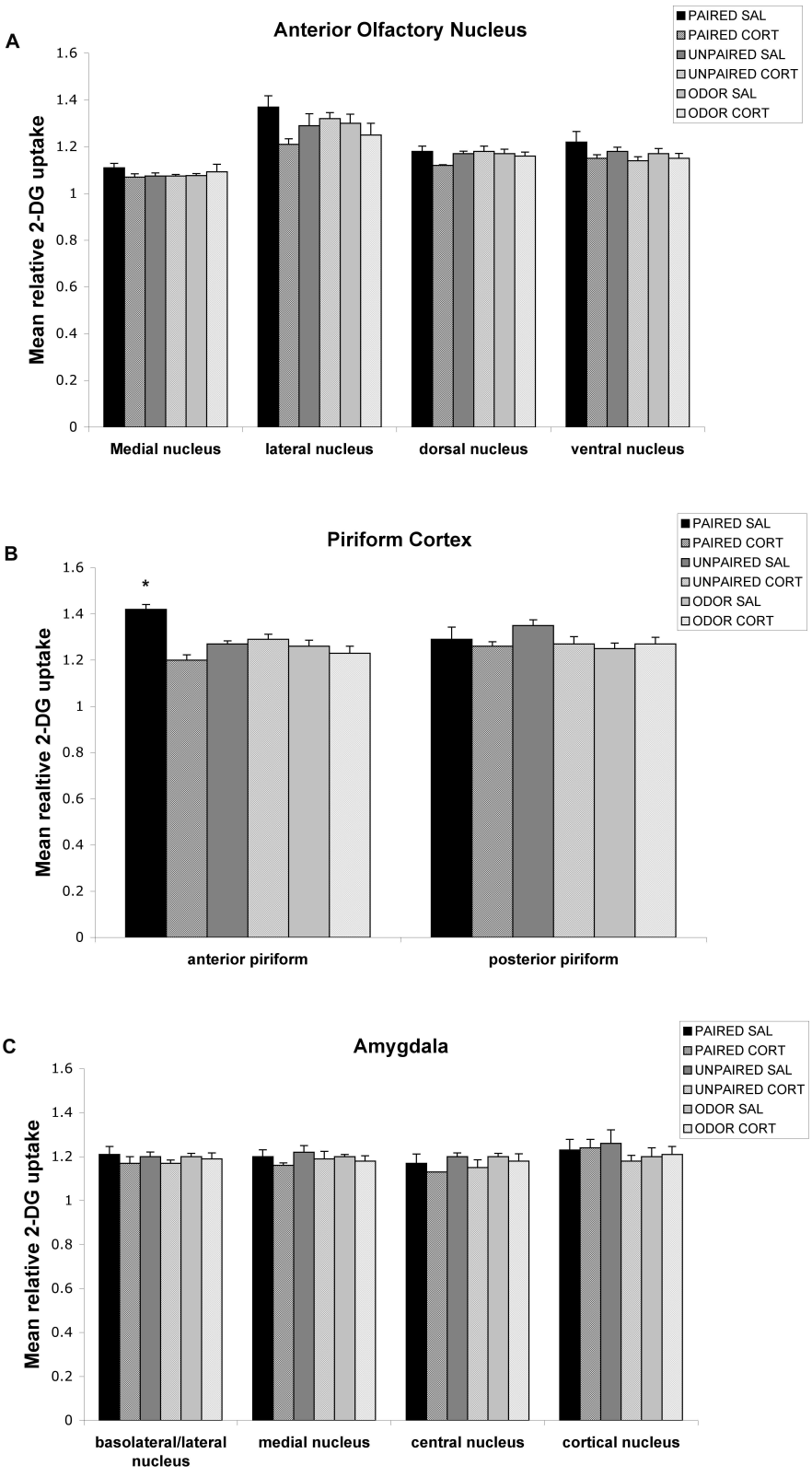

A
Anterior Olfactory Nucleus
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean relative 2-DG uptake
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Medial nucleus
lateral nucleus
dorsal nucleus
ventral nucleus
B
Piriform Cortex
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean realtive 2-DG uptake
*
anterior piriform
posterior piriform
C
Amygdala
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean relative 2-DG uptake
basolateral/lateral nucleus
medial nucleus
central nucleus
cortical nucleus

Figure 4

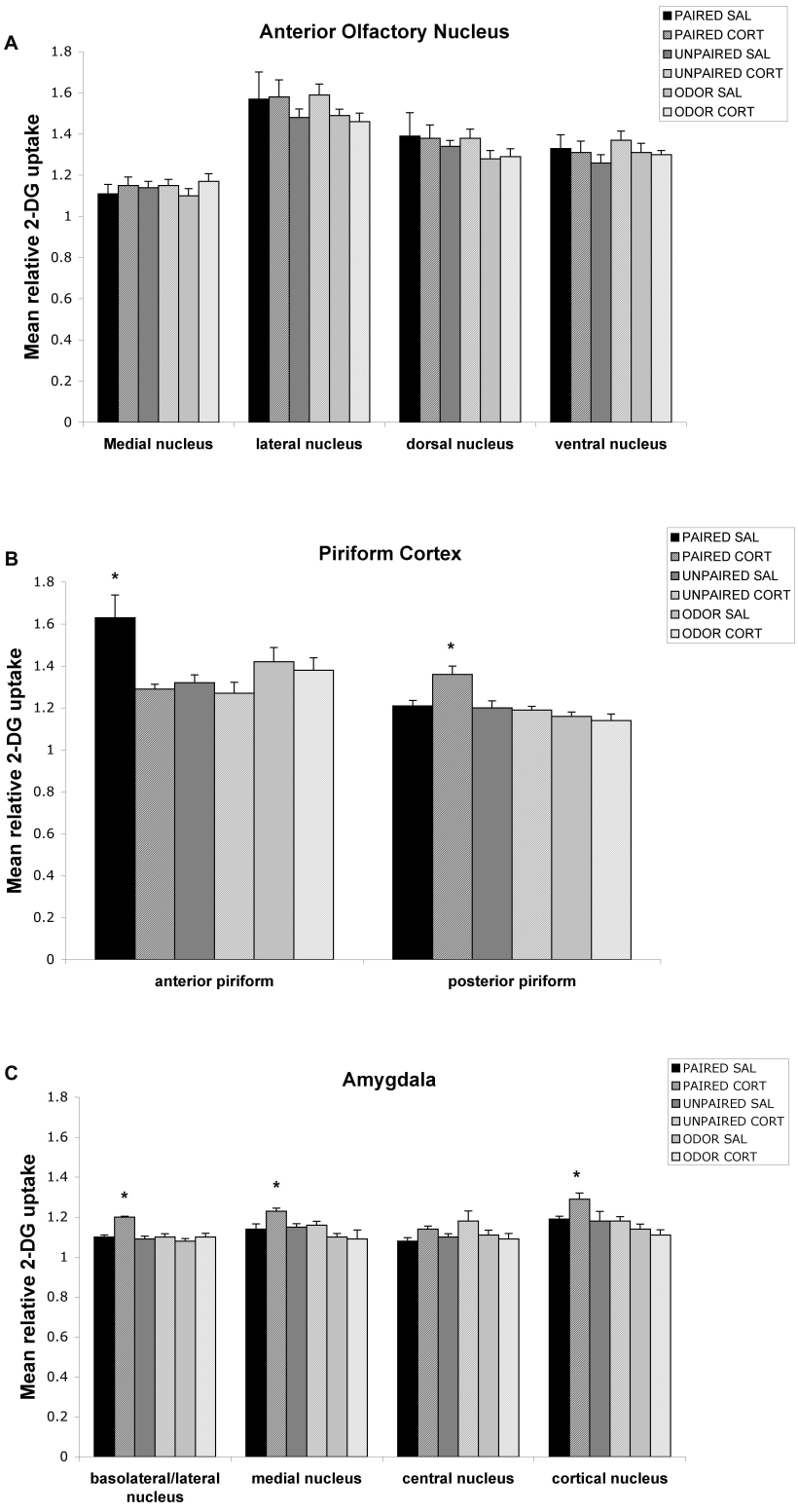

A
Anterior Olfactory Nucleus
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean relative 2-DG uptake
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Medial nucleus
lateral nucleus
dorsal nucleus
ventral nucleus
B
Piriform Cortex
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean relative 2-DG uptake
*
*
anterior piriform
posterior piriform
C
Amygdala
PAIRED SAL
PAIRED CORT
UNPAIRED SAL
UNPAIRED CORT
ODOR SAL
ODOR CORT
Mean relative 2-DG uptake
*
*
*
basolateral/lateral nucleus
medial nucleus
central nucleus
cortical nucleus

Figure 5

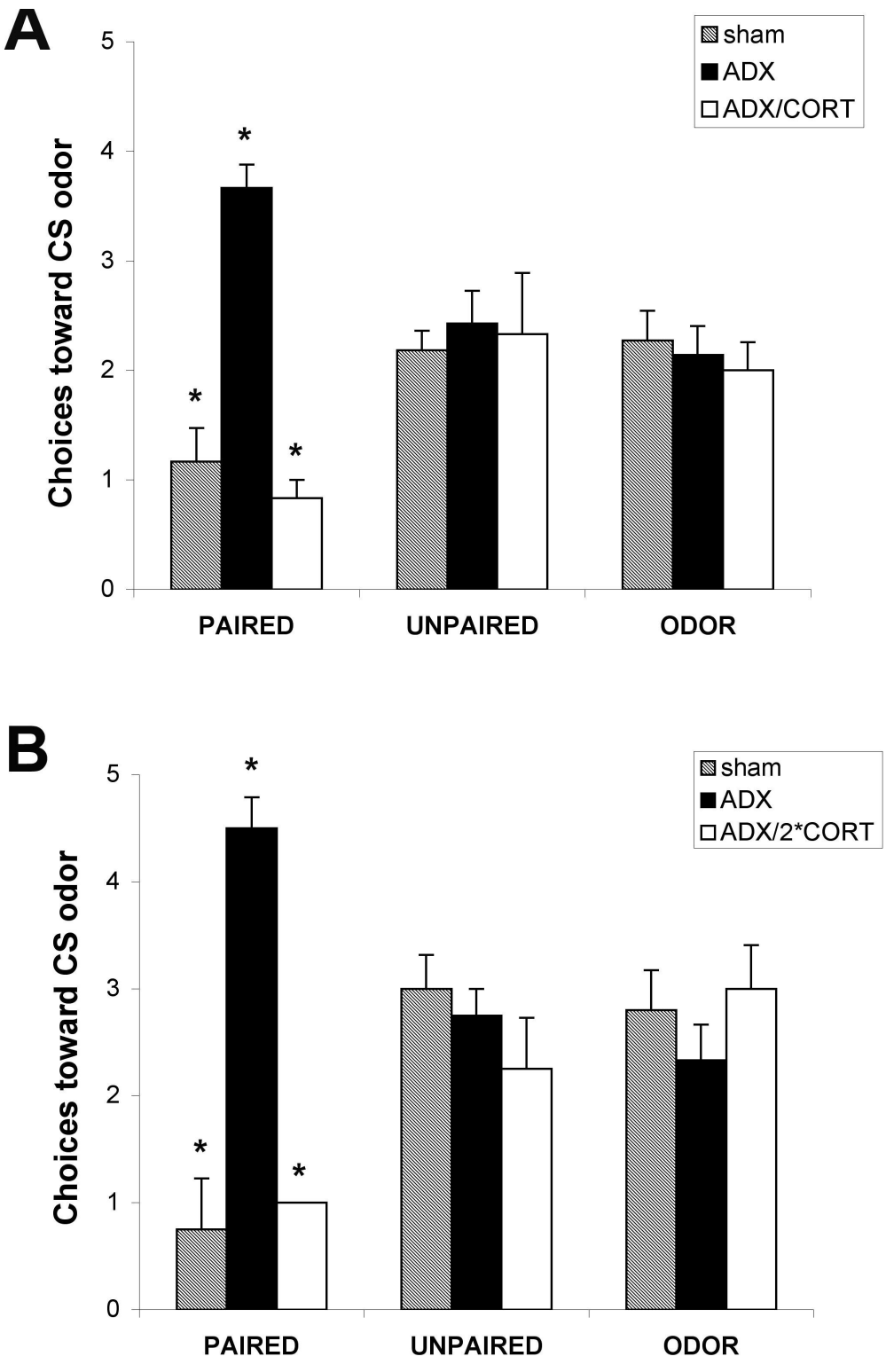

A
sham
ADX
ADX/CORT
Choices toward CS odor
0
1
2
3
4
5
*
*
*
PAIRED
UNPAIRED
ODOR
B
sham
ADX
ADX/2*CORT
Choices toward CS odor
0
1
2
3
4
5
*
*
*
PAIRED
UNPAIRED
ODOR

Figure 6

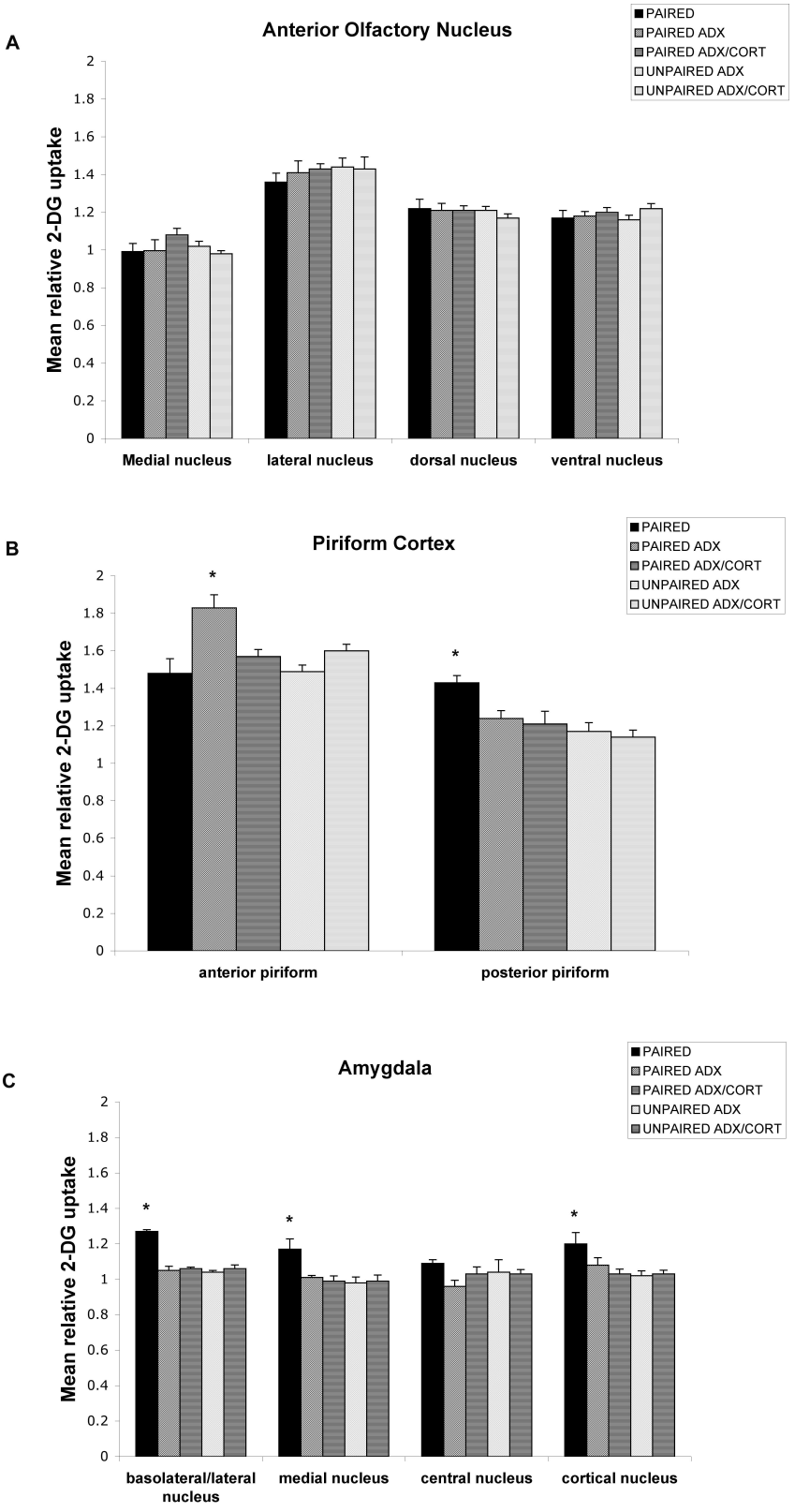

A
Anterior Olfactory Nucleus
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED ADX
UNPAIRED ADX/CORT
Mean relative 2-DG uptake
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Medial nucleus
lateral nucleus
dorsal nucleus
ventral nucleus
B
Piriform Cortex
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED ADX
UNPAIRED ADX/CORT
Mean relative 2-DG uptake
*
*
anterior piriform
posterior piriform
C
Amygdala
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED ADX
UNPAIRED ADX/CORT
Mean relative 2-DG uptake
*
*
*
basolateral/lateral nucleus
medial nucleus
central nucleus
cortical nucleus

Figure 7

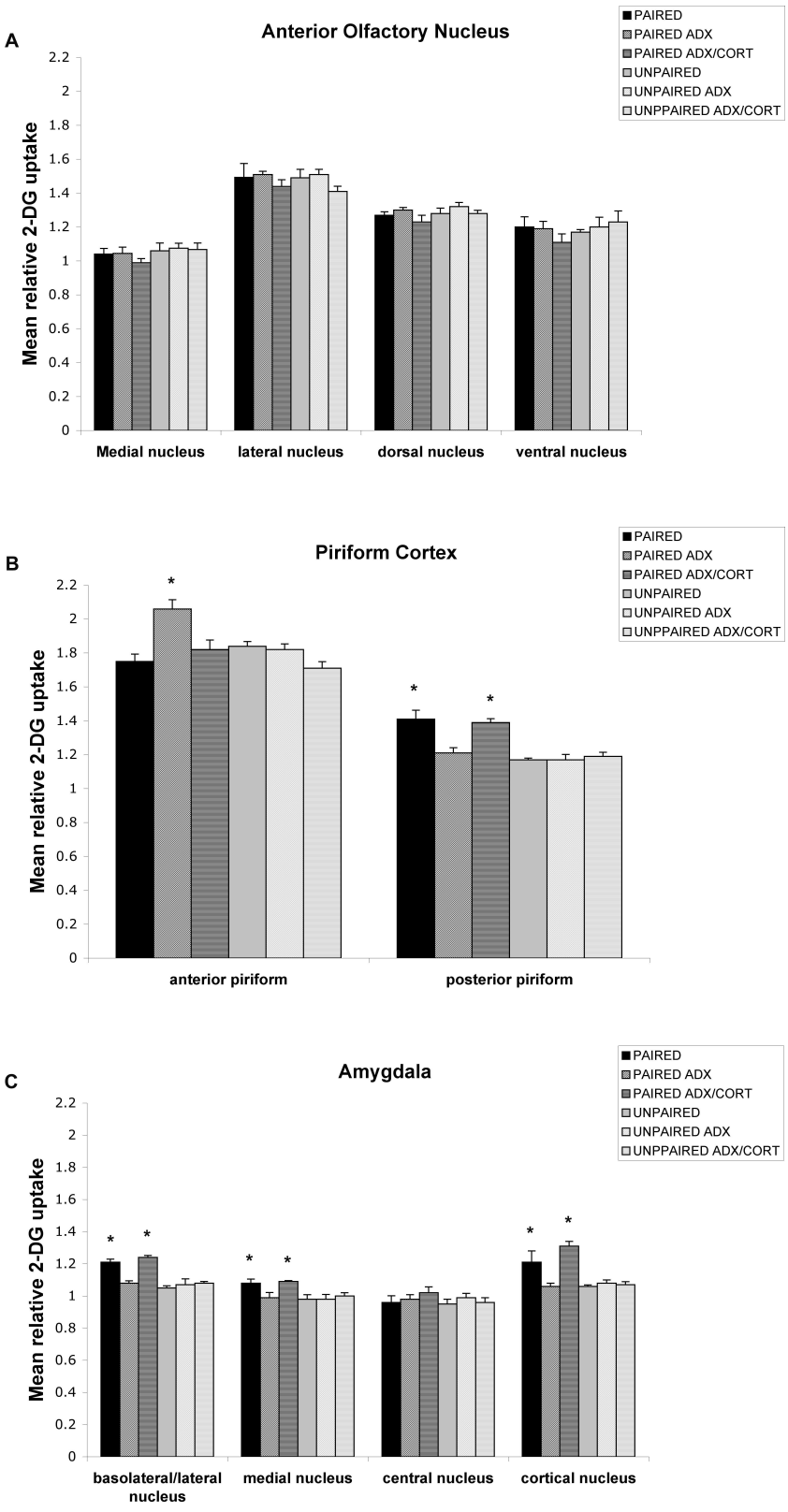
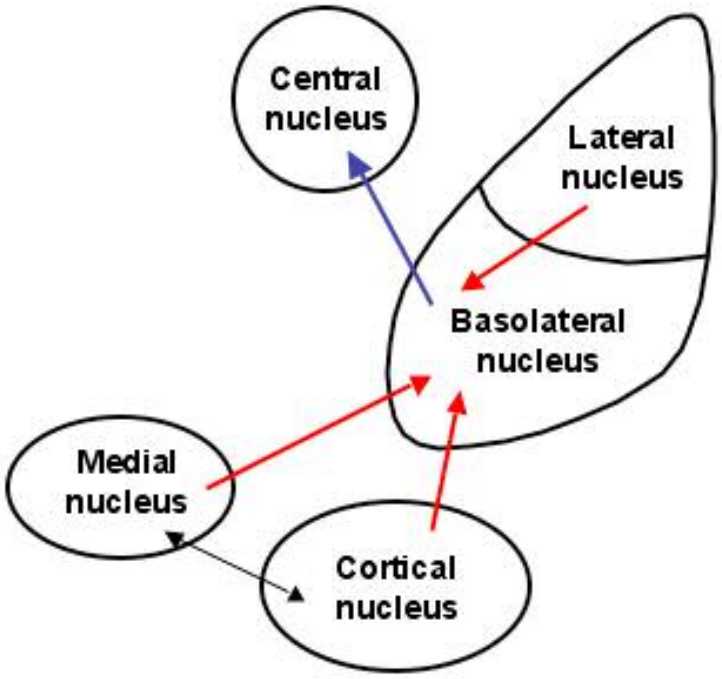

A
Anterior Olfactory Nucleus
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED
UNPAIRED ADX
UNPPAIRED ADX/CORT
Mean relative 2-DG uptake
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Medial nucleus
lateral nucleus
dorsal nucleus
ventral nucleus
B
Piriform Cortex
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED
UNPAIRED ADX
UNPPAIRED ADX/CORT
Mean relative 2-DG uptake
*
anterior piriform
posterior piriform
C
Amygdala
PAIRED
PAIRED ADX
PAIRED ADX/CORT
UNPAIRED
UNPAIRED ADX
UNPPAIRED ADX/CORT
Mean relative 2-DG uptake
basolateral/lateral nucleus
medial nucleus
central nucleus
cortical nucleus

Figure 8


Central nucleus
Lateral nucleus
Basolateral nucleus
Medial nucleus
Cortical nucleus

Chapter 6

Amygdala Corticosterone Manipulation Modifies Fear Conditioning Ontogeny

Abstract

During a sensitive period, neonatal rats express heightened approach learning and attenuated avoidance learning. This is illustrated in our mammalian model of imprinting where neonatal rats rapidly learn an odor preference following olfactory fear conditioning (odor-0.5mA shock pairing). As the sensitive period ends around postnatal day (PN) 10, the same fear conditioning produces an odor aversion. The ontogenetic emergence of fear conditioning appears to be due to functional emergence of the amygdala and an increase in corticosterone (CORT) levels. Previous work from our lab has shown that by prematurely increasing the level of CORT, neonatal pups (PN8) are able to show a precocious ontogeny of fear conditioning and the participation of the amygdala in fear conditioning. This work suggests that the increasing CORT levels are responsible for both the developmental emergence of fear conditioning and the amygdala's participation. Here we assessed whether CORT's action on the amygdala is responsible for the functional emergence of the amygdala and fear conditioning. Specifically, sensitive period pups had CORT (50 or 100ng) or vehicle (cholesterol) directly infused bilaterally into the amygdala through indwelling cannulas during odor-shock conditioning. Results showed that the vehicle infused pups expressed the age-appropriate shock-induced odor preference, while CORT-infused pups expressed a precocious odor aversion. These results suggest a unique role of CORT during the neonatal sensitive period by switching learning from preference to aversion, in contrast to CORT's role in adult learning, which is to enhance/attenuate learning.

Introduction

Fear conditioning is a commonly used paradigm that has greatly enhanced our understanding of the neurobiology of learning and memory. This model's strength lies in its wide phylogenetically representation with similar underlying neural mechanisms (Fanselow, 1994; LeDoux, 2000; Rescorla, 1988; Walters et al., 1981). Here we suggest it may also be a productive approach through which to study development.

In this developmental study, we characterize the trigger responsible for the ontogeny of olfactory fear conditioning, which is not learned until postnatal day (PN) 10 when the amygdala begins to be activated during odor-shock fear conditioning. Younger infant rats learn an odor preference from odor-shock fear conditioning (0.5 mA; Camp and Rudy, 1988; Sullivan et al., 1986a, 2000a) and this learning is correlated with the non-participation of the amygdala (Moriceau and Sullivan, in prep; Sullivan et al., 2000a). Specifically, previous infant work from our lab shows that amygdala participation is necessary to learn fear during odor-shock conditioning (Moriceau and Sullivan, in prep; Sullivan et al., 2000a). Furthermore, the amygdala' s crucial role in adult fear conditioning is well established. The amygdala is activated during fear conditioning and inactivation of the amygdala leads to the disruption of fear conditioning (Cousens and Otto, 1998; Fanselow and Gale, 2003; Honkaniemi et al., 1992; Maren, 2003; McIntyre et al., 2003; Rosenkranz and Grace, 2002; Sevelinges et al., 2004; Sullivan et

al., 2000a; Walker et Davis, 2002). Together, these data strongly suggest a critical role for the amygdala in both infant and adult fear conditioning.

Neurochemical manipulation, however, has distinct effects on infant and adult fear conditioning. For example, CORT can strengthen or weaken adult fear conditioning and it appears to also have a permissive role in amygdala plasticity (Hui et al., 2004; review in Roozendaal, 2000; Roozendaal et al., 1999b; Thompson et al., 2004). However CORT levels in the neonate appear more critical for learning and determine whether a preference or an aversion is learned. Specifically, during the first 10 days of life, neonatal rats learn an odor preference from olfactory fear conditioning, although this same conditioning can produce an odor aversion and amygdala participation when systemic CORT injections are given prior to conditioning. Thus, CORT permits the precocious emergence of fear conditioning (Moriceau and Sullivan, 2004b; in prep). Furthermore, we can extend the age at which odor-shock conditioning produces an odor preference by eliminating endogenous CORT by removal of the adrenal gland, which is the source of CORT (Moriceau and Sullivan, 2004b; in prep). Therefore, we suggest that the naturally occurring CORT increase at the end of the sensitive period is responsible for the developmental emergence of fear conditioning and changes odor-shock conditioning from producing odor preferences to producing odor aversions. Due to the importance of CORT and the amygdala in both infant and adult learning, we assessed the effects of CORT

on the developmental emergence of fear conditioning by directly infusing CORT into the amygdala.

The developmental assessment of CORT's effect on pups' ability to learn fear is important because it may enable us to understand the neurobiology of the unique learning abilities displayed by pups when learning the maternal odor. Specifically, the normally low levels of CORT during the sensitive period prevent pups from learning to avoid odors paired with pain and thus prevent pups from learning to avoid the maternal odor. It should be noted, however, that pups do have occasional increases in CORT during the sensitive period (i.e., due to prolonged maternal deprivation, cold; Gilles et al., 1996; Levine, 2001) and functional CORT receptors are present throughout the brain, including in the amygdala (Alexis et al., 1990; Diorio et al., 1993; Kitraki et al., 1996; Rosenfeld et al, 1993). This suggests that naturalistic variations in the infants' CORT levels may alter termination of the pups' sensitive period for learning.

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 × 29 × 17 cm) lined with pine shavings and were kept in a temperature (23°C) and light (0700-1900 hr) controlled room. Food and water were available ad libitum. The day of parturition was considered PN0 and litters were culled to 12 on PN0-1. No more than one male and one female from a litter were used in each experimental condition. All procedures followed University of Oklahoma and NIH standards for animal treatment.

Surgery. On PN5-6, 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 amygdaloid complex through holes drilled in the overlying skull. Stereotaxic coordinates, derived from an atlas and previous work from our lab (Paxinos et al., 1991; Sullivan and Wilson, 1993), were used for implanting cannulas into the amygdaloid complex (caudal – 0.80 mm; lateral ±3.00 mm from bregma). The cannulas were lowered 5.0 mm from the surface of the skull, placing the tip near the amygdala. 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 training. Following recovery from surgery (generally within 30 min), pups were returned to the dam and littermates for a 2-day recovery period until conditioning.

Pharmacological treatment. On the day of the training, bilateral cannulas were attached via PE10 tubing to a Harvard syringe pump driving two Hamilton microliter syringes. The cannulas were filled (16 sec at 0.5 µl/min) with either drug (described below) or control. During the first 20-min training period, pups with amygdaloid cannulas received either corticosterone (50 ng or 100 ng, Sigma,) or cholesterol (control; Sigma). Drug or control was infused at 0.1µl/min, for a total infusion volume of 2.0 µl as previously described (Sullivan et al., 1992; 2000b; Moriceau and Sullivan, 2004a). Following training, pups were disconnected from the syringe pump and returned to the nest until testing, the following day.

Odor-Shock Conditioning. On PN7-8, pups were randomly assigned to one of the three following conditioning groups: 1) paired odor-shock, 2) unpaired odor-shock, and 3) odor only. Pups were placed in individual 600-ml 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-sec peppermint odor (CS) and a 1-sec 0.5 mA tail shock (US; Lafayette) with an intertrial interval of 4 min. Peppermint odor was delivered by a flow dilution olfactometer (2 L/min flow rate) at a concentration of 1:10 peppermint vapor. Paired odor-shock pups received a shock overlapping with the last 1 sec of the 30-sec odor presentation. Unpaired odor-shock pups received the shock 2

min after each odor presentation. Odor only pups received only the peppermint odor presentation.

To monitor acquisition and general health of pups during conditioning, the number of limbs moving was recorded (0= no movement of the extremities; 5= movement of all 5 extremities) for 20 sec before the odor presentation as well as 20 sec during the odor presentation (Moriceau and Sullivan, 2004b; Sullivan et al., 1994).

Assessing learning. The day following conditioning, pups were given a Y-maze test consisting of a habituation chamber (8.5 cm width, 10 cm length, 8 cm height) and two arms (8.5 $\times$ 24 $\times$ 8 cm) separated via two doors. This test required pups to choose between two arms of a Plexiglas Y-maze, one containing the peppermint odor CS (25 μl of peppermint odor placed on a KimWipe) and the other containing the familiar odor of pine shavings (20 ml of clean shaving in a petri dish). The placement of the CS odor at the end of each arm was counterbalanced. During 5 sec before the door to each arm was opened, a pup was placed in the start box (habituation chamber). Each pup was given 60 sec to choose an arm as determined by a pup's entire body passing the entrance to the alley. Pups received 5 trials with 30 sec between trials and the floor was wiped clean between each trial (Sullivan and Wilson, 1991). The testing was done blind to the training condition and no drugs were infused during testing.

Drug diffusion. In order to characterize the extent of drug diffusion within and outside of the amygdaloid complex, additional pups were used, although the same surgical and drug infusion paradigms were used. The pups were infused with 2 μl of a saline solution of [^{3}H] CORT (1 μCi/μl; NEN Research Products). Twenty min after infusion, the brains were quickly removed and frozen in methylbutane at -45°C and sliced in 20 μm coronal sections. The slides were apposed to a tritium storage phosphor screen (Amersham Biosciences, USA). After 14 days exposure, the screen was scanned at a pixel density of 50 μm (5000 dots per cm^2) with a STORM 820 Phosphor Imager (Molecular Dynamics, Sunnyvale, Calif). Phosphorimaging of the slides results in a TIFF image file (Moriceau and Sullivan, 2004a; Tucker et al., 2002).

Histology. 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 μm) in a -20°C cryostat and cresyl violet staining was used to verify amygdaloid complex cannula placements.

Statistical analysis. Comparisons were made among groups using the analysis of variance test (ANOVA) followed by post hoc Fisher tests (Winkler and Hays, 1975).

Results

Sensitive period CORT infusion into amygdala permits learning of an odor aversion. As shown in Figure 1, control pups, with vehicle infused into the amygdala during the sensitive period (PN8) showed the age specific odor preference learning. However, sensitive-period pups given 50 or 100 ng of CORT directly infused into the amygdala during a paired odor-shock conditioning exhibited a subsequent, precocious aversion for the peppermint odor. ANOVA analysis revealed a significant main effect of training condition ($F_{(2.67)}$ = 10.525, p = 0.0001), a main effect of drug treatment ($F_{(2.67)}$ = 24.899, p < 0.0001), and a significant interaction between training condition and drug treatment ($F_{(4.67)}$ = 17.966, p < 0.0001); post-hoc Fisher tests revealed that pups infused with 50ng or 100ng CORT into the amygdala spent significantly less time over the CS odor than each of the other groups (p< 0.05).

Analysis of behavior during odor-shock conditioning demonstrated that the acquisition curves were significantly different for trial and condition (Figure 2; repeated measure ANOVA; $F_{(20.670)}$ = 7.036, p < .0001) but not by trial and drug ($F_{(20.670)}$ = 1.243, p = .2118).

Drug diffusion. As demonstrated in Figure 3, the volume of drug infused into the amygdala diffused less than 1 mm from the amygdala.

Histology. Cannula tip placements at the amygdala are shown in Figure 4. All tip placements were within 1 mm of the amygdala and targeted the basolateral nucleus, the lateral nucleus or the central nucleus of the amygdala.

Discussion

The present study confirms the importance of CORT in the emergence of fear conditioning but extends these results to suggest that CORT can work directly on the amygdala. Specifically, these results showed that during the sensitive period, local CORT infusion into the amygdala is sufficient to permit the learning of an odor aversion following an olfactory fear conditioning.

The variability of our cannula placements and the drug's spreading limit our assessment of CORT's action within specific amygdala nuclei. However, based on our previous analysis of learning induced changes within different amygdala nuclei and the neonatal CORT distribution pattern, a working hypothesis may be generated. Specifically, we hypothesize that the low sensitive-period CORT levels in the amygdala complex are responsible for pups' inability to learn shock-induced odor aversion and for amygdala nonparticipation.

Pups limited fear conditioning learning relies on the non-participation of the amygdala during neonatal fear conditioning (Sullivan and Wilson, 1993; Sullivan et al., 2000a; Roth and Sullivan, 2005; Moriceau and Sullivan, in prep; Wilson and Sullivan, 2003). Amygdala lesions during the sensitive period do not prevent the acquisition of an odor preference, although slightly longer training is required (Sullivan and Wilson, 1993). However, at the end of the sensitive period, the amygdala participates in fear conditioning (Sullivan et al., 2000a) and in adults, amygdala lesions impair fear learning, confirming is crucial role in adult

fear conditioning (Cahill et al., 1999; Doron and LeDoux, 1999; Fanselow and LeDoux, 1999; Fanselow and Gale, 2003; Goosens and Maren, 2001; Maren, 2003).

Additionally, the amygdala possesses glucocorticoid receptors and they are present in the neonate and the adult rat amygdala (Alexis et al., 1990; Cintra et al., 1993; Kitraki et al., 1996; Morimoto et al., 1996; Sousa et al., 1989). Specifically, systemic CORT injections during the sensitive period permitted the precocious participation of the amygdala in the learning of an odor aversion following an olfactory fear conditioning (Moriceau and Sullivan, 2004, in prep). In adults, CORT acts through amygdala receptors to produce a cascade of physiological and behavioral responses to prepare the animal for danger. Specifically, systemic CORT injection or exposure to a psychological stressor increases the expression of corticotropin-releasing-factor (CRF) mRNA into the central nucleus of the amygdala and increases the fear-conditioned response (Makino et al., 1999; Thompson et al., 2004). Previous findings combined with the present results suggest that CORT's presence permits the activation of the amygdala and ensures the learning of an odor aversion during an painful stimulus. However, the cellular mechanisms of CORT action on the amygdala underlying fear conditioning remain undetermined in neonates.

Pups' sensitive-period low CORT levels are referred to as the Stress Hyporesponsive Period when most stressful stimuli (restraint, shock) do not

produce an increase in CORT levels (Grino et al., 1994; Levine, 1962, 2001; Rosenfeld et al., 1992). Pups' low CORT levels appear to be controlled by sensory stimulation from the mother (Levine, 2001). Specifically, removal of maternal sensory stimulation, such as occurs when pups are separated from the mother for a prolonged period of time (24 hrs) results in an elevation of pups' CORT (Levine, 2001). Replacement of maternal stimulation reinstates pups' low CORT levels (Stanton and Levine, 1990; Suchecki et al., 1993). Cold also appears to produce an increase in CORT levels, even without maternal separation (Gilles et al., 1996), suggesting complex environmental control over CORT regulation that correlates well with the mother's treatment of her pups.

Until the end of the sensitive period (PN10), neonatal rats have an attenuated ability to learn fear, as demonstrated by the learning of an odor preference during an odor-shock paradigm (Sullivan et al., 2000a; Moriceau and Sullivan, 2004b). The shock-induced odor preference learning seen in sensitive-period pups is not due to pups' inability to feel pain since pups' pain threshold does not change as the sensitive period ends, as indicated by vocalizations and escape behaviors in response to shock (Barr, 1995; Emerich et al., 1985; Stehouwer and Campbell, 1978; Sullivan et al., 2000).

Restrictions on pup learning are not limited to fear conditioning since other learning paradigms that produce an avoidance or inhibition of behaviors are also attenuated in infant rats, such as inhibitory conditioning and passive avoidance

(Bialik et al., 1984; Blozovski and Cudennec, 1980; Camp and Rudy, 1988; Collier et al., 1979; Myslivecek, 1997). For at least some of the attenuated learning of sensitive-period pups, the neonate's low CORT levels appears to be a common underlying factor. For example, preventing the increase of CORT by adrenalectomy is sufficient to block the normal emergence of inhibitory conditioning until PN17 (Bialik et al., 1984). This suggests that low CORT levels during the sensitive period may prevent pups from learning to avoid or inhibit learned odors associated with the mother. From an evolutionary perspective it would be maladaptive for pups to learn to avoid maternal odor since approach to maternal odor is required for the milk and warmth provided by the dam.

Low CORT levels may underlie pups' inability to show unlearned fear as well as learned fear. Specifically, pups fail to show fear to natural predator odors during the sensitive period (Moriceau et al, 2004; Takahashi, 1994a), when the amygdala is not yet functional (Weidenmayer and Barr, 2001). Furthermore, Takahashi (1994) was able to alter the developmental expression of unlearned fear (predator odor) through manipulations of the CORT system (Moriceau et al., 2004, Takahashi, 1994). We extended Takahashi's work by demonstrating CORT's ability to modify the amygdala's participation in this behavior (Moriceau et al., 2004). For example, increasing the neonatal CORT level permitted the behavioral expression of fear through freezing and the activation of the basolateral complex of the amygdala during presentation of an adult male odor.

Also, depletion of CORT retarded the normal expression of fear and the participation of the basolateral complex of the amygdala.

During the neonatal period, CORT seems to have a unique effect, by changing odor-shock conditioning from preference learning to aversion learning. This is in sharp contrast to CORT effects on adult learning which only modify how well a behavior is learned (Corodimas et al., 1994; Pugh et al., 1997; Roozendaal et al., 1996). In adults, CORT injection permitted the expression of more fear-conditioned freezing and removal of the source of CORT reduced fear conditioning (Hui et al., 2004; Pugh et al., 1997; Thompson et al., 2004).

It should be noted that sensitive-period pups are able to learn an odor aversion following odor-illness conditioning either via LiCl injection or very high shock levels (1.0-1.5 mA; Haroutunian and Campbell, 1979; Rudy and Cheatle, 1977; Sullivan and Wilson, 1995). Although odor/taste-illness learning is also restricted since suckling during this conditioning will prevent the learning of an odor aversion (Thiels and Alberts, 1991). LiCl illness-induced avoidance learning does not appear to require the amygdala, although it has been shown to be capable of participating in this conditioning (Bermudez-Rattoni and McGaugh, 1991; Schafe et al., 1998; Yamamoto et al., 1995).

To conclude, our results suggest that CORT permits odor aversion learning during the sensitive period though its action on the amygdala. This

suggests that low CORT levels normally prevent pups from learning to avoid the maternal odor that guides pups' interactions with the mother and underlies nipple attachment. It is possible that naturalistic modulation of pups' CORT levels though alterations in maternal care may result in either an extension or reduction in the length of the sensitive period.

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Figure captions

Figure 1: Mean (± SEM) number of choices toward the conditioned stimulus (CS) odor during the Y-Maze test. Training infusion of amygdala CORT permitted the learning of a relative odor aversion compared with each of the other groups. Asterisks represent significant differences from the control groups ($p<0.001$).

Figure 2. Mean (± sem) number of responses (behavioral activity) during the odor conditioned stimulus (CS) presentation during infusion of amygdala CORT or cholesterol during an odor-shock acquisition.

Figure 3: Section from a PN8 pup counterstained with cresyl violet and characterizing the extent of H^3 CORT drug diffusion within the amygdala. This data is preliminary and for illustration purpose only.

Figure 4: Locations of cannula tips (solid circles) in rats used for the experiment shown in Figure 1. The amygdala is marked by the horizontal arrows. Corresponding sections from the adult stereotaxic atlas of Paxinos et al. (1991) were determined, and relative distance from bregma for each coronal section based on the adult atlas is noted on the right.

Figure 1

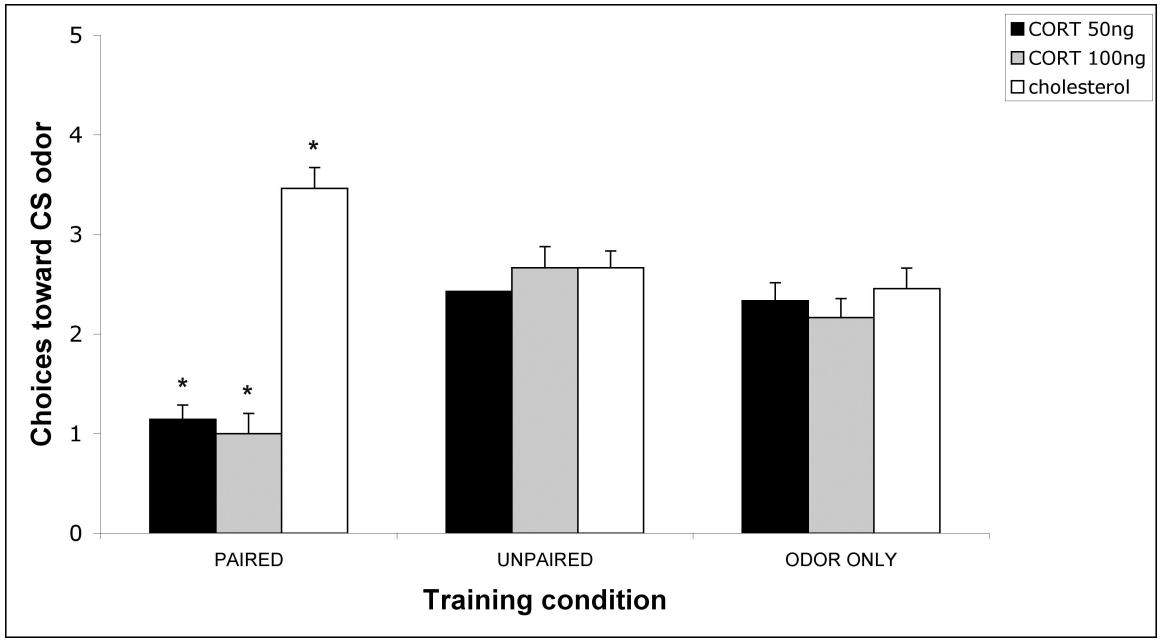

CORT 50ng
CORT 100ng
cholesterol
Choices toward CS odor
0
1
2
3
4
5
*
*
*
PAIRED
UNPAIRED
ODOR ONLY
Training condition

Figure 2

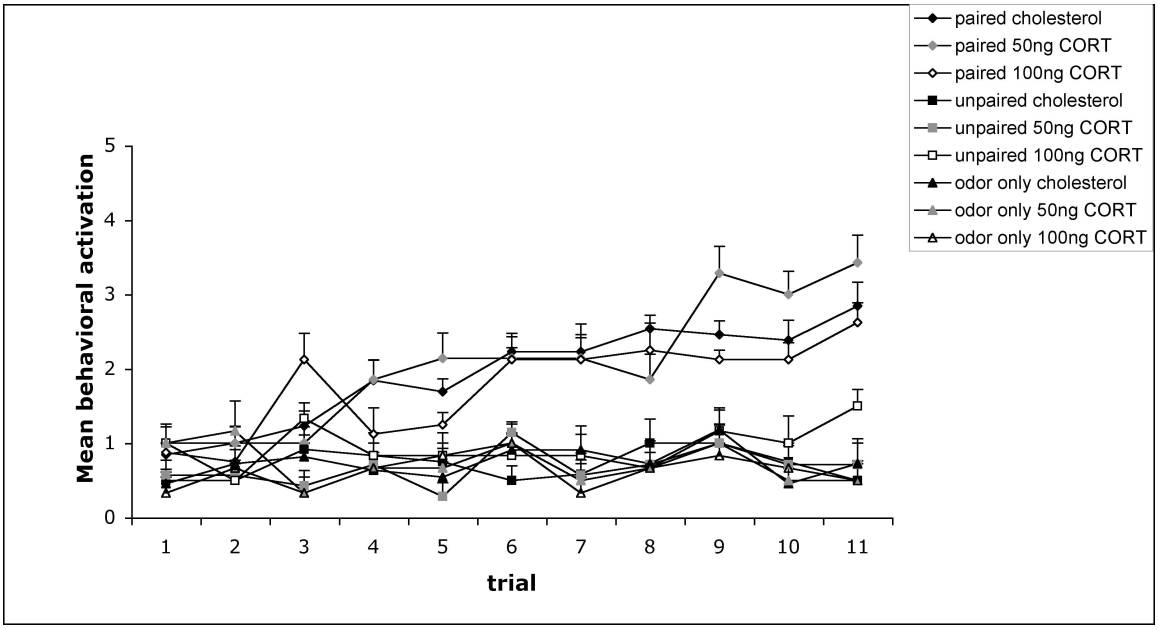

paired cholesterol
paired 50ng CORT
paired 100ng CORT
unpaired cholesterol
unpaired 50ng CORT
unpaired 100ng CORT
odor only cholesterol
odor only 50ng CORT
odor only 100ng CORT
Mean behavioral activation
0
1
2
3
4
5
1
2
3
4
5
6
7
8
9
10
11
trial

Figure 3

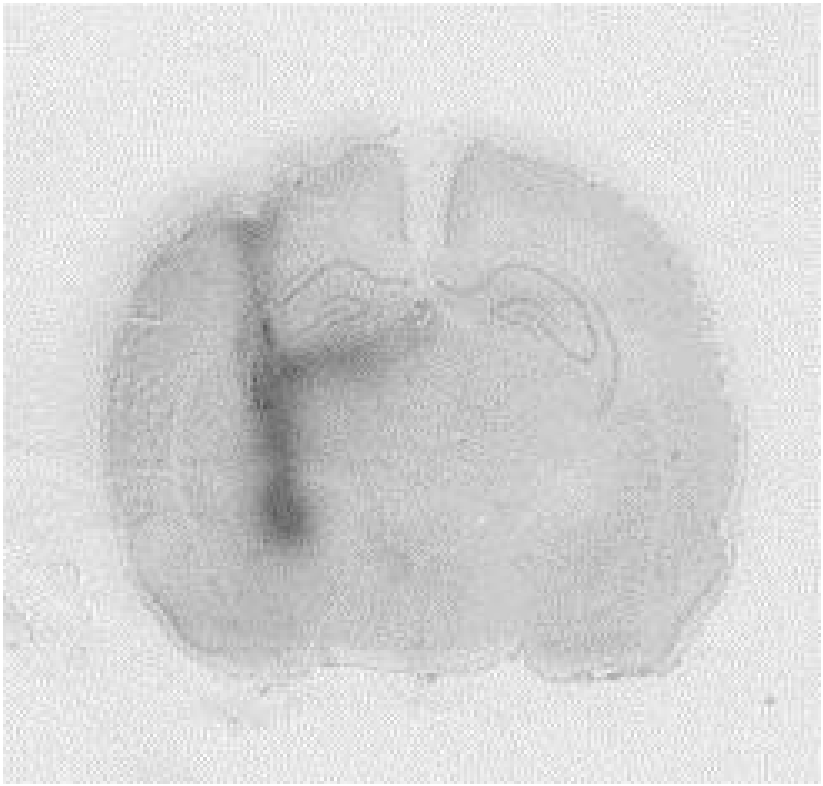
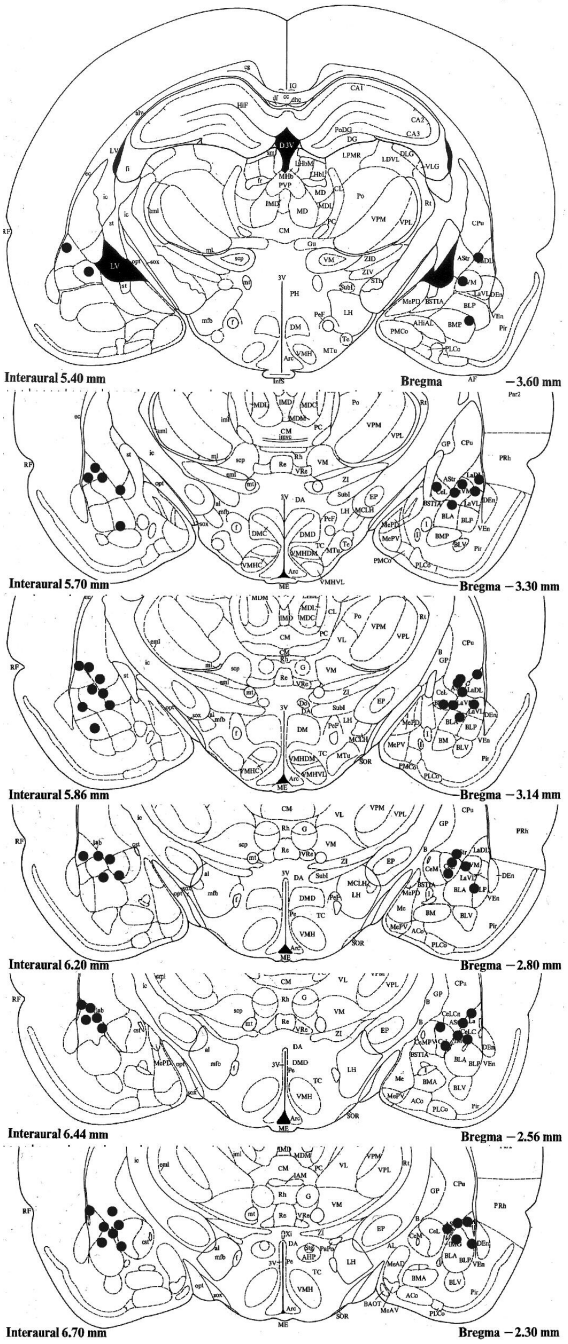


Figure 4


Interaural 5.40 mm
Bregma −3.60 mm
Interaural 5.70 mm
Bregma −3.30 mm
Interaural 5.86 mm
Bregma −3.14 mm
Interaural 6.20 mm
Bregma −2.80 mm
Interaural 6.44 mm
Bregma −2.56 mm
Interaural 6.70 mm
Bregma −2.30 mm

Chapter 7

Research Summary

Altricial species, such as humans and rats, must be able to identify, learn, and remember their caregiver in order to support the early attachment to their caregiver. This early attachment is characterized by an enhanced ability to acquire preferences and a decreased ability to acquire aversion presumably to constrain the infant to learning only preferences to the caretaker. This attachment learning has a wide phylogenetic representation and permits altricial animals to easily form a repertoire of proximity-seeking behaviors to the primary caregiver, regardless of the quality of the care they receive. For example, in avian imprinting, a chick will continue to follow its caretaker even while being shocked, but at the end of the imprinting period, the same shock will provoke an aversion (Hess, 1962). A similar experiment in young dogs showed that puppies display a strong attachment to a handler providing rough treatment or neglect. Nonhuman primates and human children also demonstrated strong attachment to their caretaker (Harlow and Harlow, 1965; Helfer et al., 1997; Rajecki et al., 1978; Sanchez et al., 2001).

While it may be adaptive for an immature organism to form an attachment to its caregiver, regardless of the quality of caregiving, this system becomes maladaptive as the infant matures and begins to become more independent. Thus, maturation of the animal necessitates the end of the attachment learning of the sensitive period and requires a more complex learning system more suitable to the life outside the nest. While there is some understanding of the neural mechanisms which underlie the sensitive period (reviews: mammals, Sullivan, 2001; avian, Rogers, 1993), there is very little known about what neural

mechanisms are supporting the sensitive period learning or its termination. The research presented in this dissertation represents an attempt to advance our understanding of the neural mechanisms involved in the transition from the attachment learning period to the end of this period.

To answer this question we used our neonatal rat paradigm that follows the characteristics of attachment seen in other species. In effect, during what we called the sensitive period (until postnatal day 10), neonatal rats exhibit an enhanced ability to acquire learned odor preferences and a decreased ability to acquire learned odor aversions even while receiving rough treatment from the mother inside the nest. These unique learning abilities, which ensure attachment during the sensitive period, are due to unique neural circuitry distinct from an adult rat.

First, it was well known that the enhanced preference learning is due to the strong noradrenergic input from the locus coeruleus (LC) to the olfactory bulb during the acquisition of olfactory associative learning and that this input was necessary and sufficient for learning. Specifically, the acquisition of a conditioned odor preference is blocked either by blocking norepinephrine (NE) receptors in the bulb or by destroying the LC. Also, the association of an odor with the activation of olfactory bulb NE β-receptors results in the learning of an odor preference. Increasing olfactory bulb NE by stimulating the LC during an odor presentation is sufficient to support odor learning. During the sensitive period, NE

is required for the maintenance of the prolonged mitral cell response necessary for acquisition of an odor preference and olfactory bulb learning-induced changes. At the end of the sensitive period, the LC's changing role in learning appears to be caused by developmental changes in the LC. In effect, neonates show prolonged excitation of the LC and it releases enormous amounts of NE compared to the level released after the sensitive period. *My research* has established a role for the maturing LC in terminating the sensitive period's rapid and robust preference learning. At the end of the sensitive period, the functional emergence of LC $\propto$2 inhibitory autoreceptors and the downregulation of LC $\propto$1 excitatory autoreceptors underlie the dramatic reduction in NE release. We have shown that pups learned an odor preference when a novel odor was paired with either olfactory bulb infusion of a β-receptor agonist (isoproterenol) or direct LC cholinergic stimulation combined with $\propto$2 antagonists and $\propto$1 agonists, which attempts to recreate neonatal levels of LC autoreceptor activity. These data indicate that the sensitive period, at least in part, is terminated through functional changes in LC autoreceptors.

Second, the amygdala is a brain area required for fear conditioning in preweanling and adult rats. It was previously established that, during the neonatal sensitive period for learning, the lack of participation of the amygdala may underlie neonatal pups' attenuated ability to learn to avoid odors paired with pain. In effect, sensitive period learning is characterized by the limitations put on learning passive avoidance, active avoidance, and inhibitory conditioning which

are behaviors associated with amygdala function emerging around PN10. Specifically, odor-shock conditioning results in pups learning a preference for that odor as indicated by orienting towards the odor and by failure of the amygdala to participate in preference learning.

It is also known that the sensitive period is characterized by an attenuated neonatal CORT response to stressful stimuli and behaviors normally emerging at PN10-11 can be delayed or advanced ontogenetically simply by removing the source of CORT or by prematurely elevating CORT levels. *Thus my research* has permitted the establishment of a role between the attenuated levels of CORT and the decreased ability to acquire learned odor aversions presumably due to the nonparticipation of the amygdala in learning. Using our odor-shock (0.5 mA) conditioning paradigm to examine the effects of manipulating CORT levels on learning during the sensitive period or postsensitive period, we demonstrated that CORT injections prior to PN8 conditioning prevented the learning of a shock-induced odor preference and that PN12 CORT-depleted (by adrenalectomy) pups demonstrated shock-induced odor preference learning. CORT replacement in ADX PN12 pups enabled pups to learn a shock-induced odor aversion. These data suggest that during the sensitive period, naturally low levels of CORT maintain pups' unique abilities to rapidly learn olfactory-based attachment to the mother even when interactions with the mother are painful. Furthermore, we were able to alter the developmental expression of unlearned fear (predator

odor) through manipulations of the CORT system similar to those described previously.

The impact of CORT within the olfactory neural circuitry was also established. We have shown that increasing CORT over 24 hours, during the sensitive period, induces the participation of brain areas implicated in adult fear learning (posterior piriform cortex and cortical, basolateral/lateral, and medial nuclei of the amygdala). Also, we have demonstrated that the unique neural circuitry involved in preference learning (increased olfactory bulb and anterior piriform cortex neural activity) can be maintained beyond the end of the sensitive period by experimentally maintaining low levels of CORT (ADX) in older pups and that CORT replacement in older ADX pups reinstates the normal balance of neural activation in PN12 pups (posterior piriform cortex and cortical, basolateral/lateral, and medial nuclei of the amygdala).

In summary, my dissertation research indicates that CORT appears to have an important role in ending the sensitive period. Since there are low levels of CORT during the sensitive period and these levels are maintained by the mother's stimulation of the pups, the quality of care-giving may be important in controlling sensitive period termination. Specifically, during the sensitive period, milk, nursing, and sensory stimulation from the mother keep pups' CORT levels low, although simple maternal presence can also lower CORT (Huot et al., 2002; Kent et al, 1997; Rosenfeld et al., 1991; Stanton et al., 1988; Wiedenmeyer et al., 2003). Even in postsensitive period pups, the presence of an anesthetized dam

attenuates pups' response to shock and prevents the novelty-induced increase in CORT (Richardson et al., 1989; Stanton and Levine, 1990; Wiedenmeyer et al., 2003).

In humans, the infant attachment experience sets the stage for adult mental and physical health (Connor et al 2003; Gunnar 2003; Heim and Nemeroff 2001; Teicher et al 2003; Zeanah et al 2003). There is also evidence for this in rats. Specifically, early attachment-related odors appear to retain value into adulthood, although the role of the odor in modifying behavior appears to change with development. Work done independently in the labs of Celia Moore (Moore et al., 1996) and Elliot Blass (Fillion and Blass, 1986), demonstrated that adult rats exhibited enhanced sexual performance when exposed to the natural and artificial odors learned in infancy. These results support observations on the role of early experience on adult mate preference in other species, such as occurs in imprinting. Infant-mother interactions also affect long-term changes in brain development (limbic system: hippocampus, amygdala; stress axis: locus coeruleus, hypothalamus, amygdala; and cerebellum) in both humans and animals. Indeed, there have been numerous studies over the past few decades, which clearly indicate that infant experiences strongly influence adult behaviors (Denenberg, 1963; Harlow and Harlow, 1965; Levine, 1962). Additionally, early stress greatly potentiates the expression of adult psychiatric problems and the locus coeruleus (releases NE), amygdala, and the hypothalamus-pituitary-adrenal (releases CORT) all seem to be vehicles of this stress effect (Bremner et

al., 1993; Coplan et al., 1996; Heim et al., 1997). The convergence of adult systems affected by early adverse experiences and the neural circuitry used for attachment are provocative. Indeed, it suggests that the action of NE and CORT on the structures supporting neonatal learning may underlie the long-term effects of adverse experiences in infancy. The long-term effects of early attachment will be the focus of my future research.

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