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ENDOGENOUS OPIOID MODULATION OF THE NEURAL AND BEHAVIORAL CORRELATES OF LEARNING AND MEMORY NECESSARY FOR INFANT ATTACHMENT

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ENDOGENOUS OPIOID MODULATION OF THE NEURAL AND BEHAVIORAL CORRELATES OF LEARNING AND MEMORY NECESSARY FOR INFANT ATTACHMENT

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Preface

I, Tania Roth, hold a principle author status for all the manuscript chapters in this dissertation. However, Chapters 1-6 are co-authored by my supervisors, whose contributions greatly facilitated the development of my research.

A large part of Chapter 1 consists of a review chapter co-authored by Donald A. Wilson and Regina M. Sullivan. The review chapter is an article in the 34th volume of *Advances in the Study of Behavior* (JB Slater, JS Rosenblatt, CT Snowdon, TJ Roper, HJ Brockmann, and M Naguib, Eds.), and will be published in December 2004.

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Abstract

Disorganized attachment in which a child attaches to an abusive caregiver is a significant risk factor for childhood and adult psychiatric disorders. Both animal and clinical studies suggest that disorganized attachment behaviors are often precipitated by disruptions to development of the endogenous opioid system, as occurs with prenatal opiate exposure or postnatal maltreatment. The goals of this dissertation research were to 1) contribute to the understanding of the neurocircuitry supporting attachment despite abuse, 2) to assess the role of the endogenous opioid system in this learned attachment behavior, and 3) to assess the role of opioid modulation of the attachment neurocircuitry. Experiments utilized our lab's unique model of infant caregiver abuse that capitalizes on rat pups' dependence on maternal odor learning for attachment. In the rat, infants must learn an odor preference regardless of the quality of maternal care to secure attachment, and learned odor aversions thwart attachment. Results demonstrate that endogenous opioids are necessary for the acquisition, memory consolidation, and expression of neonate odor preferences. Furthermore, opioids play a pivotal role in the memory formation of odor preferences despite abuse, as disruption to the opioid system yields odor aversions. Assessment of the attachment circuitry supporting odor – abuse conditioning suggests that cellular changes within the olfactory bulb, the anterior piriform cortex, and the lack of significant changes in the amygdala, an area intimately associated with the memory of fear and aversions in older pups and adults, contribute to readily learned odor preferences. Results also suggest that opioid modulation of the cellular activity within this attachment circuitry plays a pivotal role in securing learned odor preferences. More importantly, opioids appear to limit amygdala participation in neonate memory formation despite abuse, and disruption of opioid activity within the neonate amygdala yields odor aversions. Overall, results indicate a prominent role of the endogenous opioid system in mediating the neonate learning and memory

necessary for attachment, and thus highlight how prenatal or postnatal disturbances to the developing opioid system jeopardize infant attachment. Furthermore, these results offer an avenue into understanding how these early adverse experiences affect the attachment process and subsequent emotional development.

Chapter I

An introduction to the neurobehavioral development of infant learning and memory, the endogenous opioid system, and the importance of opioid modulation of attachment behavior

This introduction chapter reviews the current understanding of the learning and memory responsible for infant attachment in the rat neonate (*Rattus norvegicus*). The first section defines attachment, and is followed by a section that discusses the unique neural and behavioral characteristics of infant learning and memory. This is followed by a section characterizing the endogenous opioid system and its relevance to infant behavior, and the final section provides rationale for the experiments undertaken here.

I. ATTACHMENT

The environmental demands on an altricial newborn are simple: from the caregiver procure food, warmth, and protection, and rapidly form an attachment to the caregiver. While pheromones mediate this attachment in some species (reviewed in Bartoshuk and Beauchamp, 1994; Hudson and Distel, 1999; Schaal *et al.*, 1995), other species use learning, with avian imprinting being one of the most widely known examples of the latter. Similar learning during infancy has also been documented in mammals such as the human (DeCasper and Fifer, 1980; Sullivan *et al.*, 1991), sheep (Nowak and Lindsay, 1992; Nowak *et al.*, 1997) and rat (reviewed in Hofer and Sullivan, 2001). Although this infant learning shares many characteristics with adult learning, some of the neural structures supporting learning and memory consolidation in the adult are not yet fully developed in the infant, suggesting that the neural basis for learning and memory differs between infants and adults. In this chapter, we will present evidence that the infant's behavior and its brain are specifically designed to meet the demands of infancy and to ensure attachment to the caregiver.

John Bowlby (1965) originally documented attachment in human children, and the basic characteristics of human attachment and behavior that he described are briefly

reviewed here. First, children form rapid, strong attachments to their primary caregiver and seek proximity to the caregiver. Second, as highlighted in clinical reports, children will undergo considerable abuse yet remain in contact with the abusive caregiver (Helfer *et al.*, 1997), although, clinically, these children show some disturbed attachment characteristics (Hesse and Main, 2000; O'Connor and Rutter, 2000; reviewed in Morton and Browne, 1998). Finally, Bowlby's claim that the infant-caregiver relationship defines subsequent adult relationships can be documented most clearly in cases when these abused children later form adult relationships that often involve insecure attachments and abusive behavior (Mullen *et al.*, 1994; Smallbone and McCabe, 2003; Styron and Janoff-Bulman, 1997).

Bowlby's description of attachment applies to other species. Rapid attachment occurs in a variety of species, with imprinting as the classic example. Moreover, attachment despite abuse is widespread in the animal kingdom. For example, during the critical period for imprinting, chicks can be shocked while following the surrogate, yet following still occurs (Hess, 1962). This same treatment of a chick just hours after the critical period has closed produces an aversion to the surrogate. Similarly, infant dogs neglected, mishandled, or shocked by the caregiver also form a strong caregiver attachment, but the same situation produces quickly learned aversions in older dogs (reviewed in Rajecki *et al.*, 1978). Perhaps one of the more dramatic examples of infant attachment in an abusive caregiver relationship was documented by Harry Harlow in nonhuman primates (Harlow and Harlow, 1965). Specifically, maternally deprived infants were permitted to mature, mate and give birth. Lacking mothering skills compounded with having a disturbed nature, these animals severely mistreated their young, yet the abused infants still developed a strong attachment to their caregivers. More recently, a model of abuse in nonhuman primates at Yerkes National Primate Research Center has documented attachment after far more moderate abuse by

presumably healthy, normal mothers (e.g. Maestripieri *et al.*, 1997, 1999). Although it may appear maladaptive to attach to an abusive caregiver, when one considers survival in altricial species is greatly compromised without a caregiver (no food, protection or warmth), evolution may have carved a learning attachment system in some species that functions regardless of the quality of parental care.

II. UNIQUE CHARACTERISTICS OF INFANT LEARNING

For many altricial species, at least some learning is required for attachment to the caregiver. Using our rat model of attachment, we suggest that the unique infant learning characteristics documented below support this attachment system and ensure that the animal forms a repertoire of proximity-seeking behaviors directed toward the caregiver, regardless of the quality of care received. Indeed, as is illustrated in this review of the developmental learning literature on rats, the infant readily learns approach responses and not avoidance responses, ultimately supporting attachment under a variety of conditions. This period of unique infant learning ends at postnatal day (PN) 10 and is referred to as the sensitive period for learning.

A. ACQUISITION OF INFANT LEARNING

Learning involves the acquisition of a change in behavior to an environmental stimulus (reviewed in Abel and Lattal, 2001). A large body of literature on the development of learning in rodents suggests that acquiring new information appears quite similar in infant and adult rats (reviewed in Campbell, 1984; Fanselow and Rudy, 1998; Hudson *et al.*, 1998; Robinson and Smotherman, 1995; Spear and Rudy, 1991; Stanton, 2000). Nevertheless, unique learning characteristics have been documented in pups.

Rat pup learning seems to have two major periods of developmental change. The first occurs as pups make the transition from crawling to walking, which expands their environment to outside the nest. The second occurs around weaning, when parental assistance ends. We hypothesize that evolution has worked on each developmental transition to accommodate the pups' changing environment. While both transitions are described below, our emphasis will be the first transition, which has been the focus of our lab's research efforts. In addition, this review focuses on classical conditioning with a strong emphasis on olfaction due to its importance in mediating a range of neonatal behaviors, from suckling and huddling, to the formation of incentive-seeking or avoidance behaviors (e.g., Alberts, 1976; Rosenblatt, 1983). Due to the large amount of literature on infant learning, this is not a comprehensive review, but highlights the literature that demonstrates the specialization of the infant brain to support mother-infant attachment.

1. Behavioral Studies on Infant Acquisition

Before walking emerges, pups are usually confined to the nest and appear to have learning that predisposes them to preference learning. Both olfactory and somatosensory learning occur rapidly in the neonate, with robust learning resulting from as little as 10 min of conditioned stimulus (CS) – reward pairings (reviewed in Hofer and Sullivan, 2001). Stimuli that function as a reward are broadly defined. Traditional rewards that support odor preference learning include warmth and milk (Brake, 1981; Guénaire *et al.*, 1982; Thoman *et al.*, 1968; reviewed in Hofer and Sullivan, 2001); but unconventional rewards, such as tactile stimulation (called stroking – McLean *et al.*, 1993, 1999; Pedersen *et al.*, 1982; reviewed in Hofer and Sullivan, 2001), and paradoxical rewards, such as a 0.5 mA shock and tail pinch, also produce a subsequent odor preference (Camp and Rudy, 1988; reviewed in Hofer and Sullivan, 2001). This

changing reward value during development is illustrated in Figure 1.

The stimuli that function as reward become more narrowly defined as the learning system matures, coinciding with the emergence of walking around PN10 (Bolles and Woods, 1965). While traditional rewards (such as milk) retain their rewarding value throughout development, others (stroking) lose their rewarding value, and still others (shock) change their rewarding value from supporting an odor preference to supporting an odor aversion (Camp and Rudy, 1988; Johanson and Hall, 1982; Woo and Leon, 1987; reviewed in Hofer and Sullivan, 2001). Overall, during the sensitive period, the reward system appears to be designed to support rapid learning about the mother, at a time when pups are confined to the nest and the learning demands of the pup are limited to learning a preference for mother, nest, and peer odors. As the pup matures and begins to walk at PN10, more adult-like learning emerges that provides the pup with more complex learning to accommodate a complicated extra-nest environment.

Additionally, as compared to the adult or older pup, which focus on a select group of salient stimuli, the sensitive period neonate seems to learn about a wide range of stimuli (Spear *et al.*, 1989). Moreover, while pre-exposure to a CS, such as an odor, hinders subsequent conditioning to that CS in adults, CS pre-exposure facilitates subsequent learning in young pups (Hoffmann and Spear, 1989; Misanin *et al*., 1983). This broad sensitive period learning may help pups to learn about the complex features of the dam, siblings, and nest.

Perhaps the most dramatic neonate learning characteristic is the limitations put on learning passive avoidance, active avoidance and inhibitory conditioning during the sensitive period (Blozovski and Cudennec, 1980; Camp and Rudy, 1988; Collier *et al.*, 1979; Roth and Sullivan, 2001, 2003; Sullivan *et al.*, 2000a; reviewed in Myslivecek, 1997). Specifically, odor paired with moderate pain (0.5mA shock or tail pinch) results in pups learning a <u>preference</u> for that odor as indicated by orienting towards the odor and

even climbing an obstacle to approach the odor (Camp and Rudy, 1988; Roth and Sullivan, 2001; Sullivan *et al.*, 2000a). The 0.5 mA shock is an intensity similar to that used in adult fear conditioning experiments, and it should be noted that pups feel pain. Threshold to shock does not appear to change developmentally, and 0.5mA shock elicits both broadband vocalizations (indicative of pain, White *et al.*, 1992) and escape responses associated with pain (Emerich *et al.*, 1985; Stehouwer and Campbell, 1978; Sullivan *et al.*, 2000a).

The end of the sensitive period coincides with the emergence of walking (Bolles and Woods, 1965), suggesting that more adult-like learning may be needed as pups begin to venture outside the nest. However, it should be noted that neonatal rats can learn an odor aversion following odor – malaise pairings. Specifically, odor paired with illness-inducing LiCl or very strong shock (1.0 mA – 1.5 mA) results in a subsequent aversion for that odor (e.g., Haroutunian and Campbell, 1979; Martin and Alberts, 1979; Rudy and Cheatle, 1977; reviewed in Sullivan, 2001). Shock levels of 1.0 mA and above exceed those typically used in adult fear conditioning experiments (e.g. LaLumiere *et al.*, 2003; Paschall and Davis, 2002; Wilensky *et al.*, 1999). Work from the labs of Jerry Rudy and Byron Campbell suggests that until PN9-10, pups easily learn aversions based on interoceptive cues (malaise or internal shock) but not exteroceptive cues, such as a moderate intensity external shock (Camp and Rudy, 1988; Haroutunian and Campbell, 1979). Indeed, Rudy suggests that changes in the categorization of appetitive and aversive events occur in pups sometime between PN8 and PN12, coinciding with the end of the sensitive period (Camp and Rudy, 1988).

Although dramatic changes in learning occur in the 10-day-old pup, not all developmental changes in learning changes occur at this age. For example, conditioned fear emerges at different ages depending upon the sensory system: freezing (immobility) first emerges at PN10, 16 and 18 respectively for the olfactory, auditory and visual

systems (Hunt *et al.*, 1994; Hunt *et al.*, 1997; Sullivan *et al.*, 2000a), with ear opening at PN12-13 and eye opening around PN15. Additional learning abilities seem to emerge at weaning. For example, potentiated startle, which is the enhancement of a startle response by an innate (i.e. loud noise) or learned fear (odor previously paired with shock), does not emerge until PN21-PN23 (Richardson *et al.*, 2000, 2003; reviewed in Hunt and Campbell, 1999). Similarly, contextual fear conditioning, in which an animal displays a fear response to a context associated with an aversive stimulus, does not emerge until approximately PN23-25 (Rudy and Morledge, 1994; reviewed in Stanton, 2000). Overall, the learning of pups seems to change at the time of developmental landmarks, such as walking and weaning (Hassmannová *et al.*, 1985).

2. Neural Correlates of Infant Acquisition

One of the perplexing issues concerning the neural basis of odor learning in neonatal pups during the sensitive period is that they show excellent learning ability, yet brain areas identified as important in adult odor conditioning (amygdala, hippocampus, frontal cortex) appear not to participate, and major neural connections, such as amygdaloid-hippocampal and hippocampal-entorhinal cortical connections, are immature (Alvarez *et al.*, 2002; ; Astic and Saucier, 1982; Crain *et al.*, 1973; Hall, 1987; Landers and Sullivan, 1999; Litaudon *et al.*, 1997; Nair and Gonzalez-Lima, 1999; Ramus and Eichenbaum, 2000; Rudy and Morledge, 1994; Rudy *et al.*, 1987; Saar *et al.*, 2002; Tronel and Sara, 2002; Verwer *et al.*, 1996; reviewed in Fanselow and Rudy, 1998; Stanton, 2000).

This suggests that the neural structures supporting classical conditioning may be different in pups, and the literature suggests that the brain structures supporting sensitive period learning are the olfactory bulb, the noradrenergic locus coeruleus (LC), and the amygdala (see Figure 2). The piriform cortex and the anterior olfactory nucleus

(AON) also appear to influence how olfactory information is handled in the neonatal brain, and other brain areas will certainly be added to this list as more information about the developing brain emerges.

The classical conditioning learning circuit is diagramed within the olfactory system in Figure 2. Relative to other sensory systems, the olfactory system is simplistic: the information does not pass through the thalamus before going on to the primary sensory (piriform) cortex, and there is only one synapse between the olfactory receptors and the piriform cortex. Information progresses from the olfactory bulb to either the cortex or amygdala, with additional connections in the anterior olfactory nucleus (AON) (reviewed in Brunjes and Frazier, 1986; Shipley and Ennis, 1996; Wilson and Sullivan, 2003). Information about reward appears to reach the olfactory circuit via the locus coeruleus projection directly to the olfactory bulb.

a. The Olfactory Bulb and the source of norepinephrine (NE), the Locus Coeruleus (LC)

As illustrated in Figure 3, during acquisition the olfactory bulb's primary output neurons (mitral cells) exhibit a heightened excitatory response in experimental pups (receive paired presentations of odor and reward) as compared to odor control groups (Wilson and Sullivan, 1992). Indeed, while mitral cells normally quickly habituate to repeated odor presentations, this habituation is prevented when that odor is paired with a reward. This heightened mitral cell response during training may be a critical factor in induction of the behavioral and neural changes discussed below.

Norepinephrine is both necessary and sufficient for the enhanced mitral cell response to the odor and the acquisition of the learning induced neurobehavioral effects (reviewed in Sullivan and Wilson, 1994, 2003). Specifically, either blocking NE receptors in the bulb or destroying the locus coeruleus (source of NE) prevents odor learning. More importantly, NE is sufficient to support neonatal odor learning; activation of

olfactory bulb NE ß-receptors with isoproterenol paired with odor stimulation produces a learned approach response in rat pups (Langdon *et al.*, 1997; Moriceau and Sullivan, 2004; Sullivan *et al.*, 2000b; Yuan *et al.*, 2003; reviewed in Sullivan and Wilson, 2003). Moreover, increasing olfactory bulb NE by stimulating the LC during an odor presentation is sufficient to support odor learning and the learning-induced changes in the olfactory bulb that occur during the sensitive period (Sullivan *et al.*, 2000b; Moriceau and Sullivan, 2004). This is in sharp contrast to the effects of NE in the adult, where blocking/activating NE generally has only a modulatory effect on adult acquisition of such tasks as inhibitory avoidance or escape from a water maze (e.g. Harris and Fitzgerald, 1991; Liang, 1998; Sara *et al.*, 1995). However, adult learning critical for survival such as mating and infant care requires NE (reviewed in Brennan and Keverne, 1997; Fleming *et al.*, 1999; Insel and Young, 2001; Levy, 2002).

There is no endogenous NE in the olfactory bulb; its sole source of NE is the LC (McLean and Shipley, 1991; Shipley *et al.*, 1985), which releases copious amounts of NE (200-300% increase) into the neonatal bulb with almost any moderate intensity sensory stimulus (Rangel and Leon, 1995; reviewed in Nakamura and Sakaguchi, 1990). The neonatal LC also releases substantially more NE into the bulb than the adult LC (Rangel and Leon, 1995). As is illustrated in Table 1, there is a sharp contrast between the functioning of the neonatal and older LC, with LC developmental changes that dramatically reduce NE release coinciding with the termination of the sensitive period. The emergence of neonatal LC autoreceptors, receptors that are activated by the neurotransmitter released from LC neurons (NE), is the primary cause of developmental changes in the LC. During the 2nd postnatal week, excitatory α1 autoreceptor function becomes limited and no longer temporally extends the LC's heightened response to sensory stimuli, and inhibitory α2 autoreceptor function emerges, shutting down the LC

within a few msec of a response onset (Winzer-Serhan and Leslie, 1999; reviewed in Marshall *et al.*, 1991; Nakamura and Sakaguchi, 1990). These findings suggest that the infant LC is responsible for enhanced odor preference learning, and that maturation of the LC (via emergence of α2 autoreceptor function) signals the termination of the sensitive period for odor-preference learning in rat pups.

Although NE appears particularly important in neural plasticity during early development, many neurotransmitters have a role in olfactory learning in neonatal rats (cholecystokinin – Shayit and Weller, 2001; GABA – Okutani *et al.*, 2002; glutamate – Lincoln *et al.*, 1988; Mickley *et al.*, 1998; opioids – e.g. Barr and Rossi,1992; Kehoe & Blass, 1986; Panksepp *et al.*, 1994; and, serotonin [5-HT] – McLean *et al.*,1993). Nitric oxide, an intracellular messenger, also has a role in early olfactory learning (Samama and Boehm, 1999). It has also been shown that the interaction of NE and 5-HT within the bulb mediates acquisition. The NE effect on learning displays an inverted-U shaped dose-response curve (reviewed in Sullivan and Wilson, 1994) that can be shifted with manipulations of olfactory bulb 5-HT activity (McLean *et al.*, 1993, 1999). Specifically, more NE is required for learning if 5-HT is depleted, but less NE is required if a 5-HT receptor agonist is added to the bulb. It should be noted that 5-HT without NE is <u>not</u> sufficient to support learning, whereas NE alone is sufficient to support learning (Yuan *et al.*, 2003).

<u>b. The Anterior Olfactory Nucleus</u> Although there are learning-induced changes within the olfactory bulb, the mitral cell signal also leaves the bulb, suggesting that learning induced changes may occur in other brain areas, such as the anterior olfactory nucleus (AON). The AON serves as a commissural relay, or a connection between the olfactory bulbs, and receives input from both the olfactory bulbs and olfactory cortex (reviewed in Shipley and Ennis, 1996; Wilson and Sullivan, 2003). As in the adult rat, olfactory

stimulation via presentations of a novel odor increases activity in the AON of pups (Astic and Saucier, 1982; Hall, 1987). In adult rats, presentations of a conditioned odor enhance metabolic activity in the AON (Hamrick *et al.*, 1993); however, following an appetitive learning paradigm in 6-day-old pups, there appears to be little metabolic activity in the AON in conditioned pups, but there is an increase within the piriform cortex, suggesting learning-induced neural changes downstream of the olfactory bulb (Hall, 1987).

c. The Piriform Cortex In adults, the piriform cortex appears to serve at least three functions in odor perception: 1) it allows rapid filtering of background or irrelevant stimuli while maintaining responsiveness to novel stimuli (Wilson, 2000); 2) it allows experience-dependent synthetic processing of multiple odorant features and odorant mixtures into single perceptual odor objects (reviewed in Wilson and Stevenson, 2003); and 3) it serves as one of perhaps many sites involved in odor associative and contextual memory (Litaudon *et al.*, 1997; Saar *et al.*, 1999; Schoenbaum and Eichenbaum, 1995). However, relatively little is known about the functional ontogeny of this structure. Mitral cell afferent fibers are present throughout the piriform cortex (Schwob and Price, 1984) and can evoke responses in piriform cortical neurons by birth (Schwob *et al.*, 1984). Preliminary evidence from our laboratory suggests that odor-evoked responses (i.e., odors with which the pup has had no prior experience) can be seen in piriform single-units by at least the second postnatal week, the earliest time point examined (Wilson, unpublished observations). Furthermore, mechanisms responsible for cortical habituation and odor filtering (Best and Wilson, 2003) are expressed by at least PN7, as determined in *in vitro* slices (Best and Wilson, unpublished observations).

Using a unilateral odor conditioning paradigm combined with directed lesions of the anterior commissure, Kucharski and Hall (1987, 1988) argue that memory for learned

odor preferences is stored in the AON or piriform cortex. Learned odor preferences could be expressed by pups using an untrained naris following conditioning of the contralateral naris, but only if the anterior commissure was intact. The anterior commissure provides strong, direct connections between the bilateral AON's and piriform cortices. In line with this hypothesis, in a large 2-deoxyglucose (2-DG) autoradiography mapping study of neonatal associative memory by Hall (1987), PN6 pups exposed to learned odors had enhanced 2-DG uptake in both the olfactory bulb and the piriform cortex compared to control pups. These 2-DG data suggest that activity within the piriform cortex may reflect learned changes in neonates, although whether these changes are intrinsic to the piriform cortex or simply reflect the modified output of the olfactory bulb is unclear (Johnson *et al.*, 1995; Sullivan and Leon, 1986; Wilson and Leon, 1988b; Wilson and Sullivan, 1991; Wilson *et al.*, 1987).

d. The Amygdala Immature and/or limited amygdala function during the sensitive period appears to underlie pups' inability to learn conditioned fear from odor – shock (0.5mA) conditioning during the sensitive period. As is illustrated in Figure 1, pups learn to approach an odor even after that odor has been paired with a painful stimulus. As noted above, this shock-induced odor preference is not due to pups' inability to feel pain since shock threshold varies little during development (Emerich *et al.*, 1985; Stehouwer and Campbell, 1978). As shown in Figure 4, our assessment of the amygdala during acquisition (using 2-DG autoradiography) shows that the amygdala is not activated by odor – shock conditioning during the sensitive period, but is activated by post-sensitive period conditioning when pups readily learn an aversion to an odor paired with 0.5 mA shock (Sullivan *et al.*, 2000a). Additionally, lesioning the amygdala only slightly retards odor learning in PN6 rats (Sullivan and Wilson, 1993), while this procedure dramatically and permanently disrupts fear conditioning in adult rats (reviewed in Maren, 2001).

Furthermore, passive avoidance learning can be greatly potentiated in infant rats by facilitating amygdala activity (Dumery *et al.*, 1988).

The amygdala is a limbic structure involved with emotions, especially innate and learned fear (reviewed in Eichenbaum and Cohen, 2001; Fanselow and LeDoux, 1999; Maren, 2001; McGaugh, 2002; Walker and Davis, 2002). While most sensory systems send input to the amygdala via the thalamus and/or sensory cortices, olfactory bulb mitral cells synapse directly within the amygdala (cortical nucleus), with additional olfactory input via the piriform (olfactory) cortex (cortical and lateral nuclei; Schwob and Price, 1984), although it is unclear how effective these connections are during the sensitive period. Amygdala development begins during the midembryonic period, with subdivision of the major nuclei occurring around PN7 and stabilizing around PN14 (Bayer, 1980; Berdel *et al.*, 1997). Olfactory bulb afferent fibers are present in the cortical nucleus of the amygdala at birth, as are piriform cortex afferents to the amygdala (Schwob and Price, 1984). Development of synaptic terminals begins by PN5, with the most prolific increase between PN10-20, and adult levels reached by PN30 (Mizukawa *et al.*, 1989). As shown in Figure 4, our 2-DG data suggest that the amygdala can be activated by paired odor – shock stimulation as the sensitive period ends at PN10, but not before. Preliminary single-unit recordings from amygdala neurons of developing rats suggest dramatic changes in spontaneous activity and response latency to olfactory bulb stimulation from PN11 to adult, although younger ages have not yet been examined (Wilson, 2003). Our interpretation of amygdala function is supported by research on the developmental emergence of natural (unlearned) fear. Coinciding with the developmental emergence of learned fear, fear (freezing) to a natural predator odor emerges at PN10 (e.g. Moriceau *et al.,* 2004; Takahashi 1994; Wiedenmayer and Barr, 2001), as do cellular changes (activation of immediate early gene products, such as c-

fos) within the amygdala in response to a predator odor (Moriceau *et al.,* 2004; Wiedenmayer and Barr, 2001).

B. MEMORY CONSOLIDATION DURING INFANCY

Consolidation represents a post-acquisition period when a cascade of neural and molecular events, involving several transcription factors (cellular proteins that regulate gene expression) and changes in both gene expression and protein synthesis, transfers learned information into a less labile neural and molecular representation (reviewed in Abel and Lattal 2001; Davis and Squire, 1984; Dudai, 2002; Eichenbaum and Cohen, 2001; Stork and Pape, 2002). Despite the relative immaturity of the central nervous system in neonates, molecular processes similar to those that mediate consolidation in the adult are present and functional early in an infant's life, when learning an odor preference for the caretaker enhances survival (Yuan *et al.*, 2003; Zhang *et al.*, 2003; reviewed in Sullivan and Wilson, 2003). However, though the molecular events appear similar in infants and adults, the circuitry and the role of neurotransmitters appear to reflect how the infant's brain is optimized to facilitate rapid attachment to the caretaker. Unfortunately, consolidation has not been assessed for the transition occurring at weaning, thus the mechanisms associated with learning and consolidation in neonates are addressed here.

1. Behavioral Studies on Infant Consolidation

In the adult rat, several neurotransmitters have been shown to participate in memory consolidation. Both dopamine (reviewed in Jay, 2003) and glutamate (reviewed in Riedel *et al.*, 2003) enhance memory consolidation, while evidence suggests that serotonin impairs consolidation processes (reviewed in Meneses, 2003). In adult behavioral studies, particular attention has been given to the role of norepinephrine,

glucocorticoids, and endogenous opioids in the memory of arousing or emotional events. In general, post-training systemic or central administration of noradrenergic receptor agonists, glucocorticoid receptor agonists, or opioid receptor antagonists enhance memory, while noradrenergic receptor antagonists, glucocorticoid receptor antagonists, and opioid receptor agonists impair memory (reviewed in McGaugh, 2002; McGaugh and Roozendaal, 2002; McGaugh *et al.*, 1993; Roozendaal, 2002).

In neonatal rats during the sensitive period, behavioral studies indicate that memory consolidation processes emerge early in development. Pharmacological exploration of the mechanisms responsible for infant memory consolidation of odor preferences has demonstrated a role for the same neurotransmitters/hormones involved in adult consolidation. As in the adult, neonatal odor conditioning is impaired by post-training administration of a dopaminergic (Weldon *et al.*, 1991), glutamatergic (Weldon *et al.*, 1997), or noradrenergic receptor antagonist (Wilson *et al.*, 1994). However, in contrast to the adult, post-training administration of a noradrenergic receptor agonist impairs memory, even at very low concentrations (Wilson *et al.*, 1994). Thus, while the overall behavioral consolidation process appears similar in adult and neonatal rats, different mechanisms may be involved due to the prominent role of NE in infant learning and functional immaturity of certain brain areas. Indeed, these differences appear to reflect how the neonatal brain is designed to support rapid learning and memory of odor preferences that enhance attachment behaviors and, ultimately, survival.

2. Neural Correlates of Infant Memory Consolidation

In the neonate's brain, areas normally associated with consolidation in the adult do not appear functionally mature. Specifically, the amygdala, hippocampus, and neocortex are considered to be key loci of drug and hormonal modulation and/or encoding of adult memory consolidation (reviewed in Abel and Lattal, 2001; Eichenbaum

and Cohen, 2001; Fanselow and Gale, 2003; McGaugh, 2002; Packard and Cahill, 2001; Roozendaal, 2002; Schafe *et al.*, 2001). However, as discussed earlier, these structures are not fully functional in infants.

In the neonate, molecular events in the olfactory bulb are necessary for the association of an odor and a stimulus (McLean *et al.*, 1999; Zhang *et al.*, 2003; Yuan *et al.*, 2003). The infant's cascade of learning-induced molecular events is consistent with that in adults. The binding of a neurotransmitter to a receptor activates a cascade of events that in turn activates cyclic adenosine monophosphate (cAMP), a secondary messenger. cAMP then stimulates another enzyme that causes phosphorylation of the cAMP response element binding protein (CREB). CREB is a transcription factor that regulates expression of genes and ultimately proteins required for memory consolidation. Thus, changes in protein synthesis allows a long-term trace of the CS – unconditioned stimulus (UCS) association. Following odor-stroke conditioning in PN6 pups, there is a greater increase in phosphorylated-CREB (pCREB) levels in pups that learn an odor preference in comparison to pups that do not learn, and these levels are highest 10-30 min following training (McLean *et al.,* 1999). Similarly, there are marked increases in CREB levels 10-360 min following odor-shock pairings in PN11 pups (Zhang *et al.*, 2003). Additionally, CREB deficient PN11 rats do not demonstrate memory of an odor aversion when tested 24 hr following odor-shock training; however, CREB deficient pups are able to demonstrate an odor aversion 1 hr following the training, demonstrating the crucial role of CREB in long-term memory consolidation (Zhang *et al.*, 2003).

More recent work from the Harley and McLean research team demonstrated that a cAMP and pCREB response follows the shifted role of NE through manipulations of olfactory bulb 5-HT (mitral cells have both NE and 5-HT receptors) (Yuan *et al.*, 2003). Specifically, they were able to confirm the UCS's effectiveness at producing odor

learning (manipulating the strength of the reward and/or NE levels) through elevated olfactory bulb cAMP levels. Moreover, they demonstrated that ineffective UCS's (too low or too high levels of NE or 5-HT lesions) do not elevate cAMP levels and are directly correlated with the inverted-U shaped performance curve seen in pup learning data.

This cascade of molecular events associated with learning and memory consolidation has been identified in a wide variety of species at different stages of development, suggesting that the molecular biology underlying memory storage is highly conserved across both development and species (e.g., Carew, 1996; Carew and Sutton, 2001). Nevertheless, though learning-induced molecular events appear conserved, the neural circuitry involved in memory consolidation shows marked changes with development. Overall, comparison of adult and infant rats shows that during the first postnatal week similar molecular events that support adult learning and memory are already present to mediate early learning experiences. However, similarly to acquisition, neonatal consolidation has features unique to the neonate, especially with respect to the critical role of the opioid system in consolidation of odor preferences.

C. EXPRESSION OF INFANT LEARNING

Expression involves retrieval of the established memory (reviewed in Abel and Lattal, 2001; Szapiro *et al.*, 2002). Similar to acquisition and consolidation, behavioral studies have indicated that no single neurotransmitter or brain area is responsible for the expression of a memory. Neurotransmitters involved in expression in the adult include dopamine, norepinephrine, glucocorticoids, and opioids (reviewed in Barros *et al.*, 2003; Roozendaal, 2002). Brain systems implicated in expression of a memory include the amygdala (reviewed in Barros *et al.*, 2003; Eichenbaum and Cohen, 2001) and the hippocampus (reviewed in Abel and Lattal, 2001; Szapiro *et al.*, 2002), with the frontal cortex modulating expression through extinction (Milad and Quirk, 2002). Expression of

a memory also appears to require similar molecular mechanisms to those used in memory consolidation (reviewed in Abel and Lattal, 2001; Miller and Matzel, 2000; Nader *et al.*, 2000; Szapiro *et al.*, 2002).

1. Behavioral Correlates of Expression

Despite advancing knowledge on both the behavioral and neural correlates of learning and memory consolidation in infants, far less is known of the neurobiology underlying memory expression. Unlike its necessary role in infant learning and memory consolidation, NE is not necessary for the expression of an odor preference following odor conditioning (Sullivan and Wilson, 1991). Shide and Blass (1991) demonstrated that opioids are necessary for the expression of a sucrose-conditioned odor preference in neonates. Isolation from the home cage and mother prior to testing has been shown to disrupt olfactory memories in 18-day-old rats (Arnold and Spear, 1995), suggesting that stimuli from the home environment (from the mother and siblings) affect physiological/biochemical processes necessary for maintaining and expressing memories. Finally, Sandstrom *et al*. (1998) have shown that expression of a conditioned odor aversion in preweanling rats (after odor – footshock training on PN12) is impaired by scopolamine, suggesting that the cholinergic system has a role in the retrieval and expression of odor memories in young rats.

The age of training can have an enormous impact on what is expressed. This is illustrated by the fact that odor – shock pups trained during the sensitive period (who learn an odor preference) continue to express the odor preference even after the sensitive period (Sullivan *et al.*, 2000a). Thus, animals express a behavior consistent with the age of training (during the sensitive period) rather than the age of testing (post-sensitive period). A similar example can be seen in research on conditioned odor potentiation of startle, which emerges around PN23 (Richardson *et al.,* 2000). As noted

above, the startle response can be potentiated by presentation of an odor previously paired with shock. However, odor – shock conditioning cannot potentate the startle response in pups younger than PN23 (Richardson *et al.*, 2000, 2003). These results illustrate the importance of the age of learning on the expression of the learned response.

Attachment odors learned in infancy retain value into adulthood, although the role of the odor in modifying behavior changes. Research from the labs of Celia Moore (Moore *et al.*, 1996) and Elliot Blass (Fillion and Blass, 1986), demonstrates that adult male rats exhibit enhanced sexual performance when exposed to natural and artificial odors learned in infancy. These results support observations in other species of the role of early experience on adult mate preference, such as the parental and social influences on avian sexual imprinting (Slagsvold *et al.*, 2002; ten Cate and Vos, 1999).

2. Neural correlates of expression

Neonates show a modified olfactory bulb response to presentation of a learned odor. This response is expressed both during the sensitive period and lasts into adulthood (Pager, 1974; Sullivan *et al.*, in prep; Woo and Leon, 1987). Moreover, this modified olfactory bulb response is expressed to both natural maternal and artificial odors experienced in the nest (Sullivan, *et al.*, 1990), as well as to odors in controlled learning experiments (Johnson *et al.*, 1995; Sullivan and Leon, 1986; Wilson and Leon, 1988b; Wilson and Sullivan, 1991; Wilson *et al.*, 1987). This learning-associated olfactory bulb response is characterized by enhanced immediate-early gene activity (c-fos, Johnson *et al.*, 1995; Woo *et al.*, 1996), enhanced 2-DG uptake in focal, odor-specific glomeruli in response to the conditioned odor, modified single-unit response patterns of mitral/tufted cells near the enhanced glomerular foci (Wilson *et al.*, 1987; Wilson and Leon, 1988a; Wilson and Sullivan, 1990), odor-induced intrinsic optical

signals (Yuan *et al*., 2002), and olfactory bulb anatomical changes reflected in enlarged glomeruli within these foci (Woo *et al.*, 1987). As with the behavioral changes in attachment, these neural changes are retained into adulthood, with acquisition dependent upon experiences during infancy. These changes have not been found in animals conditioned after the sensitive period, suggesting that the brain may be specifically designed to give special significance to neonatal odors with hedonic value.

Additional experimental evidence indicates that the neonate's access to stored memory of odor conditioning differs developmentally (Kucharski and Hall, 1987, 1988; Kucharski *et al.*, 1990). Specifically, olfactory memories can be unilaterally stored by occluding one naris during training (yielding a trained and untrained olfactory bulb). During testing, a PN6 pup can only access memory if tested with the trained side while testing with the untrained side yields no memory of conditioning. However, due to the development of the projections of the anterior limb of the anterior commissure to and from the AON and the anterior piriform cortex, PN12 pups can access the memory through either the trained or untrained side. Indeed, this suggests the inclusion of the AON and perhaps the piriform cortex as sites of memory encoding in the olfactory pathway after the sensitive period (see above).

III. THE ENDOGENOUS OPIOID SYSTEM

A. SYSTEM OVERVIEW

1. Opioid receptors and peptides

Opioid binding sites were first discovered in brain tissue in the 1970s (Pert and Snyder, 1973; Simon *et al*., 1973; Terenius, 1973; reviewed in Snyder and Pasternak, 2003), and since then researchers have continued to characterize the receptors and peptides that constitute the endogenous opioid system. To date, four opioid receptors have been identified: μ- (from here on out referred to as MOP-R), δ- (DOP-R), κ- (KOP-

R), and the Nociceptin/orphanin FQ receptor (NOP-R) (Martin *et a*l., 1976; Meunier *et al*., 1995; reviewed in Chaturvedi, 2003; Akil *et al*., 1984; Brownstein, 1993; Janecka *et al.*, 2004; Mogil and Pasternak, 2001; Terenius, 2000; Waldhoer *et al*., 2004). Opioid receptors are classic 7-transmembrane, G-protein coupled receptors (coupled to G_i or $G_{i/o}$), mediating a series of biochemical and electrical events as a result of their presynaptic or postsynaptic locations within a region (reviewed in Christie *et al.*, 2000; Huang, 1995; Miotto *et al*., 1995; Moran *et al*., 2000; Waldhoer *et al*., 2004). Several endogenous opioid peptides elicit responses through binding with specific opioid receptors. Structurally similar endogenous opioid peptides include Leu- and Met-enkephalin (Hughes *et al*., 1975; Kosterlitz and Waterfield, 1975), β-endorphin (Bradbury *et al.*, 1976), dynorphin (Goldstein *et al*., 1981), and nociceptin or orphanin FQ (Meunier *et al*., 1995; Reinscheid *et a*l., 1995). Though with a slightly different chemical structure, endomorphin-1 and -2 (Zadina *et al.*, 1997; reviewed in Zadina, 2002) have been recently added to this list, as have deltorphin (Erspamer *et al*., 1989; reviewed in Lazarus *et al*., 1999; Negri *et al*., 2000). Opioid peptides are mainly derived from four large precursor proteins: pro-opiomelanocortin, proenkephalin, prodynorphin, and pronociceptin/orphanin FQ (reviewed in Akil *et al*., 1984; Brownstein, 1993; Höllt, 1993; Mogil and Pasternak, 2001; Waldhoer *et al.*, 2004). MOP-Rs are highly selective for endorphin, but will also bind endomorphins and enkephalin (reviewed in Brownstein, 1993; Knapp *et al*., 1995; Terenius, 2000; Waldhoer *et al*., 2004). DOP-Rs are highly selective for enkephalin, but will also bind endorphin and deltorphin (Brownstein, 1993; Knapp *et al*., 1995; Terenius, 2000; Waldhoer *et al*., 2004). KOP-Rs show the highest affinity for dynorphin (Brownstein, 1993; Nock, 1995; Terenius, 2000; Waldhoer *et al*., 2004), while NOP-Rs bind with the ligand termed nociceptin, or orphanin FQ (reviewed in Mogil and Pasternak, 2001; Terenius, 2000; Waldhoer *et al*., 2004).

Autoradiographic and immunohistochemical studies demonstrate the wide distribution of opioid receptors in the adult brain (e.g. Arvidsson *et al.,* 1995; Ding *et al.*, 1996; Garzon and Pickel, 2002; Sim-Selley *et al*., 2003; reviewed in Akil *et al*., 1984; Chaturvedi, 2003; Mansour and Watson, 1993; Meis, 2003). MOP-Rs are particularly dense in the basal ganglia, limbic system (LC, amygdala), and regions intimately associated with stress (hypothalamic nuclei), reward (nucleus accumbens), and pain regulation (periaqueductal gray, PAG). DOP-Rs are densest in forebrain structures including the cerebral cortex and amygdala. KOP-Rs are densest in the caudate, nucleus accumbens, olfactory tubercle and hypothalamus. And, NOP-Rs are highly expressed within the cerebral cortex, amygdala, hypothalamic nuclei, PAG, and LC. There are two major cell groups producing β-endorphin: cells located in the arcuate nucleus (Watson and Akil, 1979, 1980; Watson *et al.,* 1978), and those located in the nucleus of the solitary tract (Schwartzberg and Nakane, 1982). Unlike endorphins, enkephalinergic and dynorphinergic cell bodies are distributed throughout the brain (reviewed in Akil *et al*., 1984). High levels of cell bodies producing Nociceptin/orphanin FQ are found in the PAG, LC, amygdala, and hypothalamus (Witta *et al*., 2004).

Generally, endorphins and enkephalins elicit euphoria and analgesia, dynorphins elicit negative affect, and nociceptin/orphanin FQ elicit analgesia (reviewed in Akil *et al*., 1984; Heinricher, 2003; Kieffer and Gavériaux-Ruff, 2002; Meis, 2003; Terenius, 2000). Opioid peptides elicit three common and well-characterized actions via their G-protein coupled receptors: 1) an inhibition of adenylyl cyclase, and thus cAMP and associated phosphorylated proteins; 2) an activation of potassium conductance and decrease in sodium conductance; and, 3) an inhibition of calcium conductance, and thus inhibition of neurotransmitter release (reviewed in Christie *et al*., 2000; Huang, 1995; Moran *et al*., 2000; Taylor and Fleming, 2001; Waldhoer *et al*., 2004). Opioids can also indirectly lead to excitation of a neuron by disinhibition. That is, opioid receptors are often located on

GABAergic neurons, inhibit the release of GABA, and thus produce an increase in neurotransmitter release (Vaughn *et al*., 1997; reviewed in Christie *et al*., 2000; Huang, 1995; Kalyuzhny and Wessendorf, 1998).

2. Impact of exogenous opiates on the endogenous opioid system

Opioid receptors are not only activated by endogenous compounds, but also by exogenously administered opiates, such as morphine and heroin. It is well understood that the chronic effects of opiates contrasts the more subtle effects of endogenous opioid peptides functioning within their normal physiological parameters. Chronic use of opiates produces drug tolerance (requiring higher dosages of the opiate to achieve some effect) and drug dependence (drug cessation produces withdrawal symptoms - which include shaking, irritability, changes in respiration, and a multitude of other aversive behaviors), which ultimately produce the phenomenon of drug addiction (reviewed in Chao and Nestler, 2004; White, 2004). Though we lack a complete understanding of the biochemical mechanisms responsible for drug addiction, changes in the normal function of the endogenous opioid system within several areas of the brain are well characterized to contribute to the physiological and behavioral manifestations of drug addiction and withdrawal. One brain area well characterized to participate in drug addiction and withdrawal is the noradrenergic LC. Functioning within normal physiological parameters, opioids/opiates inhibit LC neurons by two mechanisms: 1) increasing a potassium conductance through MOP-R activation; and 2) decreasing the sodium conductance, which decreases levels of cAMP (reviewed in Chao and Nestler, 2004; Nestler, 1997; Nestler *et al*., 1999). Thus, the effect of acute actions is the inhibition of adenylyl cyclase and cAMP, and overall there is a suppression of neuronal activity and lowered release of norepinephrine.

As the animal develops drug tolerance, LC neuronal activity returns to normal levels (recovers from acute inhibitory effects). With drug tolerance, opioid agonists are no longer as effective at the potassium or sodium channels, due to a compensatory up-regulated cAMP system (reviewed in Chao and Nestler, 2004; Nestler, 1997; Nestler *et al*., 1999). With an up-regulated cAMP system, it is more difficult for the agonists to inhibit sodium channels, and evidence suggests that the MOP-R becomes desensitized – there is uncoupling of the opioid receptor and the associated G-protein, and possible receptor internalization (reviewed in Chao and Nestler, 2004; Nestler, 1997; Nestler *et al*., 1999). With chronic opiate use, there is also a corresponding decrease in opioid receptors and peptides throughout the brain (reviewed in Bhargava, 1991; Liu and Anand, 2001). Overall, to achieve any physiological or behavioral effect, the drug user must take higher and higher amounts of the drug.

When the animal has developed dependence on a drug and intake of the drug is ceased, the LC becomes hyperexcitable. The paragigantocellularis neurons, afferents to the LC, will continue to produce glutamate (excitatory on LC neurons) without the normal feedback inhibition provided by endogenous opioids/exogenous opiates (reviewed in Chao and Nestler, 2004; Nestler, 1997; Nestler *et al*., 1999). The up-regulated cAMP pathway within the LC operates unopposed, thus the LC will fire and release copious amounts of neurotransmitter, producing neuronal and behavioral excitability (reviewed in Chao and Nestler, 2004; Nestler, 1997; Nestler *et al*., 1999).

Of course exogenous drugs do not only target the LC and affect its neuronal activity. Another system affected by chronic use of opiates is the mesolimbic dopaminergic pathway (reviewed in Chao and Nestler, 2004; De Vries and Shippenberg, 2002; Kelley and Berridge, 2002; Van Bockstaele *et al*., 2001). μ-opioid agonists (acute) activate dopaminergic neurons located in the ventral tegmental area (VTA), and stimulate the release of dopamine within the nucleus accumbens (NAc, through

inhibition of GABAergic neurons). Following chronic administration of morphine, it has been shown that there is an increase in adenylyl cyclase and PKA activity within the NAc, an increase in tyrosine hydroxylase (rate-limiting enzyme in dopamine production), and there is a decrease in neurofilament proteins within the VTA, suggesting structural/synaptic changes (reviewed in Chao and Nestler, 2004; De Vries and Shippenberg, 2002; Kelley and Berridge, 2002; Van Bockstaele *et al*., 2001). KOP-Rs, which are located on the cell bodies of the dopaminergic neurons, normally inhibit the release of dopamine, which is thought to serve as a negative-feedback loop to control the amount of dopaminergic activity. Chronic opiate exposure upregulates this feedback mechanism (reviewed in Chao and Nestler, 2004; De Vries and Shippenberg, 2002; Kelley and Berridge, 2002; Van Bockstaele *et al*., 2001).

The mechanisms discussed above do not seem to be unique to the LC or to the VTA-NAc system, but may mediate neuronal responses in other areas that express opioid receptors and peptides (with chronic opiate exposure, the endogenous opioid system down-regulates). Other important areas include those involved in stress responses (the Hypothalamic Pituitary Axis (HPA) – activated during drug withdrawal) and learning and memory (since experience based learning is involved in drug behavior) (reviewed in Contet *et al*., 2004; Nestler, 2002; Siggins *et al*., 2003; Weiss and Koob, 2001).

B. ONTOGENY OF THE ENDOGENOUS OPIOID SYSTEM

To date, several studies have focused on the ontogeny of opioid receptors and peptides (Clendeninn and Petraitis, 1976; Kornblum *et al*., 1987, Petrillo *et al*., 1987, Spain *et al.,* 1985; Zhu *et al*., 1998; reviewed in Leslie and Loughlin, 1992; Pintar and Scott, 1993). Though they have often employed different methodological approaches, the aforementioned studies highlight two major developmental features of the

endogenous opioid system: 1) the system (its receptors and peptides) begins developing early in the fetus (around embryonic day 14 or 15); and, 2) the system continues to develop postnatally. Another feature that is consistent among these studies is differences in the development of the receptor types in specific brain regions (Clendeninn and Petraitis, 1976; Kornblum *et al*., 1987, Petrillo *et al*., 1987, Spain *et al.,* 1985; Zhu *et al*., 1998; reviewed in Leslie and Loughlin, 1992; Pintar and Scott, 1993). MOP-Rs and KOP-Rs are present at birth and even at very high levels in many areas (particularly the MOP-Rs), while DOP-Rs do not show significant expression until near the end of the 2nd postnatal week (around postnatal (PN) 9 or PN 10). Studies on the ontogeny of the endogenous peptides or their precursors (proopiomelanocortin for endorphins, proenkephalin for enkephalins, and prodynorphin for dynorphin) demonstrate that their expression parallels receptors (Bayon *et al*., 1979; Sato and Mains, 1985; Winzer-Serhan *et al*., 2003; reviewed in Marsh *et al*., 1997; Zagon *et al*., 1982).

Studies on the physiological function of the infant endogenous opioid system compliment their function in the adult. For example, as in adults, opioids exhibit inhibitory actions on LC neurons (e.g. Raymon and Leslie, 1994; Ronken *et al*., 1993). Also similar to the adult opioid system, the infant system is vulnerable to exogenous opiates, and thus plays a crucial role in mediating drug dependence and withdrawal following either prenatal or postnatal exposure. However, it should be pointed out that the cellular mechanisms mediating infant withdrawal and adult withdrawal may differ (e.g., Jones and Barr, 2001; Zhu and Barr, 2003). The consequences of prenatal opiate exposure are discussed later in this chapter.

C. OPIOIDS AND INFANT BEHAVIOR

Behavioral studies have demonstrated that in response to maternal stimuli, there is a release of endogenous opioids in the infant (Blass and Fitzgerald, 1988; Blass *et al*., 1990; Kehoe and Blass, 1986c, 1989; Panksepp *et al*., 1980; Smotherman *et al*., 1993). The release of opioids in response to maternal care is presumed to reflect the rewarding nature of maternal care and the social bond (Carter and Keverne, 2002; Fleming *et al*., 1999; Nelson and Panksepp, 1998). In the infant, behavioral responses to milk and physical contact are disrupted with opioid receptor antagonism, as are conditioned responses to presentations of a nipple and milk (Petrov *et al*., 1998, 2000; Robinson *et al*., 1993; Smotherman and Robinson, 1992). Morphine (Kehoe and Blass, 1986a; Randall *et al*., 1992) or sucrose (Shide and Blass, 1991) paired with an odor is sufficient to produce an odor preference in neonates as young as 5-days-old, and this conditioned preference is naltrexone or naloxone (opioid receptor antagonists) reversible. Following such conditioning, it has been shown that presentation of the odor alone can evoke a release of endogenous opioids (Kehoe and Blass, 1986b).

VTA injections of morphine paired with an odor are sufficient to produce a subsequent odor preference in PN 4 pups (Barr and Rossi, 1992). Research has also demonstrated that an olfactory association that is formed within the nest (a novel odor painted on the mother for several days) is disrupted with opioid antagonism (Panksepp *et al*., 1994), and recently it has been shown that MOP-R knockout mice fail to demonstrate a preference for maternal odor (Moles *et al*., 2004). Finally, the production of ultrasonic vocalizations (USV), which are alarm calls to aid the mother in locating and retrieving a pup that may have wondered or been removed from the nest, is also thought to involve the opioid system (Goodwin and Barr, 1997; Goodwin *et al*., 1994; Shoemaker and Kehoe, 1995; Winslow and Insel, 1991). In general, most reports show that opiate agonists decrease USV, though there are inconsistent reports of whether opioid

antagonism increases USV. Overall, behavioral studies indicate that the endogenous opioid system is functional in infancy, and contributes to infant behaviors associated with attachment.

IV. RATIONALE AND HYPOTHESES FOR THE PRESENT THESIS

Though behavioral studies demonstrate the important role of opioids in infant behavior, little is known of where endogenous opioids work in the neonate brain, and particularly, of their role within the circuitry supporting the learning necessary for attachment. Prenatal opiate exposure and early adverse experiences (such as stress or abuse) both have negative impact on the endogenous opioid system and infant attachment. As deficits in attachment are known to leaves a child vulnerable to psychiatric disorders, behavioral changes related to fear and anxiety, and alterations in neural circuits regulating stress and emotion, an understanding of the role of the endogenous opioid system in the neural circuitry and behavior supporting attachment offers an avenue into understanding the complexity between prenatal or postnatal disturbances to this system and subsequent behavioral deficits.

A. IMPACT OF PRENATAL OPIATE EXPOSURE

Each year in the United States, children are born following prenatal exposure to illicit (such heroin, morphine, cocaine, amphetamines) and licit (such as alcohol and nicotine) substances. Overall, clinical studies as well as studies using mammalian models (rats and mice) suggest that prenatal drug exposure compromises both prenatal and postnatal development of the central nervous system of the infant, yielding behavioral deficits that are not only limited to the immediate postnatal period, but throughout the life span. Methadone, a synthetic opiate that has longer-lasting physiological effects than morphine or heroin, has become the drug of choice used by

doctors to treat drug abusing females and their infants, and it is recognized that methadone treatment produces far better outcomes in infants, with symptoms intermediary to those of infants with no treatment and prenatal exposure and of non-exposed infants (reviewed in Greene and Goodman, 2003; Kandall, 1999; Johnson *et al*., 2003; Joseph *et al*., 2000).

1. Infant behavioral outcome

Most studies report that infants born to drug using mothers have significantly lower birth weights and head circumferences than control-matched non-exposed infants (Lester *et al*., 2002; reviewed in Hans, 1992; Kaltenbach and Finnegan, 1992). Methadone-treated women give birth to children that have intermediary birth weights and head circumferences, that is, these values fall somewhere between those for an unexposed infant control and a heroin- or morphine-exposed infant (reviewed in Hans, 1992; Kaltenbach and Finnegan, 1992). Opiate-exposed infants demonstrate several characteristics, which are displayed as early as the 1st postnatal week or as late as four to six months following birth: 1) they are easily aroused by stimuli that in control infants evoke no response, 2) they have higher pitched cries, 3) they have difficulty in self-quieting (infants often can quiet themselves without mother intervention; however opiate-exposed infants display difficulty in this task), 4) they have disrupted sleep patterns, 5) they are less often in an alert, non-agitated behavioral state, and 6) they show disorganized motor control (reviewed in Hans, 1992). Studies also show that substance-abusing parents are at a higher risk for child maltreatment, and due to prenatal exposure and/or maltreatment, these children have disorganized and even avoidant attachment behaviors (Goodman *et al*., 1999; Hans, 1996; Jaudes *et al*., 1995; Kelley, 1992, 2003; Mikhail *et al*., 1995).

Of opiate-exposed infants, 60-90% suffer from drug withdrawal, with symptoms that include: 1) wet-dog shakes, 2) tremors, 3) frantic fist sucking, 4) hyperactivity, 5) high-pitched crying, 6) agitation, 7) fevers, 8) seizures, 9) diarrhea, 10) vomiting, 11) changes in respiration, 12) difficulty in entering into quiet sleep, and 13) skin mottling (D'Apolito and Hepworth, 2001; reviewed in Chasnoff, 1988; Fabris *et al*., 1998; Levy and Spino, 1993; Zagon and McLaughlin, 1992). The onset of this syndrome has been reported to vary between infants, with a display of symptoms as early as the 1st postnatal week to four to six months following birth (reviewed in Hans, 1992; Hutchings, 1982; Zagon and McLaughlin, 1992). Prenatal opiate exposure has also been identified as one of the leading risk factors for Sudden Infant Death Syndrome (SIDS) (reviewed in Hans, 1992; Legido, 1997).

All of these characteristics displayed by the prenatally exposed infant suggest that development of the central nervous system has been compromised. However, human studies are often confounded (i.e., polydrug use by the mother, the mother's lifestyle during and after the pregnancy, socioeconomic status, the quality of maternal care), and it is difficult to dissect the effects of opiate exposure from other factors that may amplify the drug impact. To remedy this, researchers have commonly used rodent models. In parallel with clinical studies, studies in rat neonates demonstrate that prenatal opiate exposure produces: 1) lower birth weights and head circumference; 2) increased infant mortality rates, 3) drug dependence, 4) drug withdrawal, 5) increased vocalizations (similar to the human cry), urination, defecation, wet-dog shakes/tremors, 6) altered heart and respiratory rates, and 7) the appearance of seizures (reviewed in Barr and Jones, 1994; Zagon and McLaughlin, 1992).

2. Neural consequences of prenatal opiate exposure

Rodent neural studies have shown that prenatal opiate exposure down-regulates several neurochemical systems, including the endogenous opioid, noradrenergic, dopaminergic, and cholinergic systems (Belcheva *et al*., 1998; Robinson, 2002; Schindler *et al*., 2004; Slamberova *et al*., 2003; Slotkin *et al*., 2003; Tempel et al., 1995; reviewed in Zagon and McLaughlin, 1992). Chemicals shown to be necessary for the control of and regulation of both prenatal and postnatal synaptic development, such as nerve growth factors and brain derived nerve factors, are decreased following prenatal opiate exposure (Wu *et al*., 2001; reviewed in Hammer and Hauser, 1992; Levitt, 1998; Zagon and McLaughlin, 1992). In addition, data show that prenatal opiate exposure alters brain DNA content, cell birth and synapse number, neural structure (the number and size of dendritic spines) and neural activity, with some areas showing hyperactivity while others show suppression in activity (reviewed in Hammer and Hauser, 1992; Malanga and Kosofsky, 1999; Zagon and McLaughlin, 1992). Overall, rodent studies have shown that the brain's neural architecture, messengers, and functioning are all altered by prenatal opiate exposure (again, with methadone treatment producing less detrimental effects). These changes provide a framework for understanding the behavioral disruptions displayed by the postnatal infant.

3. The effects of prenatal opiate exposure in the older animal

Clinical and experimental research suggests that the effects of prenatal opiate exposure are not limited to infancy. Indeed, toddlers show lower scores on visual and auditory assessment tasks if their mothers used opiates during pregnancy (Bunikowski *et al*., 1998; Moe, 2002; reviewed in Hans, 1992). These children also often display attention deficits, psychiatric disorders, lower performances on intelligence tests, math and verbal deficits, and a general underperformance in learning tasks (Suess *et al*.,

1997; reviewed in Hans, 1992; Hutchings, 1982). With prenatal opiate exposure, school-aged children show inappropriate adult-peer relationships (seek the attention of adults more than non-exposed children), they have trouble forming peer bonds, and overall, appear inept in social situations; and these problems persist into adulthood (reviewed in Hans, 1992; Zagon and McLaughlin, 1992).

Similarly, use of rodent models has further demonstrated the persistence of adverse consequences throughout development. Use of rats during their post-weaning period has demonstrated that prenatal opiate exposure disrupts normal play behavior, a behavior important in both rats and humans for development of peer relationships and later development of sexual behavior (Hol *et al*., 1996; reviewed in Vathy, 1995). Prenatally exposed rats have learning deficits in adulthood, changes in responses to stressful situations (and behavior in an open-field task), altered responses to drug administration, and changes in behavior that may be sex-specific (Gagin *et al*., 1997; Rimanoczy *et al*., 2003; Slamberova *et al*., 2001; reviewed in Zagon and McLaughlin, 1992).

B. IMPACT OF EARLY ADVERSE EXPERIENCES ON NEURAL AND BEHAVIORAL DEVELOPMENT

It is well understood that infant adverse experiences produce long lasting changes in behavior, and researchers have only recently begun to document the neural modifications that presumably underlie the behavior in both humans and animal (reviewed in Glaser, 2000; Grossman *et al.*, 2003; Gunnar, 2001; Levine, 2001; Machado and Bachevalier 2003; Meaney *et al.,* 2002; Sanchez *et al.*, 2001; Schore, 2001, 2002; Teicher *et al.*, 2003). Based upon the animal literature, it is becoming increasingly clear that an early stressful environment canalizes brain development to prepare the infant to accommodate a lifetime within a stressful environment, while an

enriched infant environment prepares the brain to successfully cope with any environment. Clinical data support such an interpretation. For example, an abused child is better at detecting angry faces, and is more likely to interpret a situation as negative or dangerous (Pollak and Kistler, 2002).

1. The effects of early maltreatment on brain and behavioral development

Neural changes have been documented in adults abused as children, most notably in the temporal lobe including the amygdala, the frontal cortex, locus coeruleus, hippocampus and cerebellum (Perry *et al.*, 1995; Teicher *et al.*, 1997; Vythilingam *et al.*, 2002). In addition to attachment models, other animal models such as ones of deprivation and maternal caregiving (reviewed in Kuhn and Schanberg, 1998; Caldji *et al.*, 1998; Levine, 2001; Liu *et al.*, 2000; Meaney *et al.*, 2002; Sanchez *et al.*, 2001) are helping to differentiate between causation and correlations, and they are providing a more systematic assessment of the specific maternal behaviors regulating infant physiology and behavior (for reviews see Hofer, 2002; Hofer and Sullivan, 2001; Levine, 2001). Work from these models suggests that the LC, amygdala, hippocampus, frontal cortex, and HPA axis are all affected by maternal behaviors, and thus offer potential sites to understand the damaging effects of infant stress and maltreatment on infant attachment, subsequent behavioral development, and the etiology of psychiatric disorders (Dent *et al*., 2001; reviewed in Francis *et al.*, 1999; Gutman and Nemeroff, 2002; Heim and Nemeroff, 2001; Levine, 2001; Sanchez *et al.*, 2001).

2. Common mechanisms between the impact of prenatal opiate exposure and maltreatment

Disorganized infant attachment is a significant risk factor for childhood and adult psychiatric disorders, and both animal and clinical studies suggest that disorganized and

avoidant attachment behaviors are often precipitated by disruptions to the development of the endogenous opioid system, whether they be prenatal or postnatal disturbances. Indeed, prenatal or postnatal stress is sufficient to alter the developing opioid system in the infant brain (Carden *et al*., 1996; Insel *et al*., 1990; Sanchez *et al*., 1996). Thus, compromised function of the endogenous opioid system appears to surrender the infant susceptible to altered attachment behaviors, and thus subsequent behavioral deficits. An understanding of the role of the endogenous opioid system in the neural circuitry and behavior supporting attachment offers an avenue into understanding the complexity between prenatal or postnatal disturbances to this system and subsequent behavioral deficits. The work in this dissertation evaluates the role of the endogenous opioid system in learned behaviors responsible for infant attachment in the rat, and assesses their role within the neurocircuitry responsible for attachment.

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Figure captions

Figure 1. During the 1st postnatal week, a wide range of stimuli supports odor preference learning. Milk, stroking, and shock each can produce an odor preference in PN6 pups. However, after the sensitive period, tactile stimulation no longer serves as a rewarding stimulus, and thus does not produce an odor preference. Conversely, shock in PN12 pups produces an odor aversion, illustrating a change in the hedonic value of shock. In the bottom graphs, the total number of choices toward peppermint represents the number of choices out of 5 testing trials in a Y-maze. (Modified from Sullivan and Wilson, 1994; Hofer and Sullivan, 2001)

Figure 2. Schematic representation of the olfactory system (not drawn to scale). Information progresses from the olfactory bulb to either the piriform cortex or amygdala. A small percentage of neurons also make connections in the anterior olfactory nucleus. Locus coeruleus fibers terminate in the olfactory bulb, providing rich noradrenergic input that is both necessary and sufficient for neonate learning.

Figure 3. During acquisition the olfactory bulb's primary output neurons (mitral cells) exhibit a heightened excitatory response in experimental pups (odor – reward groups) as compared to control groups. As indicated by mitral/tufted cell single unit responses, control pups (Odor Only) habituate to the odor, while pups receiving contiguous odor presentations and stimulation of the medial forebrain bundle/lateral hypothalamus as a reward (Paired) maintain odor responsiveness. (Modified from Wilson and Sullivan, 1992)

Figure 4. Amygdala activity as measured by 2-DG autoradiography (quantitative optical densitometry). The amygdala 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 odor aversions are easily learned. (Modified from Sullivan *et al.*, 2000a)

TABLE I
EVIDENCE THAT DEVELOPMENTAL CHANGES IN THE FUNCTIONING OF THE LOCUS COERULEUS (LC) UNDERLIE TERMINATION OF THE UNIQUE LEARNING ABILITIES DISPLAYED DURING THE SENSITIVE PERIOD

Physiological and cellular characteristics	Infant LC	Adult LC	References
Response to sensory stimuli	More responsive to sensory stimuli, including both noxious and non-noxious stimuli	Less responsive, particularly to non-noxious stimuli	Aston-Jones and Bloom, 1981; Aston-Jones et al., 1994; 1999; Harley and Sara, 1992; Kimura and Nakamura, 1985; Nakamura and Sakaguchi, 1990; Sara *et al.*, 1995
Response to repeated sensory stimulation	Fails to habituate	Habituates	Kimura and Nakamura, 1985; Nakamura and Sakaguchi, 1990; Vankov *et al.*, 1995
Response to sensory stimulation	20-30sec response	Few msec response	Kimura and Nakamura, 1985; Nakamura and Sakaguchi, 1990
	Electronically coupled LC	Less electronically coupled LC	Christie and Jelinek, 1993; Marshall *et al.*, 1991
	Large output of NE due to excitatory autoreceptors	Smaller output of NE due to emergence of inhibitory autoreceptors	Kimura and Nakamura, 1985; Nakamura and Sakaguchi, 1990
	Produces 200-300% increase in olfactory bulb NE levels	Produces no increase in NE olfactory bulb levels	Rangel and Leon, 1995
Tyrosine hydroxylase levels	Greater levels, with a transient peak at postnatal day 10	Decreased levels	Bezin *et al.*, 1994 a, b

Figure 1

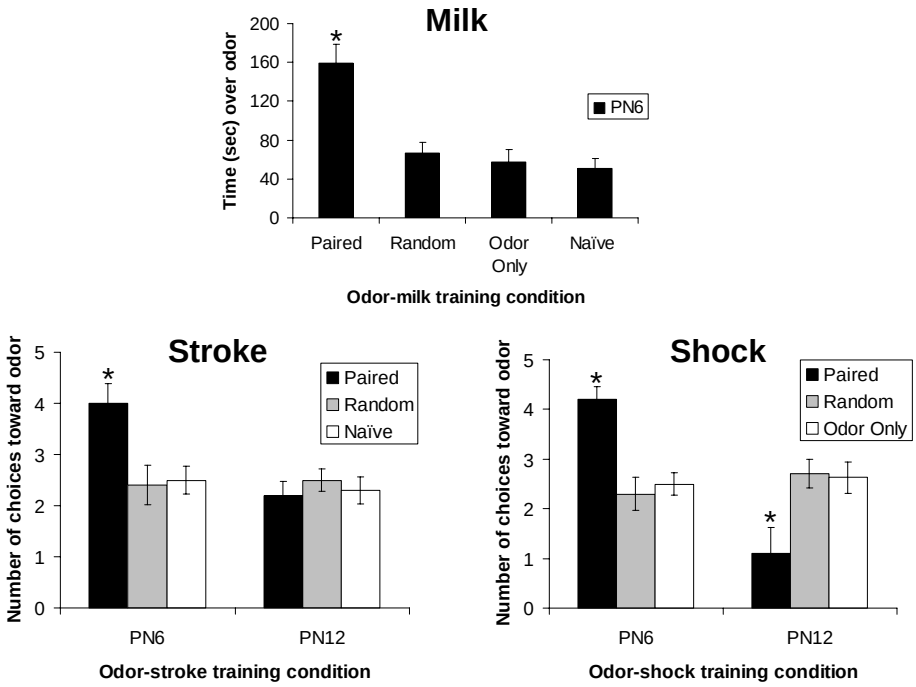

Milk
Time (sec) over odor
200
160
120
80
40
0
*
PN6
Paired
Random
Odor Only
Naïve
Odor-milk training condition
Stroke
Number of choices toward odor
5
4
3
2
1
0
*
Paired
Random
Naïve
PN6
PN12
Odor-stroke training condition
Shock
Number of choices toward odor
5
4
3
2
1
0
*
*
Paired
Random
Odor Only
PN6
PN12
Odor-shock training condition

Figure 2

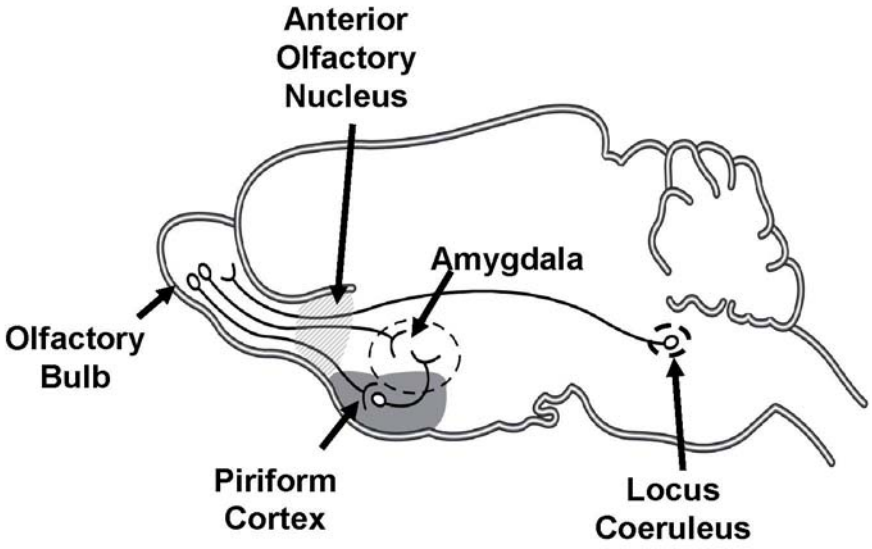

Anterior
Olfactory
Nucleus
Amygdala
Olfactory
Bulb
Piriform
Cortex
Locus
Coeruleus

Figure 3

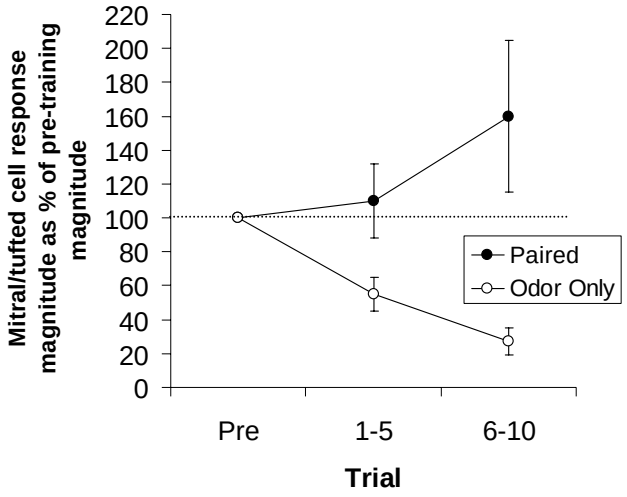
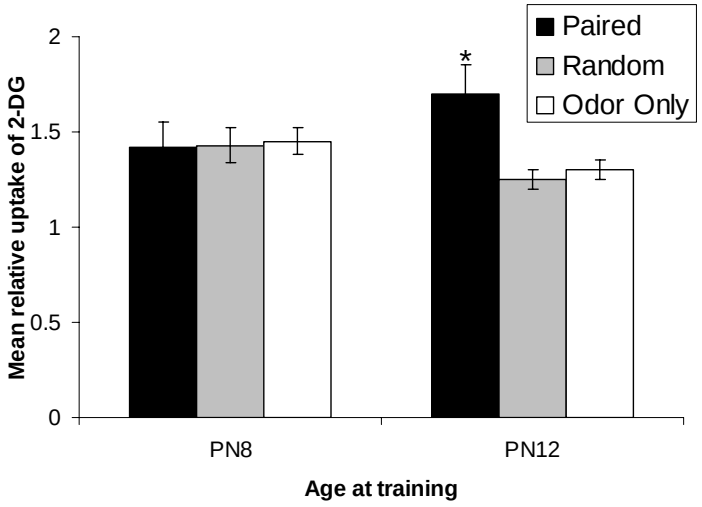

Mitral/tufted cell response magnitude as % of pre-training magnitude
220
200
180
160
140
120
100
80
60
40
20
0
Pre
1-5
6-10
Trial
Paired
Odor Only

Figure 4


Paired
Random
Odor Only
*
2
1.5
1
0.5
0
Mean relative uptake of 2-DG
PN8
PN12
Age at training

Chapter 2

Memory of early maltreatment: Neonatal behavioral and neural correlates of maternal maltreatment within the context of classical conditioning

Background: A child maltreated by their caregiver still attaches to the caregiver. Yet neurobehavioral development is compromised, leaving the child susceptible to mental illness. To better understand the child's paradoxical attachment to the abusive caregiver, we developed an abusive rodent model that capitalizes on rat pups' dependence on maternal odor learning for attachment.

Methods: We used a classical conditioning paradigm pairing a novel odor with a stressed mother that predominantly abused pups. Additionally, we used Fos immunohistochemistry to assess brain areas involved in learning this pain-induced odor preference within a more controlled maltreatment environment (odor–shock conditioning).

Results: Odor – maternal maltreatment pairings within a natural setting and odor – shock pairings both resulted in odor preferences. Pain-induced learning resulted in changes in gene expression in the olfactory bulb, and for the first time, we show processing of the odor by the olfactory cortex (excluding the amygdala).

Conclusions: Our new maternal maltreatment paradigm suggests that caregiver maltreatment can result in attachment due to unique learning circuitry that uses the anterior piriform cortex but not the amygdala. A fuller understanding of unique infant brain function may provide insight into why early maltreatment affects psychiatric well-being.

In altricial species, evolution appears to have ensured that infants will quickly learn and demonstrate robust preferences to stimuli associated with parental care, regardless of the quality of care (Sullivan 2003). Indeed, clinical data show that abused and neglected children exhibit strong, albeit disordered, attachment to their caregiver, with abuse and neglect producing distinct clinical outcomes (Bowlby 1965; Carlson et al 1990; Cicchetti and Toth 1995; Helfer et al 1997; Hesse and Main 2000; Morton and Browne 1998; Schore 2002). Though most functional imaging studies have focused on adults with a history of childhood maltreatment, such studies suggest that neglect or abuse during childhood compromises brain development, most notably for the limbic system (hippocampus, amygdala), stress axis (locus coeruleus, hypothalamus, amygdala) and cerebellum (Bremner 2003; Glaser 2000; Teicher et al 2003; Schore 2002).

Research using animal models of maternal deprivation in rodents and nonhuman primates parallel human imaging studies suggesting that child neglect also produces long-term compromises in the limbic system, stress axis, and cerebellum (Dettling et al 2002; Glaser 2000; Huot et al 2002; Liu et al 2000; Pryce et al 2004; Rosenblum et al 1994; Sanchez et al 2001; van Oers et al 1998). These findings are further supported by models of enrichment correlating high levels of maternal care (licking/grooming, nursing) with the enhancement of pups' neurobehavioral outcome (Caldji et al 1998; Liu et al 2000; Meaney 2001; van Oers et al 1998). Overall, such studies demonstrate that maternal behaviors affect development of these brain areas, and thus offer potential sites to understand the damaging effects of early maltreatment on subsequent behavioral development (Bremner 2003; Dent et al 2001; Francis et al 1999; Heim and Nemeroff 2001; Kaufman et al 2000; Levine 2001; Nemeroff 2004; Sanchez et al 2001; Teicher et al 2003).

Trauma within the attachment system leaves the infant particularly vulnerable to

childhood and adult psychiatric disorders, behavioral changes in fear and anxiety, and alterations in neural circuits, particularly those regulating stress and emotion (Connor et al 2003; Gunnar 2003; Heim and Nemeroff 2001; Teicher et al 2003; Zeanah et al 2003). The overlap in the aforementioned brain structures and those active in the attachment system suggests a possible mechanism for the commanding effects of early adverse experiences on the etiology of psychiatric disorders. Using an infant animal model that induces maternal abuse within the context of attachment offers a more direct approach to understanding the damaging effects of maltreatment on brain development, especially concerning the overlap in neural circuitry supporting attachment and the etiology of childhood psychiatric disorders. Furthermore, use of such an animal model that approximates an environment in which an infant receives both abusive and positive caregiving may yield animal data that are more relevant to the clinical data.

In the rat, neonates form memories of odors associated with both pleasant (milk or warmth) or aversive stimuli (tail-pinch or shock) that simulate aspects of maternal care (Camp and Rudy 1988; McLean et al 1993; Sullivan et al 1986, 2000a), as odor learning is critical to the development of mother-infant attachment (Hofer and Sullivan 2001). These memories are expressed as odor preferences, as indicated by pup orientation towards the odor and even the climbing of an obstacle to approach the odor (Camp and Rudy 1988; Roth and Sullivan 2001; Sullivan et al 2000a). The neural circuitry responsible for this early olfactory learning and how the neonate's brain is specialized to secure attachment have begun to be elucidated. This circuitry includes the locus coeruleus, the olfactory bulb, and an immature and/or limited functioning amygdala (Sullivan 2003). A similar neural circuit has been characterized for attachment related to reproduction in the adult (Carter et al 1995; Fleming et al 1999; Insel and Young 2001; Kendrick et al 1997).

To assess how abuse within the mother-infant dyad supports attachment in the rat, we used two approaches to examine the neurobiology contributing to the memory of neonate odor preferences. First, we examined neonate learning and memory with a new paradigm that uses maternal abuse and odor within the context of attachment. Second, we used immunohistochemical marking of the immediate early gene *c-fos* to examine neonate brain areas activated following a more controlled maltreatment environment (odor – shock conditioning, a fear-conditioning paradigm used in adults).

Methods and Materials

Subjects and Husbandry

Both male and female pups, born of Long-Evans rats (Harlan, IN) in the animal vivarium at the University of Oklahoma, were used for experiments. Mothers were housed in polypropylene cages with wood shavings, kept in a temperature (20 °C) and light (12h:12d) controlled environment, and had food and water continually available. Day of parturition was termed 0 days of age, and litters were culled to 5 males and 5 females on postnatal (PN) 1 or 2. The University of Oklahoma Institutional Animal Care and Use Committee approved all procedures.

Exp 1 Odor - maternal maltreatment conditioning. Forty-eight pups from 7 litters were used in the maternal maltreatment experiment. On PN 7 – 8 (13.9 – 18.8 g) pups were assigned to one of 4 training conditions: 1) Paired (n=13) – pups received both maltreatment by a mother and a novel odor (peppermint); 2) Unpaired (n=8) – pups received the odor 30 min before receiving the maltreatment; 3) Odor Only (n=9) – pups received only the odor; and 4) Maltreatment Only (n=8) – pups received only maltreatment by a mother. The training apparatuses consisted of a 45.5 x 30.5 x 45 (l x w x h) cm opaque Plexiglas boxes. To serve as an effective stressor for the mothers, we

provided limited clean aspen shavings (100 ml) on the floors of the chambers (Gilles et al 1996). Chambers were lit with a red light and the lid was covered with Privacy Mirror Film to ensure that behavior was not disturbed by experimenter observations (Gila, CPFilms Inc., Martinsville, VA).

Mothers (non-biological to the experimental pups) were placed in the chambers only 5 min prior to receiving the pups (1 male and 1 female were trained simultaneously within each condition). This short time also served as a stressor for induction of abuse. Odor was presented with a kimwipe (25 µl of pure McCormick peppermint extract; kept in a fume hood for 5 minutes) placed on the lid of the chamber. A training session lasted 30 min, during which maternal and pup behaviors were recorded in 5 min intervals. Mothers exhibited several behaviors during each observation period (7 total periods). Maternal behaviors observed and classified as abusive were: (1) stepping: the mother steps or jumps on the pup with one foot or both feet; (2) throwing: the mother throws the pups some distance; (3) dropping: the mother drops a pup during retrieval or transport; (4) dragging: the mother drags a pup across the chamber; (5) pushing away/actively avoiding: the mother runs from a pup's approaches or pushes a pup away from her, crushing the infant onto the floor; and (6) rough handling: the mother aggressively grooms a pup or transports a pup by an arm or leg. These behaviors typically elicited pup vocalization, a measure indicative of neonate distress and pain (White et al 1992). Additionally, several of these behaviors are categorized as abusive in primates (Brent et al 2002; Maestripieri 1998), qualitatively different from those that fall within the normal repertoire of maternal behaviors (Alberts and Cramer, 1988; Denenberg et al 1969; Fleming and Rosenblatt 1974), and may harm the infant or interfere with development (Maestripieri and Carroll 1998; Righthand et al 2003; Zigler and Hall 1990). Less frequently, positive maternal behaviors were observed, and included pup grooming, anogenital licking, and nursing. These behaviors did not elicit pup vocalization.

Following training, pups were placed in a 30 °C incubator for 15 min, and then returned to the biological mother until testing in a 2-odor-choice test the following day.

For comparative purposes, an additional 25 pups (PN 7-8; 13.7 – 20.2 g) were trained using a similar training paradigm as described above; however, pups received pairings with mothers who were non-abusive, thus representing a more normal and positive learning environment. In this natural paradigm, mothers were placed inside the training chambers, and were given 2 treatments that prevented the stress-induced maltreatment: copious shavings on the floor (2 cm layer) and a 1 hour habituation time before receiving pups. The 4 training conditions were: 1) Paired (n=7) – pups received both maternal care and peppermint; 2) Unpaired (n=6) – pups received peppermint 30 min before receiving the care; 3) Odor Only (n=6) – pups received only peppermint; and 4) Maternal Care Only (n=6) – pups received only care from a mother.

Exp 2 Immunohistochemistry (IHC) and odor – shock conditioning. To assess the neonate brain under exclusively painful learning conditions, pups were trained using odor – shock conditioning. Pups feel pain, and vocalize and try to escape in response to shock (Barr 1995; Emerich et al 1985; Stehouwer and Campbell 1978; Sullivan et al 2000a). Eleven pups from 4 litters were used for IHC neural analysis. On PN 7 – 8 (15.0 – 19.4 g) pups were randomly assigned to a condition: 1) Paired odor – shock (n=4); 2) Unpaired odor – shock (n=4); and 3) Odor Only (n=3). Once placed inside the training apparatus (individual 600 ml beakers), pups were given a 10 min period to recover from handling. During a 1 h training session, pups received 14 presentations of a 30 sec peppermint odor and a 1 sec 0.5 mA tail-shock, with an intertrial interval of 4 min. Paired odor – shock subjects received 14 pairings of the 30 sec odor with shock during the last second of the odor, while Unpaired odor – shock subjects received a 1 sec shock 2 min after an odor presentation. Odor Only subjects received only the

peppermint odor. Peppermint odor was delivered with a flow-dilution olfactometer at 2L/min and at a concentration of 1 peppermint vapor:10 clean air. Following training, pups were placed in a 30 °C incubator, and after 90 min their brains were removed and frozen in 2-methylbutane (-45 °C), and placed in -70 °C until cutting and post-fixation for IHC.

To verify associative learning for the IHC neural assessment, we recorded behavioral responses to the odor during training to construct acquisition curves. Due to motor immaturity, pups' generalized movements were recorded (0, no movement of the extremities – 5, movement of all 5 extremities excluding the tail; Hall 1979). We also trained 19 additional pups (PN6 –7, 13.3 – 18.1 g; Paired n=6, Unpaired n=6, and Odor Only n=7) to test for an odor preference the next day in a Y-maze.

Behavioral testing

2-odor-choice. One day following training in Experiment 1, pups were tested in a 2-odor choice test. The testing apparatus was a Plexiglas arena (24 x 14 cm, l x w) with a wire mesh floor. The floor was divided into 2 areas by a 2 cm midline: one area contained the conditioned odor (Kimwipe scented with 25 µl of peppermint extract placed in a ventilation hood for 15 min), and the other contained 100 ml of clean aspen shavings. The time spent on each side was recorded (Videomex-V, Columbus Instruments, Columbus, OH). Each pup received 3, 60 sec trials, with a counterbalanced orientation for each trial. The floor was wiped clean between trials.

Y-maze. For the 19 additional pups trained in Experiment 2, on the day following training they were removed from the mother and tested using a Y-maze. The Y-maze consisted of a habituation chamber (7 cm long and 9 cm wide) and 2 alleys (22 cm long and 9 cm wide) extending at 45° angles. The habituation chamber was separated from

the alleys via 2 removable doors. One arm of the maze contained aspen wood odor (20 ml of clean, aspen shavings in a petri dish), while the other arm contained the peppermint odor (25 μl of peppermint extract on a kimwipe placed in a ventilation hood for 5 min). Each pup was placed in the starting chamber and given 5 sec for habituation before the doors to the alleys were removed. Each subject had 60 sec to make a choice, which required the pup to enter one of the alleys. Each subject was given 5 sequential trials, and the floor was wiped clean (using a cloth with water) between each trial. The orientation of the pup was counterbalanced between trials when placed in the habituation chamber. Observations of each pup were made blind to the training condition.

Fos IHC

Immediate early genes serve as markers of changes in neuronal activity, and thus are indicative of changes in neuronal plasticity reflective of learning and memory (Dragunow and Bilkey 2002; Herrera and Robertson 1996; Kaczmarek 2002; Tischmeyer and Grimm 1999). Brains (from pups trained for IHC in Experiment 2) were coronally sectioned (20 μm) with a cryostat, and every 6th section was collected on pre-treated slides (Fisherbrand Plus, Fisher) for Fos processing, and every 7th section was collected for cresyl violet staining. Fos sections were post-fixed for 1 hour in 4% paraformaldehyde/0.1 M phosphate buffer (PB, pH 7.2), and then rinsed in 0.1 M PB (pH 7.2) and dried in a cool air-stream. Slides were stored in boxes with Drycap capsules (Ted Pella Inc., Redding, CA) in -20 °C until Fos processing. To eliminate peroxidase activity, sections were incubated in 0.1 M phosphate buffer saline (PBS; pH 7.2) containing 3% H_2O_2 and 10% methanol. Following PBS rinses and incubation in 0.2% Triton X-100, slides were incubated in 3% Bovine Serum Albumin for 1 hr. After

additional PBS rinses, the slides were treated overnight at 4 °C with the primary antibody (*c-fos*, sc-52, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in PBS. Afterwards, they were rinsed in PBS, incubated in the secondary biotinylated antibody (goat anti-rabbit, Vector Laboratories, Burlingame, CA) for 2 hrs at room temperature, and then incubated for 90 min in avidin-biotin-peroxidase (ABC) complex solution. Following, slides were treated with PB containing 0.1% 3,3'-diaminobenzidine and H_2O_2. Slides were then dehydrated in alcohol and Histoclear, and coverslipped for microscope examination.

Fos-positive cells were counted using a microscope (Olympus with 10x objective) equipped with a drawing tube. Brain areas were outlined using the corresponding cresyl violet sections and a stereotaxic atlas (Paxinos and Watson 1986), and all Fos-positive cells were counted bilaterally without knowledge of the training condition. A Fos-positive cell was distinguished from the background by the density of staining, the shape, and the size of the cell. The mean number of Fos cells per brain area for an animal was determined by averaging the counts from all sections (2 sections counted for each brain area). Brain areas examined were the: granule cell layer of the olfactory bulb, the anterior and posterior piriform cortex, and the basolateral/lateral and central amygdaloid nuclei.

Statistical analysis

We used the analysis of variance (ANOVA) and *post hoc* Fisher tests to analyze differences between training conditions and drug treatment groups for both behavioral and Fos experiments. In addition, we used paired and unpaired t-tests to compare the frequency of abusive and normal maternal behaviors in Experiment 1.

Results

Neonates learn a preference from odor – maternal maltreatment conditioning

To our knowledge, this is the first study to use physical abuse from rat mothers within a neonate classical conditioning paradigm. As shown in Figure 1, pups that received contiguous presentations of maltreatment and peppermint odor learned an odor preference, as demonstrated by the significant amount of time spent over the odor during the test relative to control subjects [$F(3,34) = 4.745$, $p < 0.01$]. *Post hoc* tests indicate that the Paired pups spent significantly more time over the peppermint odor ($p < 0.05$). Likewise, pup training within the natural paradigm resulted in a learned odor preference [$F(3,21) = 4.395$, $p < 0.02$; Fig. 2]. *Post hoc* tests indicate that pups receiving simultaneous odor and maternal care spent significantly more time over the odor ($p < 0.05$).

Maternal behaviors observed and classified as abusive included stepping/jumping, throwing, dropping, or dragging, pushing away or actively avoiding, and rough handling. Since behaviors were only a few seconds in length and mothers exhibited several within each observational period, we analyzed the frequency of behaviors displayed over the course of the training session. To show there were differences in maternal behavior within and between the two training paradigms, t-tests were used to compare the frequency of behaviors displayed during the 7 total observation periods for all the mothers used. As illustrated in Figure 3, within the maltreatment paradigm, there were significantly more abusive behaviors observed than normal maternal behaviors [Paired t-test, $t(14) = -3.932$, $p<0.01$], producing an adverse learning environment. In contrast, in the natural paradigm mothers were rarely abusive [Paired t-test, $t(9) = 9.390$, $p<0.01$], creating a positive learning environment. Between the paradigms, mothers were significantly more abusive towards infants in the maltreatment paradigm than the natural paradigm [Unpaired t-test, $t(23) = 9.253$,

p<0.01]. In addition, these mothers displayed significantly less normal and non-abusive behaviors [Unpaired t-test, t(23) = -5.933, p<0.01].

Table 1 provides a comparison of the frequencies of maternal and pup behaviors observed within the paradigms. Infant stepping/jumping on and rough handling were the most frequently observed abusive behaviors, followed by pushing away/avoiding, infant dragging, dropping and throwing. Within the maltreatment paradigm, 45% of the time pups emitted audible vocalizations in response to mother – infant interactions. Mothers were also observed displaying normal behaviors toward infants within the maltreatment paradigm. Thus, odor preference learning may also be attributable to these behaviors, though the occurrence of abusive behaviors far exceeds those of non-abusive behaviors. Within the natural paradigm, there was the rare observation of a mother stepping on a pup and rough handling a pup. In sharp contrast to the maltreatment paradigm, mothers spent significant time displaying normal behaviors towards neonates, such as frequent licking and nursing. Within the natural paradigm, pups vocalized during less than 3% of the mother-infant interactions.

Neural circuitry supporting odor-pain conditioning

To examine changes in the neonate brain only attributable to pain-induced learning, we used odor – shock conditioning. In a more controlled learning environment, pups learn odor preferences following conditioning with only painful stimuli. Figure 4 indicates that tail-shock served as an effective stimulus in producing a conditioned odor preference [$F(2,16) = 10.708$, $p < 0.01$]. Analysis of acquisition behavior of the pups used for IHC neural analysis indicated that all subjects had similar pre-conditioning behavior, [$F(12,48) = 0.450$, $p = 0.934$; Fig. 5A]. However, analysis of activity in response to the odor indicated a significant effect of training condition, [$F(12,48) = 2.981$, $p < 0.01$; Fig. 5B]. *Post hoc* tests showed that subjects receiving Paired presentations of

odor and shock had significant acquisition in comparison to control subjects ($p < 0.05$), indicative of learning.

Figures 6 and 7 demonstrate learning-induced changes in Fos expression following odor – shock conditioning. Cellular staining was not observed without the primary antibody. Ninety min following the conditioning, there were experience-induced changes in the number of Fos-positive cells in the granule cell layer of the olfactory bulb [$F(2,7) = 8.151$, $p < 0.02$; Fig. 7]. Analysis with *post hoc* Fisher tests revealed that the Paired presentations of the odor – shock, which again generate a behavioral odor preference, induced significantly less Fos in the granule cell layer in comparison to control presentations ($p < 0.05$).

As changes in mitral cell activity (output neurons of the olfactory bulb) have been suggested to reflect learned associations in pups (Sullivan and Wilson 2003; Wilson et al 1987; Yuan et al 2003), we were interested in the activity of cortical areas known to process olfactory information transmitted from the bulb. ANOVA analysis revealed a training effect on Fos expression in the anterior piriform [$F(2,7) = 8.779$, $p < 0.02$; Fig. 7], but not the posterior piriform cortex [$F(2,7) = 0.018$, $p = 0.982$; Fig. 7]. *Post hoc* Fisher tests showed that Paired odor – shock presentations evoked significantly more Fos expression in the anterior piriform than Unpaired odor – shock or Odor Only presentations ($p < 0.05$). In agreement with autoradiography data (Sullivan et al 2000), learned odor – shock associations did not induce significant Fos expression in the basolateral/lateral or [$F(2,8) = 0.648$, $p = 0.545$; Fig. 7] central amygdaloid nuclei [$F(2,7) = 0.065$, $p = 0.937$; Fig. 7].

Discussion

Functional imaging studies suggest that child maltreatment generates changes in brain areas that regulate stress, cognition, and emotion. Behaviorally, short- and long-

term effects of child abuse and neglect include problems in self-regulation, excessive anxiety and fearfulness, aggressive behaviors (abuse, violence), substance abuse, mood disorders, and post-traumatic stress disorder (Brown 2003; Connor et al 2003; Pollak and Tolley-Schell 2003; Righthand et al 2003; Teicher et al 2002; Zeanah et al 2003). Changes in brain development possibly contribute to the etiology of the psychiatric problems. How maltreatment produces the changes in the brain and subsequent behavior remain unclear, hindered by our limited understanding of the age at which brain function emerges within specific behavioral systems. What is evident within the attachment system is that the neural circuitry of the infant brain is unique and optimized to support mother-infant attachment. Thus, use of an animal model with abuse in the context of attachment offers an approach to assessing how the unique circuitry of the infant brain responds to early maltreatment, how an infant can still form an attachment to an abusive caretaker, and ultimately how changes in the infant's brain contribute to childhood and adult psychiatric disorders.

Attachment within an adverse environment

In rat neonates, a painful stimulus (tail- or foot-shock or tail-pinch) paired with an odor induces a conditioned odor preference (Camp and Rudy 1988; Roth and Sullivan 2001; Sullivan et al 1986, 2000a). This study has provided additional validity for such models by demonstrating that actual physical abuse from the mother in the presence of an odor supports a learned odor preference. Both a novel environment with limited bedding and the lack of habituation time before receiving pups were sufficient stressors to induce maternal abuse far beyond what might naturally occur within the nest, such as the mother briefly stepping on the pups as she enters or leaves the nest. Indeed, a stressful environment serves as a significant risk factor for potentiating infant maltreatment, particularly in humans (Cicchetti 1990; Field 1983; Maestripieri and Caroll

1998; Righthand et al 2003; Schapiro and Mitchell 1983). In our maltreatment paradigm, pups received multiple counts of abusive behaviors. It is well documented that neonates feel pain; yet, pups pursued contact with the abusive mothers. This attachment was further reflected during testing as pups that had received the abuse in the presence of peppermint spent significant amounts of time over that odor during the testing period. A similar increase in attachment to a caretaker following abuse has been documented in primates (Arling and Harlow 1967; Maestripieri 1998), chicks (Hess 1962), and infant dogs (Fisher 1955, cited in Rajecki et al 1978).

Within our maltreatment paradigm, mothers also displayed normal and positive maternal behaviors, such as pup retrieval, licking and nursing, though their occurrence was far less than abusive behaviors. Such a paradigm approximates situations in which parents maltreat offspring but also provide positive parenting (Cicchetti 1998). Thus, the learned odor preference is attributable to both abusive and normal maternal behaviors, providing a very naturalistic model to assess the effect of early adverse experiences on the brain. Overall, clinical and experimental data suggest that the limbic system and stress axis are affected by maternal behaviors, suggesting sites to understand the damaging effects of maltreatment on behavioral development (Bremner 2003; Dent et al 2001; Francis et al 1999; Heim and Nemeroff 2001; Kaufman et al 2000; Levine 2001; Nemeroff 2004; Sanchez et al 2001; Teicher et al 2003). Our model of maternal maltreatment in rat pups offers another avenue to evaluate how early maltreatment, particularly abuse, affects the aforementioned neurocircuitry and extends the analysis to its involvement in early learning and memory processes responsible for mother-infant attachment.

The unique neurobiology responsible for attachment

During the first 10 days of a rat neonate's life, the brain is optimized to ensure rapid and robust odor preference learning, regardless of the hedonic value of stimuli. This learning is expressed by neonates' enhanced ability to acquire learned odor preferences and a decreased ability to acquire learned odor aversions. At least two brain structures underlie this unique odor learning: the hyperfunctioning noradrenergic locus coeruleus (LC) underlies heightened preference learning, and the hypofunctioning amygdala appears to underlie attenuated aversion learning (Moriceau and Sullivan 2004a, b; Okutani et al 1998; Rangel and Leon 1995; Sullivan and Wilson 1993; Sullivan et al 2000a, b; Yuan et al 2003). As the sensitive period ends, maturation of LC autoinhibition (reducing the release of norepinephrine, NE) greatly attenuates the rapid odor preference learning, and amygdala participation permits fear conditioning (Moriceau and Sullivan 2004a, b; Nakamura and Sakaguchi 1990; Rangel and Leon 1995; Sullivan et al 2000a; Sullivan 2003).

To understand how painful experiences influence attachment, we assessed the neonate's olfactory neural circuit using Fos immunohistochemistry and odor – shock conditioning. Odor – shock conditioning offers a more controlled maltreatment environment, as pups only receive painful shock. It should be noted that pups do feel pain, and vocalize and try to escape in response to shock (Barr 1995; Emerich et al 1985; Stehouwer and Campbell 1978; Sullivan et al 2000a). Thus, brain changes reflect a learned association based exclusively upon aversive stimulation. This the first study to examine changes in gene expression in the neonate brain following painful learning conditions.

Olfactory bulb. We found learning-induced changes in the olfactory bulb, further confirming that the olfactory bulb encodes odor learning (Johnson et al 1995; McLean et al 1999; Sullivan and Wilson 1995; Wilson and Sullivan 1992; Wilson et al 1987; Woo

and Leon, 1996; Yuan et al 2003, 2004). The olfactory bulb learning-associated changes are dependent upon the reward causing the LC to release high levels of NE, which prevent mitral cell habituation to repeated odor presentations (Okutani et al 1998; Sullivan and Wilson 1994, 2003; Yuan et al 2003), and are only acquired during the early attachment period (Moriceau and Sullivan, 2004b; Sullivan and Wilson 1995; Woo and Leon 1987).

Piriform cortex. The axons of olfactory bulb mitral cells project directly to the piriform cortex and other areas of the olfactory cortex (Haberly 2001; Schwob and Price, 1984). The present results suggest that a section of the olfactory cortex, the anterior piriform cortex, also encodes for neonatal odor learning and therefore adds another brain area to the neonate's neural circuitry for odor learning and memory. The piriform cortex has also been implicated in adult odor perception and learning (Barkai and Saar 2001; Datiche et al 2001; Linster and Hasselmo 2001; Litaudon et al 1997; Mouly et al 2001; Ressler et al 2002; Schoenbaum and Eichenbaum 1995; Tronel and Sara 2002; Wilson and Stevenson 2003; Wilson et al 2004; Zinyuk et al 2001).

Amygdala. Consistent with previous results, we found that the neonate amygdala does not appear to participate in odor learning (Moriceau and Sullivan 2004a; Moriceau et al 2004; Sullivan et al 2000a; Sullivan and Wilson 1993). Olfactory information projects directly to the amygdala, as well as via the piriform cortex (Schwob and Price, 1984). In adult and older pup learning, the amygdala is involved in odor learning, including preference and aversion conditioning (Ressler et al 2002; Rosenkranz and Grace 2002; Tronel and Sara 2002; Fanselow and Gale 2003; Maren 2003; McIntyre et al 2003; Packard and Cahill 2001; Schafe et al 2001; Walker and Davis 2002). The lack of amygdala participation in learning is not due to immaturity since olfactory bulb afferent fibers are present in the amygdala at birth, as are piriform afferents to the amygdala (Schwob and Price 1984). Moreover, in other studies from our lab, we have been able

to prematurely incorporate the amygdala into the neonate's odor learning circuitry and produce fear conditioning by increasing pups corticosterone levels (Moriceau and Sullivan 2004a; Moriceau et al 2004) or decreasing pups' opioid activity (Roth and Sullivan, in prep).

Shared circuitry of attachment and maltreatment

The neurobiological consequences of child maltreatment and the neurobiology mediating attachment exhibit shared circuitry. For example, childhood maltreatment produces long-term changes in several brain areas, including the amygdala and locus coeruleus, suggestive that altered development of these areas contributes to the emergence of psychiatric disorders. Indeed studies continue to associate changes in amygdala function with numerous psychiatric disorders (Liberzon and Phan 2003; Machado and Bachevalier 2003; McEwen 2003; Pujol et al 2004; Rothbaum and Davis 2003). We have shown the importance of the amygdala and locus coeruleus in supporting infant attachment, implying that compromises in their development may jeopardize attachment. The lack of amygdala participation during neonatal odor-pain learning and its susceptibility to changes induced by childhood maltreatment suggests a common mechanism for attachment despite abuse and the etiology of psychiatric disorders. An understanding of this shared circuitry should provide insight into the relationship between altered mother-infant attachment and subsequent emotional health in maltreated children.

In summary, the infant forms an attachment to their caregiver regardless of the quality of the parental care (Bowlby 1965; Hofer and Sullivan 2001). This is true for a wide range of species including chicks, dogs, nonhuman primates and rats, suggestive that our model is useful in assessing principles with wide phylogenetic importance (Fisher 1955 cited in Rajecki et al 1978; Harlow and Harlow 1965; Helfer et al 1997;

Hess 1962; Salzen 1970). Our assessment of the neural basis of neonatal learning in the context of maltreatment showed cellular changes within the olfactory bulb and adds the anterior piriform cortex to the neonatal learning circuit, indicating cortical processing in relation to memory in neonates. Our results further confirm that a brain area important for emotional memory and processing, the amygdala, does not appear to be involved in the neonate learning circuit. Moreover, we suggest that our new maltreatment paradigm may provide an understanding of how abusive treatment of offspring affects the neural circuitry responsible for attachment, how an infant can still form an attachment to an abusive caretaker, and the effect of long-term experiences with maternal maltreatment on brain development and psychiatric well-being.

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Table 1. Frequency of abusive or normal maternal behaviors observed during mother – infant interactions within Experiment 1.

Percent of observation periods in which behaviors occurred		
Behaviors	**Maltreatment Paradigm**	**Natural Paradigm**
Abusive		
Step or jump on	41.9%	1.4%
Throw	1.0%	0%
Drop	6.7%	0%
Drag	8.6%	0%
Push away/avoid	14.3%	0%
Rough handling	24.8%	5.7%
Pup vocalization	44.8%	2.9%
Normal Maternal		
Retrieve	36.2%	37.1%
Lick/Anogenital Lick	28.6%	71.4%
Nurse	19.0%	27.1%

Figure Captions

Figure 1. Training within the maltreatment paradigm resulted in a conditioned odor preference. Mean total time (sec±SEM) spent over the peppermint odor during testing is shown for pups that received Paired presentations of odor – maltreatment, Unpaired presentations of odor – maltreatment, Odor Only, or Maltreatment Only. * $p < 0.05$ between these groups.

Figure 2. Training within the natural paradigm resulted in a conditioned odor preference. Mean total time (sec±SEM) spent over the peppermint odor during testing is shown for pups that received Paired presentations of odor – maternal care, Unpaired presentations of odor – maternal care, Odor Only, or Maternal Care Only. * $p < 0.05$ between these groups.

Figure 3. Assessment of maternal behaviors in the two training paradigms. Mothers within the maltreatment paradigm predominately displayed abusive behaviors toward neonates, thus providing an adverse conditioning environment. Within the natural training paradigm, mothers were rarely abusive, and displayed normal and positive behaviors toward neonates. Bars represent the mean (±SEM) frequency of behaviors observed during training sessions.

Figure 4. Odor – shock conditioning effectively induces an odor preference in neonates. Mean (±SEM) number of approaches toward the peppermint odor during Y-maze testing is shown for the 19 additional pups that received Paired presentations of peppermint and tail-shock, Unpaired odor - shock presentations, or Odor Only presentations. * $p < 0.05$ between these groups.

Figure 5. Assessment of behavioral activation indicates that pups used for the IHC neural analysis demonstrated learning. (A) Pre-odor activity for pups that received either Paired or Unpaired odor – shock presentations, or Odor Only presentations. Each data point represents the summation of behavior from 2 consecutive trials; vertical lines indicate SEM. Conditioning treatment had no effect on activity before odor presentations. (B) Acquisition in response to the odor. Paired subjects showed significant acquisition (learning) in response to the odor in comparison to the control subjects.

Figure 6. Representative images of Fos expression 90 min following odor – shock conditioning in neonates (10x, scale bar = 100 μm). A-C illustrates Fos expression in the granule cell layer of the olfactory bulb in pups with Paired odor – shock (A), Unpaired odor – shock (B), or Odor Only (C) presentations. Likewise, D-F illustrates Fos expression in the anterior piriform cortex following conditioning. (GCL) granule cell layer; (MCL) mitral cell layer; (ant PIR) anterior piriform cortex; (I) Layer I; (II) Layer II; (III) Layer III; arrows represent examples of Fos-positive cells in each image.

Figure 7. Odor – shock abusive conditioning induces Fos expression in neonate olfactory circuitry. Paired presentations of odor and shock induce significant Fos-labeling in both the granule cell layer (GCL) of the olfactory bulb and the anterior piriform cortex (ant PIR). Conditioning did not produce significant changes within the posterior piriform cortex (post PIR) or the basolateral/lateral (BLA/LA) or central (CeA) amygdaloid nuclei. Bars represent the mean (±SEM) number of Fos-positive cells counted bilaterally in each brain area. Paired n=3-4, Unpaired n=3-4, Odor Only n=3. * $p < 0.05$ between these groups.

Figure 1

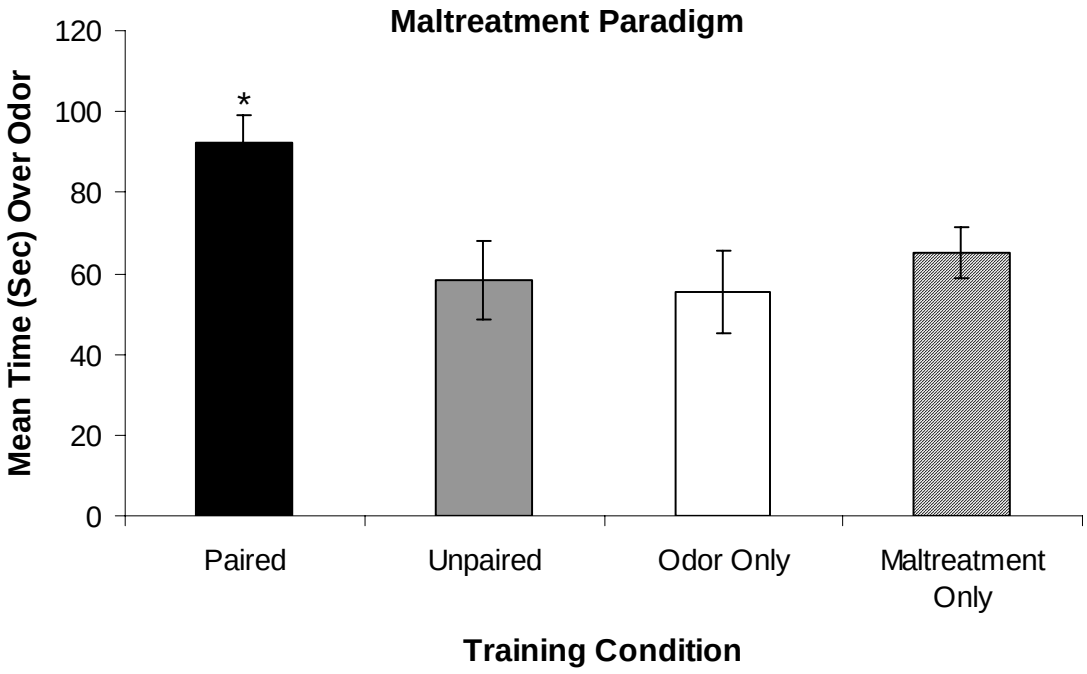

Maltreatment Paradigm
Mean Time (Sec) Over Odor
120
100
80
60
40
20
0
*
Paired
Unpaired
Odor Only
Maltreatment Only
Training Condition

Figure 2

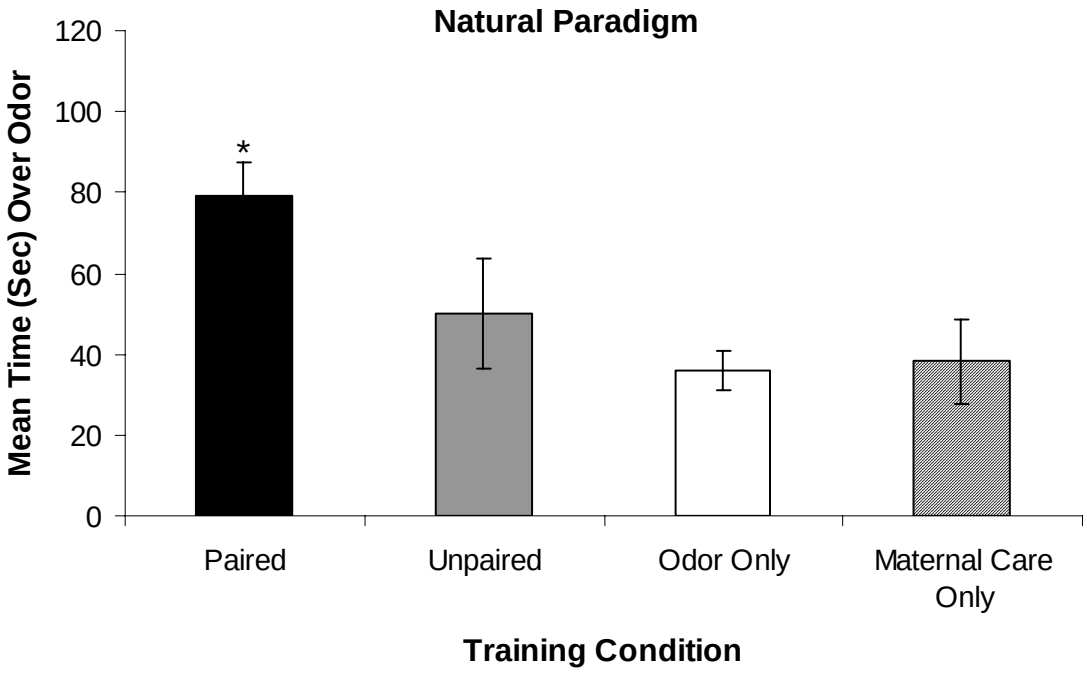

Natural Paradigm
Mean Time (Sec) Over Odor
120
100
80
60
40
20
0
*
Paired
Unpaired
Odor Only
Maternal Care Only
Training Condition

Figure 3

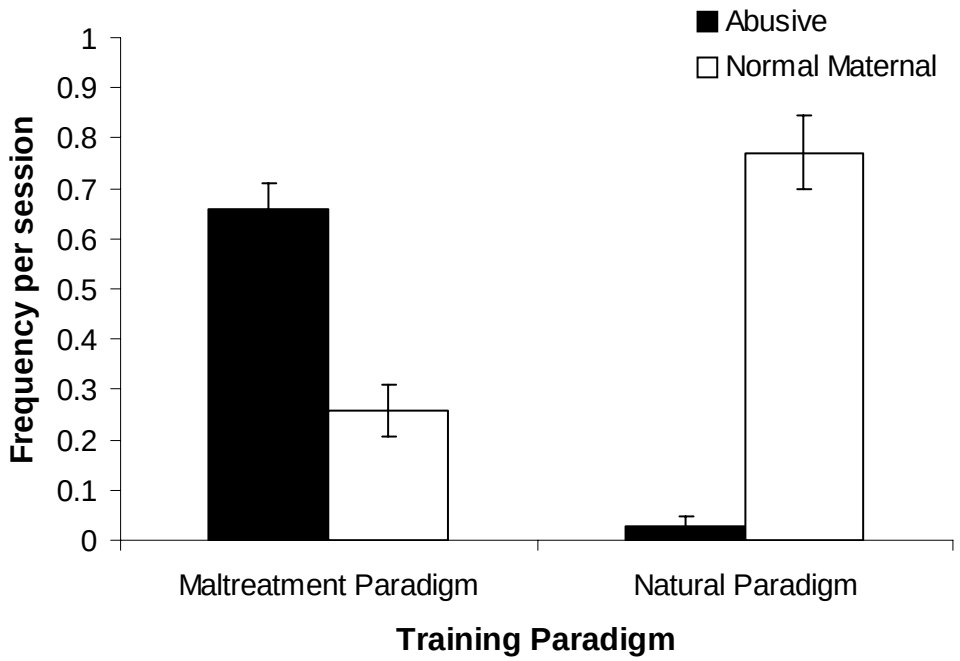

Abusive
Normal Maternal
Frequency per session
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Maltreatment Paradigm
Natural Paradigm
Training Paradigm

Figure 4

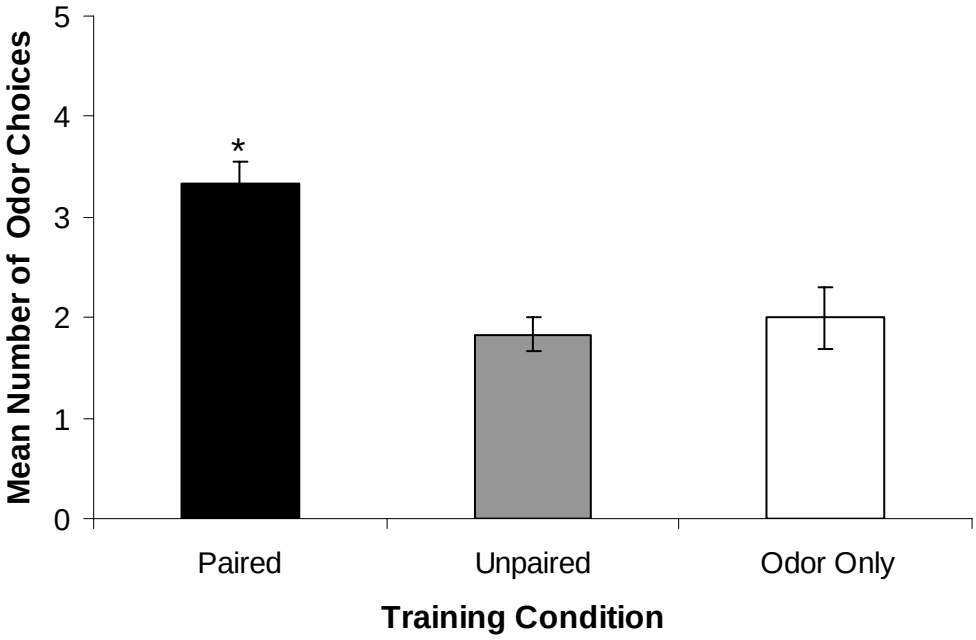

Mean Number of Odor Choices
5
4
3
2
1
0
*
Paired
Unpaired
Odor Only
Training Condition

Figure 5

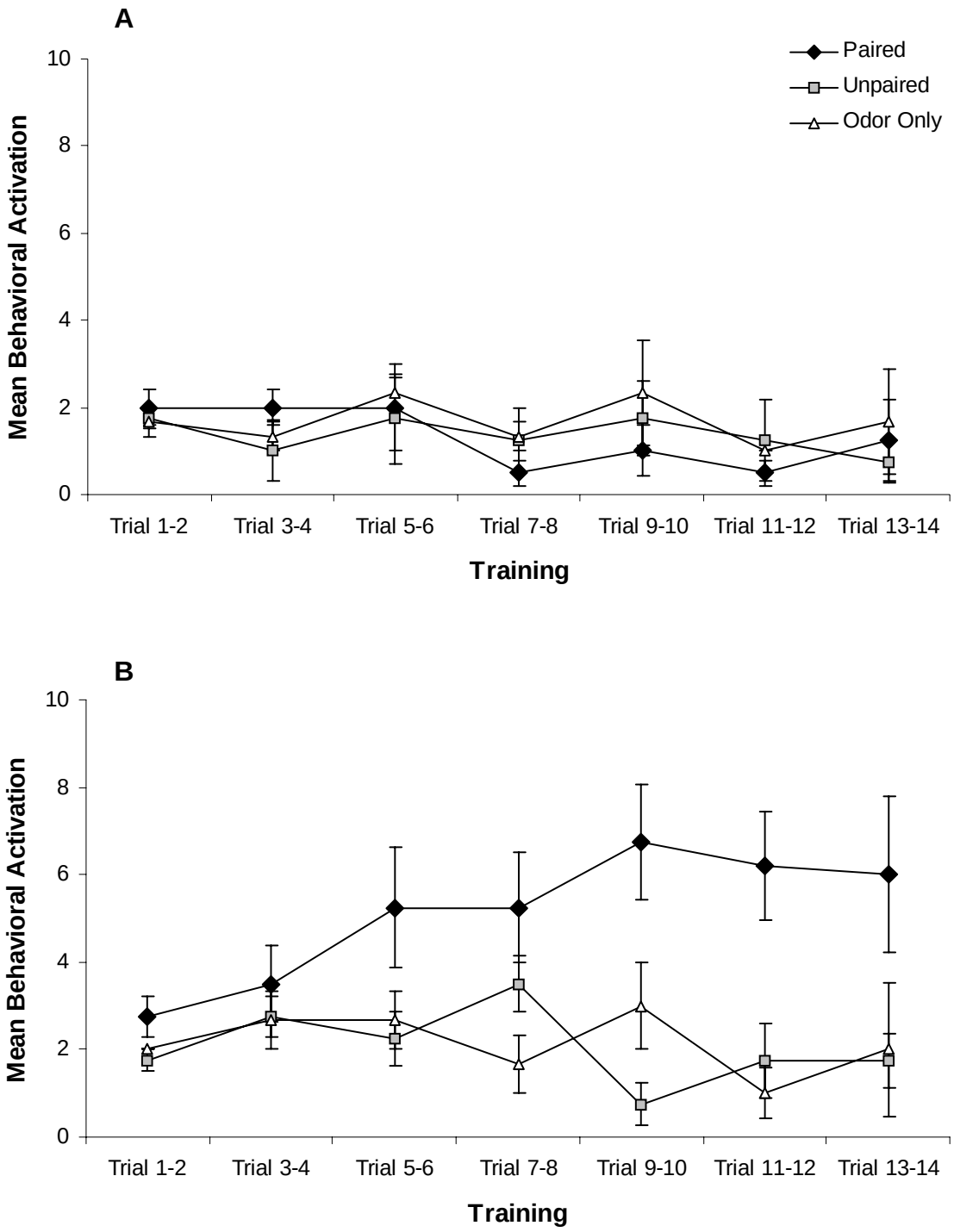

A
Paired
Unpaired
Odor Only
Mean Behavioral Activation
10
8
6
4
2
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
Training
B
Mean Behavioral Activation
10
8
6
4
2
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
Training

Figure 6

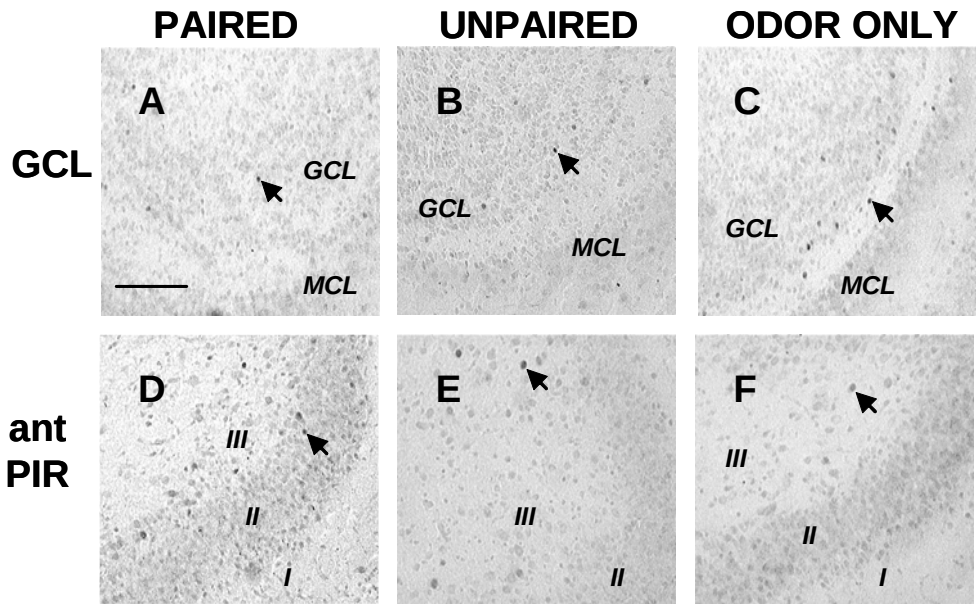
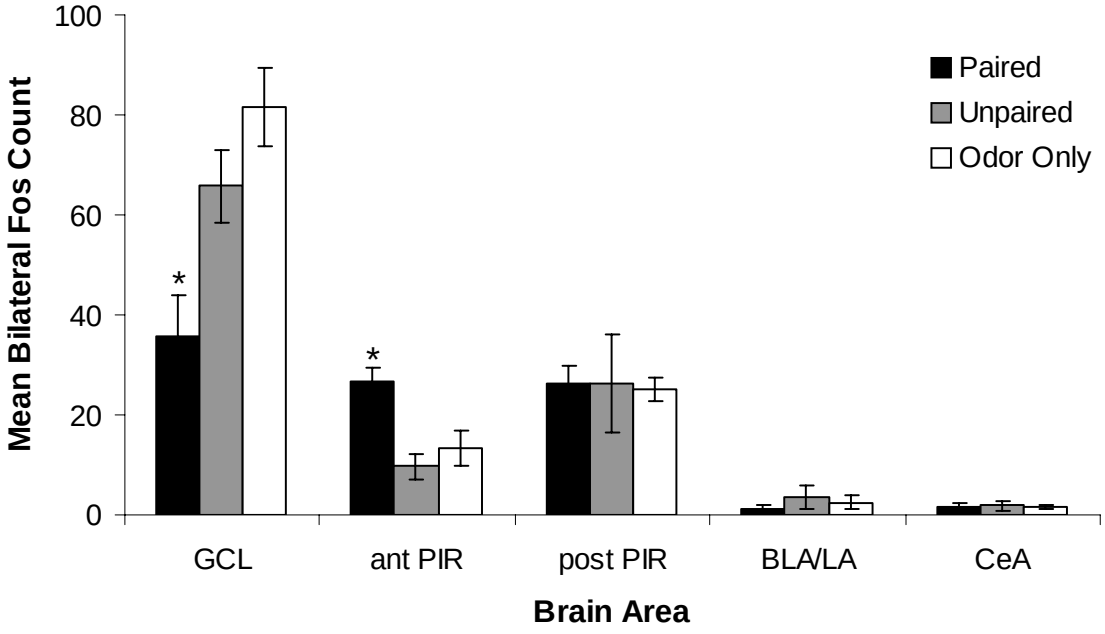


Figure 7


100
80
60
40
20
0
Mean Bilateral Fos Count
*
*
Paired
Unpaired
Odor Only
GCL
ant PIR
post PIR
BLA/LA
CeA
Brain Area

Chapter 3

Endogenous opioids and their role in odor preference acquisition and consolidation following odor-shock conditioning in infant rats

Abstract

We assessed the neurochemical basis of olfactory learning induced by presentations of odor and moderate shock in infant rats. Paradoxically, shock conditioning produces an odor preference in 8-day-olds but an odor aversion in 12-day-olds. Studies have demonstrated the importance of opioids in early olfactory learning; their specific role remains undefined. In this study, postnatal day (PN) 8 and PN12 pups were systemically injected with naltrexone, a non-specific opioid antagonist, or saline and received either paired or backward presentations of odor-moderate shock, or odor only presentations. Blocking the opioid system during conditioning disrupted acquisition of the PN8 odor preference, but not the PN12 odor aversion. Additional PN8 pups were given naltrexone post-training. Naltrexone not only blocked consolidation of an odor preference, but also yielded an odor aversion. These results suggest that the opioid system has a critical role in both olfactory learning and consolidation of odor preferences during the sensitive period.

Altricial rodents enter the world without functional visual and auditory sensory systems. The neonate relies on a still developing olfactory system to respond to the olfactory cues that are critical for survival – the maternal odor and those odors associated with the nest environment. A variety of stimuli that approximate the natural nest conditions, including noxious stimuli, support odor preference formation in neonates (Johanson & Teicher, 1980; Johanson & Hall, 1982; Pedersen, Williams, & Blass, 1982; Camp & Rudy, 1988; Sullivan, Landers, Yeaman, & Wilson, 2000). Some of these stimuli, particularly noxious stimuli, lose their ability to produce odor preferences in older infants, suggesting a sensitive period for odor conditioning during the first 9 days of life (Woo & Leon, 1987; Sullivan & Wilson, 1990; Sullivan et al., 2000). This period also corresponds to when pups begin walking (Bolles & Wood, 1964). The neurochemical basis of this paradoxical infant learning, in which a preference is learned for a noxious stimulus during the sensitive period, is not understood. Many neurotransmitters have important roles in early olfactory learning, and include norepinephrine (Sullivan, Zyzak, Skierkowski, & Wilson, 1992; Sullivan, Wilson, Lemon, & Gerhardt, 1994), serotonin (McLean, Darby-King, Sullivan, & King, 1993; Price, Darby-King, Harley, & McLean, 1998), dopamine (Weldon, Travis, & Kennedy, 1991), glutamate (Weldon & Fedorcik, 1993), GABA (Okutani, Yagi, & Kaba, 1999), and opioids (e.g., Blass & Fitzgerald, 1988; Kehoe, 1988; Shide & Blass, 1991; Aroyewun & Barr, 1992; Barr & Rossi,1992; Smotherman & Robinson, 1992; Petrov, Varlinskaya, Becker, & Smotherman, 1998). Since endogenous opioids mediate physiological and behavioral responses to pain and both rewarding and stressful situations, this neurotransmitter/neuromodulator system was chosen as a candidate system involved in this paradoxical infant learning.

Opioid receptors (μ, κ, and δ), are distributed throughout the brain in both infants and adults. Opiate receptors are present in 14-day-old fetuses and begin reaching adult levels by the 3rd postnatal week (Clendeninn, Petraitis, & Simon, 1976). μ and κ

receptors are present at birth, while δ receptors are not present until the 2nd postnatal week (Spain, Roth, & Coscia, 1985; Kornblum, Hurlbut, & Leslie, 1987; Petrillo, Tavani, Verotta, Robson, & Kosterlitz, 1987). μ receptors are morphine and enkephalin selective, mediating nociception and reward. δ receptors are enkephalin selective, mediating affective behaviors and are found primarily in the limbic system, while κ receptors are dynorphine selective, and mediate less addicting analgesia as well as affective behaviors (reviewed in Kehoe, 1988; Tseng, 1995).

Pairings of morphine with saccharine or odor show that the endogenous opioid system is present and functional in pups as early as 5 days old, and these pairings produce a conditioned preference to the conditioned stimulus (Kehoe & Blass, 1986). In PN5 pups, pairings of low doses of morphine with odor produce odor preferences, while high doses produce odor aversions (Randall, Kraemer, Dose, Carbary, & Bardo, 1992). Injections of morphine into the ventral tegmental area (an area associated with the adult reward pathway) paired with an odor in pups as young as PN4 is sufficient for an odor preference (Barr & Rossi, 1992). Intraoral infusions of sucrose paired with odor produce an odor preference in 6-day-old pups, and both the acquisition and expression of the odor preference is naltrexone reversible, suggesting the role of endogenous opioids in odor preference formation (Shide & Blass, 1991). Similarly, intraoral infusion of milk activates endogenous opioids (Blass & Fitzgerald, 1988; Kehoe, 1988). Other work shows that μ receptors are involved in suckling responses (Petrov et al., 1998), and that nipple-milk conditioning involves endogenous opioids (Smotherman & Robinson, 1992; Robinson, Arnold, Spear, & Smotherman, 1993; Robinson & Smotherman, 1997). Overall, these studies demonstrate a role of endogenous opioids in normal mother-infant attachment.

Our study investigated the role of endogenous opioids using another mammalian model of mother-infant attachment, odor-shock conditioning. This model approximates the unpleasant events that neonates encounter in their natural nest environment, which include rough transport, and being stepped upon when the mother enters and leaves the nest. Specifically, PN8 and PN12 pups were given systemic injections of naltrexone, a non-specific opioid antagonist, or equal volumes of saline before odor-shock conditioning. Additionally, in a separate experiment PN8 pups were first conditioned, and then given systemic injections of naltrexone or saline. All subjects were later tested in a behavioral Y-maze for a subsequent odor preference or aversion.

EXPERIMENT 1: PARADOXICAL ODOR-SHOCK CONDITIONING

Odor-shock conditioning in PN9 pups and younger produces a paradoxical odor preference (Camp & Rudy, 1988; Sullivan et al., 2000). Pups older than 9 days show a subsequent odor aversion after the same training paradigm. There are no differences between shock thresholds in these ages (Haroutunian & Campbell, 1979; Emerich, Scalzo, Enters, Spear, & Spear, 1985; Sullivan et al., 2000). Our purpose in Experiment 1 was to replicate previous results, and to extend preference-conditioning results to a new behavioral test, which requires the subject to climb either upon or over a barrier to demonstrate an odor preference.

Methods

***Subjects*.** Male and female pups, born of Long-Evans rats (Harlan Sprague-Dawley, IN) in the animal vivarium at the University of Oklahoma, were used. Dams were housed in polypropylene cages with wood shavings, and kept in an environment with controlled temperature (23 °C) and light (12h:12d). Food and water were available *ad libitum*. All procedures were approved by the University of Oklahoma Institutional Animal Care and

Use Committee, which follows standards certified by the NIH Guide for the Care and Use of Laboratory Animals. Subject numbers used in each experimental condition are listed in the figure captions of the corresponding figures.

Training for Experiment 1A. A total of 33 pups derived from 7 litters were used in Experiment 1A. PN8 (18.1 g – 20.9 g) and PN12 (20.3 g – 30.9 g) pups were removed from each litter (only healthy pups with similar weights were chosen from each litter) and randomly assigned to a training condition: 1) paired odor-shock, 2) backward odor-shock, and 3) odor only. Pups were marked for identification using indelible ink. During a 1 h training session, pups received 14 presentations of a 30 sec peppermint odor (CS) and a 1 sec 0.5 mA tail shock (US), with an intertrial interval of 4 min. Paired odor-shock subjects received 14 pairings of the 30 sec odor with shock during the last 29 sec, while backward odor-shock subjects received a 1 sec shock 2 min after an odor presentation. Odor only subjects received a 30 sec presentation of the peppermint odor. Peppermint odor was presented with a flow-dilution olfactometer at 2L/min and at a concentration of 1:10 peppermint vapor. Pups were trained in 600 ml glass beakers, and given a 10 min habituation period in the glass beakers prior to beginning training to recover from experimenter handling. Following training, pups were returned to their mother until tested.

Behavioral testing in a Y-maze. Pups were tested with a behavioral Y-maze the following day. The Y-maze consisted of a habituation chamber (7 cm long and 9 cm wide) with 2 alleys (22 cm long and 9 cm wide) extending at 45° angles. One arm of the maze contained pine wood odor (20 ml in petri dish), while the other arm contained the peppermint odor (25 μl of peppermint extract placed on a kimwipe for 5 min in a

ventilation hood). Each pup was placed in the starting chamber and given 5 sec for habituation before the doors to the alleys were removed. Each subject had 60 sec to make a choice, and a choice was counted when a pup had placed its nose 3 cm or 6 cm down the alley, for PN8 and PN12 respectively. Each subject was given 5 trials, and the floor was wiped clean (cloth with water) between each trial. A 30 sec intertrial was used between testing trials, and the orientation of the pup when placed in the habituation chamber was counterbalanced between trials. Observations of each pup were made without knowledge of the training condition.

Training for Experiment 1B. A total of 23 pups derived from 4 litters were used in Experiment 1B. PN8 pups were trained with the same protocol followed in Experiment 1A, with the exception that pups were trained in 400 ml beakers and odor was presented with a flow-dilution olfactometer at 1L/min and at a concentration of 1:10 peppermint vapor.

Behavioral testing in a 2-odor-choice climbing test. Pups were tested on PN10 with a 2-odor-choice climbing test. The testing apparatus consisted of a plexi-glass arena (23.5 cm long x 15 cm wide) placed on a metal tray. The arena was divided into three chambers (7 cm, 5.5 cm, and 7 cm) via wooden bars (1 cm high x 2 cm wide) covered in aluminum foil to prevent absorption of any odor. One 7-cm side contained 100 ml of clean wood shavings, and the other 7-cm side contained 100 ml of shavings treated with peppermint odor (0.05 ml of peppermint extract, placed in a ventilation hood 15 min). Each pup was placed in the 5.5-cm starting chamber (the area between the two odor sides) and given 60 sec, in which the total number of choices to either side and the total time in either side (if the pup crawled over the barrier) were recorded. Criteria for a choice consisted of placement of the head and/or front paws onto the wooden bars, or

crawling over the barriers into the shavings. Each pup received 2 trials, with the orientation of the pup counterbalanced between trials. The floor was wiped clean between trials, and a 30 sec intertrial was used between testing trials. The total number of choices made towards the odor for the two trials was summed and divided by the total number of choices made towards both odors (preference ratio). Observations of each pup were made without knowledge of the training condition.

Analysis. We used the analysis of variance test (ANOVA) and post-hoc Fischer tests to analyze differences between training conditions.

Results

Y-maze – *PN8 subjects receiving paired presentations of odor-shock showed significantly greater number of choices towards the conditioned odor*. ANOVA analysis revealed a main effect of training condition, $F(2,13) = 4.69$, $p<0.03$ (Figure 1A). Post-hoc tests showed that subjects receiving paired odor-shock conditioning had significantly more choices towards the conditioned odor during the Y-maze test in comparison to subjects that received backward odor-shock or odor only conditioning ($p<0.02$). Post-hoc tests showed no difference between the number of choices from backward odor-shock and odor only subjects.

2-odor-choice climbing test – *PN8 subjects receiving paired presentations of odor-shock showed a significant preference ratio towards the conditioned odor.* ANOVA analysis revealed a main effect of training condition, $F(2,20) = 5.25$, $p<0.02$ (Figure 1B). Post-hoc tests showed that subjects receiving paired odor-shock training had significantly more choices towards the conditioned odor in comparison to the subjects receiving backward odor-shock or odor only conditioning ($p<0.03$). In addition,

4 out of the 7 paired subjects climbed over the wooden barrier and spent time exploring the peppermint treated shavings (data not shown). Post-hoc tests showed no difference between the number of choices from backward odor-shock or odor only subjects, and none of the control pups climbed over the barrier into the peppermint-scented shavings.

PN12 Y-maze – *Subjects receiving paired presentations of odor-shock made significantly fewer choices towards the conditioned odor.* Similarly, ANOVA analysis revealed a main effect of training condition with PN12 subjects, $F(2,14) = 6.90$, $p<0.01$ (Figure 2). Post-hoc tests revealed that paired odor-shock subjects chose the conditioned odor significantly less than backward odor-shock or odor only subjects ($p<0.02$). No difference in the number of choices towards the odor was found between backward odor-shock and odor only subjects.

EXPERIMENT 2: THE ROLE OF OPIOIDS DURING LEARNING

Experiment 1 replicated results that PN8 pups receiving paired presentations of odor and moderate shock learn a subsequent odor preference, while PN12 pups receiving the same treatment learn a subsequent odor aversion. Control subjects in both age groups show neither a preference nor aversion towards the odor. Also, results indicate the newly designed behavior test may provide a more stringent testing tool for future experiments, as paired-treated subjects demonstrated a significant number of approaches (a preference) towards the odor, with some subjects even climbing over the barrier. Similar to Y-maze results, control subjects in the new test showed neither a preference nor aversion towards the odor. The purpose of Experiment 2 was to examine the role of endogenous opioids during odor preference and aversion acquisition (learning) by use of the non-specific opioid antagonist, naltrexone.

Methods

***Training and testing*.** A total of 73 pups derived from 15 litters were used in Experiment 2. PN8 (15.9 g – 20.2 g) and PN12 (25.1 g – 31.4 g) subjects were given systemic injections of naltrexone (Naltrexone HCl, Sigma) or equal volume saline before training in the conditioning paradigm described in Experiment 1A. Subjects received 0.5 mg/kg of naltrexone (Kehoe & Blass, 1986) and were given 15 min to recover undisturbed in an incubator (27°) before initiating the training protocol. We used a rating scale (0, no movement to 5, movement of all 5 extremities) to analyze conditioned behavioral activation 10 sec before and during presentation of the odor (Hall 1979). After training, pups were returned to the mother and tested in a Y-maze the following day. Observations of each pup were made without knowledge of the training condition.

***Analysis*.** We used ANOVA and post-hoc Fischer tests to analyze both acquisition and testing data. Behavior recorded both before and during the odor presentation was summed in blocks of 2 trials before analysis.

Results

PN8 – *Naltrexone blocked learning of an odor preference*. ANOVA analysis revealed a significant effect of treatment, $F(5,27) = 5.58$, $p<0.01$ (Figure 3). Post-hoc Fischer tests revealed that naltrexone significantly reduced the number of choices towards the conditioned odor in the paired training condition ($p<0.01$). Paired naltrexone subjects approached the conditioned odor in the same manner as did the backward and odor only subjects. Naltrexone had no significant effect in the backward odor-shock or odor only training conditions. No other differences were found. Analysis of learning acquisition behavior indicated that all subjects had similar pre-conditioning behavior, $F(5, 30) = 1.25$, $p>0.18$ (Figure 4A). However, analysis of conditioned behavior

indicated a significant effect of treatment, $F(5,30) = 2.37$, $p<0.01$ (Figure 4B). Post-hoc tests showed that paired saline subjects had significant conditioned behavioral activation in response to the odor in comparison to paired naltrexone subjects, as well as all other subjects ($p<0.01$). Paired naltrexone subjects were only different from the two odor only conditions ($p<0.02$). Naltrexone had no effect in backward odor-shock and odor only conditions.

PN12 – *Naltrexone did not block learning of an odor aversion*. Analysis of the results from conditioning after the sensitive period showed a significant effect of treatment, $F(5,34) = 3.67$, $p<0.01$ (Figure 5). In contrast to the results from PN8 training, Post-hoc tests revealed that paired saline and paired naltrexone subjects had no significant difference in their response to the conditioned odor ($p>0.78$). Both paired saline and paired naltrexone subjects approached the odor significantly less than all other subjects ($p<0.04$). Naltrexone had no effect in the backward odor-shock and odor only training conditions. Analysis of learning acquisition behavior indicated that all subjects had similar pre-conditioning behavior, $F(5,30) = 0.89$, $p>0.63$ (Figure 6A). Analysis of conditioned behavior revealed a significant effect of treatment, $F(5,30) = 3.10$, $p<0.01$ (Figure 6B). Post-hoc tests revealed that paired saline subjects in comparison to paired naltrexone subjects had a significant difference in conditioned behavior ($p<0.01$). However, both paired saline and paired naltrexone subjects were significantly different from all other subjects ($p<0.01$). Naltrexone had no effect in the backward odor-shock and odor-only conditions.

EXPERIMENT 3: THE ROLE OF OPIOIDS DURING CONSOLIDATION

Results from Experiment 2 suggest that blocking endogenous opioids during training affects the animal's ability to acquire a learned odor preference, but not an odor aversion. The purpose of Experiment 3 was to distinguish the role of opioids in acquisition and consolidation in PN8 pups.

Methods

***Training and testing*.** A total of 44 pups derived from 8 litters were used in Experiment 3. Immediately following training (with the same protocol as used in the previous experiments), PN8 pups (15.6 g – 19.9 g) were given systemic injections of naltrexone (0.5 mg/kg) or equal volume saline, and allowed to recover from injections and handling for 5 min (in an incubator at 27°) before being returned to the mother. On the following day, pups were tested in a Y-maze, and the observer was blind to the training condition.

Results

Blocking opioids after conditioning blocked consolidation of an odor preference and permitted an odor aversion. ANOVA analysis revealed a significant effect of treatment, $F(5,38) = 12.01$, $p<0.01$ (Figure 7). Post-hoc tests showed that paired saline subjects chose the conditioned odor significantly more times than all other subjects ($p<0.01$), while paired naltrexone subjects chose the conditioned odor significantly fewer times than all other subjects ($p<0.03$). Naltrexone had no effect in backward odor-shock or odor-only conditioning. Analysis of pre-conditioning behavior showed no differences, $F(5,30) = 0.73$, $p>0.84$ (Figure 8A), while conditioned behavior was significantly affected by treatment, $F(5,30)=13.00$, $p<0.01$ (Figure 8B). Post-hoc analysis showed no difference between behavioral conditioning in the paired saline and paired naltrexone

groups ($p>0.66$), and both paired saline and paired naltrexone subjects had significant conditioned behavioral activation in comparison to all other subjects ($p<0.01$).

GENERAL DISCUSSION

Overall, these results suggest that the endogenous opioid system is critical for odor preference formation during the sensitive period. Opioids appear to have a dual role in learning during the neonatal sensitive period: 1) opioids function in developing an odor preference; and 2) opioids function in consolidation of the preference. In sharp contrast, opioids do not appear to have a critical role in acquisition of an odor aversion after the sensitive period. However, it is quite possible that using more stringent behavioral tests to detect differences in aversion levels may produce a different outcome. Additionally, close examination of the acquisition data from Experiment 2 (Figure 4B) indicates that additional training trials may support acquisition of an odor preference. However, this was not tested, and the effect of blocking opioids during learning is apparent from the differences in the acquisition curves between naltrexone and saline treated subjects.

Other labs have investigated the role of specific opioid receptors in conditioning using other models, such as nipple-milk conditioning in fetuses. The unconditioned response to milk involves κ receptors (Smotherman & Robinson, 1992). Additionally, conditioning with milk and an artificial nipple in the rat fetus promotes κ and conditioned μ receptor activity (Robinson et al. 1993; Robinson & Smotherman, 1997). Classical conditioning of responses to an artificial nipple in the rat fetus employs both caudal κ receptor activity and rostral μ receptor activity (Petrov, Varlinskaya, & Smotherman, 2000). The role of opioids in conditioning in adults has been studied as well. Fanselow (1979) examined the effect of naloxone, another non-specific opioid antagonist, on

signaled-shock-preference conditioning to one side of a shuttlebox. Preference tests revealed that subjects who received naloxone during training did not show a subsequent side preference. Similarly, Foo and Helmstetter (2000) have suggested the role of μ opioid receptors in mediating conditioned responses to shock in adult rats. Overall, results from all of these studies suggest a role of opioid receptors in behavioral conditioning and that multiple opioid receptors mediate the conditioning.

Since naltrexone is a non-specific opioid antagonist, naltrexone may act at different receptors during the processes of acquisition (learning) and consolidation, offering a plausible explanation for the results from Experiments 2 and 3. For example, naltrexone may have blocked active κ receptors during the learning process, thus naltrexone paired pups did not show conditioned behavioral activation as the saline controls did. And when given naltrexone after acquisition (using the example κ receptors were unaffected during the training), naltrexone may have then affected conditioned μ receptors during the process of consolidation of an odor preference. If μ receptors are involved in consolidating the memory of a stimulus as being rewarding, then blocking these receptors during consolidation would affect the number of choices made towards the odor when tested. If blocking these receptors during consolidation alters cellular activity in reward or aversion areas, the consolidation could be altered from a preference to aversion.

Further evidence for this interpretation was shown by Carr, Kutchukhidze, and Park (1999); systemic injections of naltrexone in adults induce c-fos immunoreactivity, which indicates increased cellular activity, in the extended amygdala. These areas - bed nucleus of the stria terminalis (BSTLD), nucleus accumbens shell (NACshell), and the central nucleus of the amygdala (ceA) - mediate motivation and reward. Carr et al. (1999) also showed that κ receptor blockade is responsible for the immunoreactivity in

the BSTLD and CeA, while μ receptor blockade is responsible for the increased activity in the NACshell. They concluded that the CeA might be under inhibitory control of both μ and κ receptors. These results suggest that blockade of receptors in particular areas of the reward pathway is responsible for our results. Cellular activation by naltrexone may alter perception of stimuli, which Park and Carr (1998) hypothesized to account for aversion and even suppression of the positive responses towards stimuli. Blocking κ receptors is not aversive (Carr, Papadouka, & Wolinsky, 1993; Leventhal, Kirkham, Cole, & Bodnar, 1995), thus if κ receptors participate in acquisition, this may explain why pups demonstrated neither an aversion nor preference in Experiment 2. If blockade of μ receptors is aversive (Shippenberg, 1993), and these receptors are involved in the consolidation of a conditioned odor preference, then this may explain why subjects demonstrated an aversion in Experiment 3; naltrexone altered opioid secretion in areas necessary for reward, thus altering the perception of the conditioned stimulus. Taken together, our results as well as results from the aforementioned studies, suggest that odor conditioning involves several opioid receptors, and how and when developmentally they are altered may offer insight into the role of this neurotransmitter system in mother-infant attachment.

In conclusion, results from our study suggest a role of endogenous opioids in odor conditioning, with a critical, dual role during the sensitive period. Our results indicate that opioids function in both acquisition and consolidation of an odor preference. Endogenous opioids appear to be critical for preference formation to noxious stimuli that serve as reward, thus when the dam is somewhat abusive with the pups, such as during rough transport, the pups will still associate her odor with good events (milk, warmth, etc.). To further understand the role of opioids in acquisition (learning) vs. consolidation of olfactory preferences, receptor-specific drugs should be used; however as

demonstrated in this study, the mammalian model of odor-shock conditioning offers a valuable tool for investigation of their role.

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Figure captions

Figure 1. (A) Number of approaches towards the conditioned odor for PN8 rat pups receiving either paired (n=4) or backward (n=6) odor-shock presentations, or odor only (n=6) presentations in Experiment 1A. Bars represent mean values; vertical lines indicate SEM. Paired presentations of odor-moderate shock resulted in an increased number of choices towards the peppermint odor. **(B)** Preference ratio for PN8 rat pups receiving either paired (n=7) or backward (n=8) odor-shock, or odor only (n=8) presentations in Experiment 1B. Bars represent mean values, vertical lines indicate SEM. Paired presentations resulted in an increased number of choices made towards the peppermint odor. * denotes $p < 0.05$.

Figure 2. Number of approaches towards the conditioned odor for PN12 rat pups receiving either paired (n=6) or backward (n=5) odor-shock presentations, or odor only (n=6) presentations in Experiment 1A. Bars represent mean values; vertical lines indicate SEM. Paired presentations of odor-moderate shock significantly decreased the number of choices made towards the peppermint odor. * denotes $p < 0.05$.

Figure 3. Number of choices towards the conditioned odor for PN8 rat pups receiving either paired (Sal n=5, NTX n=7) or backward (Sal n=5, NTX n=5) odor-shock presentations, or odor only (Sal n=5, NTX n=6) presentations in Experiment 2. Bars represent mean values; vertical lines indicate SEM. The backward NTX condition has no SEM. Blocking opioids during paired presentations of odor-moderate shock resulted in significantly fewer choices made towards the peppermint odor. Sal = saline; NTX = naltrexone. * denotes $p < 0.05$.

Figure 4. **(A)** Pre-odor behavioral activation for PN8 rat pups receiving either paired or backward odor-shock presentations, or odor only presentations in Experiment 2. Each data point represents the summation of behavior from 2 consecutive trials; vertical lines indicate SEM. Conditioning treatment had no effect on behavioral activation before odor presentations. **(B)** Conditioned behavioral activation. The presence of an opioid antagonist during paired presentations of odor and shock disrupted learning.

Figure 5. Number of choices towards the conditioned odor for PN12 rat pups receiving either paired (Sal n=7, NTX n=7) or backward (Sal n=7, NTX n=6) odor-shock presentations, or odor only (Sal n=6, NTX n=7) presentations in Experiment 2. Bars represent mean values; vertical lines indicate SEM. Blocking opioids during paired presentations of odor-moderate shock did not affect the number of choices made towards the peppermint odor in comparison to saline controls. Sal = saline; NTX = naltrexone. * denotes $p < 0.05$.

Figure 6. **(A)** Pre-odor behavioral activation for PN12 rat pups receiving either paired or backward odor-shock presentations, or odor only presentations in Experiment 2. Each data point represents the summation of behavior from 2 consecutive trials; vertical lines indicate SEM. Conditioning treatment had no effect on behavioral activation before odor presentations. **(B)** Conditioned behavioral activation. Paired presentations of odor and shock in the presence of an opioid antagonist in PN12 pups did not disrupt learning.

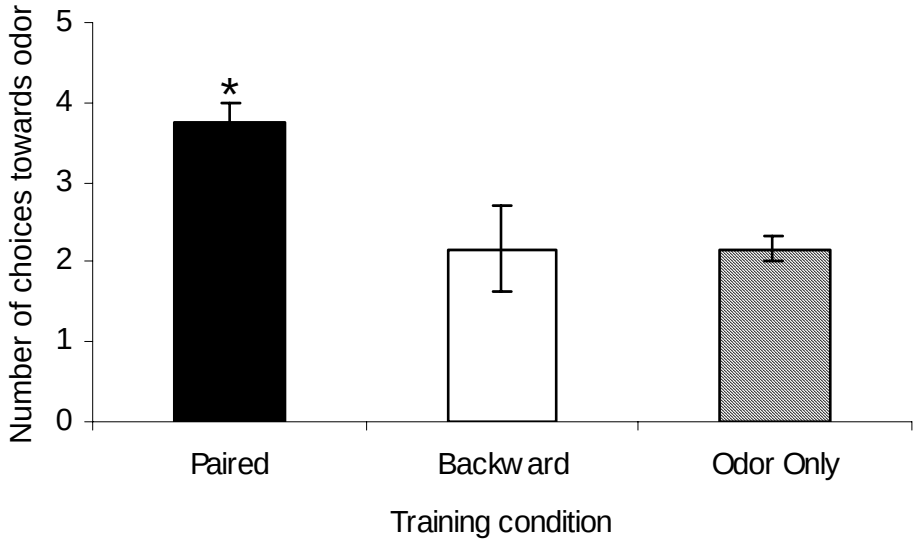
Figure 7. Number of choices towards the conditioned odor for PN8 rat pups receiving either paired (Sal n=7, NTX n=8) or backward (Sal n=7, NTX n=7) odor-shock presentations, or odor only (Sal n=7, NTX n=8) presentations in Experiment 3. Bars represent mean values; vertical lines indicate SEM. The backward NTX condition has

no SEM. Blocking opioids after paired presentations of odor and moderate shock significantly reduced the number of choices made towards the peppermint odor. Sal = saline; NTX = naltrexone. * denotes $p < 0.05$.

Figure 8. (A) Pre-odor behavioral activation for PN8 rat pups receiving either paired or backward odor-shock presentations, or odor only presentations in Experiment 3. Each data point represents the summation of behavior from 2 consecutive trials; vertical lines indicate SEM. Conditioning treatment had no effect on behavioral activation before odor presentations. **(B)** Conditioned behavioral activation. Paired presentations of odor and shock in both groups of subjects produced conditioned behavioral activation equally, thus the effects of naltrexone after conditioning can be interpreted as affecting consolidation.

Figure 1

(A)


Number of choices towards odor
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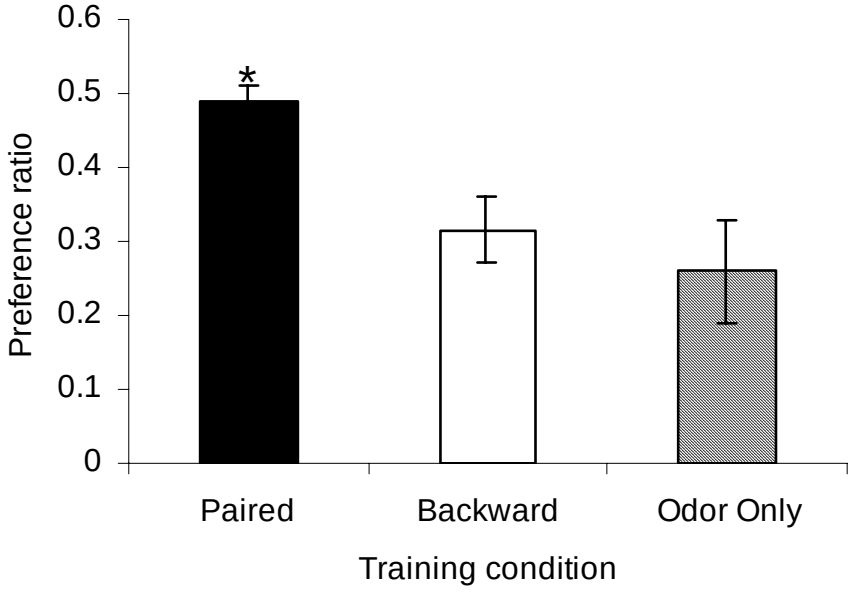
4
3
2
1
0
*
Paired
Backw ard
Odor Only
Training condition

(B)


Preference ratio
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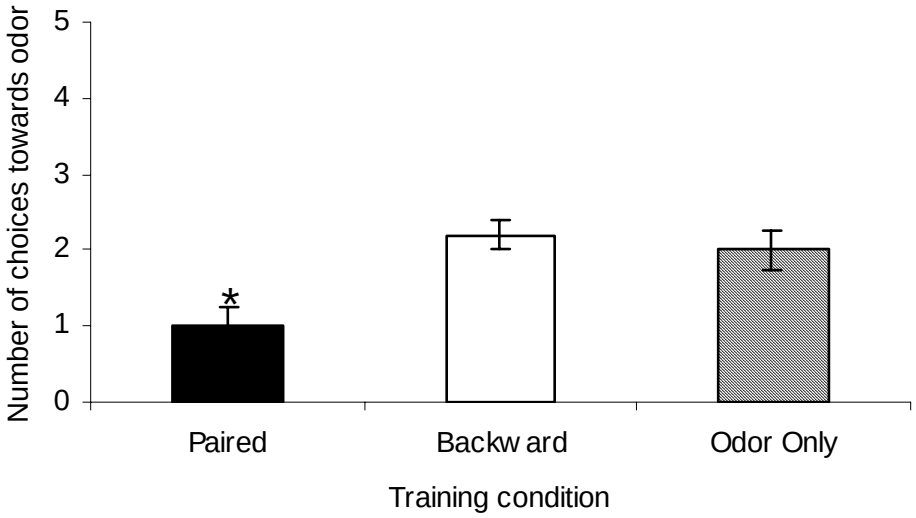
0.4
0.3
0.2
0.1
0
*
Paired
Backward
Odor Only
Training condition

Figure 2


Number of choices towards odor
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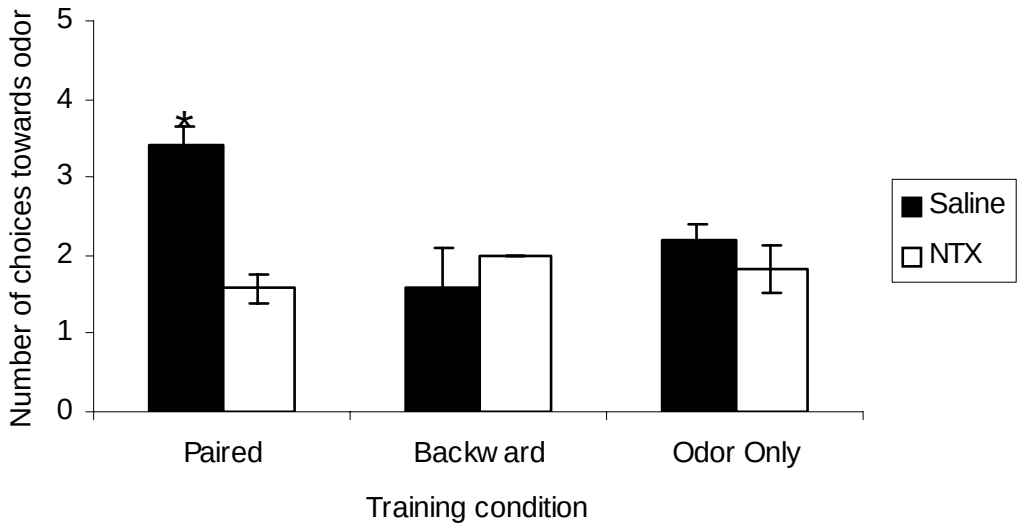
4
3
2
1
0
*
Paired
Backw ard
Odor Only
Training condition

Figure 3


Number of choices towards odor
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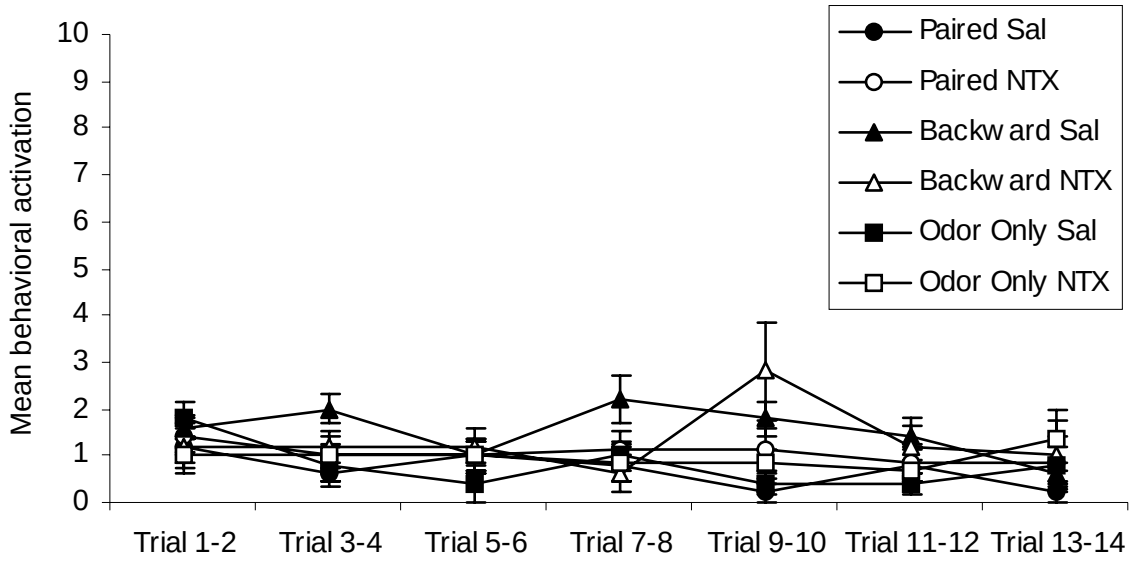
3
2
1
0
*
Paired
Backw ard
Odor Only
Training condition
Saline
NTX

Figure 4

(A)


Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
Paired Sal
Paired NTX
Backward Sal
Backward NTX
Odor Only Sal
Odor Only NTX

(B)

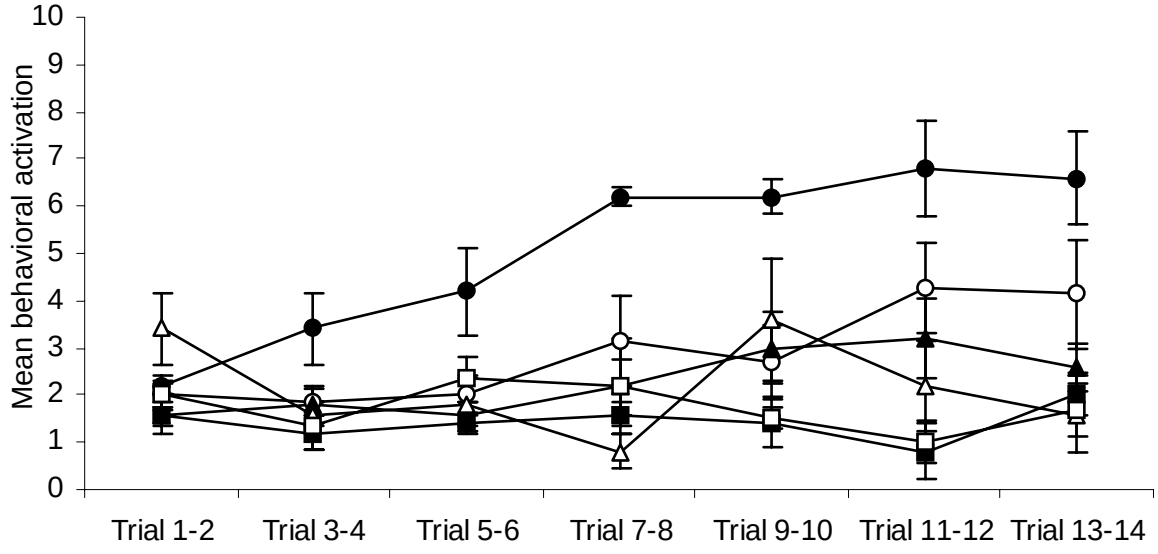

Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14

Figure 5

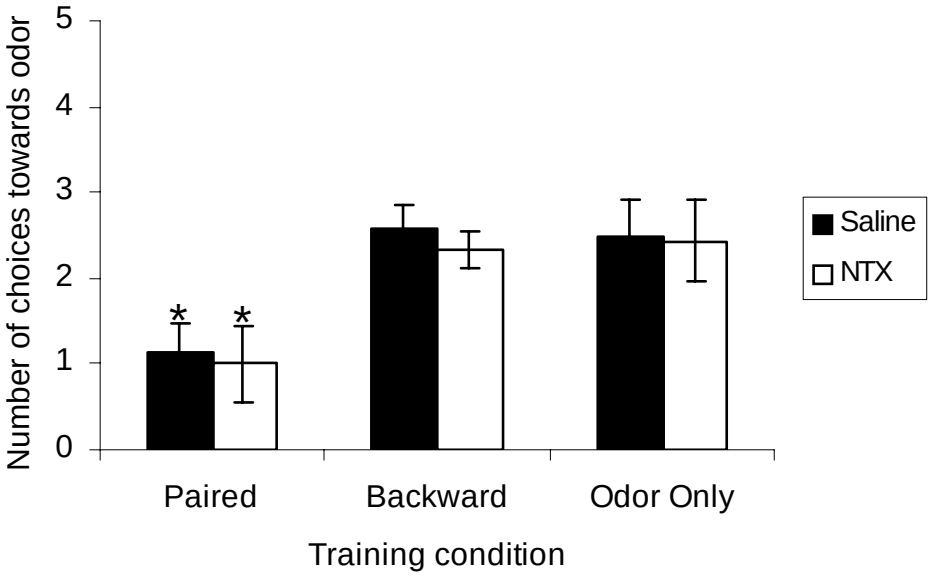
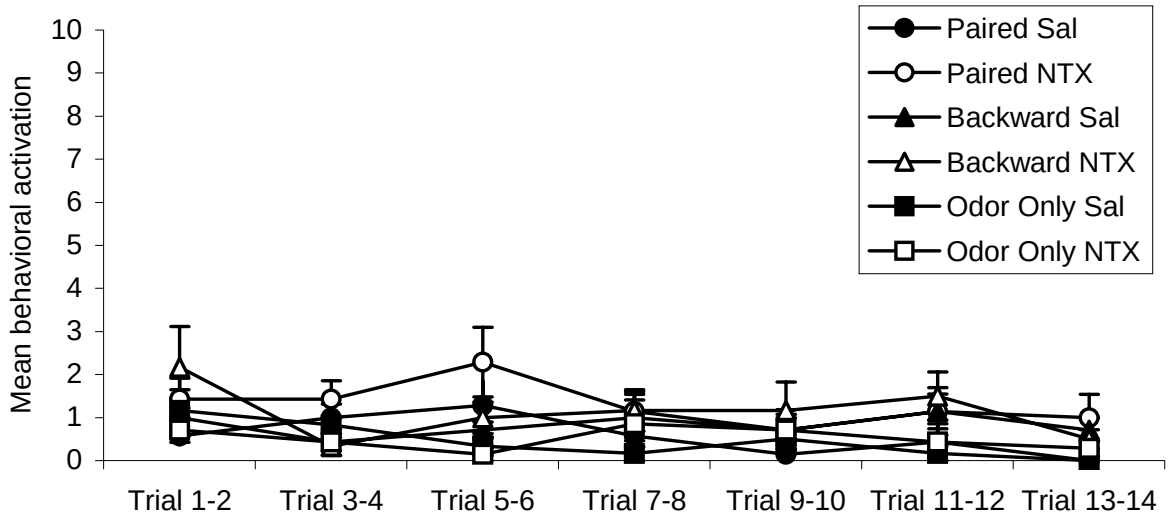

Number of choices towards odor
5
4
3
2
1
0
*
*
Paired
Backward
Odor Only
Training condition
Saline
NTX

Figure 6

(A)


Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
Paired Sal
Paired NTX
Backward Sal
Backward NTX
Odor Only Sal
Odor Only NTX

(B)

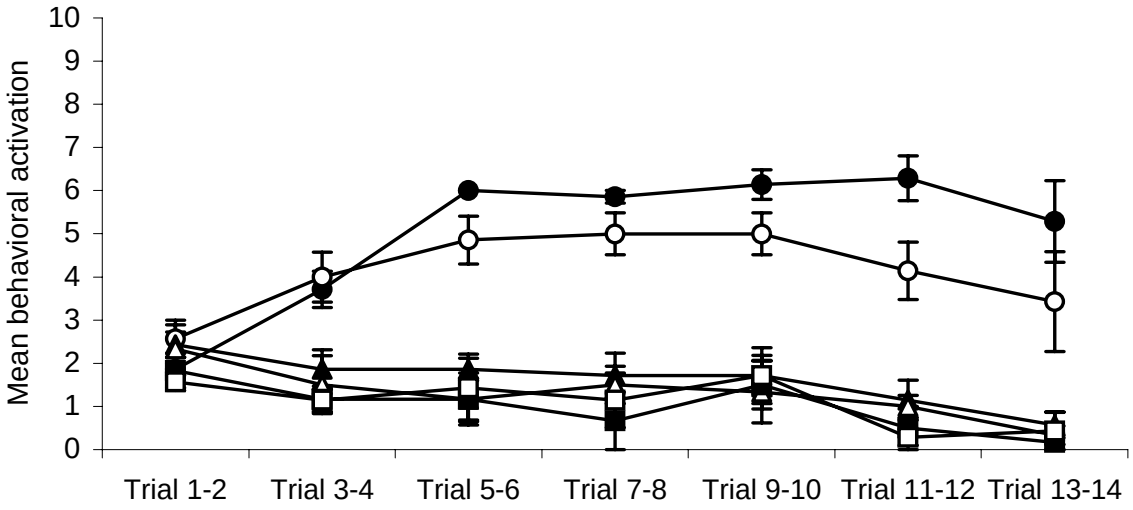

Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14

Figure 7

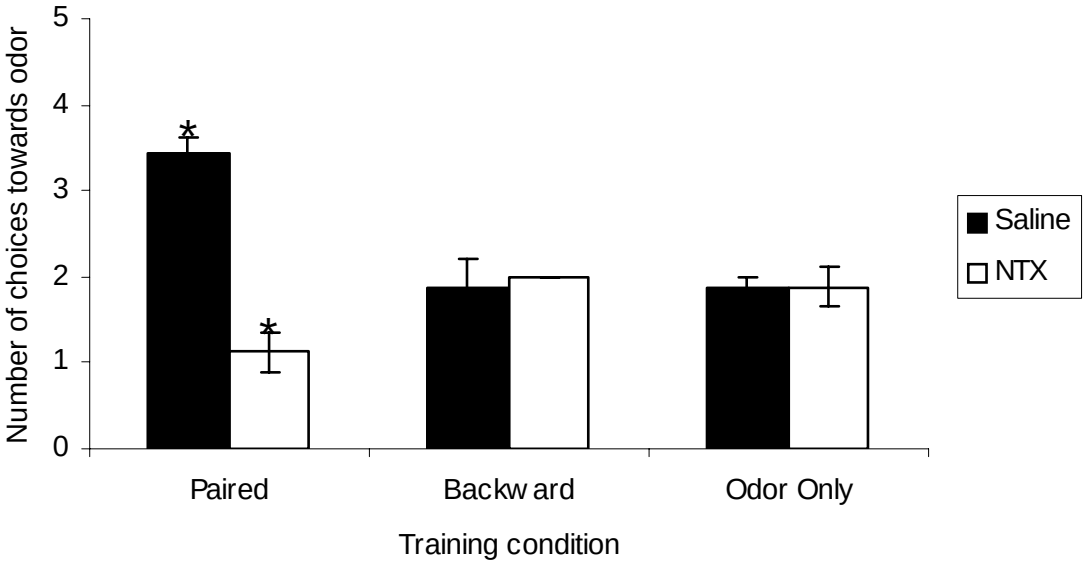
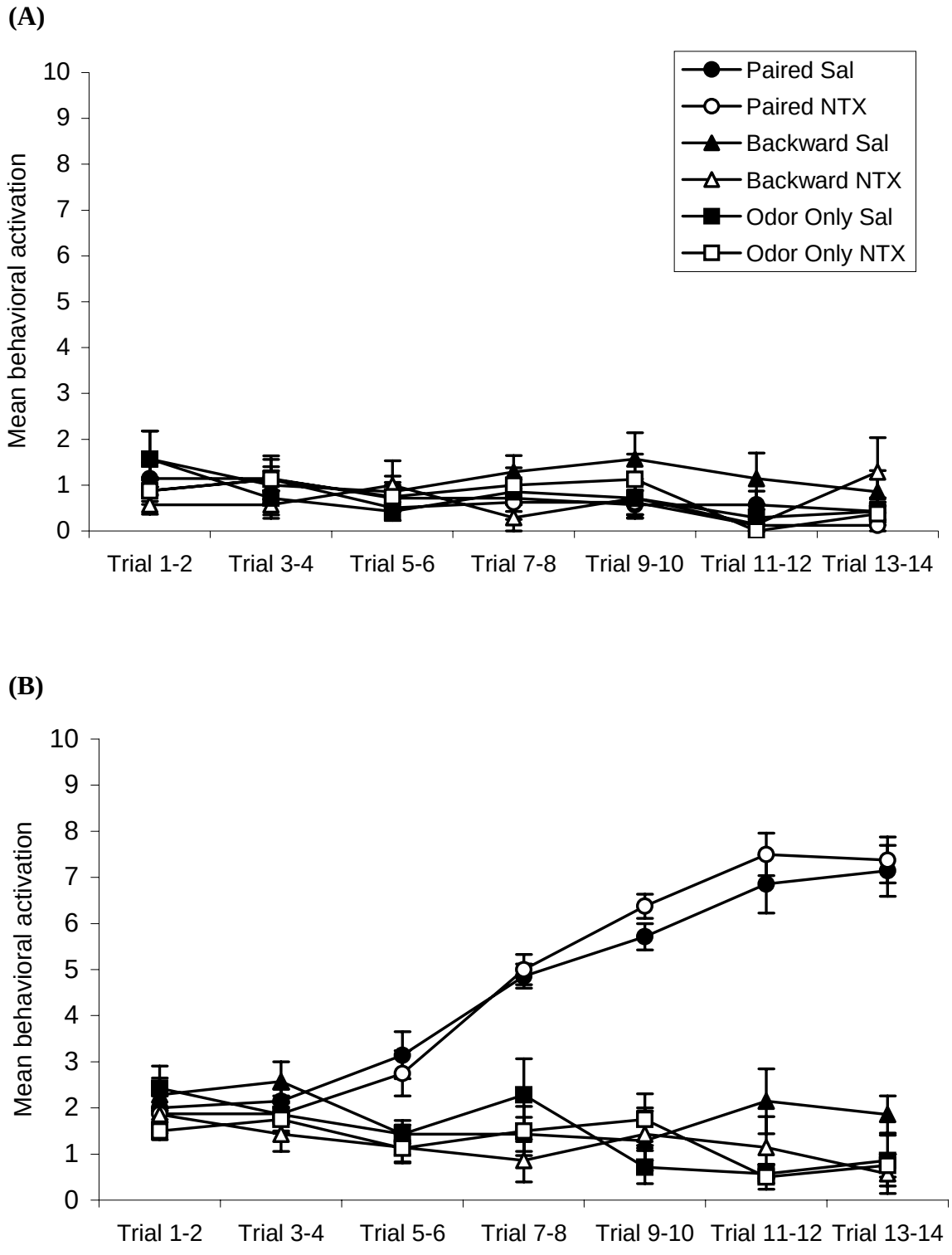

Number of choices towards odor
5
4
3
2
1
0
*
*
Paired
Backw ard
Odor Only
Training condition
Saline
NTX

Figure 8


(A)
Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Paired Sal
Paired NTX
Backward Sal
Backward NTX
Odor Only Sal
Odor Only NTX
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
(B)
Mean behavioral activation
10
9
8
7
6
5
4
3
2
1
0
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14

Chapter 4

Consolidation and expression of a shock-induced odor preference in rat pups is facilitated by opioids

Abstract

To support nipple attachment and huddling, rat pups must learn to approach and prefer maternal odor. Similar to other altricial species, rat pups have a sensitive period for learning this odor preference, which ends around postnatal day (PN) 10 and coincides with the emergence of walking. One characteristic of this sensitive period is that an odor paired with moderate shock elicits an odor preference. After PN 10, this behavioral training produces an odor aversion, although pain threshold remains unchanged. Recently, we demonstrated that the endogenous opioid system may be a key element in the acquisition of the shock-induced odor preference during the sensitive period since antagonism of this system disrupts odor preference learning. In older pups, acquisition of a shock-induced odor aversion was unaffected by opioid system manipulation. The purpose of these experiments was to further elucidate the role of opioids in infant olfactory learning through assessment of memory consolidation and expression during and after the sensitive period. In Experiment 1, we demonstrate that naltrexone (NTX), a non-specific opioid antagonist, given immediately following odor-shock conditioning during the sensitive period blocks odor preference formation and yields an odor aversion. However, the same treatment does not disrupt consolidation of an odor aversion in older pups. In Experiment 2, we demonstrate that during the sensitive period NTX disrupts expression of the shock-induced odor preference, but not the learned odor aversion in older pups. Results using this model of attachment suggest that opioids have an important role in the acquisition, consolidation, and expression of early olfactory preferences. Furthermore, since prenatal drug exposure is known to alter the endogenous opioid system, these results highlight the capacity of prenatal opiate exposure to disrupt early infant learning and attachment.

Introduction

During pregnancy, maternal opiate abuse results in fetal opiate exposure via the placenta, which not only alters the developing endogenous opioid system, but several aspects of neural development [12,27,44,48,84,85]. The opiates continue to exert their effects postnatally not only through an altered nervous system in the infant, but also through direct opiate exposure through the mother's milk and in modulation of the mother's maternal behaviors. In turn, mother-infant interactions and affiliation are altered [24,39,51]. Hence, the normal activation of endogenous opioids mediating reward, which function in concert with learning and memory circuits to facilitate mother-infant relationships, may be impaired [19]. For this reason, it is critical to elucidate the role of endogenous opioids on the neurobehavioral basis of maternal behavior and the infant's care-seeking behaviors, with the present research focusing on the infant.

Learning and memory are critical to the normal development of mother-infant relationships, and opioids have a direct impact on this learning in both infants and adults in several species [7,8,19,55,57,67,87]. Specifically in the infant rat, opioids appear to have a facilatory role in odor preference acquisition [3,40,57,62,67,73], and nipple-milk conditioning [59,60,64,65,74,75]. In addition, opioids appear to mediate isolation-induced ultrasonic vocalizations used to help the dam localize a displaced pup [10,25,42,56,86]. Overall, results from infant studies cohesively provide support for opioid facilitation of infant behaviors toward the mother.

However, recent data from our lab suggests that opioids may have a unique role in neonatal rat pup learning that is temporally limited to the sensitive period when pups rapidly, and easily learn an odor attraction. The unique role for opioids was uncovered using a mammalian model of imprinting in which the neonatal rat paradoxically learns an odor preference for an odor previously paired with a moderately painful shock [9,79,80]. This paradoxical odor preference learning does not reflect a higher pain threshold in the

neonate, is temporally limited to the sensitive period, and is characteristic of a number of other species, including dogs, chicks, nonhuman primates and potentially humans [18,28,29,31,32,47,63,68]. It is possible that this paradoxical infant learning system developed through evolution to ensure that altricial animals develop an attachment to the mother regardless of the quality of care [33].

Learning may be pharmacologically manipulated during acquisition, when information is acquired [1] or during consolidation following acquisition, when memory is stored [49,52,54,69,71]. In addition, expression of learned memories may be manipulated prior to testing [16,37]. To elucidate the role of the endogenous opioid system in infant learning, we compared opioid effects on odor-shock conditioning in rat pups younger than 9-days-old (during the sensitive period when odor-shock training produces an odor preference), and in older pups (after the sensitive period when odor-shock conditioning produces an odor aversion, [9,79,80]). Using this odor-shock conditioning, we recently demonstrated that pretraining, systemic injections (0.5 mg/kg) of naltrexone (NTX) disrupt odor preference acquisition in young rat pups (sensitive period), but not acquisition of an odor aversion in older pups [67]. We also demonstrated that the infant-learned odor preference from this conditioning paradigm is reversible to an odor aversion if NTX is administered immediately following the training [67]. This suggests not only a critical role of opioids in infant olfactory learning during the sensitive period, but that their role may be unique to this developmental period. The goal of these experiments was to further examine potential developmental differences in the role of opioids in olfactory associations using odor-shock conditioning. In the following experiments, we examined the effect of systemic NTX on olfactory memory consolidation and expression both during and after the sensitive period.

Materials and Methods

Animals. Subjects were both male and female pups, born of Long-Evans rats (Harlan Sprague-Dawley, IN) in the animal vivarium at the University of Oklahoma. Mothers were housed in polypropylene cages with wood shavings, and kept in an environment with controlled temperature (23 °C) and light (12h:12d). Food and water were available ad libitum. Cages were checked daily, and the day of parturition was termed 0 days of age. On PN1-2, litters were culled to 5 males and 5 females each. The numbers of subjects used in each experiment are listed in corresponding figure captions. All procedures were approved by the University of Oklahoma Institutional Animal Care and Use Committee and follow NIH guidelines.

General Training Procedure. On the day of training, pups were removed from the mother and randomly assigned to a training condition: 1) paired odor-shock, 2) backward odor-shock, and 3) odor-only. Pups were marked for identification using indelible ink, weighed and placed in individual 600 ml glass beakers, and given a 10 min adaptation period to recover from experimental handling. During a 1 h training session, pups received 14 presentations of a 30 sec peppermint odor (CS) and a 1 sec 0.5 mA tail shock (US), with an intertrial interval of 4 min. Paired odor-shock subjects received 14 pairings of the 30 sec odor with shock during the last second of the odor presentation, while backward odor-shock subjects received a 1 sec shock 2 min after an odor presentation. Odor-only subjects received only the peppermint odor presentations. Peppermint odor was presented with a flow-dilution olfactometer at 2L/min and at a concentration of 1:10 peppermint vapor.

During training, we recorded the number of limbs moving (0, which indicates no movement of the extremities to 5, which indicates movement of all 5 extremities) 10 sec before presentation of the odor, as well as during presentation of the odor [26]. Due to

motoric immaturity, our rating scale measures generalized behavioral activity and provides a general assessment of learning during training. Following training, pups were returned to the mother.

Y-Maze Testing Procedure. On the day following training, pups were removed from the mother and tested using a Y-maze. The Y-maze consisted of a habituation chamber (7 cm long and 9 cm wide) and 2 alleys (22 cm long and 9 cm wide) extending at 45° angles. The habituation chamber was separated from the alleys via 2 removable doors. One arm of the maze contained the familiar pine wood nest odor (20 ml of clean, pine shavings in a petri dish), while the other arm contained the peppermint odor (25 μl of peppermint extract placed on a kimwipe that had been placed in a ventilation hood for 5 min). Each pup was placed in the starting chamber and given 5 sec for habituation before the doors to the alleys were removed. Each subject had 60 sec to make a choice, which required the pup to enter the alley. Each subject was given a total of 5 trials, and the floor was wiped clean (using a cloth with water) between each trial. A 30 sec intertrial interval was used between testing trials, and the orientation of the pup was counterbalanced between trials when placed in the habituation chamber. Pups that failed to make at least 3 choices in the Y-maze were excluded from the study. Observations of each pup were made blind to the training condition.

Drug Treatment. Pups received NTX (0.5 mg/kg s.c.) or vehicle treatment either immediately following the odor-shock training in Experiment 1, or before testing in a Y-maze in Experiment 2. A detailed description of drug treatment is described below within the results sections for each experiment.

Data Analysis. In Experiment 1, we used an unpaired t-test to compare consolidation effects of NTX on the two groups trained during the sensitive period. For all other experiments, we used the analysis of variance (ANOVA) and post-hoc Fisher tests to analyze differences between training conditions and drug treatment groups. ANOVA repeated measures and post-hoc Fisher tests were used to analyze behavioral acquisition data from training. The two ages in each experiment were not trained together, and different litters were used for each age group. For this reason, the data from different ages were analyzed separately, although presented together to simplify data presentation.

Results

Experiment 1

This experiment assessed whether blocking the opioid system immediately post-training would alter consolidation of the newly acquired olfactory memory. Our previous work had suggested that opioids may be altering acquisition through its action on consolidation [67], but we had not examined the effects of opioids on memory consolidation in older pups.

Subjects. A total of 62 pups derived from 18 litters were used at either PN7-PN8 (13.1 g – 19.9 g) or PN11-PN12 (22.2 g – 31.5 g). Only healthy pups with similar weights were chosen from each litter and no more than one male and one female from a given litter were used for a given treatment/drug/age condition. Since the PN7- PN8 data was a replication of previously published results [67], pups were trained with only paired presentations of odor and shock as described above to reduce the number of animals required for this experiment as recommended by NIH Institutional Animal Care and Use Committee guidelines. All conditioning groups were used for older pups. Immediately

following odor-shock training, pups were injected (subcutaneous at the nape of the neck) with either 0.5 mg/kg NTX (Naltrexone HCL, Sigma Chemical, St. Louis, MO) or equal volume isotonic saline. Pups were given 15 min to recover from injections in a 27 °C incubator before being returned to the mother. The following day they were tested using a Y-maze as described above.

Results. During the sensitive period, a post-training injection of NTX blocked the consolidation of an odor preference, which replicated our previous results [67]. Specifically, an unpaired t-test showed a significant effect of drug treatment following paired presentations of odor-shock, with NTX producing significantly fewer choices toward the conditioned odor [$t(12) = -5.26$, $p<0.01$] (Figure 1a). This difference between paired NTX and saline treated pups was not due to differences in training behavior, since pups responded similarly to odor and shock presentations, and indeed, both groups showed acquisition curves that did not differ significantly (data not shown).

After the sensitive period, NTX had no effect on consolidation (Figure 1b). *A*NOVA analysis showed a significant main effect of training condition [$F(2,42)=10.65$, $p<0.01$]. There was no main effect of drug treatment, or a training condition x drug effect. Post-hoc Fisher tests showed that both saline and NTX-treated subjects in the paired odor-shock groups chose the peppermint odor significantly less than all other subjects in the experiment, indicating a shock induced odor aversion ($p<0.05$). Analysis of behavior during odor-shock conditioning indicated that pups from both the paired NTX and saline conditions showed significant acquisition curves [$F(12,252)=7.316$, $p<0.01$], and that acquisition curves were not significantly different from each other (data not shown).

Experiment 2

This experiment assessed whether blocking the opioid system during testing would alter the expression of the learned behavior.

Subjects. A total of 78 pups derived from 15 litters were used. PN6 - PN7 pups (11.5 g – 19.2 g) and PN11 - PN12 pups (24.4 g-32.4 g) were trained without any drug manipulation during odor-shock conditioning. Pups were returned to the mother until testing. On the following day, subjects received systemic injections of 0.5 mg/kg of NTX or saline15 minutes before Y-maze testing.

Results. NTX delivered just prior to testing eliminated the expression of the shock-induced odor preference learned during the sensitive period (Figure 2a). ANOVA analysis revealed a significant main effect of training condition [$F(2,36)= 6.25$, $p<0.01$], a main effect of drug treatment [$F(1,36)=16.24$, $p<0.01$] and a significant interaction between training condition and drug treatment [$F(2,36)=30.59$, $p<0.01$]. Post-hoc tests showed that NTX had no significant effect in the odor-only or backward groups, while NTX treated subjects in the paired odor-shock condition chose the conditioned odor significantly less than their saline controls in the paired condition ($p<0.01$). NTX treated subjects in the paired condition were not significantly different from the other control subjects, with the exception of the NTX treated subjects in the odor-only condition ($p<0.05$), although the odor control group did not differ from other control groups. This difference between paired NTX and saline treated pups was not due to differences in training behavior, since pups responded similarly to odor and shock presentations. Indeed, both groups showed acquisition curves [$F(12,216)=23.12$, $p<0.01$] which were not significantly different from each other (data not shown).

NTX delivered prior to testing had no effect on the learned odor aversion after the sensitive period (Figure 2b). ANOVA analysis showed a significant effect of training condition [$F(2,30)=14.82$, $p<0.01$]. There was no main effect of drug treatment or any interaction effects between the independent variables. Post-hoc tests revealed that both saline and NTX-treated subjects in the paired odor-shock training condition made significantly less choices to the odor in comparison to all other subjects in the experiment ($p<0.05$). There was no effect of drug treatment in the backward and odor-only conditions, and saline-treated subjects in the paired condition were not significantly different than NTX-treated subjects in the paired condition. Analysis of behavior during odor-shock conditioning indicated that pups from both the paired NTX and saline conditions showed acquisition curves [$F(12,180)=4.56$, $p<0.01$] which were not significantly different from each other (data not shown).

Discussion

Our data suggest that the opioid system has a uniquely important role during the sensitive period when pups exhibit a heightened learning ability. Specifically, in neonatal rats, the opioid system appears necessary to acquire [67], consolidate, and express odor preferences (present studies). After the sensitive period, disruption of the endogenous opioid system still permits acquisition [67], consolidation and expression of novel olfactory aversions. These data suggest that opioids modulate neurocircuitry mediating pup learning of maternal odor. This odor learning is critical for pup orientation and nipple attachment, which provide pups with the food and warmth necessary for survival.

Opioid role in infant's attachment to the mother. Our results suggest that opioids play a crucial role in infant-care seeking behaviors at a time when learned odor aversions would thwart proper mother-infant bonding. As pups grow older,

crawling/walking emerges [6], and they prepare for an environment without care from the mother, disruption of the opioid system does not appear to disrupt acquisition, memory formation, or expression of novel odor aversions. These data suggest that the brain is designed for the needs of specific developmental periods, and changes accordingly when pups begin to leave the nest.

Previously, we demonstrated the important role of the opioid system in the shock-induced odor preference in neonatal rats [67]. Systemic NTX delivered before odor-shock training disrupted odor preference formation. In addition, delivery of NTX post-odor-shock conditioning yielded an odor aversion, and our results in Experiment 1 replicated these previous results. In this study, we now demonstrate that the expression of the shock-induced odor preference is disrupted by alteration of the opioid system as well. Similarly, Shide and Blass [73] demonstrated that NTX delivered before testing of an odor preference induced by intraoral infusions of sucrose or corn oil prevented preference expression, and experimental pups reacted to the odor in the same manner as control pups that had not received training. Overall, results from use of this odor-shock model of attachment and an opioid antagonist have provided further evidence for the paramount role of the endogenous opioid system in odor preference learning and memory formation during the sensitive period.

We previously gave PN12 pups systemic injections of NTX (0.5 mg/kg) before odor-shock conditioning, and found that pups still demonstrated an odor aversion although their acquisition behavior from training was significantly different from their saline controls in the paired condition [67]. In this study, we demonstrate that NTX delivered to the same age pups after conditioning did not disrupt memory formation of an odor aversion, nor did NTX disrupt expression of the odor aversion when delivered to the pups before testing. Indeed, by day 10, adult-like behavior and learning emerge. Pups begin walking [6], and noxious stimuli lose their ability to produce conditioned odor

preferences [9,79,80]. Additionally, by the second and third postnatal weeks, the opioid system is more adult-like [13,43,58,76]. It is important to note that we did not detect any significant enhancement in the aversive learning as would be expected in comparison to adult studies. Specifically, studies in adults suggest that learning is disrupted by opiates [34,36,38,72] and facilitated by injections of opioid antagonists [20,21,22,34,35,36], suggesting that we would have expected to see a stronger odor aversion in our older pups. However, it is important to note that the Y-maze used in testing and the behavioral rating scores we employed during training may not adequately reflect increased or decreased intensities of olfactory aversions.

Comparison of adult and neonatal learning circuitry. Data from adult studies support arguments that opioids modulate memory storage through norepinephrine (NE) released from the locus coeruleus (LC), which activates β-adrenergic receptors in the amygdala [11,22,23,35,50]. The modulatory role of (NE) in adults is in sharp contrast to the necessary and sufficient role of NE in infant learning [45,79]. Additionally, work from our lab suggests that the amygdala may not be activated during odor-shock conditioning in 9-day-old or younger pups [79,80]. This suggests pups are using a learning circuitry distinct from the adult, and the altered behavior produced in the sensitive-period pups in our study may not be from opioid antagonism within the amygdala. One possible site of this opioid action in these younger pups is the LC [82]. Indeed, neonate olfactory learning is dependent upon (NE) from the LC [33,79], while in older pups, the LC appears to have a more modulatory role in learning [30,66,70]. Opioids have been shown to inhibit NE in as early as gestational day 17 cortical slices [14]. Given the importance of NE in neonatal acquisition and consolidation during the sensitive period but not after the sensitive period [33], blocking opioids may have altered NE levels, thus offering an explanation for our results.

An alternative explanation is that opioid antagonism altered any reward normally associated with the odor-shock conditioning during the sensitive period, thus a conditioned odor preference would not be expected if areas normally associated with mediating reward were altered in normal activity. Opioid antagonism in conditioned place paradigms, both in the adult [15,46,53] and infant [3,61], as well as in juvenile social play paradigms [83] disrupt formation of preferences. Indeed, the development of the endogenous opioid system shows tremendous changes corresponding to the end of the sensitive period, particularly in areas known to mediate olfaction, reward, and emotional learning in adults [13,43,58,76], suggesting that our results may be due to disruption of the neural circuitry mediating reward.

However, since we delivered the drug systemically, we cannot rule out pain-induced activation of the endogenous opioid system as a plausible mechanism mediating our results. Nociceptive and anti-nociceptive responses differ in neonates and adults [2] due to immature spinal sensory processing [18], which recently Beland and Fitzgerald [5] have suggested may be due to downregulation of opioid receptors in dorsal root ganglia during postnatal development. Behavioral studies on the ontogeny of nociception suggest that the infant rat responds to noxious stimuli in the same manner as an adult. Flexor-withdrawal reflex in response to pinching or heating is present at birth, but C-fiber mediated processes, such as the response to mustard oil, do not emerge until PN10 [2]. However, the threshold [78] and behavioral response [17] to shock does not change with the end of the sensitive period. Research on the efficacy of morphine as an effective analgesic in neonates has produced various results. Morphine was found to be an effective analgesic in PN5 pups [77], but in other studies it was found not to be effective until PN12-14 [4]. Kehoe and Blass showed an analgesic response at PN10 [41], and Thorton et al. [81] demonstrated that morphine was less effective at PN3, but reached peak potency at PN9, suggesting an age-dependent increase in anti-

nociception. Overall, the differences in behavioral studies with noxious stimulation and the studies with morphine-induced analgesia overall reflect the fact that the nociceptive system is not fully mature until close to weanling [2,18].

Significance. In conclusion, our results suggest that the opioid system mediates both neural and behavioral mechanisms to facilitate survival in the nest at a time when olfactory preference formation is of paramount concern. It appears that during this time, opioids function to modulate mother-infant interactions, particularly olfactory preferences, and to enhance the retention and expression of this behavior in pups. Future experiments are necessary to examine the location of this opioid action, and to distinguish between central and peripheral effects, particularly with the odor-shock conditioning paradigm. Since prenatal opiate exposure has been demonstrated to produce several maladaptive consequences in infant neural development and behavior, particularly with development of the endogenous opioid system, our results highlight the capacity of prenatal opiate exposure to alter neural mechanisms mediating postnatal learning, specifically mediating mother-infant relationships.

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Figure Captions

Figure 1. Effect of opioid antagonism on post-training memory consolidation following odor-shock conditioning. **(a)** – Number of approaches toward the conditioned odor for postnatal day (PN) 7-8 rat pups receiving NTX (n=8) or SAL (n=6) immediately following paired presentations of odor-shock. **(b)** – Number of approaches toward the conditioned odor for PN11 or PN12 rat pups receiving either paired (SAL n=9, NTX n=10) or backward (SAL n=7, NTX n=6) odor-shock presentations, or odor-only (SAL n=8, NTX n=8) presentations. Bars represent mean values; vertical lines indicate SEM. There is no error bar for the odor-only saline condition in the older pups. SAL=saline; NTX=naltrexone

Figure 2. Effect of opioid antagonism on expression of a shock-induced olfactory preference or aversion. **(a)** – Number of approaches toward the conditioned odor for postnatal day (PN) 6-7 rat pups receiving either paired (SAL n=6, NTX n=8) or backward (SAL n=7, NTX n=7) odor-shock presentations, or odor-only (SAL n=7, NTX n=7) presentations. Drugs were only present during testing on PN7-8. **(b)** – Number of approaches toward the conditioned odor for PN11-12 rat pups receiving either paired (SAL n=6, NTX n=7) or backward (SAL n=4, NTX n=7) odor-shock presentations, or odor-only (SAL n=5, NTX n=7) presentations. Drugs were only present during testing on PN12-13. Bars represent mean values; vertical lines indicate SEM. SAL=saline; NTX=naltrexone

Figure 1

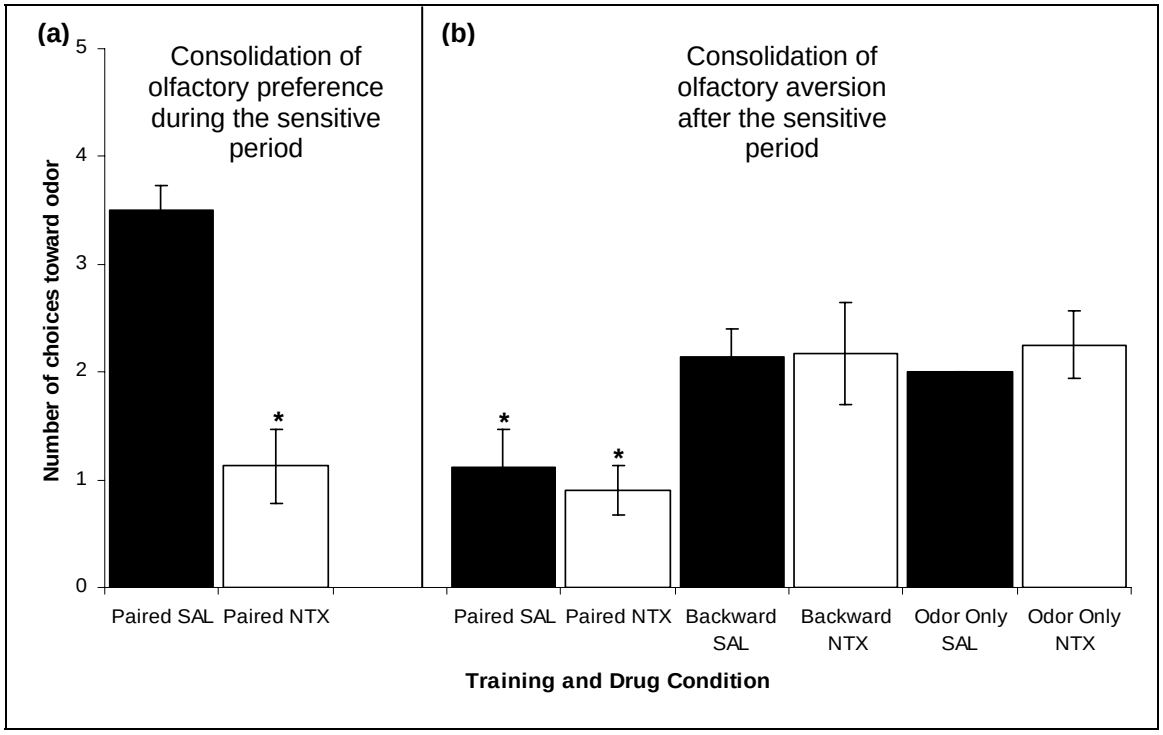
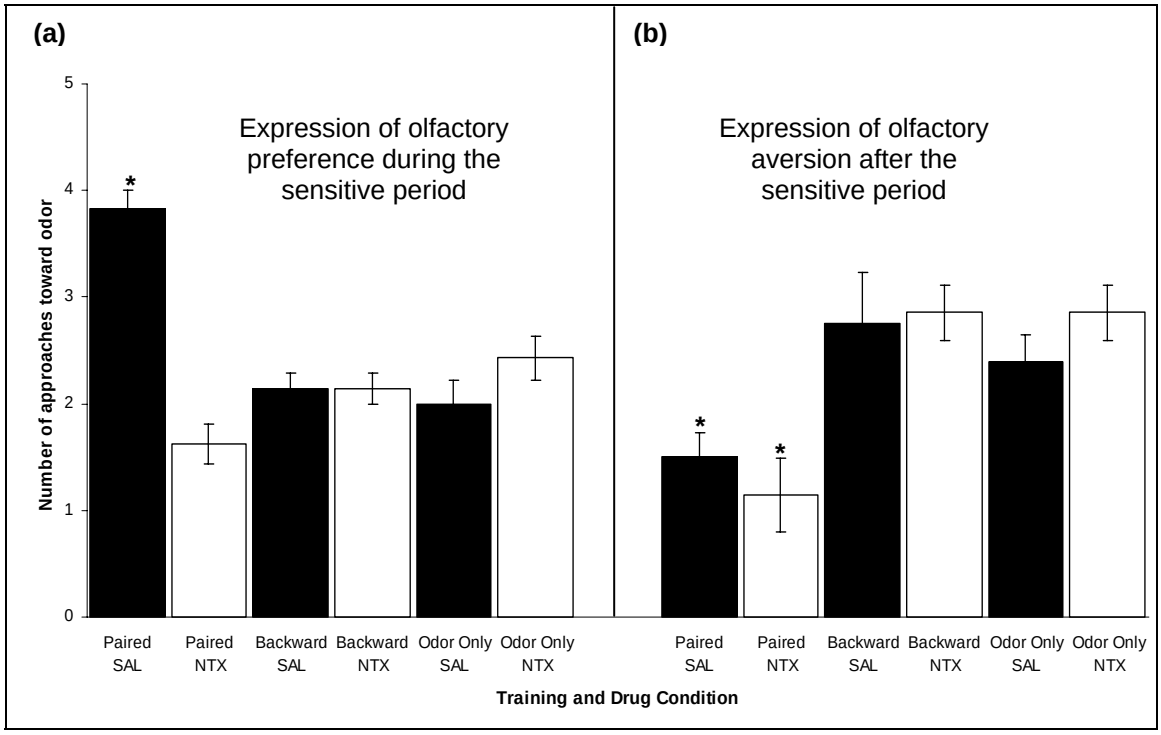

(a)
Consolidation of olfactory preference during the sensitive period
(b)
Consolidation of olfactory aversion after the sensitive period
Number of choices toward odor
0
1
2
3
4
5
*
*
*
Paired SAL
Paired NTX
Paired SAL
Paired NTX
Backward SAL
Backward NTX
Odor Only SAL
Odor Only NTX
Training and Drug Condition

Figure 2


(a)
(b)
Expression of olfactory preference during the sensitive period
Expression of olfactory aversion after the sensitive period
Number of approaches toward odor
0
1
2
3
4
5
*
*
*
Paired SAL
Paired NTX
Backward SAL
Backward NTX
Odor Only SAL
Odor Only NTX
Paired SAL
Paired NTX
Backward SAL
Backward NTX
Odor Only SAL
Odor Only NTX
Training and Drug Condition

Chapter 5

Examining the role of endogenous opioids in learned odor – stroke associations in infant rats

Maternal touch is a profound regulator of infant neural and behavioral development, and supports learned odor associations necessary for infant attachment. Endogenous opioids are known to mediate the calming and analgesic properties of maternal touch; yet their role in learned odor – touch associations is unknown. Here rat neonates were either administered naltrexone (NTX), an opioid receptor antagonist, before classical conditioning with peppermint odor and tactile stimulation (stroking) or immediately following conditioning. Results indicate that odor – stroke conditioning produces odor preferences susceptible to disruption by opioid receptor antagonism during acquisition or consolidation. These results provide additional evidence for the modulatory role of opioids in neonate learning and memory. Disturbances to this system may alter the impact of touch on infant development, particularly in the realm of learning necessary for attachment.

Sensory stimuli associated with the mother (touch, odor, milk, warmth) are substantial regulators of infant physiology and behavior (Hofer 1984, 1996; Myers et al., 2004; Stanton et al., 1987). Indeed, studies of maternal separation in rats and primates highlight the importance of mother-infant interactions, as isolation of an infant from the mother compromises both brain and behavioral development (Brake et al., 2004; Carden et al., 1996; Gunnar, 2003; Ladd et al., 2000; Meaney et al., 1996; Schanberg et al., 2003; van Oers et al., 1998, Zimmerberg et al., 2003). Studies utilizing various forms of supplemental tactile stimulation of both rat and human neonates further demonstrate the importance of maternal touch during development. Such treatment in non-deprived rats significantly alters hormones (particularly those involved in stress responses) and gene expression, and in deprived pups is sufficient to reverse the neural and behavioral severities generated by maternal separation (Jutapakdeeful et al., 2003; Lucion et al., 2003; Schanberg & Field, 1987; van Oers et al., 1998). In humans, stroking and skin-to skin contact causes positive changes in the neonate's physiology and behavior, especially in preterm or low-birth weight infants (Anisfeld et al., 1990; Bystrova et al., 2003; Ferber & Makhoul, 2004; Schanberg & Field, 1987; Pelaez-Nogueras et al., 1996; Vickers et al., 2000). Overall, these studies demonstrate the importance of maternal tactile stimulation in normal infant development.

Tactile stimulation also serves as a reward and supports learning in newborns. Specifically, human infants can associate tactile stimulation with an odor during the first few hours of life (Sullivan et al., 1991), indicative that the neural mechanisms necessary for affective learning and memory are already present and functional. Similar associative conditioning has been well documented in rat neonates (Dominguez et al., 1999; McLean et al., 1993; Sullivan & Hall, 1988; Weldon et al. 1991). Neurochemical systems involved in mediating the neonate's response to touch include cholecystokinin

(Weller & Feldman, 2003), opioids (Panksepp et al., 1980), oxytocin (Insel, 1997; Nelson & Panksepp, 1996), and serotonin (McLean et al., 1993), although norepinephrine (NE, Sullivan & Wilson, 1994) has a particularly prominent role in mediating learned odor – stroke associations in rat neonates. It is well known that the calming (attenuation of stress) and analgesic effects of mother-infant interaction is mediated through endogenous opioids (Blass et al., 1990; Gray et al., 2000; Mooncey et al., 1997; Weller & Feldman, 2003); however, their role in learned odor – touch associations has not been examined. The goal of this research was to examine the role of opioids in the associative learning and memory of an odor preference following odor – stroke conditioning in neonatal rats.

Materials and Methods

Animals

All procedures were approved by the University of Oklahoma Institutional Animal Care and Use Committee and follow NIH guidelines. Subjects were both male and female pups, born of Long-Evans rats (Harlan Sprague-Dawley, IN) in the animal vivarium at the University of Oklahoma. Mothers were housed in polypropylene cages lined with aspen wood shavings, and kept in an environment with controlled temperature (20 °C) and light (12h:12d), with food and water available ad libitum. The day of parturition was termed 0 days of age, and litters were culled to 5 males and 5 females each 1-2 days later.

Training Procedure

On the day of training, 7-8-day-old pups were randomly assigned to a training condition: 1) Paired odor – stroke, 2) Unpaired odor – stroke, and 3) Odor Only. After

being marked for identification (indelible ink), weighed, and placed in individual 600 ml glass beakers, pups were given a 10 min adaptation period to recover from experimental handling. During a 1 h training session, pups received 14 presentations of a 30 sec peppermint odor and stroke, with an intertrial interval of 4 min. Paired odor – stroke subjects received 14 pairings of the 30 sec odor with stroke during the last 20 second of the odor presentation, while Unpaired odor-stroke subjects received stroking approximately 2 min after an odor presentation. Odor-only subjects received only the peppermint odor presentations. Peppermint odor was presented with a flow-dilution olfactometer at 2L/min and at a concentration of 1:10 peppermint vapor. Using a small painter's brush, strokes were delivered in a rostral-caudal direction on the dorsal surface of the pup.

To assess pups' learning during odor – shock training, limb movements (0, no movement – 5, movement of all limbs) 10 sec prior to an odor presentation and during the odor presentation were recorded (Hall, 1979). This rating scale allows us to measure generalized behavioral activity and to provide a general assessment of learning during training. Following training, pups were returned to the mother.

Drug Treatment

For Experiment 1, pups received systemic NTX (Naltrexone Hydrochloride, Sigma, St. Louis, 0.5 mg/kg) or equal volume saline 15 min prior to odor – stroke conditioning. For Experiment 2, pups received systemic NTX or saline immediately following conditioning. For both experiments, pups were given 15 min to recover from injections in a 30 °C incubator.

Y-Maze Testing Procedure

One day after training, pups were removed from the mother and tested using a Y-maze. The Y-maze consisted of a start box (7 cm long and 9 cm wide) and 2 alleys at 45 ° angles (22 cm long and 9 cm wide). The start box was separated from the alleys via removable doors. One arm of the maze contained the familiar aspen wood nest odor (20 ml of clean, aspen shavings in a petri dish), while the other arm contained the peppermint odor (25 μl of peppermint extract placed on a Kimwipe that had been placed in a ventilation hood for 5 min). Each pup was placed in the starting box and given 5 sec for habituation before the doors to the alleys were removed. Each subject had 60 sec to make a choice, which required the pup to enter the alley. Each subject was given 5 trials, and the floor was wiped clean (using a cloth with distilled water) between each trial. A 30 sec intertrial interval was used between testing trials, and the orientation of the pup was counterbalanced between trials when placed in the habituation chamber. Observations of each pup were made blind to the training condition.

Data Analysis

We used the analysis of variance (ANOVA) and post-hoc Fisher tests to analyze differences between training conditions and drug treatment groups for both experiments. To analyze learning curves, we used repeated measures ANOVAs and post-hoc Fisher tests.

Results

ANOVA analysis of behavior during conditioning in Experiment 1 indicates that all pups had similar behavior before an odor presentation, $F(12, 246) = 1.37$, $p = .18$ (data not shown). Analysis of behavior during odor presentations indicates there was a

training condition x drug interaction effect, $F (12, 246) = 2.59$, $p < .01$ (Fig. 1A). Only SAL treated pups in the Paired odor – stroke condition demonstrated significant acquisition (learning) during the course of the training session, $p < .05$. Pups given NTX within the Paired odor – stroke condition were not different from control pups, $p > .05$. As illustrated in Figure 1B, ANOVA analysis of Y-maze results revealed a main effect of training condition, $F (2, 41) = 5.45$, $p < .01$, and a training condition x drug interaction, $F (2, 41) = 3.86$, $p < .03$. Post-hoc analysis indicates that pups that received Paired presentations of odor and stroke learned an odor preference ($p < .05$), while pups that received NTX prior to Paired presentations of odor and shock failed to learn the preference. NTX had no effect in either the Unpaired odor – stroke or Odor Only conditions.

For Experiment 2, ANOVA analysis of behavior during conditioning indicates that all pups had similar behavior before an odor presentation, $F (12, 252) = .75$, $p = .70$ (data not shown). Analysis of behavior during odor presentations indicates there was a significant effect of the training condition, $F (12, 252) = 4.07$, $p < .01$ (Fig. 2A). All pups in the Paired odor – stroke condition demonstrated significant acquisition (learning) during the course of the training session, and were significantly different from control pups, $p < .05$. ANOVA analysis of Y-maze results (Figure 2B) revealed a main effect of drug treatment, $F (2, 42) = 4.69$, $p < .04$, and a training condition x drug interaction, $F (2, 42) = 5.44$, $p < .01$. Post hoc analysis revealed that pups that received Paired presentations of odor and stroke had an odor preference, $p < .05$. NTX post-training blocked the formation of an odor preference, as these pups did not differ from controls. NTX had no effect in either the Unpaired odor – stroke or Odor Only conditions.

Discussion

The present study examined the role of opioids in odor – stroke associative preference learning and memory in rat neonates. Blockade of opioid receptors during acquisition prevented pups from learning, as indicated by their failure to display learning curves or an odor preference during the Y-maze test. Likewise, administration of an opioid receptor antagonist immediately following training prevented memory consolidation of the odor association. Overall, these results demonstrate the role of opioids in neonate learned odor – touch associations for both acquisition and consolidation.

This early odor learning is thought to be important in securing mother-infant bonding in the postnatal environment (Hofer & Sullivan, 2001; Sullivan, 2003). Endogenous opioids play a prominent role in the postnatal attachment process. Specifically in the infant rat, opioids facilitate odor preference learning (Barr & Rossi, 1992; Kehoe & Blass, 1986a; Panksepp et al., 1994; Randall et al., 1992; Roth & Sullivan, 2001, 2003; Shide & Blass, 1991), and nipple-milk conditioning (Petrov et al., 1998, 2000; Robinson et al., 1993; Robinson & Smotherman, 1997). Suggestive of their rewarding value in infants, opioids are sufficient to alleviate separation distress (Carden et al., 1991; Goodwin et al., 1994; Kehoe & Blass, 1986c; Panksepp et al., 1978). Additionally, Moles et al. (2004) have recently demonstrated that mice neonates lacking μ-opioid receptors fail to show preferences toward maternal odor and do not show distress when separated from the mother, indicative that opioids play a key role in modulating the rewarding experience of mother-infant interactions.

We have previously demonstrated that opioids play a critical role when neonates learn about pain. Specifically, in rat neonates up to PN9, presentations of odor and shock (0.5 mA) result in a learned odor preference (Camp & Rudy, 1988; Roth & Sullivan, 2001; Sullivan et al., 2000a). Indicative of the aversive nature of this

conditioning paradigm, a shock intensity of 0.5 mA is similar to that commonly used in adult fear conditioning experiments to elicit avoidance (e.g. LaLumiere et al., 2003; Paschall & Davis, 2002; Wilensky et al., 1999). Additionally, threshold to shock does not appear to change developmentally, and 0.5mA shock elicits both pain vocalizations (White et al., 1992) and escape responses (Emerich et al., 1985; Stehouwer & Campbell, 1978; Sullivan et al., 2000a). Thus, neonates display unique learning characteristics about pain. Using this paradigm, we have shown that opioid receptor antagonism during odor – shock conditioning in neonates prevents acquisition of an odor preference (Roth & Sullivan, 2001). In sharp contrast, opioid receptor antagonism immediately following the training (only during the memory consolidation process) produces an odor aversion instead of the typical odor preference (Roth & Sullivan, 2001; 2003).

Together, results from our previous behavioral studies and the present suggest that opioids play a pivotal role in ensuring learned odor preferences, especially in response to aversive stimuli. Indeed, neonates do not readily learn aversions typically produced from passive avoidance, active avoidance or inhibitory conditioning (Blozovski & Cudennec, 1980; Camp & Rudy, 1988; Collier et al., 1979; Myslivecek, 1997; Roth & Sullivan, 2001, 2003; Sullivan et al., 2000a). However, it should be pointed out that neonates can learn aversions following odor – malaise pairings, such as with LiCl or very strong (1.0-1.5 mA) shock, although even this learning can by blocked if pups nurse during acquisition (e.g., Haroutunian & Campbell, 1979; Martin & Alberts, 1979; Rudy & Cheatle, 1977). The limitations on aversive learning corresponds to a developmental period when the pups are confined to the nest (Bolles & Woods 1965) and learned odor aversions would hinder proximity seeking of the mother, and thus the nutrition, warmth and protection necessary for survival (Hofer & Sullivan, 2001; Sullivan, 2003).

Opioids have been well characterized to mediate pain and analgesic responses (Basbaum & Fields, 1984; Blass, 1997; Kehoe & Blass, 1986b; Riedel & Neeck, 2001), as well as reward responses (Blass & Fitzgerald, 1988; Kehoe & Blass, 1986a; Kelley & Berridge, 2002; Van Ree et al., 2000) in both infants and adults. Experimental and clinical data suggest that the adult central nervous system may respond to both rewarding and aversive stimuli similarly, that is, there is some shared neural circuitry regardless of the hedonic value of the stimulus (Becerra et al., 2001; Berkley & Hubscher, 1995). Such areas include the ventral tegmental area (VTA), amygdala, periaqueductal gray (PAG), nucleus accumbens, and orbitofrontal cortex (Barrot et al., 2002; Gallagher et al., 1999; Kalivas & Nakamura, 1999; Mason, 1999; O'Doherty et al., 2001; Schoenbaum et al., 1998). Our understanding of the neural circuitry mediating infant perception of rewarding and aversive stimuli remains premature, though our present behavioral results and those from odor – shock conditioning suggest that neonates can perceive and assign hedonic value to the stimuli, as we are only able to induce a neonate odor aversion when using an aversive conditioning paradigm with opioid receptor manipulation.

Brain areas activated by odor – stroke or odor – milk conditioning in rat neonates include the locus coeruleus, olfactory bulb, and piriform cortex (Hall, 1987; Langdon et al., 1997; Rangel & Leon, 1995; Sullivan & Wilson, 1994; Sullivan et al., 2000b; Yuan et al., 2002; 2003). Likewise, brain regions activated by odor – shock conditioning that produces odor preferences include the locus coeruleus, olfactory bulb and piriform cortex (Moriceau & Sullivan, 2004; Roth & Sullivan, unpublished). Additionally, learning during odor – stroke or odor – shock conditioning in neonates does not appear to involve the amygdala (Sullivan & Wilson, 1993; Sullivan et al., 2000a; Roth & Sullivan, unpublished). The role of areas believed important in assessing the hedonic value of stimuli, such as the VTA, PAG, nucleus accumbens, or orbitofrontal cortex remains

undetermined in the neonate. Furthermore, though opioids are known modulators of neural activity within these brain areas in the adult brain (Introini-Collison et al., 1989; Kalyuzhny & Wessendorf, 1998; Perez et al., 1989; Riedel & Neeck, 2001; Shepherd & Greer, 1998; Van Ree et al., 2000; Watanabe et al., 2003), their function within the neonate remain undetermined. An assessment of both the areas involved in perception of rewarding and aversive stimuli and the modulatory role of opioids within the neonate circuitry involved in both positive and aversive conditioning paradigms should offer avenues into understanding how the neonate brain assesses the hedonic value of stimuli and forms positive or aversive memories.

Overall, our results suggest that opioids play a prominent role in infant learning about rewarding and aversive stimuli, ultimately securing learned odor preferences rather than aversions. Additionally, results from this study together with our previous studies suggest that the neurocircuitry mediating neonate responses to rewarding and aversive stimuli differ. These results highlight that disturbances to the normal development of the endogenous opioid system, such as maternal drug use or postnatal stress and abuse, may affect infant response to maternal touch and thus render the infant susceptible to altered attachment mechanisms.

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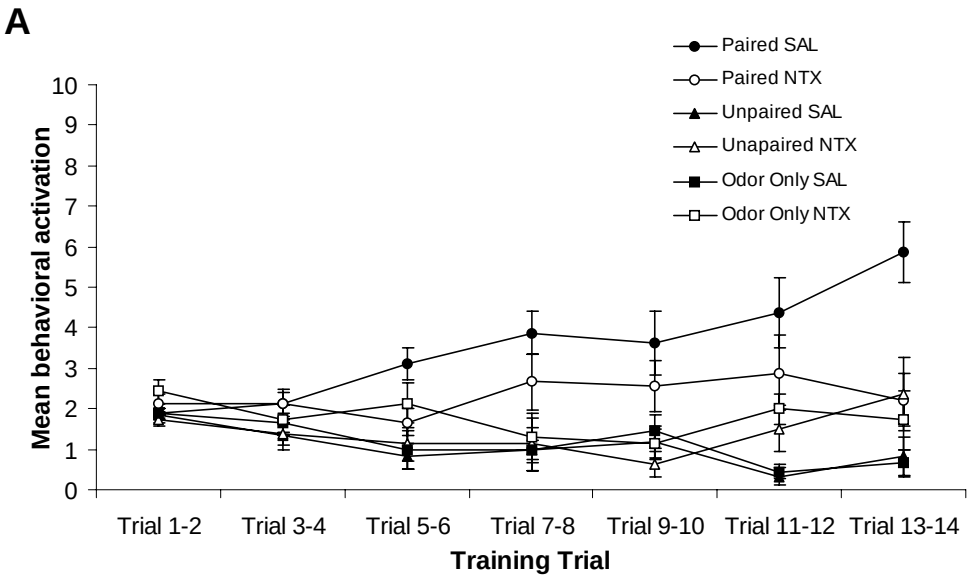
Figure legends

Figure 1. Opioid receptor antagonism blocks the learning of a stroke-induced odor preference in rat neonates. A) Behavioral acquisition in response to the odor during conditioning for rat neonates receiving NTX or SAL prior to paired (NTX n=9; SAL n=8) or unpaired (NTX n=8; SAL n=6) presentations of odor-stroke, or odor only presentations (NTX n=7; SAL n=9) on postnatal day 7 or 8. Each point represents the mean of 2 summated trials; vertical lines indicate SEM. B) Number of approaches toward the conditioned odor in the Y-maze test. Bars represent mean values; vertical lines indicate SEM. SAL=saline; NTX=naltrexone. * indicates $p < 0.05$.

Figure 2. Opioid receptor antagonism blocks the consolidation of an odor preference following odor – stroke conditioning in rat neonates. A) Behavioral acquisition in response to the odor during conditioning for rat neonates receiving NTX or SAL immediately following paired (NTX n=9; SAL n=9) or unpaired (NTX n=7; SAL n=6) presentations of odor-stroke, or odor only presentations (NTX n=8; SAL n=9) on postnatal day 7 or 8. Each point represents the mean of 2 summated trials; vertical lines indicate SEM. B) Number of approaches toward the conditioned odor in the Y-maze test. Bars represent mean values; vertical lines indicate SEM. SAL=saline; NTX=naltrexone. * indicates $p < 0.05$.

Figure 1


A
Paired SAL
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Odor Only SAL
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Mean behavioral activation
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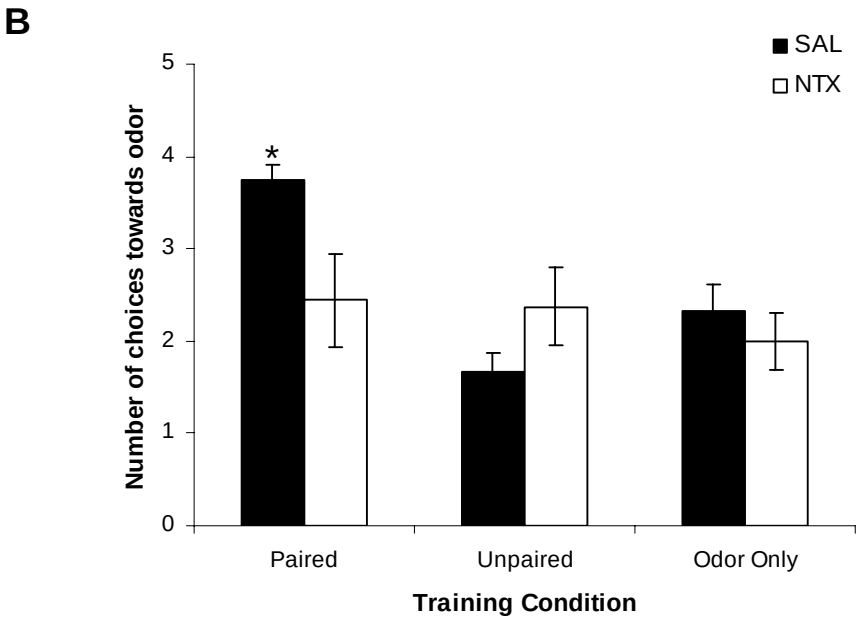
Trial 11-12
Trial 13-14
Training Trial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Number of choices towards odor
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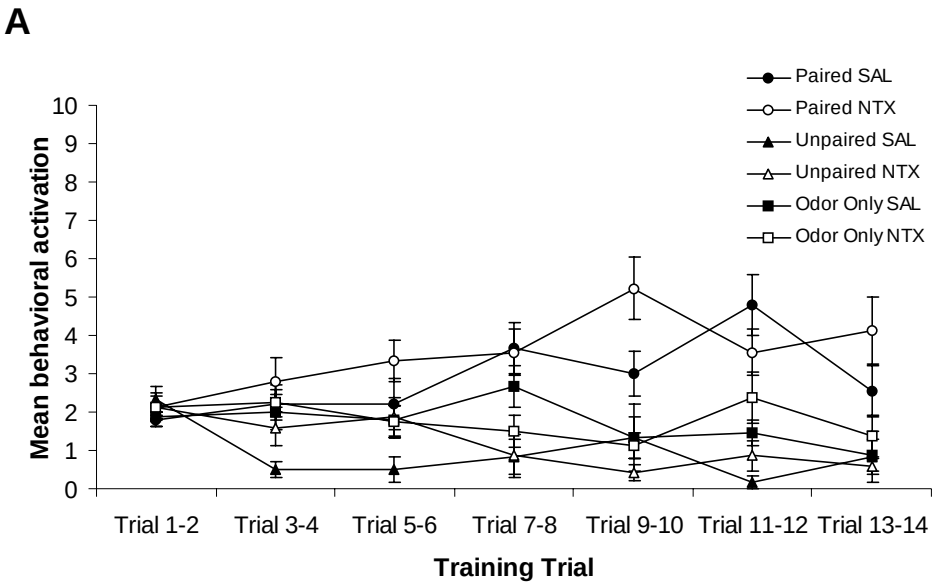
Paired
Unpaired
Odor Only
Training Condition

Figure 2


A
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Mean behavioral activation
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Trial 9-10
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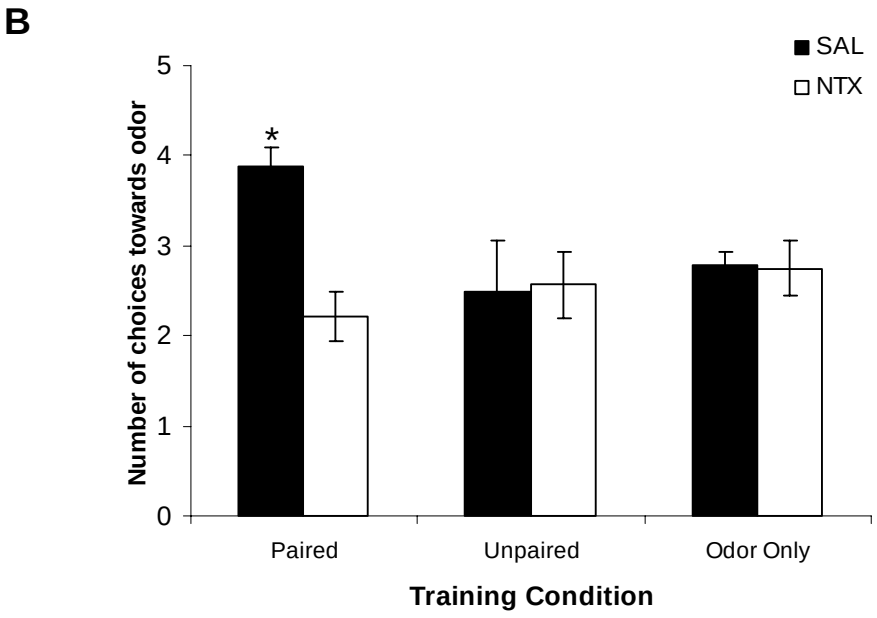
Trial 13-14
Training Trial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Number of choices towards odor
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Chapter 6

Opioid modulation of neural circuitry supporting attachment behavior in rat pups

Abstract

Ambivalent attachment in which a child attaches to an abusive caregiver is a significant risk factor for childhood and adult psychiatric disorders. Both animal and clinical studies suggest that disorganized attachment behaviors are often precipitated by disruptions to development of the endogenous opioid system. The mechanism responsible for this attachment remains unclear, hindered by limited understanding of the neurocircuitry responsible for infant attachment. In the rat neonate, odor learning and memory are necessary for attachment, and previous research has established an important role of the endogenous opioid system in supporting this behavior. These experiments were designed to address how opioids modulate the neurocircuitry involved in learned attachment behavior in rat neonates using a mammalian model of attachment under adverse conditions, odor – shocking conditioning. In Experiment 1, Fos immunohistochemistry (IHC) was used to assess the role of opioids within olfactory circuitry involved in the memory consolidation of neonate conditioned odor preferences. Opioid receptor antagonism altered Fos expression in the olfactory bulb, both anterior and posterior piriform cortex, and amygdala. In Experiment 2, we began to assess the potential sites of opioid effects on learning. Neonates received infusions of an opioid receptor antagonist into the olfactory bulbs during odor – shock conditioning. Opioid receptor antagonism within the bulbs disrupted the pups' ability to learn an odor preference. Overall, behavior and IHC results suggest that opioids directly modulate neonatal learning throughout the olfactory learning circuit: the amygdala, piriform and olfactory bulb. Prenatal or postnatal disturbances to this neuromodulatory system may produce altered attachment behaviors via disruptions in the learning and memory circuitry, and thus increase infant susceptibility to psychiatric disorders.

While the endogenous opioid system is well characterized to modulate adult learning and memory (Castellano et al., 1999; Izquierdo et al., 1991; McGaugh et al., 1993; McNally et al., 2004), far less is understood of opioid modulation of infant learning and memory. The importance of understanding the role of opioids is underscored by the impact of prenatal or postnatal disruption to the developing opioid system. Use of opiates during pregnancy alters several neurotransmitter systems, including down-regulation of the endogenous opioid system (Kuhn et al., 1992; Lesage et al., 2000; Vathy, 1995), and produces an array of behavioral problems, such as attention and learning deficits, separation anxiety and attention deficit-hyperactivity disorder (Hans, 1996; Kaltenbach and Finnegan, 1992; Šlamberová et al., 2001; Zagon and McLaughlin, 1983; Zhu and Stadlin, 2000). Prenatal or postnatal stress also impedes normal development of the opioid system (Insel et al., 1990; Sanchez et al., 1996). Overall, these changes compromise brain development and contribute to long-term behavioral abnormalities (Niesink et al., 1999; Šlamberová et al., 2003; Vathy, 1995).

One poorly understood consequence of prenatal opiate exposure is its negative impact on mother-infant attachment. Substance-abusing parents are at a higher risk for child maltreatment, and prenatal exposure produces children with disorganized and even avoidant attachment behaviors (Goodman et al., 1999; Hans, 1996; Kelley, 1992, 2003; Mikhail et al., 1995). Clinical studies demonstrate that children still attach to their abusive parent but are vulnerable to psychiatric disorders, behavioral changes related to fear and anxiety, and alterations in neural circuits regulating stress and emotion (Connor et al., 2003; Green and Goldwyn, 2002; Gunnar, 2003; Heim and Nemeroff, 2001; Schore, 2002; Teicher et al., 2003; Zeanah et al., 2003). Previous data using an animal model (Roth and Sullivan, 2001, 2003) suggests that there is overlap in the endogenous opioid system and the attachment neural circuit, suggesting a potential mechanism for attachment problems in prenatally exposed infants. An understanding of the role of the

endogenous opioid system in the neural circuitry and behavior supporting attachment offers an avenue into understanding the complexity between prenatal or postnatal disturbances to this system and subsequent behavioral deficits.

We have developed an animal model of attachment under adverse conditions. During the first 9 days of life, rat pups given odor – moderate painful shock pairings will subsequently approach that odor (Camp and Rudy, 1988; Roth and Sullivan, 2001; Sullivan et al., 2000a). Older pups readily learn to avoid odors paired with this shock, although there is no difference in shock threshold between the ages (Emerich et al., 1985; Stehouwer and Campbell, 1978; Sullivan et al., 2000a). The difficulty of younger pups to learn fear conditioning from odor – shock conditioning appears to be due to the failure of amygdala participation (Roth and Sullivan, submitted; Sullivan et al., 2000a). Using this model of mother-infant attachment, we have demonstrated that systemic manipulation of the opioid system before or after olfactory conditioning in rat neonates disrupts this shock-induced odor preference learning and memory (Roth and Sullivan, 2001, 2003). Failure to learn neonatal odor preferences compromises mother-infant attachment and hence survival (Hofer and Sullivan, 2001). Other olfactory preference conditioning paradigms with pleasant rewards and opiate manipulation have produced comparable results (e.g., Barr and Rossi, 1992; Kehoe and Blass, 1986; Shide and Blass, 1991). Together, behavioral studies suggest that opioids play an important role in neonate attachment behavior; however, their role within the neurocircuitry is unknown.

Our goal was to explore the role of opioids within the neonatal neurocircuitry. Brain areas implicated in supporting learned odor preferences and thus attachment in rat neonates include the olfactory bulb, piriform cortex, locus coeruleus (LC), and the amygdala (Hall, 1987; Kucharski and Hall, 1987; Moriceau and Sullivan, 2004; Roth and Sullivan, submitted; Wilson and Sullivan, 1992; Yuan et al., 2003; for a review see Roth et al., 2004). First, we assessed the effects of post-training opioid receptor antagonism

on molecular mechanisms within the olfactory circuitry involved in memory consolidation of neonate odor preferences. Second, since each of the brain areas in the identified olfactory pathway contain opioid receptors, and due to the prominent role of the olfactory bulb in infant learning, we began to assess the effect of opioids in learning within the olfactory bulbs.

Materials and Methods

Subjects. The University of Oklahoma Institutional Animal Care and Use Committee, which follows guidelines from the National Institutes of Health, approved all animal care and experimental procedures. We used male and female pups born of Long-Evans rats (Harlan, IN) in the animal vivarium at the University of Oklahoma. Mothers were housed in polypropylene cages with aspen wood shavings, and kept in a 20 °C environment with a 12:12 light cycle. Food and water were available ad libitum. Litters were culled to 12 males and females on postnatal day (PN) 1 or 2, with PN0 designated as the day of birth. Subject numbers for following experiments are listed in the corresponding figure legends.

Experiment 1

We have previously established that opioid receptor antagonism after odor – shock conditioning disrupts consolidation of the neonate odor preference, and yields an odor aversion (Roth and Sullivan, 2001, 2003). Our goal with this experiment was to assess the effects of post-training opioid antagonism on molecular mechanisms within the olfactory circuitry (olfactory bulb, piriform cortex, and amygdala) involved in memory consolidation following odor – shock conditioning.

Training. PN7-8 pups were randomly assigned to a training condition: 1) Paired odor – shock, 2) Unpaired odor – shock, and 3) Odor Only presentations. Pups were

given a 10 min recovery period following placement inside individual 600 ml beakers. During the training session, pups received 14 presentations of a 30 sec peppermint odor (CS) and a 1 sec 0.5 mA tail-shock (US), with an intertrial interval of 4 min. Paired odor – shock subjects received 14 pairings of the 30 sec odor with shock during the last second of the odor presentation, while Unpaired odor – shock subjects received a 1 sec shock approximately 2 min after an odor presentation. Just odor presentations were given to the Odor Only subjects. A flow-dilution olfactometer at 2/L min and at a concentration of 1 peppermint vapor:10 clean air was used.

To assess pups' learning during odor – shock training, limb movements (0, no movement – 5, movement of all limbs) 10 sec prior to an odor presentation and during the odor presentation were recorded (Hall, 1979). Immediately following training, pups received systemic injections of NTX (0.5 mg/kg) or isotonic saline, were placed in a 30 °C incubator, and their brains removed as described below. Behavioral acquisition was determined across training and drug conditions using repeated-measures ANOVA and post-hoc Fisher tests.

c-fos Immunohistochemistry. Ninety min after training and drug treatment, brains were removed and frozen in 2-methylbutane (-45 °C) and placed in a -70 °C freezer until cutting and post-fixation. Every 6^{th} section (coronal sections, 20 µm) was collected on pre-treated slides (Fisherbrand Plus, Fisher) for Fos processing, and every 7^{th} section was collected for cresyl violet staining. Fos sections received a 1-hr post-fix in 4% paraformaldehyde/0.1 M phosphate buffer (PB, pH 7.2). To eliminate peroxidase activity, sections were incubated in 0.1 M phosphate buffer saline (PBS; pH 7.2) containing 3% H_2O_2 and 10% methanol for 5 min. Following PBS rinses and 15-min incubation in 0.2% Triton X-100, slides were incubated in 3% Bovine Serum Albumin for 1 hr. Slides were treated overnight at 4 °C with the primary antibody (c-fos, sc-52, Santa

Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in PBS. Afterwards, they were incubated in the secondary biotinylated antibody (goat anti-rabbit, Vector Laboratories, Burlingame, CA) for 2 hrs at room temperature, and then were incubated for 90 min in avidin-biotin-peroxidase (ABC) complex solution. Slides were then treated with PB containing 0.1% 3,3'-diaminobenzidine and H_2O_2 and subsequently dehydrated in alcohol and Histoclear, and coverslipped for microscope examination.

Data evaluation. Fos-positive cells were counted bilaterally using a microscope (Olympus with 10x objective) equipped with a drawing tube. With aid of a stereotaxic atlas (Paxinos and Watson, 1986), brain areas were outlined using the corresponding cresyl violet sections. All Fos-positive cells were counted without knowledge of the training condition. Fos-positive cells were distinguished from the background by density of staining, shape, and size of cells. The mean number of Fos cells per brain area for an animal was determined by averaging the counts from all sections (2 sections counted for each brain area). Brain areas examined were the granule cell layer (GCL) of the olfactory bulb, the anterior and posterior piriform cortex (ant and post PIR), and the basolateral/lateral (BLA/LA) and central nucleus (CeA) of the amygdaloid complex. The number of Fos positive cells within each brain region were determined across training and drug conditions using ANOVA and post-hoc Fisher tests. To simplify the analysis and graph, the Unpaired and Odor Only conditions were collapsed after no differences were observed between the training conditions.

Experiment 2

We have previously established that opioids are necessary for infant learning during odor – shock conditioning (Roth and Sullivan, 2001). With Experiment 2, we begin to assess the potential sites of opioid effects on learning, beginning with the

olfactory bulb due to its prominent role in infant learning (e.g. Wilson et al., 1987; Woo et al., 1996; Yuan et al., 2003; Zhang et al., 2003).

Surgery. On PN5, pups were anesthetized with Isoflurane (until elimination of tailpinch reflex) and placed in a stereotaxic apparatus with bregma and lambda coordinates in the same horizontal plane. Stainless steel cannulas (30-gauge tubing) were implanted bilaterally in olfactory bulbs through holes drilled in the overlying skull, as previously described (Sullivan et al., 1992). The olfactory bulbs were located visually, and cannulas were placed in the middle. Cannulas were attached to the skull with dental acrylic and anchor wires to prevent removal by the mother between surgery and training. Additionally, clear nail polish (Onyx Laboratories, Little Rock), which has a foul taste, was painted around sutures to prevent removal by the mother. Pups were given approximately 1 hr for recovery in a 30 °C incubator before return to the home cage.

Training and Testing. On PN7, pups were placed in individual glass beakers, and their cannulas were connected by PE10 tubing to Harvard syringe pumps driving Hamilton 10 µL syringes. The cannulas were filled (volume of 1 µL) with isotonic saline, 10, or 100 µM Naltrexone Hydrochloride (NTX; Sigma Chemical, St. Louis) in saline. The infusion was continued during a 10 min adaptation period for pups to recover from experimental handling, and the 1st 10 min of the training at a rate of 0.1 µL /min, yielding 2 µL /bulb (Sullivan et al., 1992).

Pups were randomly assigned to one of three training conditions: Paired odor – shock, Unpaired odor – shock, and Odor Only. Pups were trained in separate 600 ml glass beakers. During a 20 min training session, pups received 10 presentations of a 30 sec odor and a 1 sec 0.5 mA tail shock, with an intertrial interval of 2 min. Paired odor – shock subjects received shock the last second of the odor presentation, while Unpaired odor – shock subjects received shock approximately 1 min following odor presentation. Just odor presentations were given to Odor Only subjects. Peppermint odor delivery

was controlled with a flow-dilution olfactometer as described in Experiment 1. Pups were given a 10 min habituation period prior to initiation of training to allow time. Following training, pups were returned to the home cage until testing the next day.

Pups were tested the following day (in the absence of any drugs) in a Y-maze, which consisted of a 7cm X 9cm habituation chamber, and 2 22cm X 9 cm alleys. One arm of the maze contained peppermint odor and the other arm contained the familiar odor of clean aspen wood. A pup was placed in the startbox for 5 s, and then given 60 s to make a choice. A choice was counted when the subject had entered an alley at least 3 cm. Pups were given 5 sequential trials, and start direction of the pup was counterbalanced between trials to eliminate any turn biases. Distilled water was used to clean the maze floor between testing trials. Behavioral testing was performed without knowledge of training condition. Following testing, brains were removed and stained with cresyl violet to verify cannula placement within olfactory bulbs. The number of choices toward peppermint odor was determined across training and drug conditions with both ANOVA and post-hoc Fisher tests.

Results

Opioid antagonism post-training alters Fox expression

To verify learning of the pups used for IHC neural analysis acquisition curves were constructed and indicated that all subjects had similar pre-conditioning behavior (Figure 1a), while analysis of activity in response to the odor indicated a significant training condition x training trial interaction, ($F_{(6,132)} = 25.992$, $p < 0.01$; Figure 1b). Post hoc tests showed that subjects receiving paired presentations of odor and shock had significantly higher behavioral responses to the odor in comparison to control subjects ($p < 0.05$), and there was no difference in subjects within the Paired odor – shock condition.

Ninety min following aversive conditioning, experience-induced changes were observed in the number of Fos-positive cells in the GCL of the olfactory bulb (training condition effect, $F_{(1,16)} = 12.320$, $p < 0.01$; Fig. 2). Analysis with post hoc Fisher tests revealed that the paired presentations of odor – shock induced significantly less Fos in the GCL in comparison to control presentations ($p < 0.05$). Additionally, NTX post-training in the Paired odor – shock condition, which generates a behavioral odor aversion, induced an even further decrease in Fos ($p < 0.05$).

ANOVA analysis revealed a training condition x drug interaction effect on Fos expression in the ant PIR ($F_{(1,19)} = 5.286$, $p < 0.04$; Fig. 2), and a training condition effect in the post PIR ($F_{(1,16)} = 9.223$, $p < 0.01$; Fig. 2). Post hoc Fisher tests showed that paired presentations of odor – shock evoked significantly more Fos expression in the ant PIR than all other subjects ($p < 0.05$). NTX post-training prevented the increase following paired presentations of odor – shock. Additionally, NTX treatment post-training induced significantly more Fos expression in the posterior piriform than in the control conditions, but not the saline-treated subjects in the Paired odor – shock condition ($p < 0.05$).

No effects of training condition or drug treatment were observed within the BLA/LA. However, a training condition x drug interaction was observed in the CeA ($F_{(1,16)} = 6.761$, $p < 0.02$; Figure 2). In pups that demonstrated a behavioral odor preference (Paired odor –shock SAL), and in the controls, there was no significant activity. However, in pups that demonstrate an odor aversion (Paired odor – shock NTX) a significant increase in Fos expression was observed in the CeA ($p < 0.05$).

Bulbar opioids facilitate the acquisition of an odor preference

ANOVA analysis revealed a significant effect of training condition, $F_{(2,40)} = 4.84$, $p < 0.02$, and drug treatment $F_{(2,40)} = 3.913$, $p < 0.03$ (Figure 3). Post-hoc comparisons

showed that the saline-treated subjects in the Paired training condition chose peppermint during the Y-maze test significantly more times than all other subjects ($p < 0.05$), with the exception of 10 μM NTX subjects in the Paired odor –shock condition. Subjects given paired odor-shock presentations and 10 μM NTX did not differ significantly from either the Paired saline or 100 μM Paired odor – shock conditioned subjects ($p > 0.05$). No drug effect was observed in Unpaired odor –shock or Odor Only conditions.

Discussion

Opioid modulation of neonate learned odor behavior

Numerous experiments have demonstrated a present and functional opioid system during learning in infancy. In rat neonates, pairing morphine with an odor produces an odor preference (Kehoe and Blass, 1986; Randall et al., 1992). Likewise, odor paired with injections of morphine into the ventral tegmental area is sufficient for an odor preference (Barr and Rossi, 1992). Both the acquisition and expression of an odor preference following pairings of odor – sucrose is NTX reversible (Shide and Blass, 1991). Supporting the role of opioids in mediating the rewarding aspects of mother-infant interactions, opioid receptor antagonism prevents the learning of an odor preference for an odor paired with maternal care in older pups (Panksepp et al., 1994). Furthermore, mice lacking μ-opioid receptors fail to show a preference toward maternal odor or display distress when separated from their mother (Moles et al., 2004).

To gain a better understanding of the neurocircuitry mediating infant attachment, we use odor – shock conditioning as a mammalian model of attachment under adverse conditions. This is a fear conditioning paradigm used in older pups and adults, but paradoxically in rat neonates up to PN9 this paradigm produces conditioned odor preferences (Camp and Rudy, 1988; Roth and Sullivan, 2001, 2003; Sullivan et al.,

2000a). We have shown that the acquisition, consolidation, and expression of the shock-induced odor preference is dependent upon the presence of opioids (Roth and Sullivan, 2001, 2003). Specifically, opioid receptor antagonism prior to odor-shock conditioning in neonates prevents the learning of an odor preference, while opioid receptor antagonism after odor-shock conditioning not only prevents memory of an odor preference but yields an odor aversion. Overall, studies from our lab as well as others demonstrate a role of endogenous opioids in the learning and memory of odor preferences, which are critical to the development of mother-infant attachment in the rat.

Opioids modulate the neurocircuitry involved in neonate odor learning and memory

Using odor-shock conditioning and Fos IHC we have recently shown that pups that learn an odor preference exhibit post-training changes in neural activity in several areas of the olfactory circuit (Roth and Sullivan, submitted), as replicated in this study. Specifically, Paired presentations of odor and shock produce less Fos expression in the GCL of the olfactory bulb (in comparison to controls), with an increase of Fos expression in the ant PIR. Additionally, no learning-induced changes of Fos expression are observed in either the post PIR, BLA/LA, or CeA, all presumably attributing to an odor preference.

Pups that receive NTX immediately following odor – shock conditioning display an odor aversion rather than the expected odor preference, indicative of the prominent role of opioids in neonate memory consolidation (Roth and Sullivan, 2001, 2003). In Experiment 2, we show that post-training opioid receptor antagonism alters Fos expression in several regions of the aforementioned olfactory circuit. In subjects that received paired presentations of odor and shock followed by NTX, we observed an even further decrease in Fos expression within the GCL. Within the ant PIR, we observed no

learning-induced increase in Fos, while in the post PIR we observed a significant increase in Fos expression in comparison to controls. We observed no post-training NTX effect on Fos expression in the BLA/LA; however, NTX induced a significant increase of Fos expression in the CeA. Overall, these changes in concert appear to contribute to the NTX-induced behavioral aversion.

Learning induced changes in the neonate olfactory bulb are due to a large influx of norepinephrine (NE), released by the LC (Rangel and Leon, 1995; Moriceau and Sullivan, 2004b; Sullivan et al., 1992, 1994, 2000b). It is well documented that endogenous opioids provide inhibitory control of the LC, thus modulating the amount of NE released in response to a stimulus (Christie, 1991; De Vries et al., 1990; Valentino and Van Bockstaele, 2001; Williams and North, 1984). In the piriform, opioid receptors are co-localized with gamma-aminobutyric acid (GABA) neurons (Kalyuzhny and Wessendorf, 1998), and within both the BLA and CeA opioids modulate noradrenergic activity (Introini-Collison et al., 1989; Watanabe et al., 2003). It is plausible that our post-training opioid receptor antagonism altered NE levels in the neonate brain. The further decrease of granule cell activity in Paired NTX subjects further supports this idea, as NE inhibition of GABA activity in the olfactory bulb plays a prominent role in neonate learning (Okutani et al., 1998; Sullivan and Wilson, 1994). Changes in GABA activity in pups has been shown to affect whether they learn an odor preference or aversion, suggesting that the hedonic value of an odor depends upon control of mitral cells during learning and memory formation (Okutani et al., 1999). However, direct actions of increased NE levels on mitral cells cannot be disregarded (Yuan et al., 2003; Zhang et al., 2003).

The NTX-induced amygdala activity in the neonates offers another plausible explanation for the odor aversion, as under normal conditions the amygdala does not appear to participate in learning (Sullivan and Wilson, 1993; Sullivan et al., 2000a),

presumably underlying their decreased ability to learn odor – shock aversions. Learned aversions are intimately associated with amygdala activity in older pups and adults (Fanselow and Gale, 2003; Maren, 2003; McIntyre et al., 2003; Moriceau and Sullivan, 2004a; Packard and Cahill, 2001; Schafe et al., 2001; Sullivan et al., 2000a; Walker and Davis, 2002), mediated through NE input (Liang et al., 1990; McIntyre et al., 2003; McGaugh et al., 1988). Indeed, opioid receptor antagonism in the adult amygdala potentiates noradrenergic input (Quirarte et al., 1998). The BLA/LA receives input from multiple sensory systems, thus is considered to have a critical role in fear conditioning through converging information about stimuli (LeDoux et al., 1990; Pitkanen et al., 2003). Controlling conditioned behavioral responses, the CeA receives the integrated information and serves as an interface to motor output areas (Fendt and Fanselow, 1999; LeDoux and Muller, 1997). We failed to detect any NTX-induced changes in Fos expression in the BLA/LA; however, this may reflect the sensitivity of Fos expression to the time between training and brain removal. Thus, we cannot rule out any effects of post-training opioid receptor antagonism on the BLA/LA. However, we did see changes within the CeA. The significant Fos activity in the CeA suggests that NTX removed any inhibitory control (either directly or indirectly) of the neonate amygdala. Indeed, opioid agonists inhibit neurons within the CeA (Freedman and Aghajanian, 1985; Zhu and Pan, 2004). Overall, changes in amygdala activity appear to have contributed to the NTX-induced disruption of odor preference consolidation and the memory of an odor aversion.

Opioid receptors in the olfactory bulb modulate neonate odor learning

Physiological and cellular changes within the olfactory bulb support the learning of odor associations in rat pups (e.g. Wilson et al., 1987; Woo et al., 1996; Yuan et al., 2003; Zhang et al., 2003). Results from Experiment 1 demonstrate that opioid activity

within the olfactory bulb plays an important role in odor learning as pups that received NTX limited to their olfactory bulb failed to form the association between the odor and shock. This parallels our previous results where systemic NTX disrupted odor-shock learning (Roth and Sullivan, 2001). It should be noted that naltrexone does not affect gross odor perception (Kehoe and Blass, 1986), thus deficits in learning do not appear to reflect differences in perception of the peppermint odor between the drug and non-drug treated subjects.

Previously it has been shown that in neonates and young pups that manipulation of bulbar NE (Sullivan et al., 1992), GABA (Okutani et al., 1999), and serotonin (5-HT, McLean et al., 1993; Price et al., 1998) disrupt odor learning. In addition, bulbar infusion of NE (Sullivan et al., 1991) or a glutamate receptor agonist (Rumsey et al., 2001) is sufficient for olfactory preference learning in neonates. NE appears to mediate learning through molecular changes in the granule and mitral cells of the olfactory bulb, although 5-HT to some degree can be substituted for NE (Sullivan and Wilson, 2003; Yuan et al., 2003). The location of opioid action within the neonate olfactory bulb is unknown.

The infant and adult olfactory bulbs have opioid receptor binding, although neonates have higher concentrations of receptors (Unnerstall et al., 1983; Clendeninn et al., 1976; Kornblum et al., 1987; Petrillo et al., 1987; Spain et al., 1985). In addition, within olfactory bulb layers there are changes in opioid receptor distribution during postnatal development (Kornblum et al., 1987; Unnerstall et al., 1983). In the adult, both mu and kappa opioids modulate mitral/tufted cell activity (Perez et al., 1989; Shepherd and Greer, 1998). The exact action of opioids on cells within the neonate olfactory bulb is unknown; however, our results suggest that opioids facilitate the cellular changes within the olfactory bulb necessary for learned odor associations.

Conclusions

In conclusion, our results demonstrate that opioid modulation of cellular activity in the neonate olfactory bulb, piriform cortex, and amygdala is important in the learning and memory of conditioned odor preferences. Additionally, disruption of opioid activity produces deficits in the normal learning and memory necessary for attachment. The etiology of childhood and adult psychiatric disorders is often precipitated by altered caregiver-infant relationships (Green and Goldwyn, 2002; Schore, 2002; Teicher et al., 2003; Zeanah et al., 2003). Thus, our results not only highlight the important role of the endogenous opioid system in the neurocircuitry supporting attachment, but also suggest that negative impacts on the development of this system would compromise attachment and render the child prone to behavioral disorders.

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Figure Captions

Figure 1. Behavioral activation scores indicate that pups used for IHC neural analysis had significant acquisition, indicative of learning. (A) Pup activity before odor presentations during conditioning with either Paired odor – shock presentations (SAL n=4; NTX n=7) or control presentations (SAL n=6; NTX n=9). Each data point represents the summation of behavior from 2 consecutive trials; $\bar{x}$±SEM. There was no effect of conditioning treatment on pup activity before odor presentations. (B) Pup activity during odor presentations indicates that only Paired presentations of odor – shock produced resulted in learning. SAL = saline; NTX = naltrexone.

Figure 2. Post-training antagonism of opioid receptors alters Fos expression in neonate olfactory circuitry. Opioid receptor antagonism produced a significant decrease of Fos expression in the granule cell layer of the olfactory bulb and the anterior piriform cortex. Opioid receptor antagonism also induced a significant increase of Fos expression in the posterior piriform cortex and central nucleus of the amygdala. Bars represent the number ($\bar{x}$±SEM) of Fos-positive cells counted bilaterally in each brain area for subjects receiving Paired presentations of odor – shock (SAL n=4; NTX n=4-6), or control presentations (SAL n=5-6; NTX n=6-7). SAL = saline; NTX = Naltrexone; GCL = granule cell layer of olfactory bulb; ant or post PIR = anterior or posterior piriform cortex; BLA/LA or CeA = basolateral/lateral or central nucleus of the amygdaloid complex. * indicates $p < 0.05$.

Figure 3. Antagonism of opioid receptors within the neonate olfactory bulb disrupts odor preference learning. Bars represents the number of choices ($\bar{x}$±SEM) towards peppermint during Y-maze testing for pups that received Paired (SAL n=10; 10 μM NTX n=4; 100 μM NTX n=5) or Unpaired (SAL n=8; 10 μM NTX n=5; 100 μM NTX n=5)

presentations of odor –shock, or Odor Only presentations (SAL n=5; 10 μM NTX n=3; 100 μM NTX n=4). Saline (SAL); Naltrexone (NTX). * indicates $p < 0.05$; NS = non-significant.

Figure 1

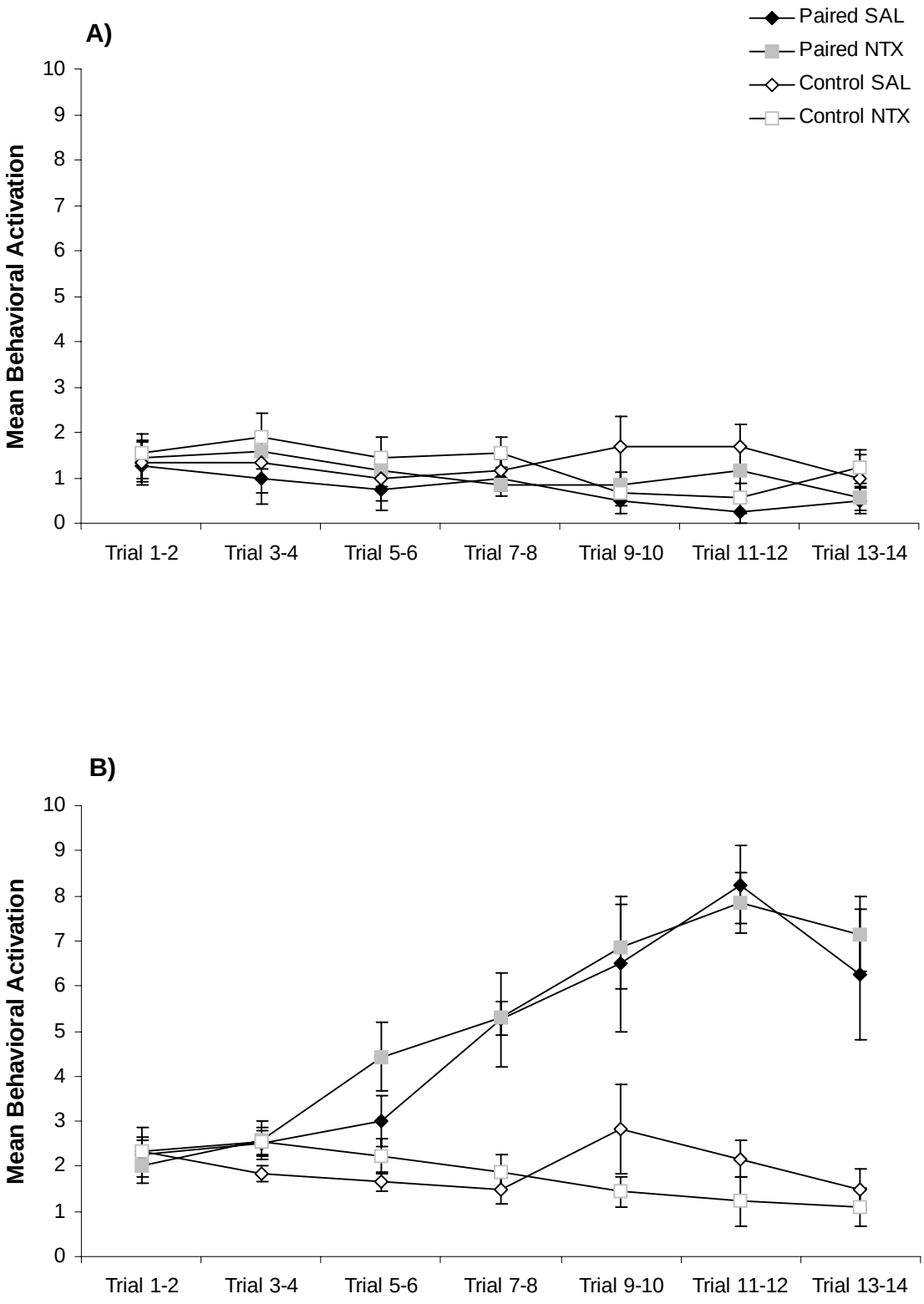
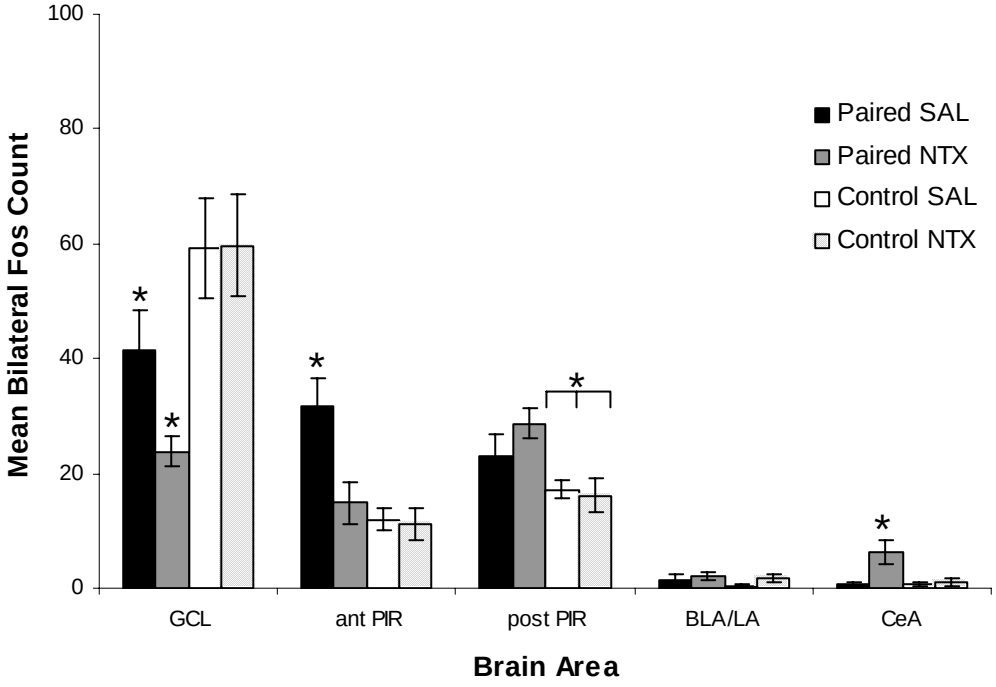

A)
Paired SAL
Paired NTX
Control SAL
Control NTX
Mean Behavioral Activation
0
1
2
3
4
5
6
7
8
9
10
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14
B)
Mean Behavioral Activation
0
1
2
3
4
5
6
7
8
9
10
Trial 1-2
Trial 3-4
Trial 5-6
Trial 7-8
Trial 9-10
Trial 11-12
Trial 13-14

Figure 2


100
80
60
40
20
0
Mean Bilateral Fos Count
Paired SAL
Paired NTX
Control SAL
Control NTX
*
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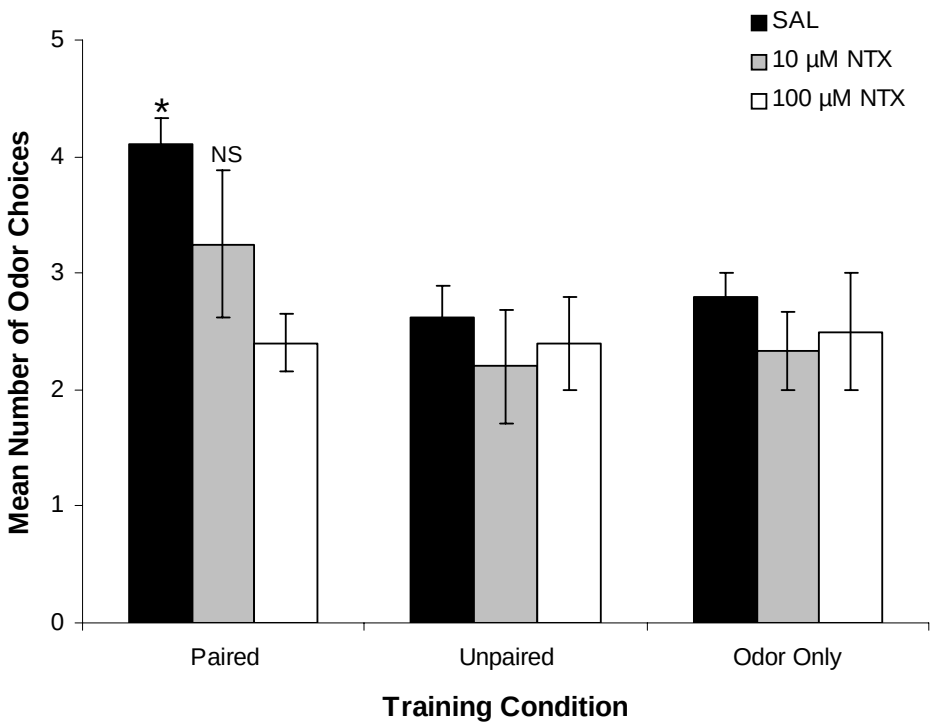
post PIR
BLA/LA
CeA
Brain Area

Figure 3


SAL
10 µM NTX
100 µM NTX
Mean Number of Odor Choices
0
1
2
3
4
5
*
NS
Paired
Unpaired
Odor Only
Training Condition

Chapter 7

Research Summary

The research in this dissertation has advanced our understanding of how the neonate brain supports the behavior necessary for infant attachment, that is, readily learned odor preferences and not odor aversions. First, my research has provided additional evidence of the learning circuitry responsible for attachment behavior despite abuse. Using two approaches to examine the behavioral and neural correlates of attachment behavior, I have demonstrated that there are cellular changes within the olfactory bulb and the anterior piriform cortex following odor conditioning. This is the first documentation of both changes in gene expression and cortical processing in relation to odor – abuse memory in neonates. I also confirm that the amygdala, a brain area intimately associated with the memory of fear in older pups and adults, does not appear to participate significantly in the neonate learning circuitry, presumably contributing to readily learned odor preferences and not aversions in neonates. Moreover, I have developed a new maltreatment paradigm that may provide an understanding of how abusive treatment of offspring affects the neural circuitry responsible for attachment, how an infant can still form an attachment to an abusive caretaker, and the effect of long-term experiences with maternal maltreatment on brain development and psychiatric well-being.

Second, my research has established a role of the endogenous opioid system in the learning and memory necessary for infant attachment. Using two learning models (odor – shock and odor – stroke conditioning) to examine the role of the endogenous opioid system in learned attachment behavior in rat neonates, I have demonstrated that endogenous opioids are necessary for the acquisition, memory consolidation, and memory expression of neonate odor preferences. Specifically, disruption of the opioid system during conditioning prevents the acquisition (learning) of an odor preference, and disruption of the opioid system during testing prevents the expression of the conditioned odor preference. Furthermore, disruption of the opioid system during memory

consolidation following odor – abuse conditioning permits a learned odor aversion instead of the typical neonate odor preference. Disruption of the opioid system following odor – stroke conditioning is not sufficient to permit an odor aversion. The results from these two conditioning paradigms not only indicate that opioids mediate neonate learning and memory necessary for attachment behavior, but that opioids play a pivotal role in securing odor preferences instead of aversions despite abuse.

Third, my research has shown that opioid modulation of cellular activity within the attachment circuitry plays a pivotal role in learned odor preferences in neonates. I have used two approaches to assess opioid modulation of the neurocircuitry supporting infant attachment despite maltreatment: brain cannulas and Fos immunohistochemistry (IHC). I have shown that disruption of the opioid system within the neonate olfactory bulb, a site pivotal in the learning of odor preferences, prevents a conditioned odor preference. Using IHC, I have demonstrated that disruption of the opioid system following the behavioral training (which again behaviorally yields an odor aversion, not the expected preference) produces significant changes in Fos expression in the neonate brain. Specifically, opioid receptor antagonism post-training produces a decrease in Fos expression in the olfactory bulbs and anterior piriform cortex, but a significant increase in the posterior piriform and central nucleus of the amygdala. This suggests that opioids modulate cellular activity and more importantly, appear to limit amygdala participation in neonates despite abuse.

Overall, the results from my dissertation research indicate a prominent role of the endogenous opioid system in mediating neonate learning and memory, and thus highlight how prenatal or postnatal disturbances to the developing opioid system may jeopardize infant attachment. Specifically, as discussed in the introduction chapter, prenatal opiate exposure or postnatal maltreatment disrupts the normal development of the endogenous opioid system, especially in areas concerned with emotion and reward,

such as the amygdala. Additionally, clinical studies indicate that such experiences produce altered attachment behaviors and subsequent emotional development. Thus, my results, indicating a pivotal role of the endogenous opioid system in facilitating the learning and memory necessary for attachment in our rat model of abusive mother-infant attachment, offer an avenue into understanding how these early adverse experiences affect the attachment process and emotional development.

My dissertation research offers several directions for further exploration. Although my research suggests a role of opioid modulation of the attachment circuitry, that is opioids appear to modulate cellular activity within the olfactory bulb, piriform cortex, and amygdala, the mechanisms of modulation within each brain area remain unclear. Of particular importance is understanding the mechanism of opioid modulation within the amygdala, as this area is profoundly impacted by prenatal drug exposure or postnatal maltreatment. As discussed in Chapter 6, in the adult opioids modulate the amygdala by inhibition of noradrenergic input to the basolateral/lateral amygdala, as well as opioids inhibit neurons within the central nucleus of the amygdala. Thus, my post-training induced odor aversion via antagonism of the opioid system may be attributable to disruption of normal opioid inhibition of the neonate amygdala, thus enhancing its participation in the memory consolidation process. Indeed, opioid receptor antagonism in the adult amygdala enhances fear memory via potentiating noradrenergic input. It remains unclear whether opioids inhibit the neonate amygdala as in the adult, and whether noradrenergic input to the neonate amygdala serves a role in the memory consolidation process.

I have preliminary data that suggests opioid receptor antagonism limited to the neonate amygdala post odor – abuse conditioning yields an odor aversion, suggesting that normal opioid activity within the neonate amygdala plays a pivotal role in consolidation of an odor preference and not an aversion. I also have data that increased

noradrenergic levels (via systemic administration of a noradrenergic agonist) post odor – abuse training yields an aversion, and that systemic administration of a noradrenergic receptor antagonist prevents the naltrexone induced-odor aversion, supporting the idea of an interaction between the opioid and noradrenergic system during neonate memory consolidation. Preliminary data from our lab suggests that norepinephrine within the neonate amygdala does appear to have a role in odor preference formation, but it remains undetermined how high levels of noradrenergic activity within the amygdala impact the memory process. Thus, further direct examination of opioid modulation of the neonate amygdala should be promising in providing answers to how neonates form positive memories despite abuse.

Other promising directions include assessing the role of endogenous opioids within the drug-reward circuit (ventral tegmental area, nucleus accumbens, prefrontal cortex) in the learning and memory necessary for neonate attachment despite abuse, and in determining the effects of repeated maternal abuse on opioid modulation of the attachment circuitry, the development of the reward pathway, and the subsequent effects on later emotion development, social behavior (mating) and drug use.