ATTACHMENT-BASED LEARNING AND *BDNF* GENE EXPRESSION IN THE INFANT RAT OLFACTORY BULB

by

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iii

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TABLE OF CONTENTS

| LIST | OF FI | GURES | vii |
|------|---------|---|------|
| ABS | ΓRAC | Τ | viii |
| 1 | NT | PODUCTION | 1 |
| 1 | | | |
| | 1.1 | Early reliance on attachment-based olfactory learning | 1 |
| | 1.2 | Classical olfactory conditioning | 2 |
| | 1.3 | Sensitive period for early olfactory learning | |
| | 1.4 | Mechanisms supporting neonatal olfactory learning | |
| | 1.5 | Olfactory learning circuit in the infant rat | 7 |
| | | 151 Locus coeruleus (LC) | 7 |
| | | 1.5.2 Olfactory bulb (OB) | 9 |
| | | 1.5.2 Onactory oute (OD) | 12 |
| | | 1.5.5 Americi princini conex (ur c) | 12 |
| | 1.6 | Neurotransmitter systems in neonatal odor preference learning | 13 |
| | | 1.6.1 Norepinephrine (NE) | 13 |
| | | 1.6.2 Glutamate | 14 |
| | | 1.6.3 Serotonin (5-HT) | 15 |
| | | 1.6.4 Gamma-aminobutyric acid (GABA) | 16 |
| | | 1.6.5 Opioids 17 | |
| | 1.7 | Learning-induced molecular changes in the OB | 18 |
| | 1.8 | A role for BDNF in early odor preference learning | 19 |
| | 1.9 | Rationale for current research | 21 |
| 2 | METHODS | | 22 |
| | 2.1 | Subjects | 22 |
| | 2.2 | Bedding preparation and room setup | 22 |
| | 2.3 | Odor-stroke conditioning | 23 |
| | 2.4 | Two-odor choice behavioral testing | 25 |
| | 2.5 | Biochemical Assays | 25 |
| | 2.6 | Statistical Analysis | 26 |
| 2 | DEC | | 77 |
| 3 | IVE 3 | 00118 | 21 |

| 3. | Odor Preference Learning Behavior | 27 | |
|------------|--|----|--|
| 3. | 2 <i>Bdnf IV</i> Gene Expression | 28 | |
| 4 D | SCUSSION | 31 | |
| 4. | Twenty-four-hour Odor Preference Learning is Significantly | | |
| | Increased in Paired Pups Compared to Non-Learning Controls | 31 | |
| 4. | 2 Bdnf IV Gene Expression in the Infant Rat Olfactory Bulb is Increased | | |
| | Following Olfactory Associative Conditioning | 32 | |
| 4. | <i>Bdnf IV</i> Gene Expression May Have Implications for Odor Preference | | |
| | Behaviors and Memory | 33 | |
| 4. | Future Directions to Investigate Epigenetics and Early Odor | | |
| | Preference Learning | 34 | |
| 4. | 5 Conclusion | 37 | |
| | | | |
| REFERENCES | | | |

LIST OF FIGURES

Figure 1. Time spent (sec) over peppermint-scented bedding during the two-odor choice test of PN7-9 pups 24 hours post-conditioning. Results indicate that paired pups spent significantly more time over peppermint-scented bedding than did stroke-only and odor-only control (*p<<0.05) and unpaired pups (p**<0.01). Time spent over peppermint-scented bedding was not significantly different between odor/stroke-only control and unpaired pups (p>0.05). n=6-8/group; subjects derived from 4 litters; error bars =SEM.

Figure 2. Fold change of *bdnf IV* mRNA in the olfactory bulbs of PN6-8 pups 30 minutes post-conditioning. Paired and unpaired mRNA levels were compared to stroke-only and odor-only controls. A fold change of 1.0 represents the mRNA level of the stroke-only and odor-only controls. Paired pups had significantly more *bdnf IV* mRNA in their olfactory bulbs than the stroke-only and odor-only controls (p*<0.05). Unpaired pups' *bdnf IV* mRNA levels were not significantly different from stroke-only and odor-only controls (p=0.2576). *Bdnf IV* mRNA levels were higher in paired pups but were not significantly different from levels in unpaired pups (p=0.0708). n=11-12/group; subjects derived from 6 litters; error bars = SEM.

ABSTRACT

Infant-caregiver experiences are known to impact neurobiological and behavioral development. The olfactory and tactile sensory systems are crucial to infant rats' survival, as they are born blind and deaf. Maternal attachment-based olfactory learning allows for vital behaviors such as orientation to the mother and nipple attachment. Learning occurs throughout development and requires the interaction of the environment and brain, suggesting epigenetic regulation in the brain as a possible mechanism. The brain structures required for olfactory learning prior to postnatal day 10 include the olfactory bulb (OB), locus coeruleus, and anterior piriform cortex. Brain-derived neurotrophic factor (bdnf) is a gene crucial to neuroplasticity, learning, and memory. Environmentally induced epigenetic modifications such as DNA methylation of *bdnf* result in altered levels of transcription and may lead to altered *bdnf* gene expression in the OB following odor learning. In this study we employ an odor-stroke conditioning paradigm to promote attraction to peppermint odor. When stroking was paired with presentation of peppermint odor, pups demonstrated an attachment-based preference for peppermint odor 24 hours post-conditioning. This learning paralleled increased expression of bdnf mRNA in the OB 30 minutes postconditioning. Taken together, these data provide evidence that maternal attachmentbased associative learning in the sensitive period rat affects *bdnf* gene expression in the OB. Future studies will determine whether this reflects experience-dependent epigenetic regulation of *bdnf*.

Chapter 1

INTRODUCTION

1.1 Early reliance on attachment-based olfactory learning

Infant rats are born blind and deaf and their visual and auditory sensory systems remain underdeveloped until the second postnatal week. Because of the deficits in these sensory systems, neonates rely profoundly on the olfactory system to associate particular odors with maternal care (Morrison et al. 2013). It is essential to pups' survival that they learn a preference for maternal odor, as the mother is their vital source of warmth and milk. Pups depend on olfactory learning to locate and attach to the mother's nipples and to suckle (Wilson and Sullivan 1994). Without a preference for the maternal odor, pups' contact with the mother is greatly reduced and pups fail to nurse, leading to lower survival rates (Sullivan and Holman 2010). Neonates readily acquire a preference for an odor that is paired with an unconditioned stimulus that imitates maternal care (Morrison et al. 2013) or physical contact with littermates (Woo and Leon 1987). This classically conditioned behavior reflects the evolutionary importance of proximity to the dam and nest (Sullivan et. al 2000). At birth, rats possess the neural mechanisms to remember the association between an odor and tactile stimulation, which serves as reward (Roth and Sullivan 2006). Similarly, human infants are capable of associating an odor and tactile stimulation during the first hours of life (Varendi and Porter 2001; Romantshik et al. 2007).

Maternal separation of the infant rat from its mother compromises both longterm behavioral and neurobiological development. Early maternal separation has been

associated with endocrine abnormalities, aberrant gene expression, and altered regulation of the hypothalamic-pituitary-adrenocortical (HPA)-axis later in life (Moriceau, Roth, and Sullivan 2010). The HPA axis is a highly conserved neuroendocrine response that helps an organism survive when faced with a challenge, and ultimately leads to the release of glucocorticoids from the adrenal cortex. Glucocorticoids, such as corticosterone (CORT) in the rat, impact many organ systems including the brain (Lightman and Conway-Campbell 2010). In maternally deprived pups, tactile stimulation may rescue some of the behavioral and brain irregularities caused by early separation. Intriguingly, stroking and skin contact in human infants likewise promote beneficial behavior and brain development, especially in preterm babies who likely spend more time in isolation (Moriceau, Roth, and Sullivan 2010). Because disruptions to maternal attachment learning have been shown to have longlasting impacts on the regulation of gene expression and neuroendocrine physiology (Brake et al. 2004), early olfactory learning may be used to study epigenetic mechanisms underlying attachment processes as these mechanisms are known to affect gene expression.

1.2 Classical olfactory conditioning

The maternal odor of the dam is dependent on the composition of her diet, which may change day to day (Wilson and Sullivan 1994). Thus, rat pups' attachment to maternal odor is dynamic, both behaviorally and neurobiologically, over the course of the postnatal period. Researchers may take advantage of this flexibility of infant odor learning in a laboratory setting by classically conditioning pups to a variety of unconditioned stimuli. Milk infusion, tail pinching, odor of maternal saliva, mild foot shock, intracranial brain stimulation, and stroking that mimics maternal licking and

grooming have all been successful in inducing odor preferences in neonates (Yuan, Shakhawat, and Harley 2014). In fact, any method that evokes behavioral activation in pups during the presentation of a novel odor yields an odor preference. For example, milk infusions and stroking both increase pups' motor behaviors like rolling over and making robust limb movements. Interestingly, milk infusion paired with an odor will only induce a preference for that odor in infants if the milk infusion caused an increase in the behavioral activity. This suggests that an activated behavioral state paired with odor is sufficient for early odor preference learning (Sullivan, Hofer, and Brake 1986). Odor preferences learned in infancy can be preserved into adulthood and may influence future sexual behaviors (Yuan, Shakhawat, and Harley 2014).

Despite their propensity to learn an odor preference, neonates are neophobic; they will naturally avoid novel stimuli like unfamiliar odors. In a two odor-choice test, when allowed to move over unscented bedding or bedding scented with peppermint, lemon, or orange odors, postnatal day (PN) 7 pups spend more time over the unscented bedding. Because pups choose the unscented bedding more than the scented bedding, peppermint, lemon, and orange odors are considered aversive to pups. In an appetitive-learning paradigm like the present study, if naturally aversive peppermint odor is paired with the appetitive stimulus of stroking, a maternal attachment-based preference for peppermint will form in neonates, demonstrating that learning has occurred (Amiri et al. 1998).

1.3 Sensitive period for early olfactory learning

Neonates' proclivity to learn odor preferences to behaviorally activating stimuli is normally limited to a sensitive period, which spans from birth to the middle of the second postnatal week on PN10. During this early stage it is more probable that

infant rats will learn an approach response rather than an avoidance response to an odor paired with an unconditioned stimulus (Sullivan et al. 2000). Until PN10, rat pups will learn to prefer an odor paired with mild foot shock (0.5mA) despite their ability to feel pain at this age. While pups 9 days old or less will exhibit this paradoxical odor preference for the odor paired with mild shock, older pups exhibit an odor aversion following the same mild foot-shock pairing (Sullivan 2005). This enhanced probability of approach learning during infants' most vulnerable stage is evolutionarily based in maintaining contact with and forming an attachment to the caregiver regardless of the quality of care (Moriceau, Roth, and Sullivan 2010). Although the pups may experience pain in the nest from the mother's periodic stepping and rough handling, their sensitive period maximizes approach behaviors to the mother, as she is the most important resource for their survival. This sensitive period for caregiver attachment is highly conserved across species, including birds, puppies, and human infants (Moriceau, Roth, and Sullivan 2010).

The behavioral changes reflect the dynamic changes in the olfactory learning circuit as the pup begins walking around PN10 and explores its environment. Because the older, more mobile pups may encounter harmful stimuli outside the nest, it appears adaptive that pups become able to learn odor aversions to unfamiliar odors at approximately the same time that walking emerges (Sullivan 2005). After the sensitive period, it is also more difficult for older rat pups to learn an odor preference for an appetitive stimulus such as stroking. In fact rats trained with paired tactile stimulation and odor exposure on PN19 will not learn a preference for that odor (Woo and Leon 1987).

1.4 Mechanisms supporting neonatal olfactory learning

Changes normally induced by associative olfactory learning in the rat olfactory bulb (OB) such as enhanced uptake of 2-deoxyglucose (2-DG) and odor-specific response patterns of OB cells are both reduced in adult brains compared to neonates, indicating enhanced neural plasticity in the neonatal olfactory bulb (Wilson and Sullivan 1994).

Changes in the primary olfactory learning circuit underlie the transition from the sometimes-paradoxical, approach-focused sensitive period to more adult-like preference learning on PN10 (Wilson and Sullivan 1994). Rather than a merely underdeveloped version of the adult, the neonatal olfactory system involves fewer brain structures and different regulation of neurotransmission. Structures involved in the adult but not the neonatal olfactory learning circuit include the amygdala, hippocampus, cerebellum, and frontal cortex (Verwer, Van Vulpen, and Van Uum 1996; Nair and Gonzalez-Lima 1999; Stanton 2000; reviewed in Sullivan 2003). It is the failure of the amygdala to participate in odor-shock pairings in sensitive period neonates that allows for preference, rather than aversion, to be formed for odors associated with mild foot shock or other painful conditioned stimuli (Sullivan 2005; Moriceau, Roth, and Sullivan 2010).

Sensitive period olfactory learning engages three key brain regions in the neonate: the locus coeruleus (LC), OB, and anterior piriform cortex (aPC). While the noradrenergic LC is also implicated in adult olfactory learning, the sensitive period LC is more responsive to tactile stimulation, resulting in increased innervation of the OB with norepinephrine (NE) and heightened synaptic plasticity (Sullivan et al. 2000). The hyperactivity of the LC and sensitive period are quelled when α_2 -adrenoceptors develop on the LC. These receptors provide auto-inhibition to the LC starting on PN10

(Yuan, Shakhawat, and Harley 2014), effectively suppressing excitability of the LC and consequently its NE output to the OB. When α_2 -adrenoceptors on the LC are pharmacologically blocked during the post-sensitive period, auto-inhibition of the LC is prevented and the LC returns to its hyperactive state. These older pups then may learn paradoxical odor preferences to odors paired with shock, highlighting the importance of enhanced NE input to the OB for sensitive period learning behavior, and the ability of the sensitive period to be extended by LC hyperactivity (Moriceau and Sullivan 2004).

In addition to NE input, the neonatal sensitive period is reliant upon low basal CORT stress hormone levels, which are maintained by maternal interaction and milk. During the first 9 postnatal days, rats' HPA axis is "hypo-responsive." However, in post-sensitive period pups, the mother's presence can serve as a social buffer, suppressing CORT levels during stressful experiences (Moriceau, Roth, and Sullivan 2010). Hypo-responsiveness of the HPA axis and low basal CORT levels during the sensitive period prevent activation of the amygdala, possibly explaining its lack of involvement during early olfactory learning. Without the involvement of the amygdala, hypo-responsive rats do not readily form odor aversions. However, CORT levels may rise in pups if the mother is stressed or if there is prolonged maternal separation (Sullivan and Holman 2010). Stressed mothers may raise their pups' CORT levels by providing insufficient care or excessive CORT concentration in her milk (Sullivan 2005). Sensitive period pups exposed to a stressed mother, when subjected to an odor-shock paradigm, exhibit an odor aversion that is amygdala-dependent due to increased CORT levels. These results suggest that increased CORT levels before PN9 end the hypo-responsive sensitive period early through premature involvement of

the amygdala (Sullivan and Holman 2010). Furthermore, PN8 pups systemically injected with CORT learn an odor aversion instead of a normal odor preference for the odor in an odor-shock paradigm because increased CORT levels permit involvement of the amygdala in the learning pathway (Sullivan and Holman 2010). Similarly, PN12 rats depleted of CORT learn an odor preference in an odor-shock paradigm when they normally would learn an odor aversion (Moriceau and Sullivan 2004b). These experiments provide evidence for CORT level's governance over the transition from the neonatal hypo-responsive period to a more mature olfactory learning circuit involving higher limbic structures like the amygdala during adulthood (Sullivan 2005; Moriceau, Roth, and Sullivan 2010).

Coinciding with the emergence of the amygdala function and walking, PN10 pups also begin to fear predator odor (Takahashi and Rubin 1993). PN10 pups begin to fear male odor because adult male rats eat rat pups. Fear to predator odor, like conditioned odor, is strongly controlled by CORT levels. As pups physically distance themselves from the nest, there is adaptive value in fearing predator odors. Fear of predator odor on PN10 supports amygdalar emergence and reveals that CORT levels modulated by the environment can have profound effects on not only the neural circuitry of learned odor associations, but also innate odor information (Sullivan 2005; Moriceau, Roth, and Sullivan 2010).

1.5 Olfactory learning circuit in the infant rat

1.5.1 Locus coeruleus (LC)

The LC is the major noradrenergic nucleus of the rat's central nervous system, located in the rostral pons (Samuels and Szabadi 2008). With 40% of its fibers

projecting to the OB (Shipley, Halloran, and de la Torre 1985), the LC is the major source of NE for the OB. Most of these noradrenergic axons project to the internal plexiform and granule cell layers of the OB while there are less dense projections to the external plexiform and glomerular layers (Yuan, Shakhawat, and Harley 2014). These axons are present and functional during the first week of the pup's life. During the hypo-responsive sensitive period, a neonate's LC is more sensitive to sensory stimulation (Nakamura and Sakaguchi 1990). Because the OB lacks intrinsic noradrenergic neurons, the LC provides its excitatory NE input and modulates the excitability of the OB via synapses on inhibitory granule cells (Sullivan, Wilson, and Leon 1989).

Excitation of the LC paired with odor exposure during the sensitive period in pups produces a preference for that odor. Experimenters have pharmacologically mimicked LC input to the OB in neonates to observe its effect on odor preference learning. When infused directly into the OB before olfactory conditioning the β adrenoceptor agonist isoproterenol results in a learned odor preference (Sullivan et al. 2000). When half of the optimal dosage of isoproterenol is infused into the OB with half of the amount of stroking normally used in odor-stroke conditioning, an odor preference is still observed in sensitive period pups. Because odor learning may result from a combination of suboptimal reinforcing stimuli, isoproterenol and stroking appear to be additive in their effects on OB stimulation and memory formation. Interestingly, a high dose of isoproterenol and normal amount of stroking does not produce odor preference learning, possibly due to over-stimulation. These results indicate an "inverted U-shaped dose-response curve" for LC stimulation and odor preference learning in sensitive period pups (Sullivan, Wilson, and Leon 1989).

Similarly, this learned approach response is blocked by infusion of β adrenoceptor antagonist propranolol into the OB just prior to olfactory conditioning. Bilateral lesions to the LC in infant rats likewise prevent odor preference learning, highlighting the necessary role of NE input to the OB for odor preference acquisition. However, pharmacological β -adrenoceptor antagonism after training and acquisition of the odor preference does not disrupt odor memory. Thus, the noradrenergic projection from the LC to the OB is vital for acquisition but not consolidation of olfactory association memory (Sullivan et al. 2000).

1.5.2 Olfactory bulb (OB)

The infant rat OB, the brain region examined in the present study, though a relatively simple structure (Wilson and Sullivan 1994), is highly dynamic from a rat's birth to adulthood (Whitman and Greer 2009). The OB possesses the sufficient and necessary neural mechanisms for associating an odor with specific response patterns (Sullivan et al. 2000) and plasticity over a lifetime (Morrison et al. 2013).

Though infants' brains contain two OBs, until PN11, odor input is lateralized to both the OB and olfactory cortex because there is a lack of anterior commissural projections to connect them synaptically. Because of this lateralization, sensitive period pups' OBs may serve as their own controls for odor memory and related physiological changes. If one naris is occluded during olfactory training, a neonate will only have access to memory formed via the open nostril and the associated olfactory cortex on the open naris side. For example, neonates with left naris occlusions that undergo odor-stroke pairings only exhibit an odor preference when tested with the right nostril open because this is the side and OB that was available to form the long-term olfactory memory. Interestingly, once the anterior commissural

projections form, these new synaptic connections become evident in behavioral memory, as older rats have access to both OBs and olfactory cortices (Fontaine, Harley, and Yuan 2013).

Odor processing begins in the nasal cavity's olfactory epithelium where olfactory sensory neurons (OSNs) are located (Whitman and Greer 2009). Specific odor receptors are expressed on cilia of OSNs with each OSN possibly expressing a variety of receptor families (Hines 2015). OSNs are replaced throughout the lifetime and project directly to the OB, followed by the olfactory piriform cortex. This cortex also projects back to the OB and widely across the central nervous system (Whitman and Greer 2009). OSNs' axon terminals synapse on the dendrites of the OB's two types of output neurons: mitral and tufted cells (Wilson and Sullivan 1994). Most of the mitral and tufted cells that a rat will have in its lifetime are present at birth. Olfactory learning is correlated with enhanced output from mitral cells, which send olfactory information from the OB to the olfactory cortex (Jerome, Hou, and Yuan 2012). These mitral and tufted cells are scattered in spherical complexes of neuropil, or dense networks of nerve fibers and glial filaments, called glomeruli (Wilson and Sullivan 1994). Glomeruli respond as units in distinct patterns to different odors. Axons of OSNs that express the same odorant receptor in the olfactory epithelium converge onto one or two glomeruli (Whitman and Greer 2004). Juxtaglomerular neurons mediate interactions between glomerular units via dopaminergic and GABAergic transmission (Wilson and Sullivan 1994). Following olfactory learning, structural changes like increased glomerular size and an increased number of juxtaglomerular cells near activated glomeruli are observed (Yuan, Shakhawat, and Harley 2014).

A single glomerulus innervates a mitral or tufted cell via apical dendrodendritic synapses. Mitral and tufted cells develop elaborate and far-reaching secondary dendrites within the external plexiform layer (EPL) of the OB. As early as PN2, axons from mitral and tufted cells expand through the lateral olfactory tract (LOT) to a variety of structures that transmit back to the OB, including the anterior olfactory nucleus, piriform cortex, and cortical nucleus of the amygdala (Wilson and Sullivan 1994). These output neurons also form dendrodendritic synapses with granule cells in the granule cell layer in the center of the OB. Granule cells are the most numerous type of cell in the OB, outnumbering the output cells 100:1. Granule cells are unique anaxonic; they are able to transmit neuronal information only via dendrodendritic synapses with mitral and tufted cell dendrites in the EPL (Whitman and Greer 2004). In the EPL inhibitory granule cells modulate mitral and tufted cells' output signals (Yuan, Shakhawat, and Harley 2014).

Granule interneurons are the main recipients of NE input from the LC and are formed by postnatal neurogenesis, which peaks between the second and third postnatal week (Wilson and Sullivan 1994). 95% of new neurons in the OB differentiate into granule interneurons while 5% differentiate into periglomerular (PG) interneurons (Whitman and Greer 2004). Periglomerular cells are a heterogeneous group of inhibitory interneurons that modulate glomerular coding (Yuan, Shakhawat, and Harley 2014), mostly via GABA. New interneurons in the olfactory system are born in the subventricular zone (SVZ) of the lateral ventricles and travel along the rostral migratory stream into the OB. Once inside the OB the new cells differentiate into granule or PG interneurons and incorporate into the existing OB network (Whitman and Greer 2004).

During associative learning between an odor and unconditioned stimulus, mitral cells are released from inhibition and no longer habituate to the conditioned stimulus. Enhanced olfactory conditioning in the infant rat strengthens synapses between mitral and tufted cell neurons and granule cells (Wilson and Sullivan 1994). However, when later exposed to the attractive odor, pups that learned an odor preference exhibit decreased excitation of mitral and tufted cells. These results reflect that initial olfactory learning alters later output from mitral and tufted cells in response to an attractive odor in the OB (Wilson, Sullivan, and Leon 1987).

1.5.3 Anterior piriform cortex (aPC)

The aPC, the largest structure in the olfactory cortex (Onoda, Sugai, and Yoshimura 2005), receives odor information from the OB via the LOT (Morrison et al. 2013). Unlike the distinct glomeruli of the OB, the aPC exhibits spatially dispersed odor representations. Like the OB, pharmacological β -adrenergic activation of the aPC just prior to olfactory conditioning is sufficient to cause a learned odor preference. Conversely, antagonism of β -adrenoceptors before olfactory training impedes preference memory (Morrison et al. 2013). Antagonism of the N-methyl-D-aspartate (NMDA) glutamate receptor in the aPC also hinders early odor preference learning. While trained pups still exhibit heightened behavioral activation in response to the conditioned odor, preference does not result, indicating NMDA receptors' role in learning in the aPC. In addition, mild odor-shock training paradigms in infant pups reveal odor-specific uptake of 2-DG in the aPC, further suggesting its role in odor recognition and memory encoding. Long-term potentiation-like effects have been proposed following odor learning in the aPC due to increased α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor currents and increased

AMPA receptor insertion in neuronal membranes (Cui et al. 2011; Lethbridge et al. 2012; Yuan and Harley 2012). These synaptic changes in the aPC are usually driven by activity in the OB, although activation of the aPC during odor exposure directly allows for odor preference memory without noradrenergic activation of the OB. Early odor preference memory is therefore not restricted to the OB but rather is supported and even produced by forebrain cortices like the aPC (Morrison et al. 2013).

1.6 Neurotransmitter systems in neonatal odor preference learning

1.6.1 Norepinephrine (NE)

Norepinephrine is present at high levels at birth in the infant rat (Wilson and Sullivan 1994). NE released from the LC upon stimulation interacts with all types of adrenoceptors, which exist in all layers of the OB (Yuan, Shakhawat, and Harley 2014) – α_1 , α_2 , β_1 , and β_2 . Blocking all β -adrenoceptors with propranolol just prior to odor training in sensitive period pups eliminates odor preference learning, indicating that α -adrenoceptor activation on the OB alone is insufficient for odor preference learning (Sullivan, McGaugh, and Leon 1991; Sullivan et al. 1992). A more specific β_2 receptor agonist infused into the OB before odor conditioning likewise fails to produce preference learning. Thus, activation of OB β_1 adrenoceptors is necessary and sufficient for early odor preference learning. α_1 -adrenoceptors likely enhance odor learning, while α_2 adrenoceptors influence memory in other brain regions. Due to the hyperactivity of the LC in sensitive period pups, these OB adrenoceptors receive significant innervation and upon their activation initiate longstanding changes in mitral and tufted cell response patterns (Harley et al. 2006).

1.6.2 Glutamate

Glutamate signaling via ionotropic receptors is critical for early odor preference learning. NMDA receptors, "the Hebbian coincidence detectors" of the central nervous system, play a pivotal role because they only activate if the membrane in which they are docked is sufficiently depolarized (Yuan, Shakhawat, and Harley 2014). B-adrenoceptor activation is believed to disinhibit and depolarize mitral cells, increasing activation of NMDA receptors embedded in their membranes (Lethbridge et al. 2012). Once NMDA receptors become active, calcium influxes through voltagegated channels to instigate a cascade of intracellular events (Jerome, Hou, and Yuan 2012). Because of its requirement of sufficient membrane depolarization, NMDA receptors' intracellular events are effectively coupled to mitral cell depolarization caused by LC stimulation. L-type voltage-gated calcium channels of NMDA receptors have been suggested to activate transcription factors, promoting further long-term synaptic changes. These calcium channels pair intense mitral cell depolarization with gene expression through enhanced transcriptional activation (Jerome, Hou, and Yuan 2012).

NMDA glutamate receptor antagonists infused into the OB before olfactory conditioning block early odor preference learning (Lethbridge et al. 2012), revealing their crucial role. Five minutes after olfactory training, the GluN1 subunit of NMDA glutamate receptors in the glomeruli of the OB is phosphorylated (Lethbridge et al. 2012). Following olfactory learning, NMDA receptor subunits GluN1 and GluN2B become down-regulated in the OB. These subunit modifications may function to store odor memories or inhibit further change (Lethbridge et al. 2012).

While there is a significant down-regulation of NMDA receptors following odor preference learning (Jerome, Hou, and Yuan 2012), there is a significant up-

regulation of AMPA receptors (Cui et al. 2011). AMPA up-regulation is associated with long-term potentiation: augmented structural size of the synapse and increased strength of the synaptic connection between neurons in the OB. NMDA receptor down-regulation suggests decreased potential for plasticity once learning has already occurred. As rats age there is a decrease in NMDA receptors in the olfactory cortex, which is consistent with decreased olfactory learning in older rats (Yuan, Shakhawat, and Harley 2014). During olfactory training of infant rats, the GluA1 subunit of the AMPA receptor becomes phosphorylated. Phosphorylation of this subunit is specific to the association between the odor and the unconditioned stimulus, and must occur before the subunit can become embedded into the neuronal membrane. If the GluA1 subunit of the AMPA receptor is blocked form membrane insertion, 24 hour odor preference learning is prevented, confirming that AMPA receptor function is a requirement for olfactory short and long-term memory (Cui et al. 2011).

1.6.3 Serotonin (5-HT)

Serotonin (5-HT) has a variety of functions that influence sleep, motor activity, mood, and cell differentiation. In addition to NE, the OB is densely innervated by 5-HT, especially in the glomerular layer (McLean et al. 1993). Input to the OB from the raphe nuclei of the brainstem is substantial by PN7 in infant rats, and its normal innervation of the OB is critical for early odor preference learning (Wilson and Sullivan 1994). Pups depleted of 5-HT axons that innervate the OB since PN1 fail to acquire a learned odor preference from conditioning during the sensitive period. However, this deficit can be overcome by increased doses of isoproterenol. This recovery with β -adrenergic agonism reveals a facilitative role for 5-HT₂ receptors in odor preference memory (McLean et al. 1993). Furthermore, normal odor preference

learning during the sensitive period can be prevented by 5-HT_{2A/2C} antagonism (McLean, Darby-King, and Hodge 1996). 5-HT appears to mediate normal β adrenoceptor control of odor preference learning. Crucial β_1 -adrenoceptors are colocalized with 5-HT₂ receptors in the mitral cells and synergistically enhance olfactory learning (Yuan, Harley, and McLean 2003). It is also suspected that 5-HT may support NMDA receptors and long-term potentiation in the OB (McLean et al. 1993).

1.6.4 Gamma-aminobutyric acid (GABA)

Periglomerular interneurons of the OB control mitral cell input and granule interneurons regulate mitral cell output via the inhibitory neurotransmitter GABA. Both of these GABA-ergic cell types communicate via GABA-A and GABA-B receptors across dendrodendritic synapses. Pharmacological manipulation of the GABA-A and GABA-B receptors reveals GABA's complex influence in early odor association learning. Excessive GABA-A and GABA-B antagonism in the OB results in odor aversions to all odors presented to neonates (Yuan, Shakhawat, and Harley 2014). Because antagonism of inhibitory GABA receptors causes strong disinhibition of glomerular cells, these results suggest that extreme disinhibition of glomeruli results in aversive learning. As would be expected, antagonism of GABA-A receptors in granule cells of the OB during odor-milk infusion pairings impairs normal odor preference learning in neonates (Wilson and Sullivan 1994). However, antagonism of GABA-A receptors in OB glomeruli paired with an odor can induce NMDA receptordependent preference learning without the presence of another appetitive stimulus. This finding suggests that the optimal level of glomerular disinhibition can induce preference learning in neonates (Lethbridge et al. 2012). These conflicting effects of GABA receptor antagonism reflect the complexity of GABA's transmission in the OB and the importance of the extent of glomerular disinhibition in early olfactory preference learning.

1.6.5 Opioids

The importance of endogenous opioids in mediating the calming effects of maternal contact is well established in neonatal rats (Roth and Sullivan 2006). During early life, opioids mediate interactions with the mother (Roth and Sullivan 2003), causing rats to associate maternal odor with maternal contact and analgesia (Roth and Sullivan 2006). Pharmacological interference with the endogenous opioid system impedes normal odor preference learning in infant rats. When the opioid antagonist naltrexone (NTX) is injected after odor-shock training during the sensitive period, NTX blocks consolidation of the odor memory and pups fail to learn a normal odor preference. Similarly, when NTX is injected just prior to odor-shock olfactory training, normal odor learning is blocked (Roth and Sullivan 2003). Just as NTX injected before or following odor-shock training inhibited preference learning, NTX administration likewise hampers odor-stroke associations during the sensitive period (Roth and Sullivan 2006).

However, older rats' ability to acquire an odor preference or consolidate an odor memory is not affected by opioid antagonism, suggesting opioids' particular role during the sensitive period to support pups' attraction to significant odors. These changes are aligned with the end of the sensitive period when pups begin to leave the nest (Roth and Sullivan 2003) and possibly encounter threats to their survival in their environment to which odor attachments should not be made. Opioid receptors are colocalized with GABA-ergic neurons in the OB and thus are believed to influence

inhibitory activity and assign hedonic values to odors in the environment (Roth, Moriceau, and Sullivan 2006).

1.7 Learning-induced molecular changes in the OB

2-DG is a glucose molecule with a hydrogen group replacing the hydroxyl group on carbon 2. 2-DG is transported into cells via glucose transporters and its enhanced uptake is observed in the OBs of sensitive period pups that learn an odor association (Johnson et al. 1995). Pups injected with 2-DG and then subsequently exposed to an odor exhibit altered glomerular 2-DG uptake that is specific to the odor (Johnson and Leon 1996). As early as PN1, pups that receive simultaneous stroking and odor exposure exhibit specific spatial patterns of 2-DG in certain patterns of glomeruli (Sullivan and Leon 1986). When odor learning does not occur due to pharmacological blockade or non-associative training enhanced 2-DG uptake patterns are not observed (Sullivan, Wilson, and Leon 1989). 2-DG uptake patterns in pups that learned reflect a unique physiological response to learned odor in the OB.

Throughout the brain, learning is associated with an increased Fos activity. Following odor memory formation, there is an 80% increase in cells in the glomerular layer (Johnson et al. 1995). Fos levels within the OB and aPC increase following odor preference learning. However, the Fos level in the granule layer of the OB following odor associations has been shown to decrease which fits the notion that granule cell layers must be less active to support learning and memory (Roth, Moriceau, and Sullivan 2006).

In the neonate, NE from the LC raises 3'5'-Cyclic Adenosine Monophosphate (cAMP) levels in the OB, which increases activation of protein kinase A (PKA). cAMP and activated PKA levels are highest 10 minutes post-conditioning. Activated

PKA may then translocate to the nucleus and phosphorylate substrates that have been linked to learning like the GluA1 subunit of AMPA receptors (Yuan, Shakhawat, and Harlely 2014) and the transcription factor cAMP response element-binding protein (CREB) at the Ser-133 site. Phosphorylated CREB (pCREB) then triggers transcription of immediate-early genes like *brain-derived neurotrophic factor* (*bdnf*), which yield transcription factors that activate late response genes for long-term memory (Raineki et al. 2010).

1.8 A role for BDNF in early odor preference learning

BDNF is a neurotrophin present in all brain regions (Zimmerberg, Foote, and Van Kempen 2009) that has been well established to support in long-term learning and memory in adult animals (Jones et al. 2007). BDNF is most abundant in the hippocampus but it is also rich in the OB's granule, mitral, and periglomerular cells, and is up-regulated following experiential olfactory learning (Zimmerberg, Foote, and Van Kempen 2009). As a neurotrophic protein BDNF supports neurogenesis (Jones et al. 2007), cell differentiation (McLean, Darby-King, and Bonnell 2001), neural growth, cell survival, and neural plasticity due to learned events (Zimmerberg, Foote, and Van Kempen 2009). BDNF has been associated with morphological modification to dendritic spines and recruitment of scaffolding proteins required for synaptic plasticity (Jones et al. 2007). Throughout development in the infant rat, BDNF levels increase in the central nervous system (Zimmerberg, Foote, and Van Kempen 2009).

CREB, a protein up-regulated during olfactory learning, is a transcription factor for BDNF, and BDNF acts through the high-affinity tyrosine kinase receptor B (TrkB) or receptor p75 to activate a cascade of complex and still unknown intracellular events that support learning (Jones et al. 2007). Both BDNF receptor

types are present in the SVZ, the site of neurogenesis in the olfactory system (Bath et al. 2008). During the first three postnatal weeks there is drastic synaptic growth between the SVZ and OB (Whitman and Greer 2004). Starting in early life BDNF serves a critical role in OB neurogenesis, although differences in neurogenesis between wild type pups and BDNF-depleted pups are not detected until the second postnatal week (Bath, Akins, and Lee 2012). These newly generated interneurons are crucial for future olfactory discrimination (Yuan 2008).

Intriguingly, transcription of *bdnf* is particularly sensitive to environmental events. A variety of stress-inducing paradigms reveal aberrant regulation of BDNF protein, usually a decrease in expression, in the brain as a result of social stress (Zimmerberg, Foote, and Van Kempen 2009). Even prenatally stressed pups exhibit decreased hippocampal BDNF levels in adulthood (Gomez-Pinilla and Vaynman 2005). Likewise, BDNF levels dramatically decline following naris occlusion in neonatal pups. Blocking odor input through one naris significantly deprives olfactory stimulation to the ipsilateral OB and olfactory cortex. As a result of naris occlusion, there is a decrease in ipsilateral OB weight in adulthood, related to depletion of BDNF and its associated neurogenic and neuronal protective actions (McLean, Darby-King, and Bonnell 2001).

Increases in BDNF protein levels that occur following olfactory learning (Zimmerberg, Foote, and Van Kempen 2009) may be due to epigenetic regulation of the gene. DNA methylation may influence whether or not *bdnf* is transcribed. Methyl groups attached to cytosine nucleotides in or around the gene promoter may silence gene expression in cells (Blaze, Scheuing, and Roth 2013). Decreased methylation at certain exons of *bdnf* may lead to increased gene expression. Long-term memory

depends on both transcription and translation of mRNA in the OB (Yuan, Shakhawat, and Harley 2014). It is hypothesized that after translation of *bdnf* mRNA in mitral cells of pups that learned an odor preference, BDNF protein is transported to other brain regions to mediate formation and storage of the long-term olfactory memory (Zimmerberg, Foote, and Van Kempen 2009).

1.9 Rationale for current research

The *bdnf* gene is critical in synaptic plasticity and neurological development (Bath, Akins, and Lee 2012) and is epigenetically altered in response to the caregiving environment (Roth et al. 2009). For these reasons, alterations in the expression of *bdnf* mRNA would be useful to examine in the context of infant olfactory learning and memory. This study: 1) established a neonate olfactory conditioning protocol to be used in the Roth Lab at UD; and, 2) investigated the effects of odor-stroke learning on expression of *bdnf* mRNA associated with exon IV in the OB of infant rats. As abnormal BDNF regulation has been linked to many psychiatric disorders in humans (Blaze, Scheuing, and Roth 2013), studying *bdnf* gene expression in the context of maternal attachment learning may aid the creation of interventions and treatments for cases in which normal developmental trajectories have been disturbed due to dysregulation or absence of caregiver attachment.

Chapter 2

METHODS

2.1 Subjects

For this study, Long-Evans rat mothers and pups were housed in polypropylene cages (18"x9"x8") with ample beta-chip bedding (Nepco). The rats were housed in a temperature- and light-controlled colony room (12 hour light/dark cycle, with lights on at 6:00am) and had access to food and water ad libitum. All experimental procedures were performed during the light cycle. The dams were bred in the laboratory and PN0 was considered the day of birth. On PN1, litters were culled to 6 males and 6 females and each subject was randomly assigned to one of four conditions: paired, unpaired, stroke-only, or odor-only. The University of Delaware Animal Care and Use Committee approved all procedures prior to execution of the experiment.

2.2 Bedding preparation and room setup

Using a previously published ratio of 0.3mL peppermint extract/500mL aspen bedding (Fontaine, Harley, and Yuan 2013), scented bedding mixture was created beneath a fume hood and shaken vigorously for 15-20 seconds in a closed plastic container. After mixing, scented bedding was aired in the open container under the fume hood for five minutes and, following this period, was used immediately for conditioning or behavioral testing. Bedding was transported to a portable hood with containers covered with aluminum foil to prevent diffusion of peppermint odor

throughout the laboratory. This room was maintained at 23-26°C (as measured by a digital thermometer) and lights were kept dim. Pup containers were mounted upon shock-absorbing material to minimize possible vibrations emanating from the portable hood. To ensure consistent concentration of peppermint odor, fresh scented bedding was made after every conditioning session (~20 minutes) and after every 3 pups that were behaviorally tested during the 2-odor choice memory test (~20 minutes).

2.3 Odor-stroke conditioning

On PN6, 7, or 8, infant rats of both sexes from one litter were weighed, marked, and randomly assigned to one of four training conditions: paired, unpaired, stroke-only, or odor-only. Half of the pups in a single litter were sacrificed for gene expression analysis 30 minutes post-conditioning and half were behaviorally tested 24 hours post-conditioning for long-term odor preference memory. Ideally, for each of the training conditions male and female pups would be used for both gene expression and behavioral testing. In a hypothetical litter of 6 males and 6 females, 4 pups (2 males and 2 females) would be assigned to the paired, unpaired, and either the odoronly or stroke-only training conditions. Of the two pups of the same sex in the same condition, one would be sacrificed for gene expression analysis and the other tested for odor preference learning 24 hours post-conditioning.

Regardless of training condition, prior to conditioning all pups were exposed to a 10-minute habituation period on unscented aspen bedding in the mounted habituation chamber under the portable hood (Roth and Sullivan 2006). After habituation, pups were gently and quickly transferred to containers (12cm x 8cm x 5cm) filled with 150mL of the appropriate bedding for the condition (i.e. scented or unscented aspen bedding). Pups assigned to the paired condition were exposed to

vigorous and circular caudal stroking with an artist's paintbrush while lying directly upon peppermint-scented aspen bedding. Sessions lasted for 20 minutes with alternating periods of stroking (1 minute) and no stroking (30 seconds). Inclusion of this 30-second inter-trial interval decreased the possibility of over-stimulating the pup and hindering learning (Sullivan, Wilson, and Leon 1989). Unpaired pups were similarly stroked but on unscented aspen bedding. Afterward, unpaired pups were returned to the home cage for one hour. They were then re-habituated for 10 minutes and then exposed for 20 minutes to peppermint-scented bedding without stroking. The unpaired stimuli of stroking and peppermint odor should not result in odor preference learning; the unpaired condition was intended to ensure that the mere presentation of each stimulus separately did not yield a significant effect on *bdnf* gene expression or odor preference behavior as the animal should not be learning an association between the two stimuli. Odor-only pups were habituated for 10 minutes and then exposed for 20 minutes to peppermint-scented aspen bedding without any stroking. Stroke-only pups were habituated for 10 minutes and then exposed for 20 minutes to non-scented aspen bedding accompanied by alternating periods of stroking (1 minute) and no stroking (30 seconds).

Pups that did not require peppermint-scented bedding were conditioned under the portable hood first to minimize the possibility that peppermint odor was circulating under the hood and unintentionally promoting associative learning in unpaired groups. Thirty minutes post-conditioning, pups designated for gene expression analysis were removed from the home cage and sacrificed. Olfactory bulbs were obtained immediately and placed in a tube on dry ice to later be stored at -80°C until processing took place.

2.4 Two-odor choice behavioral testing

Twenty-four hours post-conditioning, pups designated for behavioral testing were given a 2-odor choice test to examine long-term odor preference memory. A 21.1cm x 27.6cm tray was filled with 150mL of unscented aspen bedding on one side and 150mL of peppermint-scented bedding on the other. There was a 4.1cm wide neutral zone in the middle of the tray and a screen lying 2cm above it, on which a 15.5cm x 25.2cm pup corral was placed to contain the pup during the test. Upon testing, a single pup (whose experimental condition was unknown to the experimenter), was removed from the home cage, transported to the behavior room, and immediately placed in the neutral zone. For five one-minute trials, the time the pup spent over the scented and unscented side of the container was measured and recorded. During the 30-second inter-trial interval, pups were gently held in the experimenters' gloved hand to maintain body temperature. Pups were placed in the neutral zone in an alternating direction each trial to account for the possibility of lateralization. After the five trials were completed, the time spent over unscented and scented bedding was totaled and comparisons were made between pups of different conditions in order to detect whether odor preference learning occurred within the litter

2.5 Biochemical Assays

Thirty minutes post-conditioning, PN6-8 rats that were designated for *bdnf* gene expression analysis were removed from their home cages with minimal disturbance and then sacrificed for brain extractions. Olfactory bulbs were obtained and placed in a tube on dry ice to later be stored at -80°C to await further processing.

Olfactory bulbs were homogenized and RNA was extracted using an Allprep DNA/RNA kit (Qiagen Inc., Valencia, CA). Quantity and quality of purified RNA samples were analyzed with a NanoDrop Spectrophotometer (2000).

RNA was then reverse-transcribed using a cDNA synthesis kit (Qiagen) and then a real-time PCR system (Bio-Rad CFX96) was used for amplification and a taqman probe was used to target *bdnf IV* mRNA or a reference gene (*tubulin*). We chose to study *bdnf* IV because expression of this transcript is activity-dependent and sensitive to environmental stimuli (Tao et al. 1998; Zheng et al. 2011; Zheng and Wang 2009). The comparative C_t method was used to quantify the relative fold change in gene expression of paired and unpaired animals versus odor-only and stroke-only controls (Livak and Schmittgen 2001).

2.6 Statistical Analysis

Behavioral data were analyzed using a one-way ANOVA and unpaired t-tests. Gene expression data were analyzed using one-sample t-tests for comparison with collapsed odor- and stroke-only controls (with a mean value of 1 representing no changes in expression when compared to controls).

Chapter 3

RESULTS

3.1 Odor Preference Learning Behavior

A two-way ANOVA (levels: training condition and sex) revealed a main effect of training condition [F(2,15)=6.61, p<0.01], but no main effect of sex [(F(1,15)=0.00, p= 0.9857]. Males and females' behavioral data were thus collapsed into one graph (Figure 1). A one-way ANOVA revealed that PN7-9 pups in the paired condition, which were predicted to have learned an association between peppermint odor and stroking, spent significantly more time over peppermint-scented bedding than did the non-learning unpaired, stroke-only, and odor-only pups when tested 24 hours postconditioning (Figure 1, [F(2,18)= 7.449, p<0.01 paired vs. unpaired, p<0.05 paired vs. odor/stroke only)]. There was no significant difference in time spent over peppermintscented bedding of stroke-only and odor-only and unpaired pups (Figure 1).



Odor Preference Learning

Figure 1. Time spent (sec) over peppermint-scented bedding during the two-odor choice test of PN7-9 pups 24 hours post-conditioning. Results indicate that paired pups spent significantly more time over peppermint-scented bedding than did stroke-only and odor-only control (*p<0.05) and unpaired pups (p**<0.01). Time spent over peppermint-scented bedding was not significantly different between odor/stroke-only control and unpaired pups (p>0.05). n=6-8/group; subjects derived from 4 litters; error bars =SEM.

3.2 Bdnf IV Gene Expression

Gene expression of *bdnf IV* was quantified in PN6-8 olfactory bulb tissue 30 minutes post-conditioning. Relative *bdnf* mRNA levels in paired and unpaired pups

were compared to mRNA levels of stroke-only and odor-only controls (which are represented by the dotted line at 1 in the Figure 2). A one sample t-test revealed a significantly higher level of *bdnf IV* mRNA in the olfactory bulbs of paired pups compared to stroke-only and odor-only pups (Figure 2, t_{11} = 2.999, p<0.05). *Bdnf IV* mRNA levels were not significantly different among unpaired pups and stroke-only and odor-only controls (Figure 2, t_{10} =1.2, p=0.2576). An unpaired t-test revealed that although *bdnf IV* mRNA was higher in paired pups, levels were not quite significantly different from unpaired pups (t_{21} =1.903, p=0.0708).



Figure 2. Fold change of *bdnf* IV mRNA in the olfactory bulbs of PN6-8 pups 30 minutes post-conditioning. Paired and unpaired mRNA levels were compared to stroke-only and odor-only controls. A fold change of 1.0 represents the mRNA level of the stroke-only and odor-only controls. Paired pups had significantly more *bdnf* IV mRNA in their olfactory bulbs than the stroke-only and odor-only controls (p*<0.05). Unpaired pups' *bdnf* IV mRNA levels were not significantly different from stroke-only and odor-only controls (p=0.2576). *Bdnf* IV mRNA levels were higher in paired pups but were not significantly different from levels in unpaired pups (p=0.0708). n=11-12/group; subjects derived from 6 litters; error bars = SEM.

Chapter 4

DISCUSSION

4.1 Twenty-four-hour Odor Preference Learning is Significantly Increased in Paired Pups Compared to Non-Learning Controls

This study aimed to establish a conditioning protocol and investigate the link between attachment-based olfactory learning and *bdnf IV* gene expression in the olfactory bulb during the sensitive period of infant rats. This was assessed in PN6-8 pups of both sexes using a within-litter model that randomly assigned neonates to one of four 20 min training conditions. Infant rats experienced one of the following conditions: paired odor and stroke, unpaired odor and stroke, stroke-only, or odoronly. Use of these four conditions allowed for confirmation that only the paired odor and stroke pups learned a long-term odor preference (24 hours later) in a 2-odor choice test. The mere presentation of either stroking or odor, or the unpaired exposure to both of these stimuli, was insufficient to produce a significant preference for peppermint odor 24 hours post-conditioning. Contiguous presentation of the stimuli was required to produce a learned odor preference.

Because peppermint odor is naturally aversive to neonates, paired pups' 24hour preference for peppermint odor indicates that learning occurred as opposed to the expression of an innate/unlearned odor preference (Amiri et al. 1998). This study confirms previous findings that stroking when paired with an odor elicits a long-term odor preference in PN6-8 pups (Sullivan, Hofer, and Brake 1986). Throughout the training sessions pups exhibited robust leg movements, rolled over, and crawled

throughout the container. These behavioral results, showing that paired pups spent the most time over scented bedding (compared to unpaired, and stroke-only and odor-only controls), are concordant with other studies demonstrating that simultaneous pairing of an odor with a behaviorally-activating stimulus produces a learned odor preference in sensitive period pups (Sullivan, Hofer, and Brake 1986). This experiment also replicates previous research indicating that male and female neonates equally form learned odor preferences (Roth and Sullivan 2006; Sullivan 2005).

While other researchers successfully produced 24-hour odor preferences in sensitive period neonates using 10 minute odor-stroke training sessions (Morrison et al. 2013; Fontaine, Harley, and Yuan 2013), we were unable to replicate this and instead here employed 20-minute sessions to elicit more robust learning as gauged by the 2-odor choice test. In the creation of the protocol for this study, other modifications to the paradigm that promoted better learning in infant rats included the use of two types of bedding – one for the home cage (beta-chip) and one for conditioning/testing (aspen). The home cage with the mother was kept in a separate room from the conditioning/testing room, and the stroking of the rats was limited to quick, circular motions at the hind region of the pup. This targeted stroking simulates the nurturing anal licking that pups experience in the home cage (Yuan, Shakhawat, and Harley 2014).

4.2 *Bdnf IV* Gene Expression in the Infant Rat Olfactory Bulb is Increased Following Olfactory Associative Conditioning

To quantify the effects of odor-preference learning on *bdnf IV* gene expression levels 30 minutes post-conditioning, biochemical assays were performed and revealed that *bdnf IV* mRNA levels were significantly increased in the olfactory bulbs of paired pups, which should have learned an attachment-based odor preference. There was no main effect of sex on *bdnf IV* gene expression, which coincides with previous research demonstrating the lack of effect of sex on olfactory learning and 24-hour odor preference memory (Roth and Sullivan 2006; Sullivan 2005).

One strength of this study is the use of not only odor-only and stroke-only controls, but also an unpaired condition to confirm that *bdnf IV* gene expression profiles of all three types of non-learning controls differed from paired pups, even when both stroking and odor stimuli are presented. Although the difference in *bdnf IV* mRNA levels between paired and unpaired pups were not quite significant (Figure 2, p= 0.0708), *bdnf IV* mRNA levels were higher in paired pups. Adding additional litters to increase sample sizes will likely flush out significance between these groups. Other studies have shown that mere exposure to odor does increase BDNF in the olfactory bulb compared to pups whose nares were occluded entirely (McLean, Darby-King, and Bonnell 2001). Although the nares of stroke-only pups used in this study were not occluded, it may be expected that the unpaired and odor-only pups may have slightly higher *bdnf IV* gene expression because of the exposure to a novel olfactory stimulus and engagement of the olfactory bulb that stroke-only pups lacked. These data support other studies' findings that BNDF is up regulated in the OB following experiential learning (Zimmerberg, Foote, and Van Kempen 2009).

4.3 *Bdnf IV* Gene Expression May Have Implications for Odor Preference Behaviors and Memory

BDNF is critically important to long-term learning and memory (Jones et al. 2007) through its promotion of cell differentiation (McLean, Darby-King, and Bonnell 2001), neural growth, cell survival, and neural plasticity due to learned events

(Zimmerberg, Foote, and Van Kempen 2009). The up regulation of *bdnf IV* mRNA in only pups that learned an attachment-based olfactory association in this study suggests that *bdnf IV* mRNA plays a role in up regulation of BDNF protein in mitral cells which then may have important downstream intracellular effects supporting long-term learning and memory (Jones et al. 2007). BDNF may be responsible for neurogenesis of interneurons responsible for discrimination of peppermint odor in these pups' futures (Yuan 2008). As BDNF receptor subtypes are prevalent in the subventricular zone, the site of neurogenesis in the olfactory system (Bath et al. 2008), increased BDNF as a result of epigenetic modulation of the *bdnf* gene following odor-stroke conditioning may stimulate increased neurogenesis. Up regulated BDNF may also be transported to other brain regions to mediate formation and storage of long-term odor memory (Zimmerberg, Foote, and Van Kempen 2009).

4.4 Future Directions to Investigate Epigenetics and Early Odor Preference Learning

There are many ways in which the findings from this experiment may be expanded to investigate the influence of epigenetics on maternal attachment-based olfactory learning. Throughout this study, OBs were obtained 30 minutes post-conditioning because cAMP and activated PKA levels peak 10 minutes post-olfactory conditioning. PKA can then translocate to the nucleus and phosphorylate CREB (Yuan, Shakhawat, and Harley 2014), which then triggers transcription of *bdnf* (Raineki et al. 2010). Longer time points after olfactory conditioning for analysis of *bdnf* mRNA, for example 2 hours or 24 hours, may reveal more drastic changes between pups in the paired and unpaired condition as increased cAMP and CREB levels may yield more *bdnf IV* in paired pups. It would also be fascinating to compare

the *bdnf IV* level 24 hours post-conditioning with littermates' behaviorally tested 24 hours post-conditioning to evaluate the effect of *bdnf IV* mRNA level on odor preference behavior at that time.

Another future direction may be to extract a more specific subset of cells from the OB. While *bdnf* transcription has been shown to increase specifically in the mitral cells of the bulb (Zimmerberg, Foote, and Van Kampen 2009), it is possible that transcription is suppressed following olfactory learning in other cell types. Because this study used the entire OBs, cell type-specific changes in gene expression may have been obscured. In addition to more specific subsets of OB cells, different brain regions crucial to sensitive period olfactory learning would also be fascinating to investigate, including the olfactory sensory neurons of the olfactory epithelium, locus coeruleus, and anterior piriform cortex. Because noradrenergic agonism of the anterior piriform cortex has been shown to be sufficient for odor preference learning, this cortical structure would be interesting to analyze in an odor-stroke paradigm since its storage of olfactory memory does not necessarily rely on the olfactory bulb (Morrison et al. 2013).

Just as distinct odors activate specific families of OSNs and glomeruli (Whitman and Greer 2009), as well as distinct patterns of 2-DG uptake within the OB (Johnson and Leon 1996), it would be interesting to add an additional odor to this study. A comparison between the *bdnf IV* mRNA level following training with peppermint odor and another odor would be fascinating to see if the specific quality of the odor yields a specific transcriptional level outcome. Likewise, substitution of the unconditioned stimulus of stroking with another appetitive unconditioned stimulus like milk infusion or opioid injection may yield unique gene expression profiles. It

may also be helpful to investigate gene expression profiles of aversive unconditioned stimuli like mild foot shock to see if there is a difference in transcriptional level of *bdnf IV* despite similar behavioral responses.

Another efficient way this experiment may be performed involves the use of naris occlusion (McLean, Darby-King, and Bonnell 2001; Fontaine, Harley, and Yuan 2013). Because prior to PN11 the infant rat olfactory system is lateralized, if one naris is occluded during conditioning then the pup will lack access to its olfactory bulb and anterior piriform cortex on its occluded side. The pup may then be used as its own control for *bdnf IV* gene expression, as one OB will have had an olfactory experience (that may have resulted in long-term learning) and the other OB will remain inactive for the ~20 minute training period. This within-subject model for comparison may be more accurate than the within-litter design because animals may have different basal levels of OB *bdnf* mRNA based on genetic reasons or other confounding factors (van Otterdijk and Michels 2016).

In addition to *bdnf IV*, other immediate early genes may also be useful to investigate and elucidate the key molecular changes in the OB that constitute a learned maternal attachment. While *bdnf* is an immediate early gene, having a direct effect on synaptic plasticity, genes like *c-fos* and *zif268* are other immediate early genes that may modulate transcription of other effector genes, which then cause synaptic changes. It would be helpful to observe the changes in gene expression of regulatory *c-fos* or *zif268* in the OB following olfactory conditioning to examine their relationships with the expression of *bdnf IV*. Other immediate early genes worth examining in the context of odor-stroke learning are *arg3.1* and *Homer* (Davis, Bozon, and Laroche 2003) known for their roles in activity-dependent learning and memory

(Lyford et al. 1995), and localization of specific target proteins in the synapse that lead to long-term potentiation (Shiraishi-Yamaguchi and Furuichi 2007), respectively. Analyzing the gene expression response of a wider variety of immediate and effector early genes will broaden understanding of the molecular changes that lead to neuronal plasticity and ultimately, long-term attachment-based olfactory memory.

Regardless of the gene in question, the next step in this line of research is to investigate DNA modifications at different time points (including the current time point) as a potential mechanism for observed changes in gene expression following learning. Presumably with the observed increase in *bdnf* mRNA in the paired pups we would expect to see less DNA methylation in that group. Although attachment of a methyl group to the 5th atom of the cytosine ring is typically associated with decreased transcription of that gene (Blaze, Scheuing, and Roth 2013), studies have shown atypical relationships between methylation and gene expression in which gene expression increases with increased methylation of a gene promoter (Costello et al. 1994). Understanding the relationship between methylation of the *bdnf IV* promoter or global methylation of bdnf following olfactory training and its correlation to gene expression changes may help pinpoint a potential mechanism for observed molecular changes in the OB. Analysis of hydroxymethylation levels in promoter regions of bdnf may also provide insight about a mechanism for these observed transcriptional changes, as hydroxymethylation is believed to be an intermediate for DNA demethylation and has unique effects on gene transcription (Guibert and Weber 2013).

4.5 Conclusion

Based on the evidence provided from this study, it is clear that sensitive period neonatal rats are capable of demonstrating an attachment-based preference for a novel

peppermint odor paired with the unconditioned stimulus of stroking when tested 24 hours post-conditioning. Analysis of *bdnf IV* gene expression suggests that levels of *bdnf IV* mRNA are upregulated 30 minutes following olfactory training in pups that learned an association between peppermint odor and the appetitive stimulus of stroking. Further research with this model and in humans will advance our knowledge of the effects of early attachment learning on gene regulation and behavioral outcomes. As the link between maternal attachment-based learning and the trajectories of neurological and behavioral health have been long established in psychiatry and developmental psychology, this research could help establish new guidelines for therapies for children whose early attachment learning has been compromised or struggle with learning disabilities.

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