Maternal Buffering of Infant's Fear
In Typically Developing Rats and In Rat Models for Psychiatric Disorders

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2021

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Dedication

I would like to dedicate this dissertation to Dr. Victoria Braithwaite, my undergraduate honors thesis advisor at Penn State University, who left this world far too soon. In addition to teaching me to be a more critical thinker, to approach the world with steadfast optimism and kindness, and to always consider the welfare of experimental animals, you were the mentor I needed at a time when I might have walked away from science. I will always strive to carry on your legacy.

Acknowledgements

This work on the social regulation of threat learning in infancy would not be possible without the social and technical support I have received over the past six years from my colleagues at the University of Michigan, friends, and family. I would first and foremost like to thank my PhD advisor, Dr. Jacek Debiec, for giving me the opportunity to study these exciting questions with him, allowing me the freedom to develop and try out new ideas, and helping me grow as an independent scientist. I would also like to especially thank the other members of my committee, Dr. Huda Akil, Dr. Audrey Seasholtz, Dr. Natalie Tronson, and Dr. Alexa Veenema. In addition to providing their insightful advice on my work, they have been excellent advocates for me and my training. I would like to thank the staff of the Neuroscience Graduate Program, especially Valerie Smith, who has always gone above and beyond to help me during my PhD.

In the lab, I have had the pleasure to work alongside a number of talented and dedicated undergraduate students. I would like to thank Freddie Delarosa, Esha Kaul, Robert Maurer, Vartika Parashar, Rachel Ritter, Emma Vasquez, and Mary Zylinski for their technical assistance and for always helping to remind me why I love science. I would also especially like to thank Joanna Hider, who trained me when I started in the Debiec Lab and has been an incredible source of support both inside and outside the lab. I would like to thank Dr. Elaine Hebda-Bauer and Alex Stefanov for their technical assistance on the bred Low-Responder project and Jennifer Fitzpatrick for having answers for almost any question I have ever had about wet lab techniques and for always letting me know when there is a fresh pot of coffee brewing. I would also like to thank past and present members of the Debiec Lab Xianli An, Julie Boulanger-Bertolus, Da-

Jeong Chang, Alana Duran, Dan Epstein, Nathan Horrell, Brad Savard, Delawrence Sykes for their technical assistance, career advice, and camaraderie. For their advice on the graph theoretical approach, I would like to thank Tony Larkin, Ishtiaq Mawla, and Dr. Michal Zochowski. Beyond the University of Michigan, I would like to thank Dr. Regina Sullivan and Dr. Maya Opendak for offering their expertise on infant rat behavior and neurobiology and their mentorship.

Finally, I would like to thank my parents, Dr. Jim White and Sheryl White, and my brother Jim White for instilling a love for science in me at an early age and encouraging it at every turn. I would like to thank the members of my cohort in the Neuroscience Graduate Program, Alex Bova, Alex Chen, James Delorme, Rob Doherty, Lucas Huffman, Krista Kernodle, Tony Larkin, Sofia Lopez, and Sijin Ren and the members of the Diversity Equity and Inclusion Task Force, Filipe Cerqueira, Alex Ethridge, Jacqueline Forson, Lindy Jensen, Jess Maung, Mads Shay, and Christa Ventresca for their support and some of the best times I have ever had. I would like to thank Meredith Collier, Lorraine Horwitz, Lindy Jensen, Dalia Murra, and Dr. Maya Opendak for being incredible friends and encouraging and inspiring me especially during the last days of my PhD. Last but not least, I would like to thank Rob Doherty for giving me a home away from home (along with Kourtney Doherty, Jack Doherty, Bowie, and Rupert), for career advice, for late night conversations, air hockey, and Ticket to Ride, and for being one of the best friends I have ever had.

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Abstract

Social buffering is broadly defined as an individual's ability to suppress the physiological, behavioral, and/or emotional consequences of adverse events in another individual. A particularly potent and vital form of social buffering is caregiver buffering, which protects the developing infant brain from the deleterious effects of stress. Rodent work has provided valuable information about the behavioral, endocrine, and neurobiological mechanisms of maternal regulation of threat and how those mechanisms may be altered by early life experiences. The ability of infant rats to acquire Pavlovian odor-shock associations generally emerges when they are about ten days old, but is under tight regulation by the mother. Previous studies have shown that when infant rats underwent an odor-shock Pavlovian threat learning experience in the presence of an anesthetized mother, they did not avoid the conditioned odor when tested, unlike pups conditioned without maternal presence. This behavioral effect was, in part, mediated by the mother's ability to suppress the infant rat's stress response and amygdala reactivity during the threat learning experience. Follow-up studies have shown that disruption of the relationship between mother and infant can affect the ability of the mother to regulate fear in her infants. In this thesis, I address several remaining questions about the functions and underlying mechanisms of maternal buffering in infant rats. In Chapter 1, I briefly review the existing human and rat literature on caregiver regulation of stress, threat learning, and neural activity and review the trajectory of infant rat development. In Chapter 2, I demonstrate that the effect of maternal presence during a threat learning experience can be observed using a robustly studied defense response – threat conditioned-induced freezing – and that female infant rats may

be more susceptible to maternal buffering of freezing than male infant rats. In Chapter 3, I examine the functional networks engaged by infant rats conditioned with and without maternal presence and apply graph theory to analyze patterns of immediate early gene expression. Overall functional connectivity was significantly increased in pups conditioned with maternal presence. A graph theoretical analysis revealed that the network engaged by pups conditioned with maternal presence was more integrated and lacked distinctive hubs; in contrast, the network engaged by pups conditioned without maternal presence was more segregated and had distinct hubs: the lateral amygdaloid nucleus, dorsolateral part, basolateral amygdaloid nucleus, anterior part, and medial amygdaloid nucleus, anterior dorsal part. In Chapter 4, I examine the ontogeny of threat learning and stress responsivity in a vulnerable phenotype prone to high-anxiety like behavior in adulthood, and then question whether maternal presence and fibroblast growth factor 2 are capable of regulating threat learning in these animals. In Chapter 5, I summarize results from each chapter and discuss future directions. The findings outlined in this thesis provide an important next step in characterizing maternal buffering and illuminate exciting topics for future inquiry.

Chapter 1 Introduction

Social cues play a powerful role in the regulation of an individual's emotions. One way in which this is accomplished is via an effect termed "social buffering." Social buffering is a well-conserved effect in social species and has been documented in zebrafish (Faustino et al., 2017), zebra finches (Emmerson & Spencer, 2017), prairie voles (Burkett et al., 2016; Smith & Wang, 2014), rats (Davitz & Mason, 1955), harbor seals (Di Poi et al., 2015), piglets (Kanitz et al., 2016), squirrel monkeys (Coe et al., 1978), rhesus monkeys (Hill et al., 1973), chimpanzees (Wittig et al., 2016), and humans (Coan et al., 2006). Social buffering may consist of different effects in different contexts; however, it can be broadly defined as an individual's ability to suppress the physiological, behavioral, and/or emotional consequences of adverse events in another individual (Gunnar et al., 2015; Gunnar & Hostinar, 2015; Gunnar & Sullivan, 2017; Hennessy et al., 2009).

Although the ability to learn fear associations is adaptive, social regulation of fear is also adaptive. Unregulated excessive fear can have maladaptive consequences, including clinical anxiety (Ressler, 2020). Many labs have studied social buffering in the context of fear, while others have studied social buffering in the context of stress alone. As fear states are accompanied by stress states, there are likely many overlapping mechanisms.

1.1 Caregiver Buffering of Stress and Fear in Humans

A particularly potent form of social buffering is caregiver buffering. Like all animals of altricial species, when infant humans are born, their physiological and emotional needs must be fulfilled by their caregivers. Parental regulation of their child's emotions exhibits long-lasting

influences on their child's neural processing of emotional stimuli (Kopala-Sibley et al., 2020) and interacts with the child's own temperament to influence how the child will respond to future stressful situations (Kiff et al., 2011). Therefore, understanding how parental buffering of stress and fear functions is critical.

In the acute laboratory setting, the presence of a parent can buffer a child's cortisol response to socially stressful events (Hostinar et al., 2015; Nachmias et al., 1996) and facilitate a child's affective regulation during an emotional go/no-go task (Gee et al., 2014). Maternal cues also have been shown to regulate neuronal activity in their children; specifically, viewing images of the mother suppressed amygdala activity (Gee et al., 2014).

Studies of children who experienced chronic stressors during early life have also shown how parental figures can play a critical role in regulating the effects of stress on their children. Children who grew up in a socioeconomically impoverished neighborhood have altered brain development, including greater left and right amygdalar volumes as adolescents (Whittle et al., 2017). Among these adolescents growing up in socioeconomically disadvantaged neighborhoods, positive maternal parenting behavior ameliorated this effect (Whittle et al., 2017). In Rwandan orphans, perceived social support from adults (and peers) was associated with less emotional distress and greater emotional well-being (Caserta et al., 2017). In economically disadvantaged children that had experienced early life trauma, maternal presence improved children's discrimination between a cue predictive of an aversive stimulus and a similar cue predictive of safety during a fear potentiated startle task (van Rooij et al., 2017).

Any disruption to the attachment between the infant and caregiver, therefore, may lead to reduced efficacy of parental buffering of stress and/or fear. Indeed, there are several pieces of evidence that suggest that this is the case. There have been many studies examining outcomes in

children who grew up in institutional settings, who have experienced the trauma of separation from the parents and may not have a caregiver in the institutional setting to provide parental buffering. Indeed, very young children who grew up in institutional settings are less likely to be securely attached, display more emotional inhibition (Zeanah et al., 2005), show less positive affect (Smyke et al., 2007), and have higher rates of internalizing and externalizing psychiatric disorders (Zeanah et al., 2009) than children who grew up in community settings. These behavioral outcomes are accompanied by accelerated maturation of connectivity between the amygdala and prefrontal cortex (Gee et al., 2013). Placement in a foster care setting after a period of institutionalization ameliorated many of the negative outcomes associated with institutionalization (Bos et al., 2011). However, the lack of parental buffering at that early life critical period may still have long-lasting consequences on buffering later in life. Individuals in early adolescence who spent their first few years of life in an institutional setting but were adopted into a family by age 5 did not experience parental buffering of cortisol response to a laboratory stressor (Hostinar et al., 2015).

In summary, caregiver buffering is critically important in human children and has long-term consequences for emotional regulation, development of neural circuitry, and risk for neuropsychiatric disorders. Studies of laboratory rodents have built on these findings in humans to allow for a deeper investigation of the behavioral consequences and neuroendocrine and neurobiological mechanisms of social buffering.

1.2 Social Buffering in Adult Rodents

Most of what is known about social buffering in rodents comes from studies of juvenile and adult males. These studies have often used Pavlovian fear conditioning, which consists of repeatedly pairing a neutral conditioned stimulus (CS), such as a tone, light, or odor, with an

aversive unconditioned stimulus (US), such as a mild shock to the footpads or tail (Maren, 2001b). The aversive stimulus causes an unconditioned response (UR) which, may be physiological (e.g., changes in heart rate) and/or behavioral (e.g., freezing, avoidance) (Maren, 2001b). After repeated pairings of the CS with the US, the animal can learn the association between the CS and the US and will begin to show defensive behavior in response to the CS alone; this is termed a conditioned response (CR) (Maren, 2001b). Although Pavlovian fear conditioning has been referred to as such for many years, there has been a recent movement to use the term "threat" instead of "fear." Fear implies the existence of a conscious emotional state, which is difficult to infer from animal studies and may rely on different circuits than those required for rapid defensive responses. Referring instead to an animal's ability to detect and respond to threatening stimuli more precisely describes what is occurring when an animal is exposed to a CS previously paired with an aversive outcome (LeDoux, 2014). Therefore, throughout the rest of this thesis I will be using the terminology "threat conditioning" and "threat responses."

In many experiments, rats underwent threat conditioning in isolation and were tested the next day in the presence or absence of a conspecific. Male and female rats showed reduced threat conditioned freezing behavior when exposed to the CS with a conspecific present (Fuzzo et al., 2015; Ishii et al., 2016; Kiyokawa & Takeuchi, 2017). Male rats also showed decreased hypothalamic-pituitary-adrenal (HPA) axis activation in response to CS presentation when accompanied by a conspecific (Kiyokawa, Hiroshima, et al., 2014). The potency of the social buffering effect could be modified by the stress state of the individual acting as the social buffer (Kiyokawa et al., 2004) and by whether the threat-conditioned individual is familiar with the individual acting as the "social buffer" (Kiyokawa et al., 2007; Kiyokawa, Honda, et al., 2014).

Investigations into the neural mechanisms of social buffering in adult rodents suggest that regions critical for threat memory formation and expression, initiation of the stress response, and olfactory processing play key roles. Social buffering of conditioned threat responses is associated with reduced c-Fos expression in the lateral amygdala (Kiyokawa et al., 2007; Kiyokawa, Honda, et al., 2014; Y. Takahashi et al., 2013) and the paraventricular nucleus of the hypothalamus (PVN) (Kiyokawa et al., 2004, 2009; Y. Takahashi et al., 2013). Reduced amplitude of CS-evoked field potentials in the lateral amygdala during social buffering has also been observed (Fuzzo et al., 2015). Sensory information about the conspecific travels to the amygdala nuclei via the main olfactory system (Kiyokawa et al., 2009, 2012; Y. Takahashi et al., 2013).

1.3 Infant Rat Development

Relatively less is known about the behavioral consequences and underlying mechanisms of social buffering of stress and threat in infant rats. There may be similar mechanisms as those seen in adult rodents but infancy is a time in which the HPA axis and the neural systems underlying threat learning are developing, so there may be some critical differences. The infant rat is very susceptible to environmental factors and adversity which may have long-lasting consequences (Lupien et al., 2009; McEwen, 2008; Sapolsky & Meaney, 1986). In humans early life adversity is associated with higher rates of neuropsychiatric disorders (Carr et al., 2013; Devi et al., 2019) and furthermore, many neuropsychiatric disorders have a developmental origin (Muris et al., 2011; Zahn-Waxler et al., 2000). A deeper understanding of maternal buffering of threat and stress, therefore, is essential. To fully appreciate this phenomenon, a summary of the unique features of infant rat development is necessary.

1.3.1 POSTNATAL DAY 0 THROUGH POSTNATAL DAY 10

Rodents, like humans, are an altricial species. When infant rats are born, their eyes and ears are closed, they lack the ability to regulate their own body temperature, and they are not yet capable of seeking out food on their own (Bolles & Woods, 1964). Therefore, infant rats rely on maternal care for survival in the first 3 weeks of life. Specifically, they rely on the mother to regulate their body temperature by covering her pups and building a nest for them and they rely on the mother to provide food for them through nursing. Pups also rely on the mother for safety and protection from predators.

In accordance with this necessity, during the first week of life infants must learn to initiate and maintain a bond with their caregiver. This is a form of learning called attachment learning. During the process of attachment learning, infants learn that specific cues, especially olfactory cues, are associated with the parent (Debiec & Sullivan, 2017b). In humans, and perhaps other species, the process of attachment learning begins in utero, as the olfactory system of the developing fetus is exposed to the odors of the amniotic fluid which are also later found in the mother's milk (Varendi et al., 1996). Indeed, rat pups in the first week of life will readily learn an association between a novel odor and maternal odor or simulated maternal contact (Regina M. Sullivan et al., 1986).

These attachment cues have critical ecological importance. If separated from the nest and/or the mother, the pup will use those attachment cues to orient towards them and approach (Bolles & Woods, 1964). The pup will also crawl or walk towards olfactory cues associated with the nest and/or the mother (Mendez-Gallardo & Robinson, 2014). Following separation from the mother, pups will emit frequent ultrasonic vocalizations (Hofer & Shair, 1978) which will elicit maternal contact (Brunelli et al., 1994). These maternal attachment cues are so powerful that

they can even ameliorate depressive-like behavior and altered amygdala functioning later in life (Sevelinges et al., 2011).

From approximately postnatal day 3 through postnatal day 10, the infant stress response system is generally suppressed; thus this period of development has been called the "stress hyporesponsive period." Baseline plasma corticosterone levels are generally very low (Henning, 1978) and if the infant rat experiences a painful stimulus during the first week of life, its corticosterone response will be minimal or absent (Schapiro et al., 1962; C. D. Walker et al., 1986, 2003; C. D. Walker & Scribner, 1991). Following extended separation from the mother, the infant rat may show a more mature corticosterone response (Ladd et al., 1996; Suchecki et al., 1993; C. D. Walker, 1995; C. D. Walker & Scribner, 1991). For these reasons, the mother has been hypothesized to be a primary regulator of the infant HPA axis. Indeed, direct maternal care as well as nutrition and tactile cues associated with the mother can suppress corticosterone release (Levine, 2001). The effects of maternal care on the HPA axis even extend into adulthood (Champagne et al., 2003).

1.3.2 POSTNATAL DAY 10 THROUGH POSTNATAL DAY 15

Once rats enter the second week of life, they begin to walk and venture outside of the nest (Bolles & Woods, 1964), although the eyes do not open until about postnatal day 14 (Bolles & Woods, 1964). Accordingly, defensive behaviors begin to emerge at this age. Pups will show a startle response following loud sounds (Bolles & Woods, 1964) around postnatal day 13. A hallmark behavioral defensive response – freezing, or total body immobility except for respiration (Fanselow, 1980) – also emerges at this age. Pups will begin to freeze when exposed to a natural threat (an adult male rat) (L. K. Takahashi, 1992, 1994; Wiedenmayer, Lyo, et al., 2003; Wiedenmayer & Barr, 1998), in contrast to the first week of life (L. K. Takahashi, 1992).

Adrenalectomy blocks this developmental onset of the predator-induced freezing response (Moriceau et al., 2004b; L. K. Takahashi, 1994), but this effect can be reversed by supplemental injections of corticosterone (L. K. Takahashi, 1994). Furthermore, precocious emergence of predator cue-induced freezing behavior can be elicited by exogenous administration of corticosterone (Moriceau et al., 2004b). Taken together, these findings suggest that changes in the HPA axis are critical for the onset of predator-induced freezing behavior.

Indeed, the infant stress response system undergoes significant changes during the second week of life. If the infant undergoes a stressful or painful experience in the absence of the mother, it begins to show a robust corticosterone response. Twelve-day old rat pups will begin to show increased corticosterone levels when exposed to predator odor (Moriceau et al., 2004b) and following repeated mild shocks (Moriceau & Sullivan, 2006). However, the infant's stress response axis is still regulated by maternal presence and by cues associated with the mother (Moriceau & Sullivan, 2006; Shionoya et al., 2007; Stanton et al., 1987; Stanton & Levine, 1990; Suchecki et al., 1993; Wiedenmayer, Magarinos, et al., 2003)

Significant neurodevelopmental events are also still occurring between postnatal 10 and 15. The brain continues to undergo drastic changes in patterns of gene expression, including those that differentiate one brain region from the other, e.g., cortex from the hypothalamus (J. D. H. Stead et al., 2006). The amygdala, PVN, and periaqueductal gray will begin to activate in response to predator exposure (Wiedenmayer & Barr, 2001) and the PVN will begin to activate in response to shock (Shionoya et al., 2007). At the level of neural networks, functional magnetic resonance imaging of two-week old rat pups suggests that some of the resting state functional networks present in adult rats are present at this developmental time stage (e.g., autonomic networks comprised of the hypothalamus, thalamic nuclei, and hippocampus) (Bajic et al., 2016).

However, other functional networks, while in part resembling the adult form, do not appear to be fully mature at this age (Bajic et al., 2016).

1.3.3 POSTNATAL DAY 16 THROUGH POSTNATAL DAY 21

At the third week of life, rat pups are highly active (Bolles & Woods, 1964), begin eating rat chow (Bolles & Woods, 1964) and are nursing less frequently (Bolles & Woods, 1964). However, pups are still not fully independent and still rely on their mother to filter their experiences with the environment. In the laboratory setting, pups are typically weaned abruptly at postnatal day 21 and subsequently housed with same sex siblings. In the wild, weaning likely begins in the third postnatal week and continues gradually over the next weeks as pups continue to eat solid food more frequently and nurse less frequently (Henning, 1981; Martin, 1984; Schweinfurth, 2020).

1.4 Infant Rat Emergence of Threat Conditioning and Maternal Regulation of Threat Conditioning

The rat's capability to learn associations between neutral stimuli and aversive stimuli develops throughout infancy. Whether learning occurs, and/or whether learning is observed, depends on the nature of the CS and US and on what behavior is used as an indicator of threat.

1.4.1 POSTNATAL DAY 0 THROUGH POSTNATAL DAY 10

Aversive Pavlovian conditioning has been observed as early as the first days of life. Caldwell and Werboff demonstrated that 1-8 hour old rat pups were capable of learning an association between a vibration applied to the chest and a 1.0 mA shock to the foreleg following 80 vibration-shock pairings (Caldwell & Werboff, 1962). Here, the CR was indicated by leg flexion in response to vibration (Caldwell & Werboff, 1962). Postnatal day 2 pups that received

a pairing of lemon odor with malaise induced by lithium chloride injection will avoid the lemon odor when tested on postnatal day 8 (Rudy & Cheatle, 1977).

Quite different patterns of results have been observed by investigators using a Pavlovian threat conditioning protocol that pairs a novel odor with shock. In the first week of life, infant rats trained on such a protocol will develop a preference for the conditioned odor (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Regina M Sullivan et al., 2000). The attraction that pups will show to an odor paired with a mild shock in early infancy is not due to the infant's inability to experience pain or due to a heightened pain threshold (G A Barr, 1995; Fitzgerald, 2005). Instead, it is thought to be due to the pup's stress hyporesponsive state at this developmental timepoint. Rat pups younger than postnatal day 10 can learn to avoid a novel odor paired with shock if they receive a systemic injection of corticosterone (Moriceau et al., 2004b; Moriceau & Sullivan, 2004). The suppression of threat learning by the mother at this developmental stage is thought to be adaptive. Even if the mother causes the infant pain (e.g., through rough handling), the infant should not learn to avoid the mother because it will not survive without her care (Perry & Sullivan, 2014). The learning that needs to be prioritized at this age is attachment learning, rather than threat learning. Accordingly, any sensory stimuli that the infant is exposed to at this age activate the locus coeruleus (Nakamura et al., 1987) which facilitates the continued process of attachment learning.

1.4.2 POSTNATAL DAY 10 THROUGH POSTNATAL DAY 15

Once infant rats reach 10-12 days of age, they can learn to avoid a novel odor paired with shock if their mother is not present during the threat learning experience (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Kucharski & Spear, 1984; Regina M Sullivan et al., 2000). Pups are also capable of learning an association between an auditory cue and footshock at postnatal

day 15, as they will suppress their activity when presented with the tone (Campbell & Ampuero, 1985; Moye & Rudy, 1985). The ability of rat pups to learn threat associations at this stage is supported by increased corticosterone release in response to shock (Moriceau & Sullivan, 2006; Shionoya et al., 2007) and the amygdala becoming functionally mature enough for induction of threat learning-induced plasticity (Thompson et al., 2008).

However, if a calm, anesthetized mother was present during an odor-shock threat conditioning experience, she is capable of buffering threat learning in her pups. Specifically, pups will not show avoidance of the peppermint odor conditioned stimulus in the y-maze test (Moriceau & Sullivan, 2006; Opendak et al., 2019; Shionoya et al., 2007). The mother is thought to block threat learning in her pups via several mechanisms. Maternal presence during threat conditioning is able to suppress shock-induced increases in circulating corticosterone (Moriceau & Sullivan, 2006; Shionoya et al., 2007). At the neurobiological level, pups undergoing threat conditioning with maternal presence show reduced activity in the amygdala (Moriceau & Sullivan, 2006; Opendak et al., 2019), PVN (Shionoya et al., 2007), and the ventral tegmental area (VTA), and ventral striatum (Opendak et al., 2019). The olfactory bulb shows increased activity in pups that underwent threat conditioning with maternal presence (Moriceau & Sullivan, 2006). The effects of corticosterone in the amygdala are particularly critical for the effect of maternal buffering. Systemic administration of corticosterone or direct infusion of corticosterone into the amygdala will drive threat learning despite maternal presence (Moriceau & Sullivan, 2006).

1.4.3 POSTNATAL DAY 16 THROUGH POSTNATAL DAY 21

The mother is less capable of regulating threat learning as pups approach weaning age. At postnatal day 18, pups that underwent threat learning with maternal presence froze less at test

than pups that underwent threat learning alone (Robinson-Drummer et al., 2019). However, freezing levels observed in pups conditioned with maternal presence were still greater than those observed in pups that were only exposed to the odor CS alone or received unpaired exposures to the odor CS and shock alone, suggesting that some learning did occur (Robinson-Drummer et al., 2019). In pups that were a few days older, maternal presence during learning did not block avoidance of the threat conditioned odor in a 2-odor choice test (Moriceau & Sullivan, 2006). Cage mates become more effective regulators of threat and stress than the mother as pups develop into adolescents (Kikusui et al., 2006).

1.5 Early Life Stress Effects on Threat Learning, Stress Responsivity, and Maternal Buffering – Implications for Vulnerable Phenotypes

There is some evidence to suggest that attachment or familiarity is important for the effectiveness of parental buffering. In guinea pigs, the mother is a better buffer of the infant's stress response than other females (Hennessy et al., 2006). Father titi monkeys are more effective buffers for their children than mother titi monkeys, perhaps because fathers are the primary caregivers in this species (Hoffman et al., 1995).

As in the studies of human children discussed above, studies of rodents have begun to uncover the behavioral, endocrine, and neurobiological sequelae of early life stress and disruption of maternal care and attachment. There is a significant body of literature that uses repeated, daily separations from the mother and looks at the short and long-term effects of this separation on her infants. In infancy (Horii-Hayashi et al., 2013) and adulthood (Aisa et al., 2008; Lajud et al., 2012), infant rats that experienced maternal separation have altered reactivity of the stress response system and altered functioning of the brain regions controlling the stress

response system in adulthood (O'Malley et al., 2011). Pups that experienced maternal separation also had fewer newborn neurons in the hippocampus (Lajud et al., 2012).

Infant rats that experience maternal separation also have a disrupted system regulating their response to threats. Threat associations can be unlearned or "extinguished" by re-exposing the rat to a conditioned stimulus without also re-exposing the rat to the shock. Adult rats that undergo extinction may show spontaneous recovery of the threat memory, however rat pups do not typically show spontaneous recovery. Once rat pups that experienced repeated maternal separation (Callaghan & Richardson, 2011) or an acute, extended separation from the mother on postnatal day 9 (Cowan et al., 2013) acquire a threat association, they are more susceptible to the return of the threat association that has been unlearned. This precocious emergence of extinction-resistant threat learning also appears to be transmitted from rat mothers that experienced maternal separation to their offspring (Kan et al., 2016).

Another experimental manipulation that has been utilized to examine the consequences of disruption of maternal care in infancy is the "limited bedding" or "scarcity-adversity" model. In this model, rat mothers are provided limited nesting materials, which leads the mother to spend more time trying to build an ideal nest, more time handling her pups roughly, and less time actively caring for her pups (Ivy et al., 2008; Perry et al., 2019; Perry & Sullivan, 2014; Raineki et al., 2010). One of the outcomes of this manipulation is that the mother is no longer capable of buffering threat learning and amygdala activity in her pups (Opendak et al., 2019) or of suppressing cortical oscillations in her pups while she is grooming or nursing them (Opendak et al., 2020). Maltreated infant pups also show reduced attachment behaviors upon reunion with the mother following a separation (Opendak et al., 2020) In the periweaning period and adolescence, rats reared in this condition show altered social behaviors and increased depressive-like behavior

(Raineki et al., 2012; Rincón-Cortés & Sullivan, 2014). These rats also showed increased amygdala activity during the test of depressive-like behavior (Raineki et al., 2012) and reduced amygdala activity during the test of social behavior (Rincón-Cortés & Sullivan, 2014). These findings point to the amygdala as a critical structure whose developmental trajectory may be altered by maternal maltreatment during infancy.

1.6 Remaining Questions

Although a number of the studies described above have begun to elucidate the role of the mother in regulating threat learning and responses, HPA axis functioning, and neural activity in infant rats, a number of questions remain. The goal of this thesis has been to address some of these. In Chapter 2, I examine whether maternal presence during a threat learning experience at postnatal day 15 is associated with a different behavioral outcome in a robustly studied defense response – threat conditioned-induced freezing. In Chapter 3, I apply graph theory to analyze patterns of immediate early gene expression and describe the functional neural network engaged by infant rats that underwent threat learning with or without maternal presence. In Chapter 4, I examine the ontogeny of threat learning and stress responsivity in a vulnerable phenotype prone to high-anxiety like behavior in adulthood, and then question whether maternal presence and fibroblast growth factor 2 are capable of regulating threat learning in these animals. In Chapter 5, I will summarize results from each chapter and discuss future directions.

Chapter 2 Maternal Buffering of Freezing to Conditioned Threat

2.1 Abstract

During infancy, rats, like humans, rely extensively on parental regulation of homeostatic needs, stress response, and emotions. As infant rats move from the first to the second week of life, they develop the ability to learn about associations between neutral and aversive stimuli and exhibit appropriate behavioral responses, such as freezing. However, infant rats are still dependent on maternal care for survival, and therefore their ability to learn about threats is still regulated by maternal cues. Previous studies have shown that pups that underwent a threat learning experience in the presence of a calm mother did not avoid the threat conditioned odor at test, in contrast to those pups that underwent a threat learning experience with no maternal presence. However, it is not clear if freezing, a hallmark defensive behavior that emerges at this age, can be used to demonstrate the effect of maternal buffering of threat learning. I found that pups who were exposed to 11 odor-shock pairings at postnatal day 13 with maternal presence tended to spend less time freezing to that odor conditioned stimulus compared to pups conditioned with no maternal presence when tested at postnatal day 18. When I split our results by sex, I found that this effect was significant in females but not in males. To delve deeper into the architecture of this freezing behavior, I divided the time pups spent freezing during CS presentations into "bouts," or periods of time longer than one second during which the pup was continuously immobile. Pups that underwent threat conditioning in the absence of a calm mother tended to enter a freezing state more frequently during CS presentations than pups conditioned with a calm mother present. The duration of freezing bout length was significantly longer in pups

conditioned without maternal presence during the third CS presentation. Although there was significant variability in the freezing data, these results confirm that the effects of maternal buffering on threat learning can be observed on a freezing test and underline the critical role of the mother in regulating threat learning in infancy. Future studies will be needed to confirm whether the ability of the mother to modulate threat learning differs between male and female pups.

2.2 Introduction

In altricial species, including rats, newborn infants spend most of their time with their mother and are wholly dependent on maternal care for protection from predation and other threats (Bolles & Woods, 1964). However, as infant rats become mobile and venture outside of the nest in the second week of life, they begin to encounter many novel stimuli on their own (Bolles & Woods, 1964). In order to survive, infant rats need to learn which stimuli are threatening and to exhibit defensive behaviors where appropriate (Debiec & Sullivan, 2017b). In accordance with this developmental necessity, rat pups generally begin to learn associations between co-occurring neutral olfactory stimuli and mild shock around postnatal day (P) 10; for example, pups of this age can learn to avoid a novel odor paired with a mild shock (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Kucharski & Spear, 1984; Regina M Sullivan et al., 2000) and will cease ongoing pursuit of a food reward when presented with an auditory threat conditioned cue (Campbell & Ampuero, 1985). When exposed to a natural predator, rat pups in the second week of life will freeze, which is a hallmark defensive behavior characterized by the cessation of all voluntary movement (L. K. Takahashi, 1992, 1994; Wiedenmayer, Lyo, et al., 2003; Wiedenmayer & Barr, 1998).

Although 10-15 day old rat pups are beginning to develop some independence, they are still reliant on maternal care. As discussed above, P10 pups can learn associations between novel stimuli and shock, but that learning ability is still under tight regulation by the mother. Specifically, pups that underwent odor-shock threat conditioning in the presence of a calm, anesthetized mother did not avoid the conditioned odor when tested in a Y-maze apparatus (Moriceau & Sullivan, 2006; Opendak et al., 2019; Shionoya et al., 2007). This is thought to be an adaptive mechanism, as the pups are not yet fully independent and any learning that takes place in the presence of the mother should facilitate approach behavior towards the mother, not defensive responses (Perry & Sullivan, 2014).

There is a dissociation in the developmental emergence of different conditioned threat responses (Campbell & Ampuero, 1985; Richardson et al., 2000), and different circuits are responsible for different behavioral responses to threat (LeDoux, 1993). Therefore, it is also possible that some threat responses may be regulated by maternal presence during threat conditioning and that other threat responses may not. A remaining question is whether the effect of maternal buffering of learned threat can be observed using a different behavioral measure, namely, the freezing response. One previous study used the freezing test to examine the effect of maternal presence on regulation of threat, however, training took place in late infancy (P18), when the effect of maternal presence is thought to be less potent (Robinson-Drummer et al., 2019). Additionally, there is an emerging literature on sex differences in expression of threat (Gruene et al., 2015) and social regulation of threat in adults (Mikosz et al., 2015); however, sex differences in maternal buffering of threat have not received much attention.

Here, I extend the findings on maternal buffering of learned threat in infant rats. I employed odor-shock threat conditioning in the presence or absence of a calm mother and

measured subsequent freezing behavior when pups were exposed to the odor conditioned stimulus. I also examined our results separately by sex in order to determine whether males and females responded differently to maternal presence during threat conditioning.

2.3 Methods

2.3.1 ANIMALS

All procedures were approved by the University of Michigan Institutional Animal Care & Use Committee in accordance with guidelines from the National Institutes of Health Breeding-age outbred Sprague-Dawley male and female rats were acquired from Charles River. Rats were kept in a 22 ± 2 °C colony room with a traditional light cycle (12 h; lights on from 0800 : 2000 h) and ad libitum access to water and standard lab chow. One male and one female rat were pair-housed for 10 days. Pups used in these experiments were born and bred in the lab. The day pups were born was designated postnatal day 0 (P0). The day after pups were born, each litter was culled to a maximum of 12 pups to achieve equal numbers of male and female pups. Dams were given ample nesting material.

2.3.2 THREAT CONDITIONING

All behavioral procedures were performed by female experimenters (Sorge et al., 2014). Threat conditioning took place when pups were 13 days old. The experimental room was heated to approximately 28 ± 2 °C and a heating pad was placed underneath the testing apparatus to maintain the pups' body temperature. Threat conditioning took place in an empty standard rat home cage (300 mm x 234 mm x 412 mm) lined with an absorbent blue pad (total n = 18; male n = 9; female n = 9). The threat conditioning protocol consisted of a 10 minute habituation period followed by 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned

stimulus (US) pairings. A 4-minute inter-trial interval separated each shock from the next odor CS presentation. Shocks were applied manually to the pup's tail (Moriceau & Sullivan, 2006). Pups were returned to their home cage immediately following the end of the threat conditioning protocol.

2.3.3 MATERNAL BUFFERING

In the maternal presence group (total $n = 15$; male $n = 7$; female $n = 8$), the rat pups' mother or a dam of equivalent postpartum age was used (Moriceau & Sullivan, 2006). Prior to threat conditioning, dams were deeply anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) delivered by intraperitoneal injection. Dams were placed in the experimental chamber when they were no longer responsive to toe pinch. Threat conditioning then began as described above.

2.3.4 FREEZING TEST

Pups underwent a freezing test at P18. Pups were retrieved from the colony room and individually placed in a testing chamber. Pups received 3 30 second presentations of the odor conditioned stimulus separated by a 2 minute inter-trial interval. Testing chambers were washed with unscented soap and water to prevent the transfer of threat via smell to other experimental groups. Freezing behavior during the test was automatically scored by Ethovision (Version XT 10, Noldus, Leesburg, VA, USA). The percent of time that each animal spent freezing during the pre-conditioned stimulus period (defined as the 30 seconds immediately before the first odor conditioned stimulus presentation) and the percent of time that each animal spent freezing during each conditioned stimulus presentation was calculated. I also analyzed the number and duration of "bouts" of freezing exhibited by pups following the first CS presentation through the duration of the test and during each CS presentation. A bout was defined as any period of time longer than

one second in which the animal was continuously immobile. Examining bouts of freezing behavior allows for a deeper characterization of pup behavior; it allows me to ask whether the effect of maternal presence during conditioning is associated with suppressed initiation of freezing behavior and/or suppressed continuation of freezing behavior during the test (Maren, 2001a).

2.3.5 DATA ANALYSIS

Statistical analyses were performed using GraphPad Prism (Version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). A two-way repeated measures ANOVA (Maternal Presence X Time) was used to investigate the effect of maternal presence on the percentage of time spent freezing during each CS presentation. Separate two-way repeated measures ANOVAs (Maternal Presence X Time) were used to investigate the effect of maternal presence on the percentage of time spent freezing during the pre-CS period and each CS presentation in males and females individually. Student's *t*-tests were used to compare the number and duration of freezing bouts between all pups conditioned with and without maternal presence and then between female pups exclusively and male pups exclusively. Two-way repeated measures ANOVAs (Maternal Presence X Time) were also used to examine whether the number and duration of freezing bouts occurring during the pre-CS period and CS presentations differed between pups conditioned with or without maternal absence. Additional two-way repeated measures ANOVAs (Maternal Presence X Time) were used to compare the number and duration of freezing bouts occurring during the pre-CS period and CS presentations in males and females individually.

2.4 Results

2.4.1 PERCENT OF TIME SPENT FREEZING DURING CS PRESENTATIONS

A two-way repeated measures ANOVA revealed a significant effect of time, such that pups tended to freeze more during each subsequent CS presentation, $F(3,69) = 9.82$, $p < 0.0001$ (Figure 2.2a). Importantly, the two-way repeated measures ANOVA also revealed a significant effect of maternal presence, such that pups that underwent conditioning in the presence of a calm mother froze less to the conditioned cue than pups that underwent conditioning without a calm mother present $F(1,23) = 4.72$, $p = 0.04$. There was no significant interaction effect, $F(3,69) = 0.79$, $p = 0.50$. The effect of maternal presence appeared to be mainly driven by differences in freezing at the third CS presentation; Sidak's multiple comparisons test revealed that pups conditioned without maternal presence froze significantly more during CS 3 than pups conditioned with maternal presence, $p = 0.01$.

I next split our conditioned freezing results by sex. There was a significant effect of time in both female ($F(3,33) = 6.75$, $p = 0.001$) and male ($F(3,30) = 3.65$, $p = 0.02$) infants. Female pups conditioned in the presence of a calm mother ($n = 6$) versus no calm mother ($n = 7$) froze less to the CS at test, $F(1,11) = 10.43$, $p = 0.008$ (Figure 2.2b). In contrast, no significant differences were observed between male pups conditioned with a calm mother ($n = 5$) vs. without a calm mother ($n = 7$), $F(1,10) = 0.10$, $p = 0.75$ (Figure 2.2c). There was no significant interaction effect between time and conditioning group in females ($F(3,33) = 0.66$, $p = 0.58$) or males ($F(3,30) = 1.50$, $p = 0.23$).

2.4.2 FREQUENCY AND DURATION OF BOUTS OF FREEZING DURING CS PRESENTATIONS

In order to further examine the behavioral patterns exhibited by infant rats during the test of conditioned freezing, I extracted bouts of freezing from the data. A bout of freezing was defined as any period of time longer than one second during which the pup was continuously immobile. When examining the average number of freezing bouts animals engaged in during CS presentations, I found significant main effects of time ($F(2,62) = 3.37$, $p = 0.04$) and maternal presence ($F(1,31) = 6.87$, $p = 0.01$) (Figure 2.3a). The interaction effect was not significant, $F(2,62) = 0.65$, $p = 0.52$. These results suggest that pups that underwent threat conditioning in the absence of a calm mother tended to enter a freezing posture more frequently during CS presentations than pups conditioned with a calm mother present.

As in our analysis of the percentage of time pups spent freezing during conditioned stimulus presentation, I also split our freezing bout data by sex to look for potential sex differences. When looking at the number of freezing bouts that occurred during CS presentations, I found a significant main effect of maternal presence ($F(1,15) = 6.61$, $p = 0.02$), such that females conditioned in the absence of a calm mother tended to engage in more freezing bouts during CS presentations than females conditioned in the presence of a calm mother (Figure 2.3b). There was no significant main effect of time ($F(2,30) = 1.93$, $p = 0.16$) and no significant interaction effect ($F(2,30) = 1.13$, $p = 0.34$). I found no significant effects of time ($F(2,28) = 1.60$, $p = 0.22$), maternal presence ($F(1,14) = 1.62$, $p = 0.22$), or interaction effect in males ($F(2,28) = 0.02$, $p = 0.98$) (Figure 2.3c).

There was also a significant interaction effect between time and maternal presence on the average freezing bout length during CS presentations, $F(2,62) = 3.8$, $p = 0.03$ (Figure 2.4a).

Main effects of time ($F(2,62) = 3.09$, $p = 0.05$) and maternal presence ($F(1,31) = 3.63$, $p = 0.07$) did not reach significance. Sidak's multiple comparisons test revealed that pups conditioned with maternal presence had significantly shorter freezing bout length during the third CS presentation than pups conditioned without maternal presence, $p = 0.004$.

When measuring the average freezing bout length during CS presentations, time ($F(2,30) = 1.53$, $p = 0.23$) and maternal presence ($F(1,15) = 3.91$, $p = 0.07$) had no significant main effect on the average freezing bout length in females (Figure 2.4b). Additionally, there was no significant interaction effect ($F(2,30) = 1.77$, $p = 0.19$). In males, a significant interaction effect was observed ($F(2,28) = 4.07$, $p = 0.03$) (Figure 2.4c). However, there was no significant main effect of time ($F(2,28) = 2.19$, $p = 0.13$) or maternal presence ($F(1,14) = 0.28$, $p = 0.61$).

2.5 Discussion

As infant rats become mobile and begin to explore the area surrounding their nest independently, they need to detect and respond to potential threats (Bolles & Woods, 1964). However, this need is balanced by an opposing need for the infant to remain dependent on the mother until it has developed enough on its own to survive independently. The consequence of this duality is that the pup's ability to learn about threat is regulated by maternal presence (Debiec & Sullivan, 2017b).

I found that P13 pups that were conditioned in the presence of a calm mother tended to freeze less during conditioned stimulus presentations than pups that were conditioned in the absence of a calm mother. Pups conditioned in the presence of a calm mother also tended to engage in fewer bouts of freezing during conditioned stimulus presentations and shorter bouts of freezing during CS 3. Increased freezing in pups conditioned without maternal presence may be driven more by their heightened tendency to enter a freezing state rather than their tendency to

remain in a freezing state. Our data complement previous work which showed that on an odor-avoidance task, pups that were conditioned in the presence of a calm mother did not avoid that odor previously paired with shock (Moriceau & Sullivan, 2006; Opendak et al., 2019; Shionoya et al., 2007) and in pups approaching weaning age, the presence of a calm mother during training was associated with less freezing (Robinson-Drummer et al., 2019).

Variability in the percentage of time pups spent freezing in our experiments was high, and the levels of freezing observed in these experiments was generally lower than those observed in experiments with adult animals. Other studies of conditioned freezing in infant rats also suggest that levels of freezing are overall low, though group differences between threat-conditioned infant rats and those exposed to unpaired CS-US presentations or those exposed to the CS alone are still observed (Robinson-Drummer et al., 2019; Yap et al., 2005). Although the ability of pups to acquire an association between a neutral odor stimulus and shock (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Kucharski & Spear, 1984; Regina M Sullivan et al., 2000) and the ability of pups to freeze in response to a natural predator at this age (L. K. Takahashi, 1992, 1994; Wiedenmayer, Lyo, et al., 2003; Wiedenmayer & Barr, 1998), are well documented, it is possible that the neural circuits involved in driving threat-conditioned freezing are not yet fully developed. There is some evidence to suggest that the conditioned behavioral response measured at test is dependent on whether the infant was capable of exhibiting that type of behavioral response at training (Richardson & Fan, 2002), so it is possible that at training (P15), some pups had developed the ability to freeze while others had not.

Previous studies of threat learning and the behavioral effects of maternal buffering in infant rats do not report sex differences. The authors of these studies either state that no sex differences were observed and therefore male and female animals were included together in all

analyses (Moriceau & Sullivan, 2006) or they include male and female animals together in all analyses but do not discuss whether they tested for potential sex differences (Opendak et al., 2019; Shionoya et al., 2007). However, some of these studies may have included too few animals to appropriately assess for sex differences (Moriceau & Sullivan, 2006; Shionoya et al., 2007).

In our experiments, I found that in female pups, those conditioned in the presence of a calm mother froze less to the cue at test. This effect was not seen in males. It is surprising and interesting to see an effect of sex so early in life. It is also remarkable since there is some evidence from studies of conditioned freezing in adult rats suggest that females freeze less than males in tests of conditioned threat (Baran et al., 2010; Gupta et al., 2001; Kosten et al., 2005; Maren et al., 1994; Pryce et al., 1999) and females may have a higher tendency to exhibit active defensive behaviors than males (Gruene et al., 2015). A recent meta-analysis of the rodent threat conditioning literature concluded that a study predicting a medium effect size would be 80% powered if each experimental group had 15 animals (Carneiro et al., 2018). Therefore, it is possible that our study did not include enough subjects to determine whether the differences between males and females that I observed are the result of a true sex difference.

In adult rats, circulating levels of sex hormones may account for differences in threat learning and threat responses (Day & Stevenson, 2020). Although levels of sex hormones are significantly lower in rat pups compared to adults, there are sex differences in circulating testosterone and estradiol in the second week of infancy (Döhler & Wuttke, 1975). It is possible that these sex steroids may lead to differences in threat learning and expression that manifest via the effect of maternal buffering. Another possibility is that differential provisioning of maternal care may result in differential effectiveness of maternal buffering. Rat mothers tend to provide more care to their male pups than their female pups (Deviterne & Desor, 1990; Moore & Morelli,

1979; Richmond & Sachs, 1984) and there is strong evidence to suggest that the quality of maternal care during infancy impacts the effectiveness of maternal buffering (Opendak et al., 2019; Robinson-Drummer et al., 2019). This leads me to conclude that, if anything, maternal buffering of threat learning should be *more* effective in male pups than female pups. It is important to note that in general, female pups that underwent fear conditioning with no maternal presence appeared to freeze more even during the pre-CS period. The failure to observe the maternal buffering effect in male animals could instead be a reflection of differences in the ability or tendency of male rat pups to show freezing behavior. Further experiments will need to explore this.

During the test of threat memory, several things may be happening. The pup may simply not retrieve any memory of the association between the odor and the shock, because no memory successfully formed during conditioning. The fact that both an odor avoidance test and a freezing test showed similar results suggests that this may be the case. Alternatively, the presentation of the odor conditioned stimulus may activate two memories: the memory of the odor-shock pairing and the memory of the odor-mother pairing. The memory of the mother may be strong enough of a safety signal to override the memory of shock.

Our results provide additional evidence for the critical role of the mother in modulating the ability of the infant rat to learn about threat and suggest that this effect can be measured using indices of learning beyond an odor avoidance task. Future studies are required to determine if the sex differences I observed represent a true difference between male and female infant rats.

2.6 Acknowledgements

This research was supported by the K08 MH014743-01A1 and NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation to Jacek Debiec. Amanda M. White was supported by NIH T-32-NS076401 and the Department of Psychiatry.

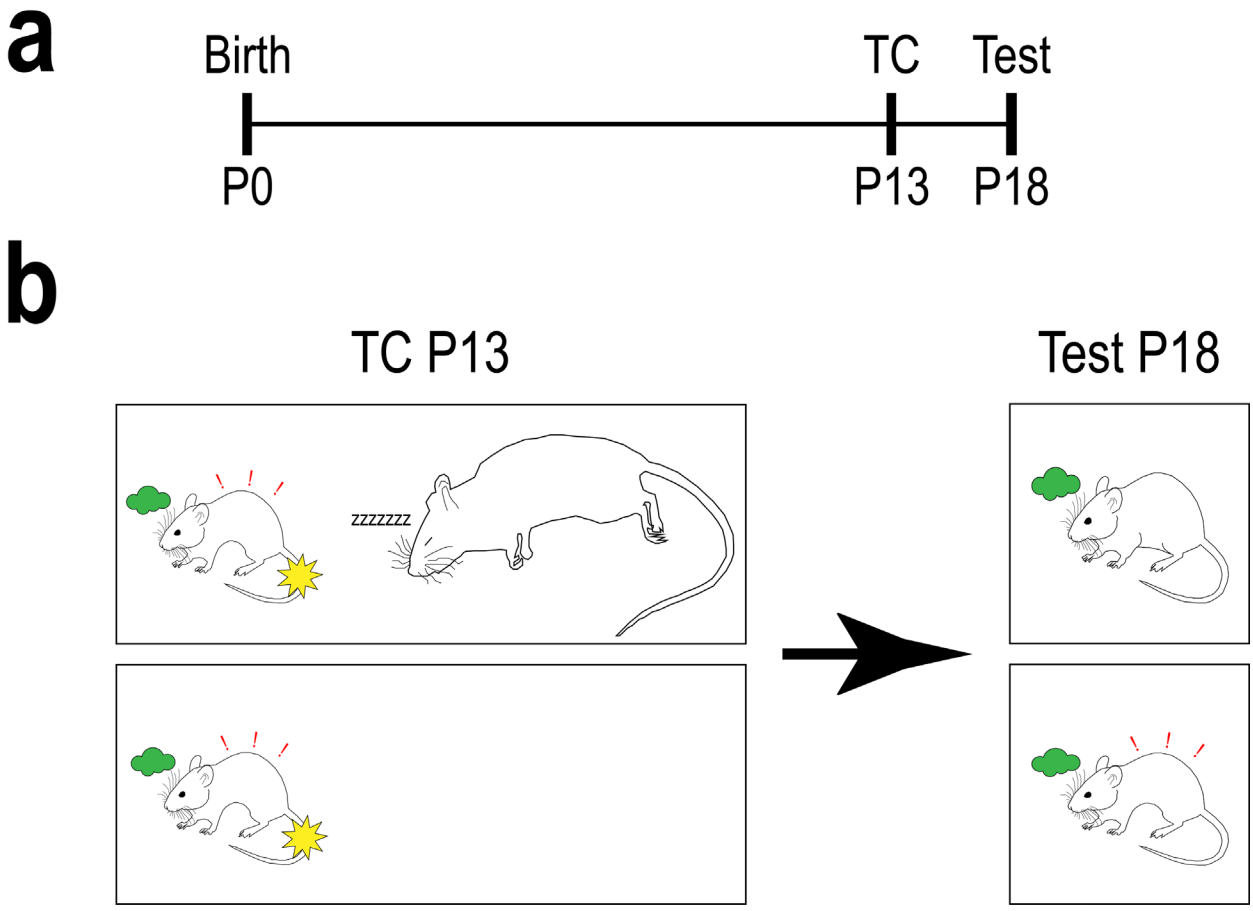

Figure 2.1 Experimental Design. (a) The day pups were born was designed postnatal day zero (P0). Odor-shock threat conditioning (TC) took place at P13 and the test of conditioned freezing took place at P18. (b) Threat conditioning took place with or without the presence of an anesthetized dam that was the pup's own mother or a mother of equivalent postpartum age. All pups received 11 pairings of a novel peppermint odor with a 0.5 mA shock to the tail. The test of conditioned freezing took place at P18. Pups received 3 presentations of the peppermint odor conditioned stimulus and freezing behavior was tracked using Ethovision.

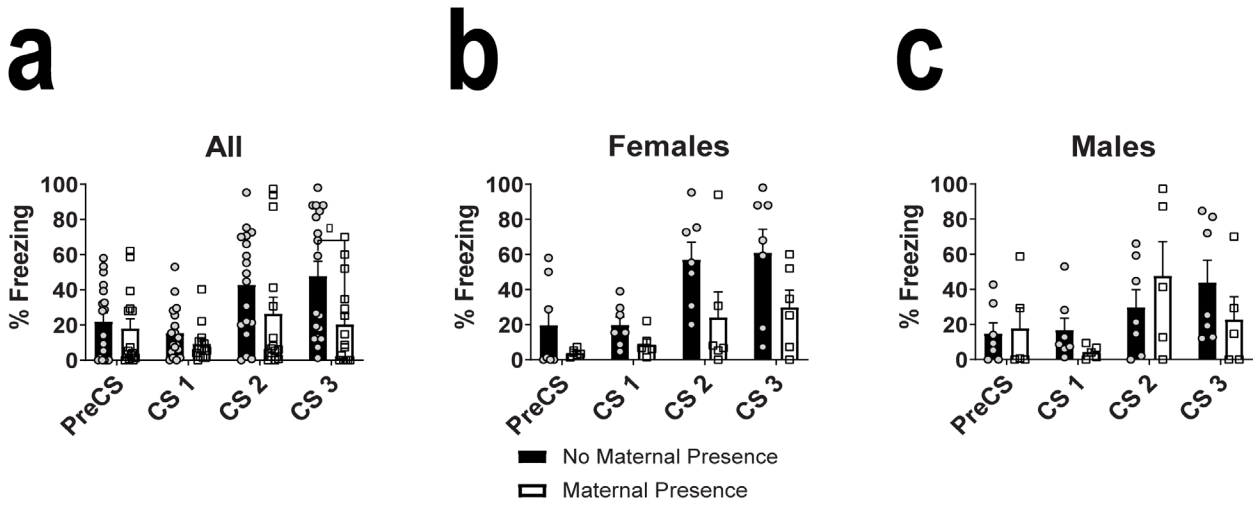

Figure 2.2 Percent time spent freezing during conditioned stimulus presentations. (a) Rat pups conditioned with maternal presence froze less to the conditioned stumulus at test relative to pups conditioned without maternal presence. (b) Female pups conditioned with maternal presence froze less to the conditioned stumulus at test relative to pups conditioned without maternal presence. (c) Male pups conditioned with maternal presence did not freeze less to the conditioned stimulus at test relative to pups conditioned without maternal presence. Error bars represent SEM. *$p < 0.05$

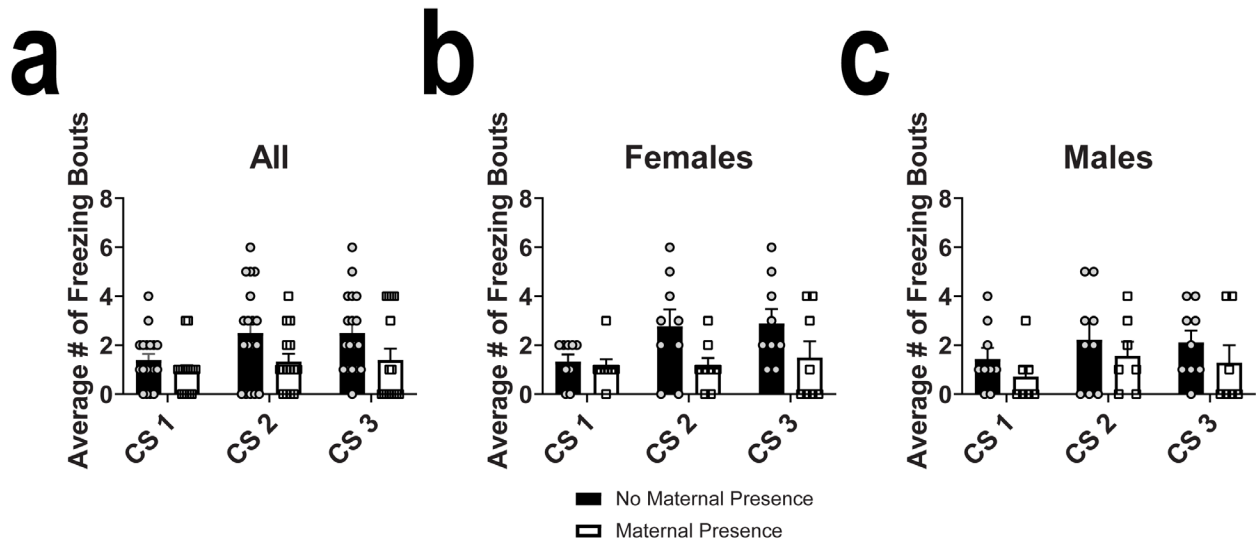

Figure 2.3 Number of freezing bouts during conditioned stimulus presentations. (a) Rat pups conditioned with maternal presence tended to enter a freezing posture less frequently during the conditioned stimulus than pups conditioned without maternal presence. (b) Female rat pups conditioned with maternal presence tended to enter a freezing posture less frequently during the conditioned stimulus than female pups conditioned without maternal presence (c) Male pups conditioned with maternal presence did not enter a freezing posture less frequently during the conditioned stimulus at test relative to pups conditioned without maternal presence. Error bars represent SEM.

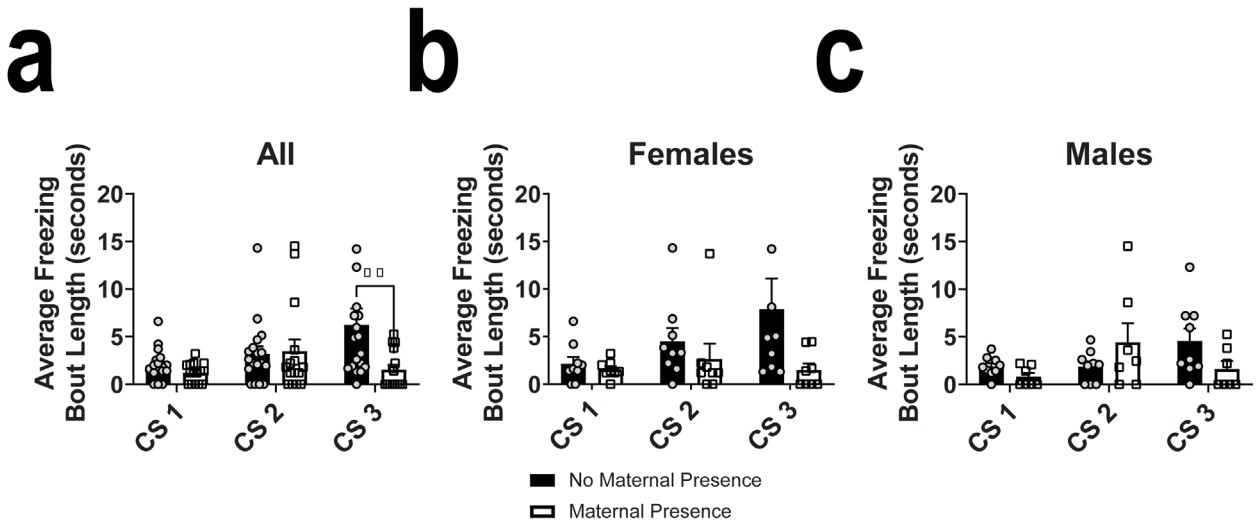

Figure 2.4 Duration of freezing bouts during conditioned stimulus presentations. (a) There was a significant interaction effect between maternal presence and time on the duration of freezing bouts during conditioned stimulus presentations. Rat pups conditioned with maternal presence tended to engage in shorter duration freezing bouts during conditioned stimulus presentations during the third conditioned stimulus presentation. (b) The duration of freezing bouts did not differ between female rat pups conditioned with and without maternal presence. (c) There was a significant interaction effect between maternal presence and time on the duration of freezing bouts during conditioned stimulus presentations in male pups. Error bars represent SEM. **$p < 0.01$.

Chapter 3 Maternal Presence During Threat Learning Modulates Functional Connectivity and Community Structure in Infant Rats

3.1 Abstract

As infant rats begin to develop independence from their caregivers and explore the areas surrounding their nest in the second week of life, the amygdala must start responding to innate threats and undergo plasticity during threat learning. However, it remains ecologically relevant for threat learning to be under the control of the mother until the infant has matured to full independence. Accordingly, previous studies have shown that pups of this age that undergo Pavlovian odor-shock conditioning in the presence of an anesthetized mother will not demonstrate threat responses to future presentations of the conditioned odor. This is an effect called "maternal buffering," and it is mediated in part by the mother's ability to suppress amygdala activity during threat learning. In recent years, there has been a growing appreciation that patterns of neuronal activity between groups of brain areas, rather than just absolute increases or decreases in activity, are essential for learning. Here, I used a graph theoretical approach to analyze patterns of immediate early gene expression and investigate whether there are differences in functional networks engaged by infant rats conditioned without and with maternal presence. Although absolute numbers of c-Fos positive cells did not differ between groups, overall functional connectivity was significantly stronger in pups conditioned with maternal presence. A community detection algorithm revealed that network structure differed between groups. The functional network engaged by pups conditioned with maternal presence was more integrated and did not appear to have distinct hub regions. In contrast, the functional network engaged by pups conditioned without maternal presence was more segregated and the

lateral and basolateral amygdala appeared to be hubs. These results demonstrate the power of the graph theoretical approach and the power of the mother's ability to modulate neural activity within her pups during a critical developmental timepoint.

3.2 Introduction

In altricial species such as rodents, caregivers provide nutrition, warmth, and safety from predators, as well as regulation of emotional responses for their infant offspring (Bolles & Woods, 1964). The mother's ability to protect the developing infant brain from the deleterious effects of stress (Lupien et al., 2009; McEwen, 2008) and to protect the infant rat from acquiring any learned avoidance of the mother (Perry & Sullivan, 2014) helps the infant survive and continue developing toward independence. This latter role is referred to as maternal buffering of stress and threat.

In rodents, Pavlovian threat conditioning has been used to explore the behavioral and neural sequelae of maternal buffering. This effect can be observed in the transitional period between postnatal day (P) 10-15, during which pups are beginning to explore the area surrounding the nest, but are still dependent on maternal care. Maternal presence during threat conditioning blunts the infant's threat response to later presentation of the conditioned stimulus (Moriceau & Sullivan, 2006; Opendak et al., 2019; Robinson-Drummer et al., 2019; Shionoya et al., 2007). Infant rats show increased activity in the olfactory bulb (Moriceau & Sullivan, 2006) and show reduced activity in the amygdala (Moriceau & Sullivan, 2006; Opendak et al., 2019), paraventricular nucleus of the hypothalamus (PVN) (Shionoya et al., 2007), the ventral tegmental area (VTA), and ventral striatum (Opendak et al., 2019) when a calm mother is present during threat conditioning. The lateral/basolateral amygdala appears to play a particularly critical role, as infusion of corticosterone directly into the infant rat's amygdala can block the ability of

the mother to regulate threat learning in the infant rat (Moriceau & Sullivan, 2006). One conceptualization of this phenomenon is that during this transitional period, the mother can "switch" the pups' ability to learn threat associations from a more mature state (learning to avoid an odor paired with shock) to an immature state (learning to prefer an odor paired with shock) (Santiago et al., 2017).

However, the amygdala and these other brain structures do not function in isolation. Within the field of learning and memory, there is growing appreciation for the idea that examining the patterns of interactions between brain areas may be just as, if not more important than, measuring absolute increases or decreases in overall activity (Buzsáki, 2010; Josselyn et al., 2015; McIntosh, 1999). It remains unclear what role the amygdala plays in the broader context of the infant brain during threat learning, and how that role may be modulated by maternal presence during threat learning. One study examined functional connectivity during threat conditioning with and without maternal presence. Here, functional connectivity refers to the strength of the entire network of interregional connections and/or between groups of brain areas defined a priori (Opendak et al., 2019; Perry et al., 2016; Wheeler et al., 2013). Functional connectivity between the VTA and amygdala was increased during threat conditioning with maternal presence (Opendak et al., 2019). A more sophisticated analysis of network structure in infant rats undergoing threat conditioning has yet to be performed.

Human neuroimaging researchers have long used network analysis to probe resting state brain networks in health and disease (Akiki et al., 2018; Keown et al., 2017; Zeng et al., 2017) and to investigate how human brain network functioning changes during experimental tasks (Cisler et al., 2018; Kim et al., 2018; Lithari et al., 2016). One widely used network analysis technique applies graph theory. Graph theoretical analysis goes beyond the functional

connectivity approach in several ways. It allows an unbiased identification of groups of regions (modules) whose activity are closely linked to each other (Rubinov & Sporns, 2010) rather than groups of regions that have been defined a priori. Additional graph theoretical metrics illuminate how information flows within a network. For example, a recent graph theoretical modeling of resting-state human brain during prenatal and early postnatal stages revealed characteristic developmental patterns (Zhao et al., 2019). Before birth, the network is fragmentized, forming a highly efficient small-world topology with distributed nodes (Zhao et al., 2019). During the third trimester of human prenatal brain development (which corresponds to the first week of life in infant rats (Semple et al., 2013)), the local primary clusters and short-range edges become enhanced resulting in a more segregated network (Zhao et al., 2019). After birth, the emergence and increase of long connections leads to increased integration of the global network (Zhao et al., 2019).

The graph theoretical approach has been recently successfully used in datasets of immediate early gene expression in adult rodent brains in order to examine the functional networks engaged during threat memory recall (Wheeler et al., 2013), social recognition (Tanimizu et al., 2017), spatial learning and execution (Babayan et al., 2017), social interactions with stressed individuals (Rogers-Carter et al., 2018), pharmacological activation of the dopaminergic system (Cruces-Solis et al., 2020), and opiate dependence (Brynildsen et al., 2020). One study (Vetere et al., 2017) identified highly connected brain regions (hubs) within a functional network that was engaged by mice during contextual threat memory recall. One of these hubs, the reuniens thalamic nucleus, was identified as a novel brain region important for contextual threat memory (Vetere et al., 2017). In an elegant demonstration of the power of the graph theoretical approach, the investigators chemogenetically silenced the identified hubs and

were able to successfully disrupt contextual threat memory consolidation, whereas silencing of non-hub regions did not disrupt contextual threat memory (Vetere et al., 2017). Thus, applying graph theoretical analysis to immediate early gene expression datasets has begun to increase the field's understanding of the contribution of coordinated neural activity across the brain to complex behaviors.

Here, I examined the functional brain network engaged by infants undergoing a threat learning experience in the presence or absence of a calm mother. I hypothesized that the functional networks activated during threat learning differ depending on whether the mother was present or not. Additionally, I hypothesized that the lateral/basolateral amygdala is a key hub in the network engaged by pups undergoing a threat learning experience in the absence of the mother, but not in the network engaged by pups undergoing a threat learning experience in the presence of the mother. Finally, I hypothesized that maternal presence may switch the pups' functional network from an integrated state to a more immature, segregated state.

3.3 Methods

3.3.1 ANIMALS

All procedures were approved by the University of Michigan Institutional Animal Care & Use Committee in accordance with guidelines from the National Institutes of Health. Rats were housed in a 22 ± 2 °C colony room with a traditional light cycle (12 h; lights on from 0800 : 2000 h). Water and standard lab chow were freely available. The subjects used in the current study were the offspring of male and female outbred Sprague-Dawley rats obtained from Charles River. Breeding pairs were housed together for 10 days and then separated. Mothers were provided with plenty of nesting material. The colony room was checked twice daily for newborn

pups; the day pups were first observed was defined as postnatal day (P) 0. On P1, litters were sexed and culled to 12 pups with the goal of having equal representation of males and females.

3.3.2 THREAT CONDITIONING

Pups (n = 5; 2 females 3 males) underwent threat conditioning between P12-15. This sample size was chosen based on sample sizes from previous studies that investigated c-Fos expression in rat pups of this age group (Gordon A Barr, 2011; Olesen & Auger, 2005; Wiedenmayer & Barr, 2001). Threat conditioning took place in an empty cage lined with an absorbent bench pad. The room was heated to 28 ± 2 °C and a heating pad was placed beneath the cage. Pups habituated to the room and testing apparatus for 10 minutes prior to the onset of the threat conditioning protocol. Threat conditioning was comprised of 11 pairings of a 30 second peppermint odor conditioned stimulus (CS) and a mild (0.5 mA) 1 second shock to the tail. Each pairing was separated by 4 minutes. The duration of the threat conditioning protocol was 55 minutes.

3.3.3 MATERNAL BUFFERING

A separate group of pups (n = 6; 3 females 3 males) underwent threat conditioning between P12-15 in the presence of an anesthetized mother. The pups own mother or a mother of equivalent postpartum age (Moriceau & Sullivan, 2006) was deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). When the dam stopped responding to toe pinch, she was placed in an empty cage lined with an absorbent bench pad and threat conditioning of the pups proceeded as described above.

3.3.4 C-FOS IMMUNOHISTOCHEMISTRY

Between 15 and 30 minutes following threat conditioning, rats were deeply anesthetized and underwent transcardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. Brains were dissected out, post-fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose solution, and then frozen at -80 C. Brains were sectioned coronally on a cryostat at 30 μm for free-floating immunohistochemistry. Sections containing the regions of interest were selected and immunohistochemistry was then performed using a rabbit anti-c-Fos antibody (1:1000) (ABE457, Sigma-Aldrich, St. Louis, MO) and a goat anti-rabbit secondary antibody (1:500) (Vector Laboratories, Burlingame, CA). An avidin-biotin complex kit (ABC kit, Vectastain Elite, Vector Laboratories, Burlingame, CA) and the chromogen diaminobenizdene (Vector Laboratories, Burlingame, CA) were used to reveal staining.

3.3.5 IMAGE ACQUISITION AND ANALYSIS

Once slides were coverslipped and fully dried, slides were imaged on a Zeiss light microscope at a 10x objective. Outlines of the regions of interest were drawn in Photoshop on the section images using the Paxinos and Watson stereotaxic rat brain atlas as a reference (Paxinos & Watson, 2009). I selected regions of interest previously shown to be critical for threat learning, defensive behaviors, and processing of social cues. The lateral amygdala receives sensory information about the shock and odor (CS and US) and projects to the basal and central amygdala nuclei (Janak & Tye, 2015). The basal amygdala nuclei have projections to and from cortical regions, including the prelimbic cortex, that are critical for acquisition of threat conditioning (Janak & Tye, 2015). Interestingly, these projections develop around P10, when pups begin to show defensive behaviors to neutral stimuli paired with shock (Bouwmeester, Smits, et al., 2002; Bouwmeester, Wolterink, et al., 2002). The central amygdala nuclei are the

main “outputs” of the amygdaloid complex; they project to the periaqueductal gray and other subcortical structures to modulate freezing behavior, heart rate, and the stress response axis (Janak & Tye, 2015). The medial amygdala nuclei connect to and from primary olfactory structures such as the olfactory bulb and secondary olfactory structures such as the piriform cortex and cortical amygdala nuclei; the medial amygdala nuclei thus receive information about the olfactory CS and maternal odors (Petrulis, 2020). Efferents to the central amygdala also project from the medial amygdala (Petrulis, 2020). The insular cortex receives sensory information from the external environment and from within the body and has reciprocal connections with the amygdala nuclei (Gogolla, 2017). Therefore, this region is well-positioned to evaluate threats and indeed, the posterior insular cortex plays an important role in safety learning (Christianson et al., 2008). A list of the regions of interest can be found in Table 3.1.

Investigators blind to experimental group quantified the number of c-Fos positive cells in each region of interest in Fiji (Schindelin et al., 2012). Inter-rater reliability between investigators performing cell counts was 0.95 or greater. In order for a cell to be identified as “positive,” it needed to meet several criteria: (1) round or oval shape with smooth edges, (2) area between 50 and 200 pixels, (3) mean signal intensity between 30 and 90 (where a lower value indicates darker color), and (4) not on the edge of the tissue. Each animal had at least 2 complete sections containing each region of interest; counts were then averaged to determine a mean cell count for each region. These mean cell counts were combined with the mean cell counts of other animals from that conditioned group and between-group comparisons were made.

3.3.6 FUNCTIONAL NETWORK CONSTRUCTION

Following quantification of mean c-Fos expression for each region in each individual animal, pairwise Pearson’s correlations were computed between all brain regions. This resulted

in two correlation matrices, one for pups conditioned with no maternal presence and one for pups conditioned with maternal presence. To investigate functional connectivity, these correlation matrices were compared statistically by averaging all unique Pearson's correlations (n = 105) and performing a Student's t-test. Here, functional connectivity refers to the strength of all interregional connections (Opendak et al., 2019; Perry et al., 2016; Wheeler et al., 2013). Correlation matrices were visualized using the ggplot2 package (Wickham, 2016) within the R computing environment (R Core Team, 2019).

In graph theoretical terminology, these correlation matrices were "weighted" rather than "binary", which means that the connections between nodes could range between -1 and 1 and that some connections would be stronger than others. These graphs were also undirected, because information obtained through quantification of c-Fos expression does not allow the experimenter to infer the directionality of connections.

The diagonal of each correlation matrix was set to 0 and each matrix was next run through a community detection algorithm for weighted, undirected graphs using the Brain Connectivity Toolbox (Rubinov & Sporns, 2010) for MATLAB (MATLAB, 2019). All of the graph theoretical measures listed below were calculated within MATLAB using the Brain Connectivity Toolbox and custom MATLAB code.

3.3.7 GRAPH THEORETICAL MEASURES: NETWORK SEGREGATION

The community detection algorithm sorts nodes, or regions of interest, based on their functional connections with other nodes in order to maximize within-group connections and minimize between-group connections (Newman, 2006; Rubinov & Sporns, 2010). Here, a functional connection was defined as the value of Pearson's correlation coefficient computed between two regions of interest. The community detection algorithm sorting process determines

how many “modules” the resulting networks contain and which nodes fall within each individual module. A modularity statistic was computed for each network, which describes the extent to which the nodes can be sorted into distinct, non-overlapping groups. The modularity statistic is a method of quantifying the degree of segregation within the functional network, where a more modular network is more functionally segregated. A network with high functional segregation suggests that specialized information can be processed locally within densely interconnected nodes, rather than processed globally (Rubinov & Sporns, 2010).

I also computed the within-module correlations and the between-module correlations by averaging all Pearson’s correlation coefficients that represent nodal connections within each identified module and averaging all Pearson’s correlation coefficients that serve as nodal links between modules. Comparison of within-module and between-module correlations provides another measure of functional network segregation; a network with stronger within-module correlations than between-module correlations has some degree of functional segregation.

Networks were visualized in R using the sna (Butts, 2019), network (Butts, 2015), and ggplot2 (Wickham, 2016) packages and custom R code. Nodes, or brain regions, were represented by circles and functional connections, or correlation coefficients, were represented by links between nodes. For visualization purposes, connections less than 0.2 were excluded from the network maps. Module assignment was indicated by node color; nodes assigned to the same module were assigned the same color.

3.3.8 GRAPH THEORETICAL MEASURES: HUB MEASURES

I next calculated graph theoretical measures that would allow us to identify potential hubs within the networks within pups undergoing threat learning with no maternal presence and pups undergoing threat learning with maternal presence. Hubs are critical nodes within a network;

they typically have functional connections with nodes throughout the network and facilitate global processing of information (Rubinov & Sporns, 2010). The participation coefficient is one measure that identifies hubs; it is a value which describes the extent to which each node has connections with nodes in modules outside of its own. Nodes with high participation coefficients likely facilitate network integration (Rubinov & Sporns, 2010).

I also calculated betweenness centrality as an additional metric of hubness. Betweenness centrality refers to the fraction of shortest paths on which a node lies (Rubinov & Sporns, 2010). Here, a "short path" refers to a stronger correlation. For information to travel from one node to another, at each node along its route it will travel the adjacent path with the strongest connection.

3.3.9 STATISTICAL ANALYSIS

Student's *t*-tests were performed in GraphPad Prism (Version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) to compare mean c-Fos expression and correlation matrices between pups conditioned without and with maternal presence. GraphPad Prism was also used to perform one-way ANOVA and Dunnett's Multiple Comparisons Test in order to compare within-module correlations and between-module correlations within each experimental group. All graph metrics were computed using the Brain Connectivity Toolbox (Rubinov & Sporns, 2010) within the MATLAB computing environment (MATLAB, 2019).

3.4 Results

3.4.1 C-FOS EXPRESSION

Mean cell counts were computed for each region of interest and are listed in Table 3.1. Unpaired *t*-tests comparing mean cell counts in the 15 regions of interest between pups

conditioned with no maternal presence and pups conditioned with maternal presence found no significant differences (p's > 0.05).

Within each experimental group, mean cell counts for each region of interest were correlated with the mean cell counts for all other regions of interest. This resulted in two matrices of Pearson's correlation coefficients (Figure 3.2a-b). Statistical comparison of these two matrices revealed that c-Fos expression was more strongly correlated in pups conditioned with maternal presence than in pups conditioned without maternal presence, $p = 0.005$ (Figure 3.2c).

3.4.2 GRAPH THEORETICAL MEASURES: NETWORK SEGREGATION

The community detection algorithm sorted nodes into modules based on their functional connections with other nodes by maximizing within-group connections and minimizing between-group connections. The algorithm identified two modules within the functional networks engaged by pups without maternal presence and engaged by pups conditioned with maternal presence. Figure 3.3 shows the network structure for each experimental group and Table 3.2 lists the module assignments. Interestingly, in the pups conditioned with maternal presence, there was a module that contained all cortical regions and a module that contained all amygdala subnuclei (with the exception of the BLV). In contrast, in the pups conditioned without maternal presence, the cortical regions and amygdala subnuclei were evenly distributed between both modules.

I next investigated the functional segregation within these identified networks. In the pups conditioned without maternal presence, within-module correlations and between-module correlations were significantly different from each other, $F(2, 207) = 125.5$, $p < 0.0001$ (Mean within-Module 1 correlation = 0.74, SEM = 0.06, Mean within-Module 2 correlation = 0.88, SEM = 0.01, Mean between-modules correlation = 0.2, SEM = 0.04). Dunnett's multiple comparisons test revealed that the within-module correlations were stronger than between

module correlations, Module 1 $p < 0.0001$, Module 2 $p < 0.0001$. In the pups conditioned with maternal presence, within-module correlations and between-module correlations were also significantly different from each other, $F(2, 207) = 37.01$, $p < 0.0001$ (Mean within-Module 1 correlation = 0.9, SEM = 0.02, Mean within-Module 2 correlation = 0.78, SEM = 0.02, Mean between-modules correlation = 0.53, SEM = 0.03). Dunnett's multiple comparisons test revealed that the within-module correlations were stronger than between-module correlations, Module 1 $p < 0.0001$, Module 2 $p < 0.0001$. The functional network of pups conditioned without maternal presence had a modularity statistic of 0.16, while the functional network of pups conditioned with maternal presence had a modularity statistic of 0.07.

3.4.3 GRAPH THEORETICAL MEASURES: HUB MEASURES

I next calculated two graph theoretical measures that allowed us to look for hubs in the functional networks engaged by pups during threat learning without and with maternal presence. The participation coefficient describes the extent to which each node has connections with nodes in modules outside of its own. In the pups conditioned without maternal presence, participation coefficients ranged from 0.07 through 0.46 (Figure 3.4a). The regions with the highest participation coefficients were the MeAD, LaDL, and BLA. In the pups conditioned with maternal presence, participation coefficients ranged from 0.35 through 0.5. The regions with the highest participation coefficients were the GI, DI, and BLV.

Another graph theoretical measure that can help identify hubs is betweenness centrality, which refers to the fraction of shortest paths within a network on which a given node lies. In both experimental groups, most nodes did not fall on any of the shortest paths (Figure 3.4b). In pups conditioned without maternal presence, betweenness centrality was highest in the LaDL, BLA,

and MeAD while in pups conditioned with maternal presence, betweenness centrality was highest in the AIP, LaDL, and BLA.

3.5 Discussion

The infant rat's ability to learn associations between threatening and neutral stimuli and respond with defensive behavior emerges as the amygdala becomes functionally mature (Thompson et al., 2008). Since threat learning in adult rodents depends not only on the amygdala but also on coordinated patterns of neural activity between multiple brain regions (Josselyn et al., 2015), I investigated the neural networks that rat pups engaged during threat learning with and without maternal presence.

I did not observe between-group differences in c-Fos expression in any of my selected regions of interest, in contrast to previous studies that used [^{14}C]-2-deoxyglucose (2-DG) autoradiography to measure neural activity during threat conditioning with and without maternal presence. There could be several reasons for this. The 2-DG method, unlike immunohistochemistry, does not offer cellular resolution and reflects glucose metabolism within brain tissue (Sokoloff et al., 1977). Quantification of cells labeled by immunohistochemistry reflects cells which produced c-Fos protein and offers high spatial and cellular resolution (McReynolds et al., 2018). Additionally, it is possible that sex differences in c-Fos expression may explain why I did not observe significant differences between pups conditioned without and with maternal presence. One study found sex differences in baseline c-Fos expression in rat pups; specifically, relative to P1 female pups P1 male pups had more c-Fos positive cells in the medial preoptic area, dorsolateral bed nucleus of the stria terminalis, ventrolateral bed nucleus of the stria terminalis, supraoptic nucleus, central amygdala, and habenula (Olesen & Auger, 2005). Males also had higher levels of Fos protein in the basomedial hypothalamus than females at P1,

P5, and P20 but not at P0 or P11 (Olesen & Auger, 2005). The authors focused on regions sensitive to sex steroids, which, with the exception of the central amygdala, were regions not included in my analyses. It is possible that these observed sex differences are limited to the regions outlined above, that task-induced c-Fos expression may not reveal any sex differences, or that task-induced c-Fos expression may mask underlying sex differences in baseline c-Fos expression. However, further studies are needed to address this. Although the number of subjects I included in each group was typical for c-Fos studies of animals of this age group (Barr, 2011; Olesen & Auger, 2005; Wiedenmayer & Barr, 2001), this analysis may have benefited from a larger sample size, especially if sex differences are a factor.

Despite a lack of significant between-group differences in the numbers of c-Fos labeled cells within each individual region of interest, striking differences began to emerge once I investigated the correlated patterns of activity between regions of interest. This is in line with what has been seen in other studies that have used the graph theoretical approach to analyze datasets of immediate early gene expression (Wheeler et al., 2013). Overall, functional connectivity was significantly stronger in pups conditioned with maternal presence. The graph theoretical approach then offered a deeper look into the structure of the functional network and revealed notable differences between experiment groups. In a previous study, at postnatal 14, exposure to odor from a natural predator was associated with increased functional connectivity between a cluster of cortical regions which included the prelimbic cortex and a cluster of amygdala subnuclei (Perry et al., 2016). This parallels, in part, what I observed when examining differences in module assignment between pups conditioned without and with maternal presence. Specifically, in the pups that were exposed to another type of threat, the prelimbic cortex was included in a module that contained the majority of amygdala subnuclei, suggesting that it was

very highly connected with those subnuclei. In contrast, in pups that were exposed to threat with maternal presence, the prelimbic cortex was in a module dominated by other cortical structures, not amygdala subnuclei. Tight functional connections between the prelimbic cortex and lateral, basolateral, and central amygdala nuclei may be common neural signatures of exposure to threatening stimuli. Anatomical tracing studies indicate that bidirectional connections between the prelimbic cortex and amygdala emerge at the age when I am conducting these experiments (Bouwmeester, Smits, et al., 2002; Bouwmeester, Wolterink, et al., 2002). Therefore in pups conditioned without maternal presence, the fact that these regions were assigned to the same module may reflect direct activation of those connections.

Next, I compared network segregation between groups, which is a network property that allows for efficient, specialized processing of information (Rubinov & Sporns, 2010). Comparison of the modularity statistic between groups revealed that the network engaged by pups conditioned without maternal presence was more segregated. Both groups showed stronger within-module correlations than between module correlations, indicating some degree of functional segregation. However, the between-module correlation was smaller in pups conditioned without maternal presence relative to pups conditioned with maternal presence, which suggests that the former network is more segregated than the latter.

Little is known about the development of functional networks in infant rats. Based on human studies of resting-state brain network development (Zhao et al., 2019) and on the idea that at P12-15, the mother is capable of switching the pups' threat learning ability to a more immature state (Moriceau & Sullivan, 2006), I hypothesized that infant rats conditioned with maternal presence would show a more segregated functional network. These findings from human infants may not translate to infant rats, or the transition from a more segregated network

to a more integrated network as the infant brain develops may only apply to resting-state networks, not networks engaged during a threat learning experience.

Since the network engaged by pups conditioned with maternal presence was more integrated, there would naturally be less variability in how much each node participates in network activity as a whole. Indeed, the participation coefficients and betweenness centrality for each region in this group were all very similar, in contrast to pups conditioned without maternal presence. In the latter group, the LaDL, and BLA were regions with the most diverse intermodular connections and had the highest weighted betweenness centrality, suggesting that they may be hub regions engaged during threat learning. This is in line with previous studies that show that the lateral/basolateral amygdala is critical for threat learning in infancy (Moriceau & Sullivan, 2006; Opendak et al., 2019).

Our results suggest that the functional network engaged by pups undergoing threat learning with maternal presence has stronger overall functional connectivity, a highly integrated structure, and no obvious hub regions relative to pups conditioned without maternal presence. This analysis could benefit from the inclusion of additional regions implicated in threat learning, social behavior, and the stress response and from a larger sample size, however these results have raised many interesting questions. Is the high level of integration observed in pups conditioned with maternal presence a general effect of maternal presence, or is it specific to maternal presence during a threat learning experience? Is strong functional segregation necessary for the formation of a threat memory in infant rats? In summary, through this work I have demonstrated the exciting potential of applying the graph theoretical approach to understand functional network activity in infant rats.

3.6 Acknowledgements

This research was supported by the K08 MH014743-01A1 and NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation to Jacek Debiec. Amanda M. White was supported by NIH T-32-NS076401 and the Department of Psychiatry. I would like to thank Esha Kaul, Robert Maurer and Emma Vasquez for excellent technical support and dedication to this project.

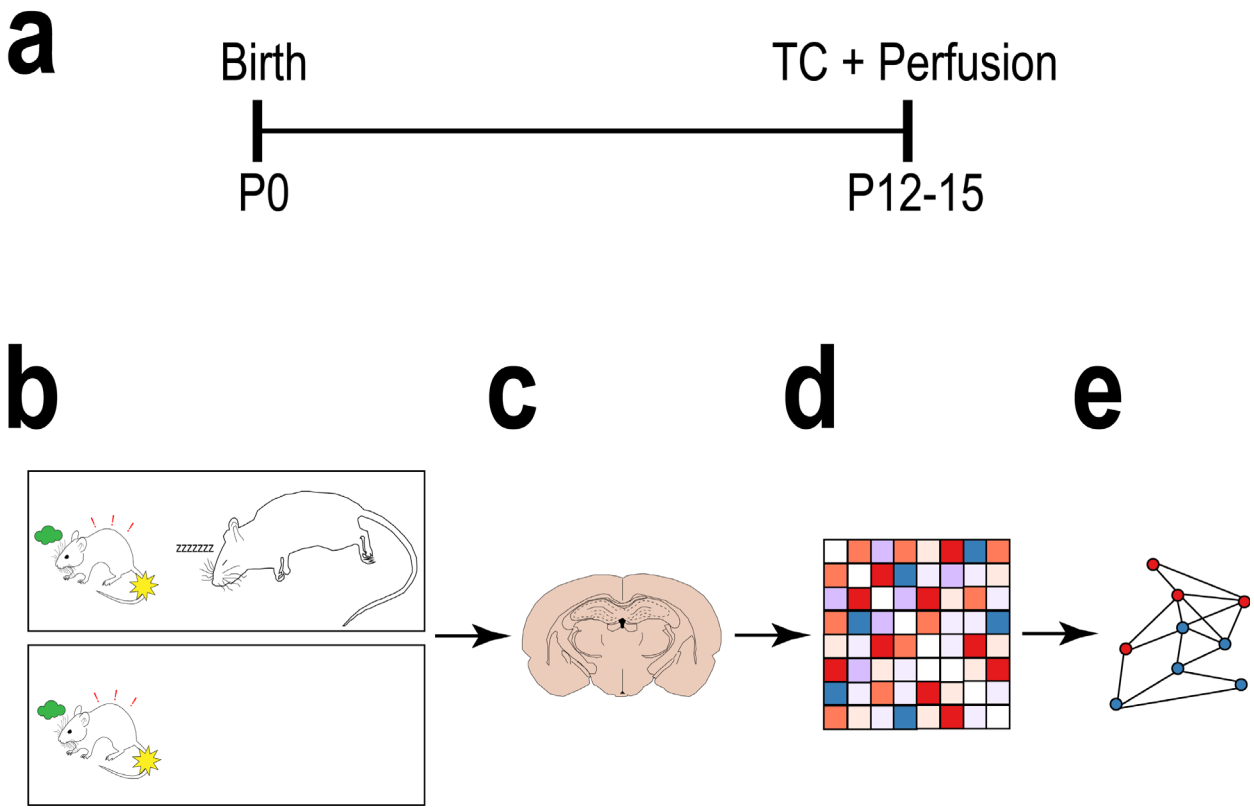

Figure 3.1 Experimental Timeline and Design. (a) The day pups were born was designed postnatal day zero (P0). Odor-shock conditioning and perfusions took place when pups were between 12 and 15 days old. (b) Threat conditioning took place with or without the presence of an anesthetized dam that was the pup's own mother or a mother of equivalent postpartum age. All pups received 11 pairings of a novel peppermint odor with a 0.5 mA shock to the tail. (c) Following threat conditioning and perfusion, pup brains were dissected out, post-fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose until brains sank, and then frozen at -80 C. Brains were then sectioned at 30 µm, underwent immunohistochemistry for c-Fos, and imaged on a light microscope. Using a rat brain atlas, structures were drawn on each image and c-Fos expression was counted by an investigator blind to experimental group assignment. (d) Correlation matrices for each experimental group were generated by computing Pearson's correlation coefficients between mean c-Fos counts in each region of interest. (e) Correlation matrices were run through a community detection algorithm in order to analyze network structure.

REGION OF INTEREST	REGION OF INTEREST CATEGORY	MEAN NO MATERNAL PRESENCE	MEAN MATERNAL PRESENCE	*P*
BLA	Amygdala	27.54	25.83	0.86
BLV	Amygdala	4.301	5.49	0.70
BMA	Amygdala	24.42	25.47	0.92
Aco	Amygdala	42.03	43.81	0.91
Pir	Cortex	159.6	197	0.61
MeAD	Amygdala	17.34	23.18	0.41
MeAV	Amygdala	13.45	11.7	0.72
CeL	Amygdala	20.19	10.46	0.23
CeC	Amygdala	29.43	23.11	0.61
CeM	Amygdala	10.25	10.06	0.96
Prl	Cortex	44.62	62.82	0.55
GI	Cortex	39.85	52.48	0.59
DI	Cortex	31.4	46.62	0.52
AIP	Cortex	52.91	60.33	0.79
LaDL	Amygdala	9.82	15.17	0.37

Table 3.1 Differences in mean c-Fos positive cells between infant rats conditioned with no maternal presence and pups conditioned with maternal presence. Abbreviations: BLA, basolateral amygdaloid nucleus, anterior part; BLV, basolateral amygdaloid nucleus, ventral part; BMA, basomedial amygdaloid nucleus, anterior part; Aco, anterior cortical amygdaloid nucleus; Pir, piriform cortex; MeAD, medial amygdaloid nucleus, anterior dorsal part; MeAV, medial amygdaloid nucleus, anterior ventral part; CeL, central amygdaloid nucleus, lateral part; CeC, central amygdaloid nucleus, capsular part; CeM, central amygdaloid nucleus, medial part; Prl, prelimbic cortex; GI, granular insular cortex; DI, dysgranular insular cortex; AIP, agranular insular cortex, posterior part; LaDL, lateral amygdaloid nucleus, dorsolateral part.

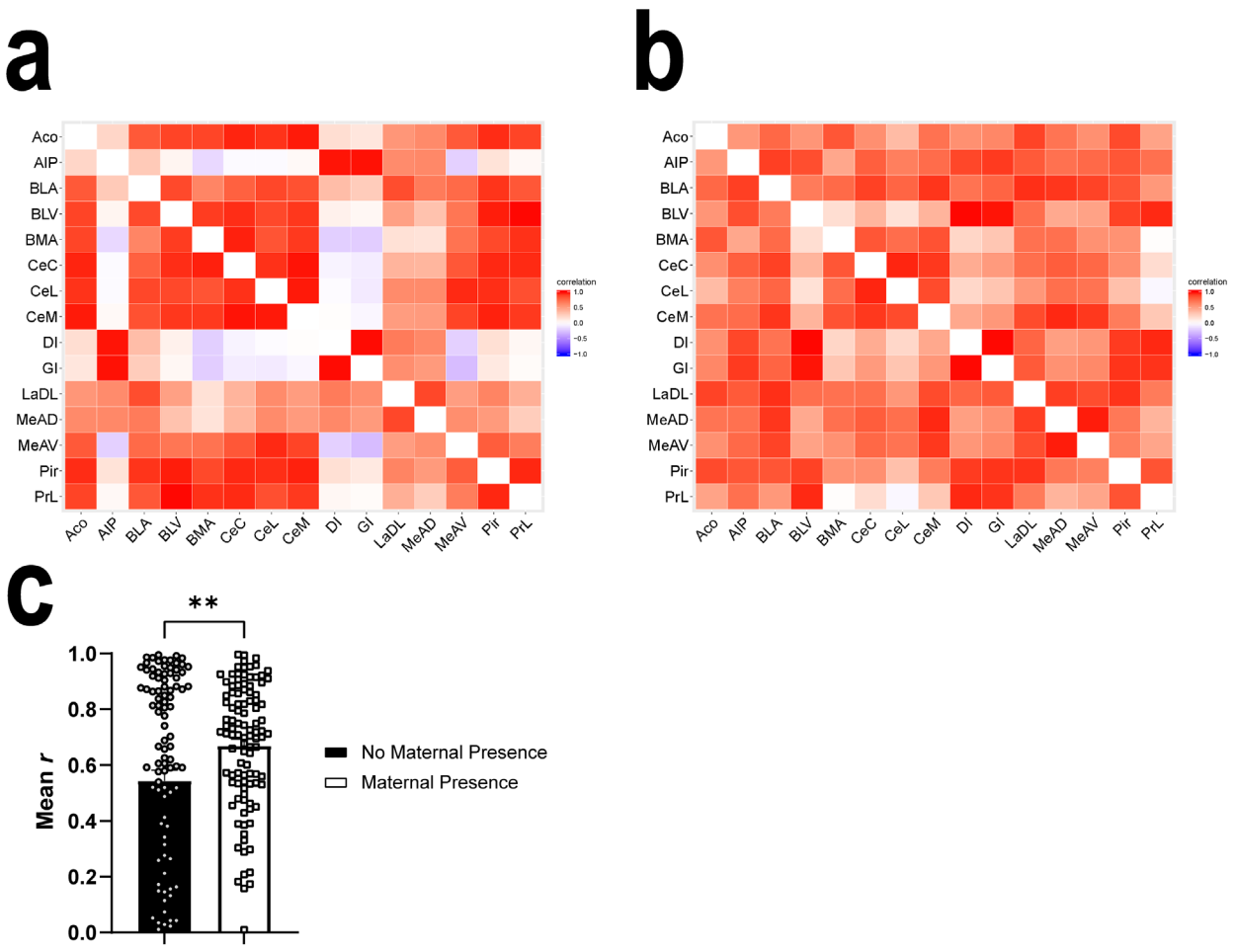

Figure 3.2 Correlation Matrices and Functional Connectivity. (a) Correlation matrix for pups conditioned without maternal presence. Each square represents the Pearson's correlation coefficient computed by correlating the mean number of c-Fos positive cells between two regions of interest. (b) Correlation matrix for pups conditioned with maternal presence. Each square represents the Pearson's correlation coefficient computed by correlating the mean number of c-Fos positive cells between two regions of interest. (c) Overall functional connectivity was compared between pups conditioned without and with maternal presence by averaging all unique correlations and performing a Student's t-test. Pups conditioned with maternal presence had stronger functional connectivity than pups conditioned without maternal presence, $p = 0.005$. Error bars represent SEM. $^{**}p < 0.01$.

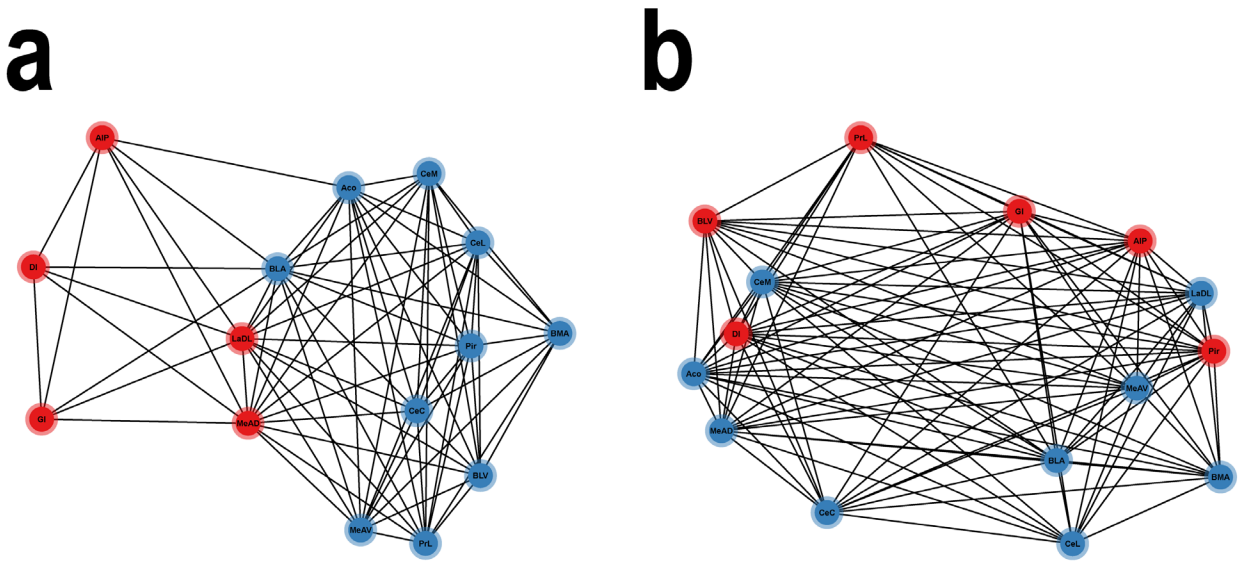

Figure 3.3 Functional network maps. (a) In the pups conditioned without maternal presence, the community detection algorithm identified two modules. Nodes within module 1 are colored red; nodes within module 2 are colored blue. The links between nodes represent the Pearson's correlation coefficient between regions; for visualization purposes, correlation coefficients less than 0.2 were excluded from network maps. (b) In the pups conditioned with maternal presence, the community detection algorithm identified two modules. Nodes within module 1 are colored red; nodes within module 2 are colored blue. The links between nodes represent the Pearson's correlation coefficient between regions; for visualization purposes, correlation coefficients less than 0.2 were excluded from network maps.

REGION OF INTEREST	NO MATERNAL PRESENCE	MATERNAL PRESENCE
BLA	2	2
BLV	2	1
BMA	2	2
Aco	2	2
Pir	2	1
MeAD	1	2
MeAV	2	2
CeL	2	2
CeC	2	2
CeM	2	2
Prl	2	1
GI	1	1
DI	1	1
AIP	1	1
LaDL	1	2

Table 3.2 Module assignment. The community detection algorithm identified two modules within the network engaged by pups conditioned without maternal presence and the network engaged by pups conditioned with maternal presence. The module assignment of each node for each experimental group is listed above.

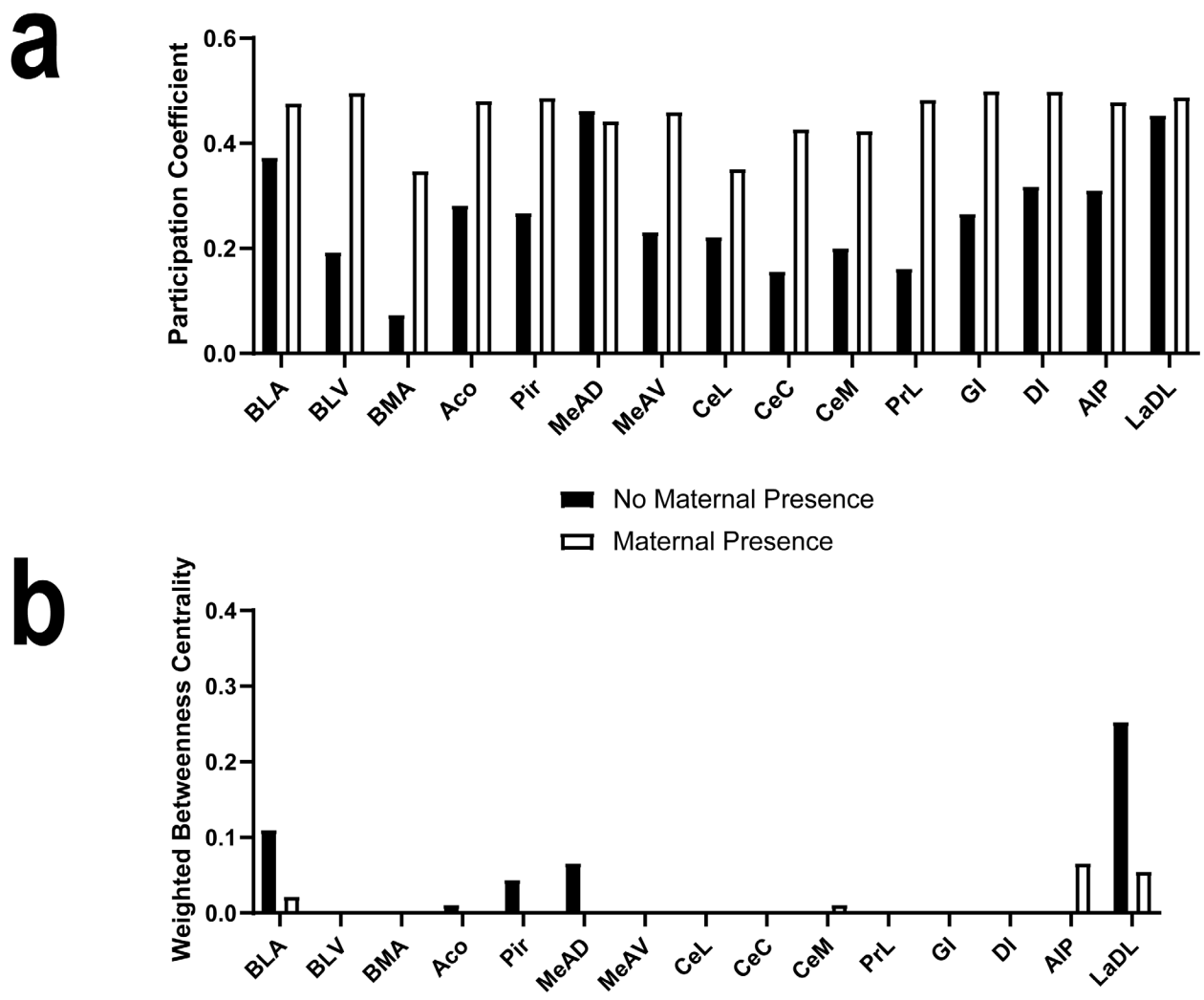

Figure 3.4 Hub metrics. (a) The participation coefficient is a graph theoretical measure which describes the diversity of connections each node has with nodes outside of its own module. Regions with the highest participation coefficients may serve as hubs. In the pups conditioned without maternal presence, the MeAD, LaDL, and BLA had the highest participation coefficients; while in the pups conditioned with maternal presence, the GI, DI, and BLV had the highest participation coefficients but there was much less variability in participation coefficient values. (b) Betweenness centrality is a graph theoretical measure that refers to the fraction of shortest paths on which each node lies. Regions with the highest betweenness centrality may serve as hubs. In the pups conditioned without maternal presence, the LaDL, BLA, and MeAD had the highest betweenness centrality; while in the pups conditioned with maternal presence, the AIP, LaDL, and BlA had the highest betweenness centrality.

Chapter 4 Early acquisition of threat conditioning in a selectively-bred anxiety-like rat phenotype: regulation by maternal presence and FGF2

4.1 Abstract

Temperament is an innate, stable predisposition towards particular emotional and behavioral responses. In humans, certain temperaments are associated with a heightened risk of developing anxiety later in life. Non-human animals, including rodents, also exhibit innate, stable dispositions; these are referred to as behavioral phenotypes. The interaction between behavioral phenotype and early life adverse events is critical for the development of maladaptive anxiety. Rodent studies of typically developing animals have identified a number of mechanisms that protect against aversive experiences in early life. One such mechanism is an early life quiescence of threat learning, which protects against the effects of stress and facilitates safety and attachment learning. However, little is known about the factors that alleviate the effects of early life aversive events on phenotypes vulnerable to pathological anxiety. Here, we examined threat learning and the stress response in selectively-bred infant rats that show an anxiety-like phenotype relative to typically developing animals. We investigated the potential roles of maternal presence and the anxiolytic neurotrophic factor fibroblast growth factor 2 (FGF2) in regulating threat learning and the stress response in infant anxiety-like phenotype animals. We observed that rats selectively-bred for anxiety-like behaviors could acquire conditioned freezing earlier in life than typically developing animals. FGF2 administration on postnatal day 1 (P1) and maternal presence during threat conditioning were both capable of suppressing this early

emergence of conditioned freezing. However, neither FGF2 nor maternal presence during threat conditioning were associated with reduced corticosterone levels during threat conditioning. Our results suggest that although an anxiety-like phenotype may be associated with early threat learning, environmental factors (such as maternal presence) and pharmacological intervention (such as modulation of the FGF2 system) may be capable of counteracting that early aversive learning. Interventions in vulnerable infants may thus decrease the impact of aversive events.

4.2 Introduction

Temperament is an innate, stable disposition that leads an individual to exhibit particular emotional and behavioral responses. Certain features of temperament are expressed very early in life and may predispose individuals for certain outcomes. For example, some children are comfortable in novel situations, while others display signs of discomfort and will cling to their parent or cease play in the presence of unfamiliar objects or individuals (N. A. Fox et al., 2005; Kagan et al., 1988). This inhibited response to novelty in early childhood appears to be stable and heritable and is referred to as behavioral inhibition (Cyphers et al., 1990; N. A. Fox et al., 2005; Kagan et al., 1988). Children who exhibit this behaviorally inhibited temperament are at a heightened risk for anxiety disorders (Muris et al., 2011). Behavioral phenotypes characterized by inhibited behavior in novel environments can also be observed in other animal species, including non-human primates (A. S. Fox & Kalin, 2014; Williamson et al., 2003) and rodents (Pawlak et al., 2008; Piazza et al., 1989).

After many generations of selectively breeding rats based on their response to a novel environment, the Akil lab has developed two lines of Sprague-Dawley (SD) rats that represent the extremes of this phenotype – those that show high activity in a novel environment and those

that show low activity in a novel environment, which the authors term “Bred Low-Responders (bLR).” In addition to exhibiting differences in their exploratory behaviors, bLR rats tend to show increased anxiety-like behaviors as adults. Specifically, comparing to animals showing high activity in novel environments, bLR rats spend less time in the open arms of the elevated plus maze, spend less time in the light area of the light-dark box, and spend less time in the center of an open field (J. D. Stead et al., 2006). Additionally, adult bLR rats show deficits in extinction of threat learning and extinction retention (Prater et al., 2017).

Rodent studies using both genetically engineered and selectively bred models have identified a number of candidate neurobiological mechanisms underlying anxious-like phenotypes (Jacobson & Cryan, 2010; Le-Niculescu et al., 2011; S. E. Walker et al., 2017; Wegener et al., 2012). Several studies have pointed to the role of neurotrophic factors in the regulation of affective-related behaviors (Chen et al., 2006; Duman & Monteggia, 2006). One of the neurotrophic factors involved in anxiety-like behavior in rats is fibroblast growth factor 2 (FGF2) (Turner et al., 2012). In typically developing adult rats, lower endogenous levels of FGF2 have been associated with greater anxiety-like behavior in the elevated plus maze (Eren-Koçak et al., 2011) and higher cue-induced freezing (Graham et al., 2017). Furthermore, knockdown or knock-out of FGF2 in typically developing rodents led to increased anxiety-like behaviors in the elevated plus maze (Eren-Koçak et al., 2011; Salmaso et al., 2016). Knock-out of FGF2 led to elevated baseline corticosterone levels, and enhanced corticosterone response to stress (Salmaso et al., 2016). The FGF2 system also appears to play a critical role in the behavioral phenotype exhibited by bLR rats, as adult bLR rats show lower endogenous levels of FGF2 mRNA in the hippocampus (Turner et al., 2011).

Early life intervention could modify some features of anxiety-like phenotypes, such as responses to stress and adversity. In typically developing animals, administration of FGF2 has been shown to influence threat learning, extinction, and anxiety-like behaviors (Eren-Koçak et al., 2011; Graham & Richardson, 2009b, 2010b, 2011, 2015; Walters et al., 2016). In bLR rats, a single subcutaneous (s.c.) injection of FGF2 on the day after birth was associated with less anxiety-like behavior in adulthood than bLR rats given a vehicle injection (Turner et al., 2011). Additionally, bLR rats that received an FGF2 injection showed improved extinction of conditioned threat responses and improved extinction retention in adulthood (Prater et al., 2017).

The bLR model is particularly useful as it can shed light on the behavioral phenotypes that predict susceptibility to anxiety disorders and on the developmental timepoints at which preventive interventions may prove most effective. This is a critical question for translational applications, as anxiety disorders typically develop in childhood and early intervention may lead to improved outcomes later in life. However, not all children with behavioral inhibition will go on to develop anxiety disorders; the interaction between behavioral phenotype and the environment is critical for the development of maladaptive anxiety. One of the known experiential risk factors for anxiety disorders is an aversive experience. Most of what is known about the neurobiology of aversive experiences comes from human and animal studies of threat learning in phenotypically normal adults, and little is known about early life aversive experiences in the anxious-like phenotypes.

Studies using outbred rat pups show that until the animals start leaving the nest around postnatal day (P) 10, the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky & Meaney, 1986; Schapiro et al., 1962) and amygdala are generally quiescent, and Pavlovian threat learning does not typically occur (Regina M Sullivan et al., 2000; Thompson et al., 2008). It is hypothesized

that the lower activity of the HPA axis prior to P10 is primarily responsible for the quiescence of threat learning at this age, as injection of corticosterone prior to threat conditioning in pups younger than P10 can drive synaptic plasticity in the amygdala and support the acquisition of threat learning until pups are P16 (Moriceau et al., 2004a, 2006; Moriceau & Sullivan, 2006). Additionally, a dam that expresses defensive responses in the presence of a threat conditioned cue can acutely elevate corticosterone levels in pups and transfer that conditioned threat to her P6-7 pups (Debiec & Sullivan, 2014). Once rat pups reach ten days of age the HPA axis becomes more reactive to stressors (Sapolsky & Meaney, 1986) and Pavlovian threat learning to olfactory cues emerges (Regina M Sullivan et al., 2000). However, if the pup undergoes threat conditioning in the presence of a calm mother, threat learning and the accompanying corticosterone response is suppressed (Moriceau et al., 2006). The low reactivity of the HPA axis in very young pups and the ability of maternal presence to extend the period of this low reactivity in pups from P10-15 are thought to be adaptive mechanisms by which the still-developing pup is protected from the deleterious effects of stress, including a learned aversion to a caretaker (Debiec & Sullivan, 2017a; Sapolsky & Meaney, 1986)

A recent work has shown that early-life adversity can alter infant HPA axis reactivity and compromise the mother's ability to alter pups' stress hormone response (Opendak et al., 2020). However, how inherited deficits associated with dysfunctional infant stress systems impact the mother's ability to regulate her pups stress hormone levels has yet to be explored. Dysfunction of pups' stress system, especially related to elevated corticosterone levels and its suppression by the mother is of substantial importance because even one day of increased stress hormone levels may have an enduring impact on later life neurobehavioral function (Mitra & Sapolsky, 2008).

Here, we examine the ontogeny of threat learning in bLR rats and compare it to the ontogeny of threat learning in outbred SD rats. We also examine whether maternal presence is capable of regulating threat learning and the response to stress in these bLR rats and whether the administration of FGF2 early in life influences the emergence of threat learning in bLR pups.

4.3 Methods

4.3.1 ANIMALS

Outbred SD (Charles River) breeding pairs and bLR breeding pairs were mated and all pups were born and raised in our colony. The room was set to a 12:12 h light:dark cycle and food and water were freely available. bLR animals were obtained from the Akil laboratory colony. These animals were initially bred from Sprague-Dawley rats obtained from multiple commercial vendors and henceforth selectively bred in the Akil laboratory. bLR pups were offspring of the F43, F48, F50, or F56 generations (J. D. Stead et al., 2006). All animal handling and behavioral experiments were conducted by a female experimenter. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

4.3.2 THREAT CONDITIONING AND FREEZING TEST

On P4, SD and bLR pups were placed in individual plastic beakers fixed with individual Plexon tubing attached to an olfactometer. A heating pad was placed underneath the beakers in order to maintain pups' body temperature. After 10 minutes of habituation, pups were exposed to 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned stimulus (US) pairings, 11 unpaired CS and US presentations, or 11 CS presentations. Shocks were manually delivered by the experimenter to the pup's tail. Each presentation was separated by a 4-minute inter-trial interval (Moriceau & Sullivan, 2006). Pups were immediately returned to the

home cage at the end of the conditioning session. Beakers were cleaned with water between groups to prevent additional cleaning odors and to prevent the transfer of threat via smell to other experimental groups.

At P11, threat memory was assessed by exposing pups to 3 30s CS presentations separated by a 2-minute inter-trial interval. Freezing behavior during the CS presentations was manually scored. Freezing behavior was defined as a clear discontinuation of any movement that is not associated with breathing or other involuntary actions.

4.3.3 CORTICOSTERONE

Pups were threat conditioned or exposed to the odor CS as described above on P4 and sacrificed immediately following the end of threat conditioning. A separate group of animals were removed from the home cage and sacrificed immediately in order to establish baseline corticosterone levels. Collection took place between 10am and 2pm. Trunk blood was collected in EDTA tubes which were subsequently centrifuged at 3000 rpm for 10 minutes at 4 C. Following centrifugation, serum was extracted and stored at -80 C until being sent to the UM Core Facility for radioimmunoassay. Radioimmunoassay was performed using the MP Bio Corticosterone Double Antibody RIA Kit (Irvine, CA, USA).

4.3.4 MATERNAL BUFFERING

Dams of the experimental animals or dams of an equivalent postpartum age maintained on an identical diet were deeply anesthetized with sodium pentobarbital prior to maternal buffering experiments. When the toe pinch response was no longer observed, dams were then placed with the experimental animals in an empty cage lined with an absorbent blue pad. Threat conditioning then commenced as described above (Moriceau & Sullivan, 2006).

4.3.5 FGF2

FGF2 (50 ng/g, Sigma) was dissolved in a vehicle solution of 0.1 M PBS with 0.1% BSA. On P1, bLR pups were injected with FGF2 (20 ng/g, s.c. in 50 ul 0.1 M PBS with 0.1% BSA) or a vehicle solution (s.c. 50 ul 0.1 M PBS with 0.1% BSA) on P1 (Turner et al., 2011). Pups were threat conditioned as described above on P4 and either returned to the home cage to undergo a freezing test at P11 or were sacrificed immediately after conditioning for corticosterone assay.

4.3.6 STATISTICS

Data were analyzed in GraphPad Prism (San Diego, CA, USA) using analyses of variance. Dunnett's multiple comparisons test and Tukey's post hoc test were additionally used where appropriate. Differences between groups were considered significant where $p < 0.05$.

4.4 Results

4.4.1 BLR PUPS SHOW AN EARLY EMERGENCE OF THREAT LEARNING AND ALTERED STRESS RESPONSIVITY DURING THREAT LEARNING

Outbred SD rat pups that underwent threat conditioning at P4 ($n = 10$) did not freeze more to the CS at test than outbred SD rat pups that were exposed to the CS alone ($n = 9$) or the unpaired CS and US ($n = 8$), $F(2,24) = 0.89$, $p = 0.42$ (Figure 4.1a). However, a 1-way ANOVA comparing mean freezing that bLR pups showed to the CS at test was significant, $F(2,27) = 5.34$, $p = 0.01$ (Figure 4.1b). Dunnett's multiple comparisons test revealed that bLR pups that underwent threat conditioning ($n = 11$) froze significantly more to the CS at test than pups that were exposed to the CS alone ($n = 9$; $p = 0.01$) or the unpaired CS and US ($n = 10$; $p = 0.04$).

We next examined the corticosterone response to the experience of threat conditioning or exposure to the CS only at P4 in outbred SD rat pups and bLR pups. In outbred SD rat pups, serum corticosterone levels differed across all 3 collection groups, $F(2,24) = 11.83$, $p = 0.0003$ (Figure 4.1c). Tukey's post hoc test was used to test for differences in corticosterone levels between each pair of experimental conditions. Pups that underwent threat conditioning showed significantly higher serum corticosterone levels than pups that were exposed to the CS alone ($p = 0.03$) or pups that were taken directly from their cage ($p = 0.0002$). There were no differences in serum corticosterone levels between pups that were exposed to the CS alone or were taken directly from the cage ($p = 0.10$).

In bLR pups, serum corticosterone levels differed across the 3 collection groups, $F(2,26) = 8.65$, $p = 0.001$ (Figure 4.1d). Tukey's multiple comparison's test revealed that relative to corticosterone levels at baseline ($n = 10$), serum corticosterone levels in pups that underwent threat conditioning ($n = 10$; $p = 0.005$) and pups that were exposed to the CS alone ($n = 9$; $p = 0.003$) were significantly higher. There were no significant differences in serum corticosterone levels between pups that underwent threat conditioning and pups that were exposed to the CS alone ($p = 0.95$).

4.4.2 MATERNAL PRESENCE DURING ACQUISITION REGULATES RETENTION OF LEARNED THREAT RESPONSES IN BLR PUPS BUT NOT CORTICOSTERONE RELEASE DURING THREAT LEARNING

We next examined whether maternal presence was capable of regulating expression of learned threat and corticosterone release during threat learning in bLR pups. These freezing data were compared with the previous freezing data from pups that underwent threat conditioning in the absence of an anesthetized mother. Average freezing to the 3 CS presentations differed

across all 4 conditioning groups, $F(3,36) = 8.74$, $p = 0.0002$. Using Dunnett's multiple comparison's test, we found that bLR pups that underwent threat conditioning in the presence of an anesthetized mother ($n = 10$) froze significantly less to the CS at test than pups that underwent threat conditioning in the absence of an anesthetized mother ($p < 0.0001$) (Figure 4.2a).

To examine whether the mother was capable of regulating corticosterone release during threat conditioning, we collected trunk blood samples from an additional group of bLR pups that were conditioned in the presence of an anesthetized mother. Serum corticosterone levels in these pups were then compared to those from bLR pups in the previous corticosterone experiment. Serum corticosterone levels differed across the 4 collection groups, $F(3,35) = 7.38$, $p = 0.0006$. As described above, serum corticosterone levels in threat conditioned pups and pups that were exposed to the CS alone were significantly higher than baseline levels, p's = 0.02. However, Tukey's post hoc test found no significant differences in serum corticosterone levels between pups that underwent threat conditioning in isolation relative to pups that underwent threat conditioning in the presence of an anesthetized mother ($n = 10$), $p = 0.42$ (Figure 4.2b). Serum corticosterone levels were also significantly higher in pups that underwent threat conditioning with maternal presence relative to baseline, $p = 0.0004$.

4.4.3 FGF2 ADMINISTRATION AT P1 BLOCKS THE EARLY EMERGENCE OF THREAT LEARNING IN BLR PUPS BUT DOES NOT MODULATE CORTICOSTERONE RESPONSE DURING THREAT CONDITIONING

Finally, we examined whether early life administration of FGF2 modulated the early emergence of threat learning in bLR pups. At P4, threat conditioning or exposure to 11 CS presentations occurred as described above. When exposed to the CS again in a freezing test at P

11, we observed that FGF2-treated pups (n = 16) froze less to the cue than Vehicle-treated pups (n = 17), $t(31) = 2.54$, $p = 0.02$ (Figure 4.3a).

In a separate group of animals, we next asked whether early life administration of FGF2 modulated the corticosterone response during threat conditioning. A two-way ANOVA revealed a significant main effect of conditioning group on serum corticosterone levels, $F(2,33) = 18.14$, $p < 0.0001$. However, the effect of FGF2 treatment was not significant, $F(1,33) = 1.64$, $p = 0.21$, nor was there a significant interaction effect, $F(2,33) = 0.19$, $p = 0.82$. Within FGF2-treated pups and vehicle-treated pups, serum corticosterone levels were significantly higher in pups that underwent threat conditioning relative to those that received exposure to the CS alone ($p = 0.009$; $p = 0.01$) and relative to those that were sacrificed immediately after removal from the home cage ($p = 0.006$; $p = 0.006$) (Figure 4.3b).

4.5 Discussion

Here, we examined the ontogeny of threat learning in a line of rats that show high anxiety-like behavior as a result of selective breeding for inhibited behavior in a novel environment. Previous studies have shown that rat pups younger than P10 typically do not show avoidance of odors that have been paired with mild shock (Regina M Sullivan et al., 2000) and that freezing behavior in response to noxious stimuli emerges as rat pups approach weaning age (Burman et al., 2014; Deal et al., 2016; Richardson et al., 2002; Rudy, 1993; L. K. Takahashi, 1992; Wiedenmayer & Barr, 2001). Consistent with these findings, we observed that typically-developing outbred SD rats did not show conditioned freezing responses when trained at P4 and tested at P11. However, we found that bLR rat pups can acquire threat learning and express that learning on a freezing test at P11. This early emergence of cued threat-induced freezing could be

diminished in bLR pups that underwent threat conditioning in the presence of an anesthetized mother and in bLR pups that received an FGF2 injection on P1.

Although there is a significant body of literature supporting the relationship between FGF2 and threat learning, the nature of this relationship differs based on the context. Administration of FGF2 prior to threat conditioning has been shown to enhance contextual threat learning in 19-day old rats (Graham & Richardson, 2009a). Furthermore, daily administration of FGF2 from P1-5 (but not a single administration on P1) was associated with a precocious emergence of contextual threat learning on P16 (Graham & Richardson, 2010a). Acute FGF2 administration has also been found to facilitate the acquisition of extinction learning and retention of extinction learning (Graham & Richardson, 2009b, 2010b). Taken together, these results suggest that acute FGF2 may enhance learning processes in general and chronic administration of FGF2 in early life may accelerate the development of contextual threat learning. Additionally, these effects of FGF2 on the early emergence of contextual threat learning were observed after multiple FGF2 injections over several days (Graham & Richardson, 2010a). It is important to note that the above-mentioned studies were performed in wild-type animals, and that here we administered FGF2 to rats of the bLR phenotype. In bLR animals, a single administration of FGF2 the day after birth suppressed the early emergence of cued threat learning. Other studies have suggested that the effects of early life FGF2 administration are indeed dependent on phenotype (Turner et al., 2016).

Although maternal presence during threat conditioning and P1 FGF2 treatment were associated with suppressed cue-induced freezing in bLR pups, these manipulations were not sufficient to suppress serum corticosterone levels during threat conditioning. These results are particularly surprising because in typically developing animals, corticosterone appears to

regulate the emergence of threat responses. Administration of corticosterone to preweanling pups can drive early expression of a freezing response to predator odor (Moriceau et al., 2004a) and the acquisition of threat learning in the presence of the mother (Moriceau & Sullivan, 2006). In bLR pups, stress reactivity and threat learning may be operating independently at this age because elevated corticosterone levels did not appear to be sufficient in supporting the acquisition of threat learning in bLR pups. Maternal buffering and FGF2 administration may instead lead to suppressed threat learning by altering neuronal activity within the network of brain areas that underlie infant threat learning or that support the conditioned freezing response (Chang & Debiec, 2016; Wheeler et al., 2013).

In contrast to what we observed in non-injected bLR pups, we found significantly elevated corticosterone levels in both FGF2 and vehicle-injected bLR pups following threat conditioning relative to following exposure to the CS alone. Baseline levels of corticosterone were also higher in injected bLR pups (regardless of injection type) relative to non-injected bLR pups. The experience of receiving a painful injection in the absence of the dam on P1 could have altered the developmental trajectory of bLR pups' stress response system. Although rat pups are considered to be in a stress hyporesponsive period until about P10, studies of typically developing animals suggest that the experience of stress or pain in the absence of the dam can induce acute corticosterone release. Several studies suggest that newborn rats that experience pain in the absence of the dam show elevated corticosterone levels between 24 hours and up to 7 days after the painful experience (Butkevich et al., 2013; Mooney-Leber et al., 2018; Victoria et al., 2014).

The bLR behavioral phenotype appears to be characterized by more robust learned threat responses in infancy in addition to increased spontaneous anxiety-like behaviors observed later

in life. Our model of early threat learning in anxiety-like phenotype demonstrates that the relationship between threat conditioning, stress response, and maternal regulation of threat conditioning may be more complex than what studies of typically developing animals show. Our results suggest that although an anxious-like temperament may be associated with early threat learning, environmental factors (such as maternal presence) and pharmacological intervention (such as modulation of the FGF2 system) may be capable of counteracting that early threat learning. Psychosocial and pharmacological interventions in vulnerable infants may therefore increase resilience to adverse events and potentially decrease the risk of developing anxiety disorders later in life.

4.6 Acknowledgements

This research was supported by the K08 MH014743-01A1 and NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation to Jacek Debiec and NIDA U01DA043098; NIH R01MH104261; Office of Naval Research (ONR) N00014-12-1-0366 and 00014-19-1-2149; The Hope for Depression Research Foundation (HDRF): the Pritzker Neuropsychiatric Research Consortium to Huda Akil. Amanda M. White was supported by NIH T-32-NS076401 and the Department of Psychiatry. I would like to thank Freddie Delarosa, Joanna Hider, Da-Jeong Chang, Elaine Hebda-Bauer, Vartika Parashar, Rachel Ritter, Alex Stefanov, Cortney Turner, and Mary Zylinski for excellent technical support and dedication to this project. I would also like to thank Huda Akil, Elaine Hebda-Bauer, Cortney Turner, and Regina Sullivan for helpful comments on previous drafts of this manuscript.

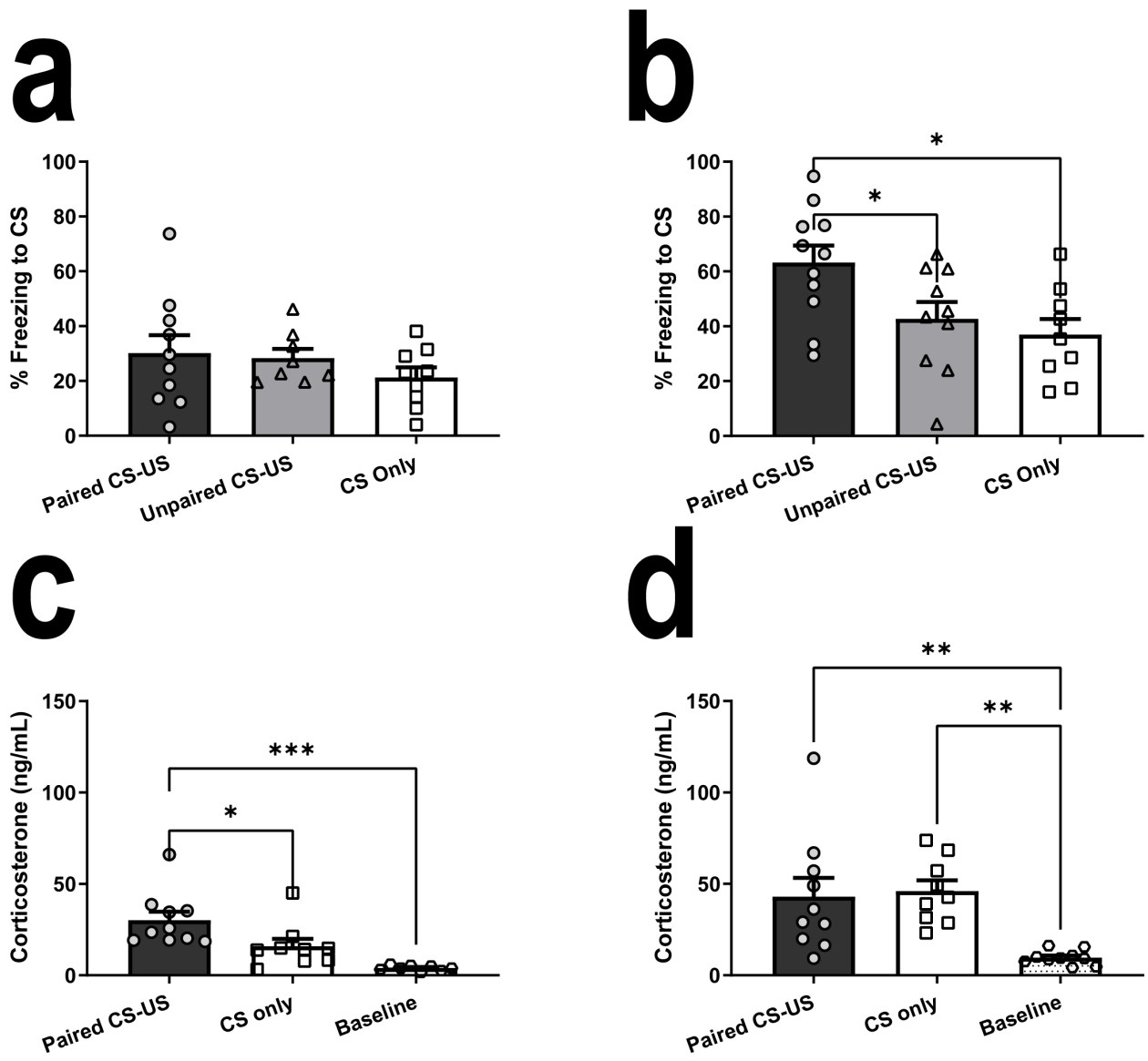

Figure 4.1 Threat Conditioning-Induced Freezing and Stress Response in Bred Low-Responder (bLR) and Typically-Developing Pups (A) Outbred Sprague-Dawley pups were exposed to 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned stimulus (US) pairings, 11 unpaired CS and US presentations, or 11 CS presentations during training at postnatal day 4, an age at which pups typically do not acquire classical threat conditioning. Average cue-induced freezing was measured during 3 odor CS presentations at postnatal day 11. Outbred SD rat pups that underwent threat conditioning at P4 did not freeze more to the CS at test than outbred SD rat pups that were exposed to the CS alone or the unpaired CS and US (B) At postnatal day 4, bLR pups were exposed to 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned stimulus (US) pairings, 11 unpaired CS and US presentations, or 11 CS presentations. Average cue-induced freezing was measured during 3 odor CS presentations at postnatal day 11. bLR pups that underwent threat conditioning froze significantly more to the CS at test than pups that were exposed to the CS alone or the unpaired CS and US. (C) Average serum corticosterone levels in outbred Sprague-Dawley pups exposed to 11 CS-US pairings or 11 CS presentations relative to average serum corticosterone levels from pups taken directly from the home cage. Pups that underwent threat conditioning showed significantly higher serum corticosterone levels than pups that were exposed to the CS alone or pups that were taken directly from their cage. (D) Average serum corticosterone levels in bLR pups exposed to 11 CS-US pairings, 11 CS presentations, 11 CS-US pairings in the presence of an anesthetized mother, or taken directly from the home cage. Pups that underwent threat conditioning and pups that were exposed to the CS alone showed significantly higher serum corticosterone levels than pups that were taken directly from their cage. Error bars represent SEM. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$

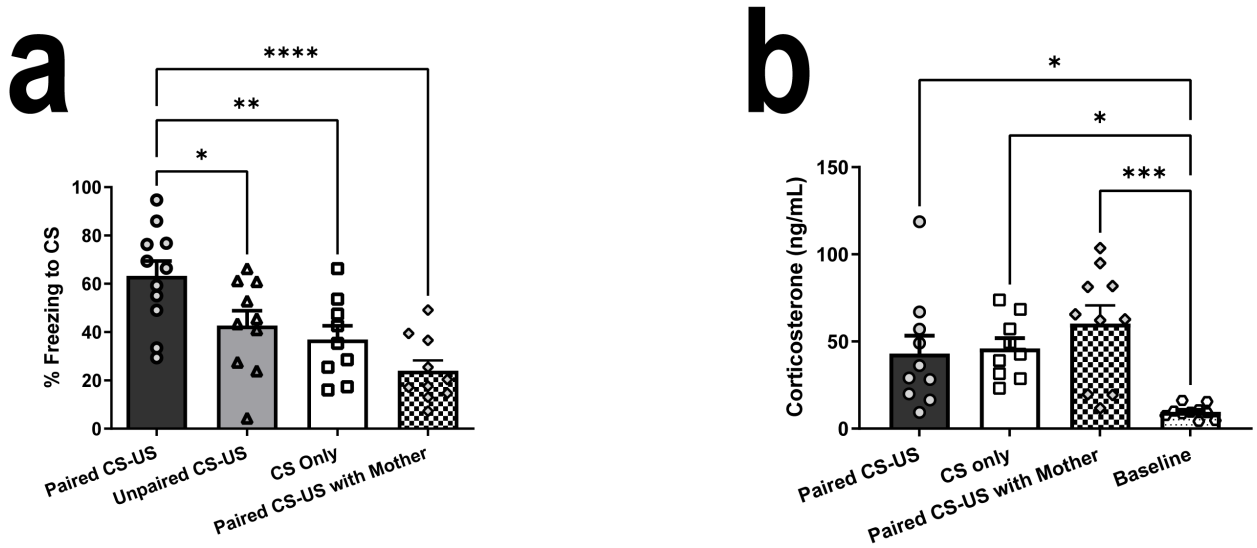

Figure 4.2 Effect of maternal presence during threat conditioning on conditioned freezing and stress response (A) At postnatal day 4, bLR pups were exposed to 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned stimulus (US) pairings, 11 unpaired CS and US presentations, 11 CS presentations, or 11 CS-US pairings in the presence of an anesthetized mother. Average cue-induced freezing was measured during 3 odor CS presentations at postnatal day 11. bLR pups that underwent threat conditioning in the presence of an anesthetized mother froze significantly less to the CS at test than pups that underwent threat conditioning in the absence of an anesthetized mother (B) Average serum corticosterone levels in bLR pups exposed to 11 CS-US pairings, 11 CS presentations, 11 CS-US pairings in the presence of an anesthetized mother, or taken directly from the home cage. There were no significant differences in serum corticosterone levels between pups that underwent threat conditioning in isolation relative to pups that underwent threat conditioning in the presence of an anesthetized mother, but serum corticosterone levels were significantly higher in pups that underwent threat conditioning with maternal presence relative to baseline. Error bars represent SEM. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$ ****$p < 0.0001$

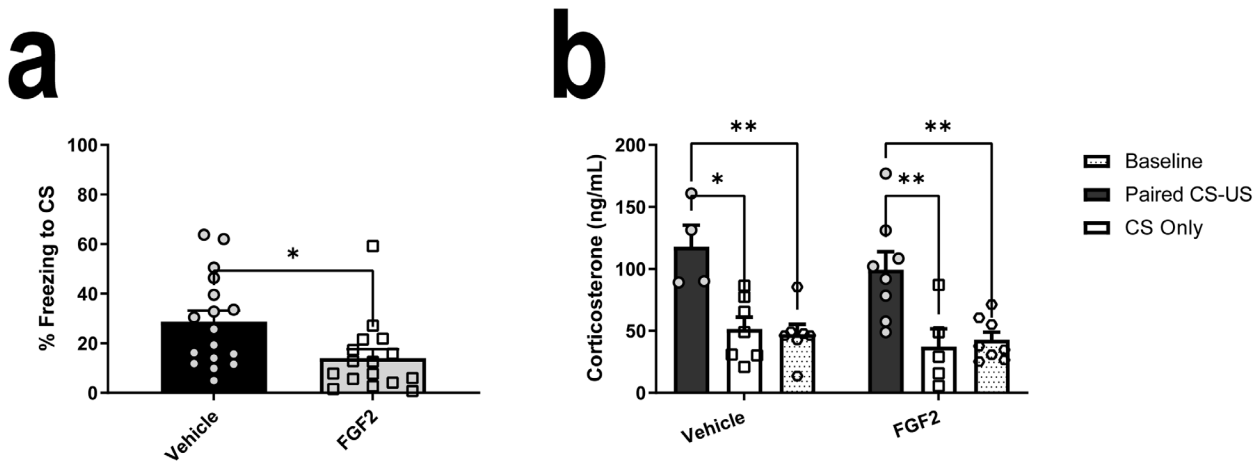

Figure 4.3 Effect of FGF2 Administration on Postnatal Day 1 on Threat Conditioned-Induced Freezing and Stress Response
(A) On postnatal day 4, FGF2-treated or vehicle-treated bLR pups were exposed to 11 30s peppermint odor conditioned stimulus (CS)-1s 0.5 mA shock unconditioned stimulus (US) pairings or 11 CS presentations. Average cue-induced freezing was measured during 3 odor CS presentations at postnatal day 11. FGF2-treated pups froze less to the CS than Vehicle-treated pups. (B) Average serum corticosterone levels in FGF2-treated or vehicle-treated bLR pups exposed to 11 CS-US pairings, 11 CS presentations, or taken directly from the home cage. Within FGF2-treated pups and vehicle-treated pups, serum corticosterone levels were significantly higher in pups that underwent threat conditioning relative to those that received exposure to the CS alone and relative to those that were sacrificed immediately after removal from the home cage. Error bars represent SEM. *$p < 0.05$ **$p < 0.01$.

Chapter 5 Summary and Future Directions

Maternal buffering is a vital function that protects the developing infant brain from the damaging effects of stress and ensures the infant remains securely attached to the mother until it is ready for independent life. Throughout this dissertation, I have shared the results of my experiments on the behavioral consequences, neuroendocrine mechanisms, and neural networks underlying maternal regulation of threat learning in typically-developing infant rats and in a vulnerable phenotype. In this chapter, I will summarize my findings and suggest future directions for research.

5.1 Maternal Regulation of Threat Learning in Typically Developing Animals

5.1.1 SUMMARY

In Chapter 1, I characterized threat conditioning-induced freezing in typically-developing pups who underwent threat conditioning with and without maternal presence at postnatal day (P) 13 and found that maternal buffering can be observed using a freezing test at P18, although freezing behavior does seem to be quite variable. Pups conditioned with maternal presence spent less time freezing during conditioned stimulus (CS) presentations and entered a freezing state less frequently during CS presentations. The duration of each freezing bout was only significantly shorter in pups conditioned with maternal presence during the third CS presentation. Taken together, these results suggest that differences in overall time spent freezing may be driven by pups conditioned without maternal presence entering a freezing state more frequently during CS presentations, rather than entering a freezing state and remaining immobile

for long periods of time. I also have preliminary findings which suggest that female pups may be more susceptible to maternal threat learning regulation than male pups.

These results bolster the findings from previous studies which demonstrated that pups conditioned with maternal presence showed preference for, not avoidance of, a conditioned odor in a Y-maze test (Moriceau & Sullivan, 2006; Opendak et al., 2019; Shionoya et al., 2007), and provide additional evidence for the important role of the mother in regulating threat learning in her pups. The defensive capabilities of rats extend beyond avoidance and freezing in response to threat-conditioned stimuli. There is a growing appreciation that conditioned suppression of ongoing behavior (Bouton & Bolles, 1980), darting (Gruene et al., 2015), conditioned flight (Totty et al., 2021), and other behaviors may be important to consider when assessing threat learning, especially since these are controlled by different neural circuits. Freezing is a behavior which is emerging during infancy (L. K. Takahashi, 1992). Analysis of these additional types of threat responses would provide a more complete understanding of threat learning and maternal regulation of these processes in infant rats. As the first study to use a freezing test to assess the effect of maternal presence during threat conditioning at P15, my work provides a next step in that direction.

5.1.2 FUTURE DIRECTIONS

The sex differences observed in threat-conditioned freezing were surprising in two ways: (1) they were present during a time in which the levels of sex steroids are much lower than in adults (Döhler & Wuttke, 1975) and (2) since prior studies have shown that male pups tend to receive more direct maternal care (Deviterne & Desor, 1990; Moore & Morelli, 1979; Richmond & Sachs, 1984), I might expect that maternal buffering would be stronger in male pups.

Despite lower levels of circulating sex steroids than adult rats, levels of sex steroids do differ between male and female pups during infancy, depending on pup age and which sex steroid is being assayed (Döhler & Wuttke, 1975). To examine whether the observed sex differences are related to differences in levels of the circulating sex steroids estradiol and testosterone, I could use antagonists to pharmacologically block signaling at the respective receptors for these steroid hormones during fear conditioning with and without maternal presence. Alternatively, I could conduct experiments with gonadectomized animals. Social buffering of conditioned freezing does not appear to depend on estrus cycle stage in adult female rats (Ishii et al., 2016), suggesting that estradiol levels do not influence how social regulation of fear responses functions in adult female rats. The potential role of testosterone signaling in social buffering in adult male or female rats has not been investigated, so targeting androgen receptor signaling would be a logical place to start.

Another possible explanation for the sex differences that were observed in my experiments is that perhaps males require more "active" maternal behaviors for effective maternal buffering of threat learning to occur. Since male pups are more likely to receive active care from their mothers than females, it is possible that the passive presence of the mother during threat learning was not sufficient for effective buffering. One way to address this question would be to perform an experiment in which the pup was exposed to a threat conditioned cue in the presence or absence of an awake, behaving mother. Examining how the pup responds to the cue, and the interactions between mother and pup during cue exposure, could better address this question.

Previous studies of infant rat behavior, including responsivity to predators (L. K. Takahashi, 1992, 1994; Wiedenmayer, Lyo, et al., 2003; Wiedenmayer & Barr, 1998), threat learning (Caldwell & Werboff, 1962; Regina Marie Sullivan et al., 2000), and maternal buffering of threat

learning (Moriceau & Sullivan, 2006; Opendak et al., 2019; Shionoya et al., 2007) only tested male animals, did not discuss whether they tested for sex differences or mentioned that they tested for sex differences and found no effect, but did not show the data. Including adequate numbers of male and female subjects in order to determine whether sex differences are a factor is a critical, yet often neglected factor in experimental design (Shansky & Murphy, 2021). My results highlight the need to consider sex as a biological variable in infant rats and future studies should consider this.

5.2 Maternal Regulation of Functional Neural Networks in Typically Developing Animals

5.2.1 SUMMARY

In Chapter 3, I investigated the functional neural network that pups engaged when undergoing threat learning with and without maternal presence using a graph theoretical approach to analyze datasets of immediate early gene expression. Despite a lack of between-group differences in c-Fos expression within brain regions involved in threat learning and responsivity to social cues, I did observe remarkable between-group differences in correlated patterns of c-Fos expression and graph theoretical measures. Pups conditioned with maternal presence had overall stronger functional connectivity than pups conditioned without maternal presence. Their network was also more integrated and did not appear to have distinct hub regions. In contrast, pups conditioned without maternal presence had a more segregated network with the lateral amygdaloid nucleus, dorsolateral part, basolateral amygdaloid nucleus, anterior part, and medial amygdaloid nucleus, anterior dorsal part standing out as hubs.

Although one group has examined functional connectivity in pups conditioned with and without maternal presence (Opendak et al., 2019), my analyses are the first to take a graph theoretical approach to characterize network structure in this context. This allowed me to go a

step beyond comparing mean correlations between a prior identified modules and use an unbiased approach to sort nodes into modules based on their inter-related patterns of activity and describe how the network structure is organized. An investigator taking a traditional approach to analyzing neural activity might stop at measuring absolute values of immediate early gene expression in a select group of brain regions and, in my study, I would have missed the above-mentioned additional information. These findings, taken together with a growing appreciation for the role of neural ensembles in learning (Buzsáki, 2010; Josselyn et al., 2015; McIntosh, 1999), underscore the importance of taking a network approach to study neural activity in infant rats.

5.2.2 FUTURE DIRECTIONS

The regions included in this analysis of functional brain networks engaged in infant rats that underwent threat conditioning with and without maternal presence are all cortical regions or amygdala subnuclei that were selected based on previous studies of threat learning, maternal buffering of threat learning, and responsivity to social cues. Ideally, this analysis would have included as many regions as possible to enable the potential identification of novel brain regions that may be important in regulating threat learning and/or maternal buffering of threat learning in infant rats. Additionally, there are other brain regions that are implicated in these behaviors that may have been important to include. A recent study found that neurons active in the infralimbic cortex while a mouse was investigating a new cagemate could alleviate defensive behaviors when those neurons were reactivated in a threatening situation (Ahuna et al., 2020). The previous study of functional connectivity in infant rats during threat conditioning with and without maternal presence found stronger functional connections between the BLA and ventral tegmental area when infant rats were conditioned with maternal presence (Opendak et al., 2019). Given

these findings, inclusion of the infralimbic cortex and ventral tegmental area should be prioritized in future experiments.

Little is known about functional networks in infant rats. An fMRI study of resting-state functional networks in two-week old infant rats found that some networks resembled the adult form while others did not, including the network that contained the amygdala (Bajic et al., 2016). I hypothesized that maternal presence may switch the infant rat's functional network from a mature state to a more immature state, which would be reflected in a switch from a more integrated network engaged during threat learning and a more segregated network engaged during threat learning with maternal presence. Future studies could examine the functional network engaged by pups undergoing threat conditioning prior to postnatal day 10, when pups generally do not show aversive responses following odor-shock conditioning (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Kucharski & Spear, 1984; Sullivan et al., 2000) and compare it to the network engaged by pups conditioned with maternal presence at postnatal day 12-15. This could better address the question of whether the mother is capable of switching the pup neural network from a more mature state to a more immature state.

5.3 FGF2 and Maternal Presence Regulating Threat Learning in Vulnerable Phenotype

5.3.1 SUMMARY

In Chapter 4, I found that bred Low-Responder (bLR) pups could show conditioned freezing to an olfactory conditioned stimulus when threat conditioning took place at postnatal day 4 and testing occurred at postnatal day 11. Threat-conditioned freezing appears to emerge earlier in bLR pups than in typically developing pups from our experiments and those from previous studies (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Sullivan et al., 2000). Both FGF2 administration on the day following birth and maternal presence during the threat

learning experience were associated with reduced threat conditioned freezing at test. In typically developing animals, exogenous administration of corticosterone can drive precocious acquisition of odor-shock associations (Moriceau et al., 2004; Moriceau & Sullivan, 2004). Interestingly, neither of the behavioral effects of FGF2 administration on P1 or maternal presence during threat learning were accompanied by blunted corticosterone release during the threat learning experience.

Although there is growing evidence to suggest that disruption of maternal care alters maternal regulation of threat learning in infancy and has long term consequences on behavior, hypothalamic pituitary adrenal axis activity, and brain (Kundakovic & Champagne, 2014; Opendak et al., 2019; Raineki et al., 2012; Rincón-Cortés & Sullivan, 2014; Robinson-Drummer et al., 2019), to date there has been no investigation of these dimensions in innately vulnerable phenotypes such as the bLR phenotype. My data suggest that although maternal buffering of threat learning appears intact in bLR animals, the neural and endocrine mechanisms underlying this effect may be different than those in typically developing animals. These findings should encourage investigators to include maternal buffering of threat learning and stress responsivity in future studies of animal models for developmental neuropsychiatric disorders, as maternal buffering is a vital aspect of infancy and a potential target for therapeutic intervention.

5.3.2 FUTURE DIRECTIONS

There are well-documented differences in the endogenous FGF2 and glucocorticoid systems of bLRs and bHRs. Compared to bHRs, bLRs have lower levels of FGF2 mRNA in the hippocampus (Clinton et al., 2012; Turner et al., 2011). Additionally, bLRs have higher levels of glucocorticoid receptor mRNA in the hippocampus; pharmacological blockade of bLR hippocampal glucocorticoid receptors was associated with less anxiety-like behavior (Kabbaj et

al., 2000). It is unclear if such drastic differences in hippocampal FGF2 and glucocorticoid receptor mRNA exist between bLRs and typically developing animals, so a logical next step would be to perform an *in situ* hybridization experiment. To address whether low levels of FGF2 signaling and/or high levels of glucocorticoid receptor signaling explain the early emergence of threat learning in bLRs, I could administer FGF2, a glucocorticoid receptor antagonist, or vehicle into the hippocampus of bLR pups prior to threat conditioning at P4 and then look for potential differences in conditioned freezing at P11. The role of the hippocampus in cued threat conditioning in early infancy has not yet been studied, however, given previous work showing the importance of corticosterone signaling in the amygdala for threat learning in infancy, I would hypothesize that blocking glucocorticoid signaling in the hippocampus of bLRs would suppress precocious threat learning at P4.

Given that maternal buffering may function differently in bLR pups, it would be an excellent idea to examine the characteristics of maternal buffering in another behavioral phenotype that models aspects of a neuropsychiatric disorder. Autism spectrum disorders (ASDs) are characterized by disrupted processing of social cues, altered social behaviors, and, are often comorbid with anxiety (Lord et al., 2018; White et al., 2009). Animal models are particularly useful tools for modeling aspects of ASDs, as they can help identify the neurobiological underpinnings of some of these symptoms (Argyropoulos et al., 2013). One such model is the prenatal valproic acid (VPA) exposure model of ASD (Schneider & Przewłocki, 2005). Developing rat fetuses exposed to VPA show altered social behaviors (Schneider & Przewłocki, 2005), increased anxiety-like behaviors, and altered threat learning and extinction as adults. However, less is known about how VPA-treated rats respond to social cues in infancy (Markram et al., 2008). Data from my ongoing project suggest that in early infancy, VPA-treated pups may

have impaired social recognition and/or may be less motivated to approach social odors. Experiments to determine whether maternal buffering of threat learning in early infancy is intact or disrupted in these pups that model aspects of ASDs are ongoing. These results could tell us more about the underlying behavioral characteristics of ASDs and could shed light on potential opportunities for intervention.

5.4 Conclusion

Social buffering is an important behavioral, physiological, and neurobiological phenomenon that regulates an individual's response to threat and stress throughout the lifespan but is especially critical in infancy. Maternal buffering helps protect the infant from unregulated excessive threat, which can lead to neuropsychiatric disorders such as anxiety (Ressler, 2020), maladaptive programming of the HPA axis (Lupien et al., 2009; McEwen, 2008) and prevents the infant from learning to avoid the mother before it is ready for full independence (Perry & Sullivan, 2014). Through the course of my thesis work, I found that female pups may be more susceptible to maternal buffering of fear, suggesting that sex differences in social regulation of emotion may emerge very early in life. Examinations of functional connectivity and community structure during threat conditioning in the presence or absence of a calm mother imply that pups may engage different networks. Although maternal buffering of threat learning is intact in bLR pups, it appears to function through different neuroendocrine mechanisms than in typically-developing pups.

Historically, maternal buffering of threat in infant rats has been described as a phenomenon that completely blocks fear learning and depends on suppression of amygdala activity and corticosterone release. Conditioned freezing in typically-developing and bLR infant rats was not wholly suppressed in those conditioned with maternal presence, suggesting that the apparent

strength of the buffering effect may depend on the behavioral assay chosen. Quantification of c-fos expression in the BLA of typically-developing infant rats and analysis of corticosterone levels in bLR pups demonstrates that suppressed responses to conditioned threat may occur despite a lack of significant differences in overall amygdala activity or suppressed corticosterone release during conditioning. The findings outlined in this thesis provide an important next step in characterizing maternal buffering and illuminate exciting topics for future inquiry.

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