Interactions of Early-Life Temperature Exposure, Offspring Genotype and Maternal Care on Later-Life Maternal Care Provisioning in Female Rats

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Cell and Systems Biology
University of Toronto

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2021

Abstract

The early-life maternal environment has a profound effect on offspring behaviour, including the transmission of maternal care across generations. Variations in rat maternal care provisioning are associated with alterations in the oxytocinergic and dopaminergic systems in the maternal brain and, in turn, maternal care received by pups can alter oxytocinergic and dopaminergic systems in the rat pup brain. Though there has been progress in elucidating the biological mechanisms underlying the developmental programming of maternal care, the mechanisms that link maternal care received to alterations in the dopaminergic and oxytocinergic systems in the offspring remains to be an active area of research. Previous work has studied the transmission of pup licking across generations, but there are other relevant factors that could interact with pup licking on later-life maternal care provisioning. In this thesis, I review early-life temperature exposure and offspring genotype as important factors also involved in the developmental programming of maternal care. One project directly manipulated early-life temperature exposure and levels of supplemental licking-like tactile stimulation. The second project investigated the main effects of observed early-life inter-individual maternal licking received and interactions with naturally occurring genetic variants in dopamine-related genes. I hypothesized that early-life temperature

exposure and offspring genotype would interact with licking-like tactile stimulation or pup licking to alter the oxytocinergic and dopaminergic systems in the offspring and also influence their later-life maternal care provisioning. I found that 1) early-life temperature exposure influenced the epigenetic regulation of the oxytocin gene in week-old female pups with changes in oxytocin transcript abundance and 2) that both early-life temperature exposure and supplemental tactile stimulation affected later-life maternal care provisioning. In addition, I found that a single nucleotide polymorphism in the dopamine receptor 2 gene interacted with inter-individual maternal licking received on 3) later-life performance on dopamine-related tasks and 4) maternal licking provisioning. Moreover, the association between maternal licking received and maternal licking provisioning was mediated by dopamine levels in the nucleus accumbens of the maternal brain. These findings suggest novel biological mechanisms of the developmental programming of maternal care that could be involved with the transmission of maternal care across generations.

Acknowledgments

The completion of this thesis would not have been possible without the many forms of support I received during my time at the University of Toronto. I am very grateful to have had the opportunity to pursue graduate education with funding from the Connaught International Scholarship in the McGowan Lab at the University of Toronto Scarborough Campus.

I would like to first acknowledge the rats and the rat pups that were the central focus of my thesis and the primary reason I pursued research as a career in the first place. I also would like to thank the animal care staff for ensuring the well-being of the rats in my research and for being open to me socializing one of their singly housed teaching rats for around a year before she passed away. I hope that my research here could also greatly benefit them as much as it benefits us.

I would also like to thank my parents, Christopher and Annette Lauby, as well as my older sister Sheryl Lauby for encouraging me to pursue whatever I wanted in my life and for instilling the importance of education, work ethic, and integrity. I was bombarded with regular messages and weekly video calls while I've been living in a different country but that's a good thing! I also extend my gratitude to my extended family who always looked forward to seeing me during my visits home and for being patient while I try to explain what I do for a living. I will be the first person in my family to receive a doctorate degree and I appreciate their unconditional love and commitment to get me to where I am today. On a similar note, I would like to acknowledge my guinea pig Kirby especially for being a rock of emotional support in the final months of writing this thesis during a global pandemic.

My heartfelt thanks extend to the friends I have made during graduate school for enriching my life with social gatherings and the occasional spontaneous adventure. I first met Dr. Roxanne Fournier at the orientation session for the CSB department and she has shared this PhD journey with me up until the thesis writing (and she will likely successfully defend a couple weeks after I do!). She encouraged me to reach for the stars and I deeply appreciate her support and friendship over the years.

I also want to thank the other members of the McGowan Lab and the "sister" Boonstra Lab. Their insights helped improve my research and clarify my ideas and they pushed me to become a more competent and compassionate scientist. I would not have gained the confidence in my

abilities needed to push the boundaries of what I was capable of doing without them. The litters in Chapters 4 and 5 were generated by Pauline Pan and I am grateful that I was able to ultimately extend and lead the project. Dr. David Ashbrook also became a helpful collaborator in starting to map out variation in the Long-Evans rat strain described in Chapter 5. This thesis would also not be possible without the undergraduate student researchers who assisted me with my work. I would like to specially mention Bryant Lim, who held a summer NSERC USRA to help me with the genotyping in Chapter 4; Tania Muthu, who worked as a D95 thesis student and facilitated the maternal care coding from many hours of video recordings; and Hannan Malik, who worked as a D98 thesis student and did all the cryosectioning and DNA extractions described in Chapter 5. They gave me the opportunities to grow as a mentor and I am also very proud of them.

Finally, I would like to express my deepest appreciation for my PhD supervisor, Dr. Patrick McGowan, for taking me on first as a summer undergraduate research intern and then as a PhD student, nominating me for the Connaught International Scholarship that financially supported me throughout my studies. He saw potential in me and the scientist I could become even when I did not see it in myself. He also allowed me the autonomy to take ownership of my projects and pursue research directions that interested me even when it did not fully overlap with his research program. With these opportunities, I also experienced personal growth as a human being who is capable of thinking for myself. I would also like to thank my committee members Dr. Suzanne Erb and Dr. Alison Fleming (also a collaborator on the project described in Chapters 4 and 5) for their mentorship and guidance in academic research more broadly.

I dedicate this thesis to all the people in my life who uplifted me and advocated for me to do something meaningful and enjoyable with my life, even when it didn't seem possible. You know who you are.

~Samantha Lauby

Table of Contents

List of Abbreviations

5HTT: Serotonin Transporter
ACSF: Artificial Cerebral Spinal Fluid
ACTB: Actin-Beta
ADHD: Attention Deficit Hyperactivity Disorder
AG: Ano-Genital (Licking)
ANOVA: Analysis of Variance
AVP: Arginine Vasopressin
AVPR1A: Arginine Vasopressin Receptor 1A
BD: Body (Licking)
cAMP: cyclic adenosine 3,5-monophosphate
cDNA: Converted Deoxyribonucleic Acid
ChIP: Chromatin Immunoprecipitation
CHRE: Composite Hormone Response Element
COMT: Catechol-O-methyltransferase
CpG: Cytosine-Guanine
CRF: Corticotropin Releasing Factor
CYP19A1: Aromatase
DA: Dopamine
DAT: Dopamine Transporter
DNA: Deoxyribonucleic Acid
DNMT1: DNA Methyltransferase 1
DNMT3A: DNA Methyltransferase 3A
DNMT3B: DNA Methyltransferase 3B
DOPAC: 3, 4-dihydroxyphenylacetic acid
DRD1: Dopamine Receptor 1
DRD2: Dopamine Receptor 2
DRD3: Dopamine Receptor 3
DRL-20: Differential Reinforcement of Low Rates—20 seconds
ELISA: Enzyme-Linked Immunosorbent Assay
ESR1: Estrogen Receptor Alpha
ESR2: Estrogen Receptor Beta
FDR: False Discovery Rate
GEO: Gene Expression Omnibus
HDAC3: Histone Deacetylase 3
HPA: Hypothalamic-Pituitary-Adrenal
HPLC: High Performance Liquid Chromotography
LG: Licking/Grooming
LSD: Least Significant Differences
MeCP2: Methyl CpG Binding Protein 2
MPOA: Medial Preoptic Area
NAcc: Nucleus Accumbens
NCOR1: Nuclear Receptor Corepressor 1
NGFI-A: Nerve Growth Factor-Induced Protein A
NRSF: Neuron-Restrictive Silencer Factor
OXT: Oxytocin
OXTR: Oxytocin Receptor

PCR: Polymerase Chain Reaction
PFC: Prefrontal Cortex
PND: Postnatal Day
PRLR: Prolactin Receptor
PVN: Paraventricular Nucleus
qPCR: Quantitative Polymerase Chain Reaction
RNA: Ribonucleic acid
SEM: Standard Error of the Mean
SMRT: Silencing Mediator of Retinoid and Thyroid Hormone Receptors
SNP: Single Nucleotide Polymorphism
SON: Supraoptic Nucleus
T3: Triiodothyronine
T4: Thyroxine
THRA1: Thyroid Hormone Receptor Alpha 1
THRB: Thyroid Hormone Receptor Beta
TRE: Thyroid Hormone Responsive Element
UBC: Ubiquitin C
VTA: Ventral Tegmental Area

List of Tables

List of Figures

Chapter 1
General Introduction

1 General Introduction

The early-life maternal environment has a profound effect on offspring behaviour. The ability for early-life exposures such as postnatal maternal care to affect the behaviour and physiology of the offspring in a persistent manner is termed 'developmental programming.' Studies using rat models suggest that early-life maternal care can influence the developmental programming of offspring in part through altered epigenetic regulation of specific genes. Understanding the altered epigenetic regulation of these genes as a biological mechanism has been important to study how animals adapt to their environments and how these developmental trajectories can be altered. Moreover, variations in maternal care provisioning can be propagated between generations of female offspring (Champagne, Francis, Mar, & Meaney, 2003; Fleming et al., 2002; Francis, Diorio, Liu, & Meaney, 1999). Variations in maternal care provisioning correspond to alterations in the oxytocinergic and dopaminergic systems in the maternal brain, and maternal care received can alter oxytocinergic and dopaminergic systems in the pup brain. This overlap suggests that alterations to these systems can provide a mechanism through which the transmission of maternal care provisioning across generations could occur. However, many studies that use conventional paradigms to manipulate and assess maternal care received in rat models have primarily examined the effect of the licking of pups on later-life behaviour outcomes of the offspring. Studying licking alone could limit the discovery of other biological mechanisms involved in developmental programming. In addition, other relevant factors have been shown to interact with licking received on later-life phenotype. In this thesis, I review early-life temperature exposure as well as offspring genotype and gene x environment interactions as important factors in the developmental programming of offspring. These two factors were shown to alter the oxytocinergic and dopaminergic systems and influence the transmission of maternal care provisioning in the female offspring.

1.1 Early-Life Maternal Environment on Rat Offspring Later-Life Phenotype

1.1.1 Developmental Programming of Early-life Maternal Care

Early-life alterations in the environment can have major downstream effects on an individual's physiology and behaviour and can influence the predisposition for later-life health issues. The ability for early-life exposures to persistently affect the behaviour and physiology of the

organism is termed ‘developmental programming.’ While considered an adaptive process (i.e., increases individual fitness; Bateson et al., 2004; St-Cyr & McGowan, 2018), there is interest in understanding the biological mechanisms underlying developmental programming in order to mitigate or prevent disease and its burdens on the individual (Matthews & McGowan, 2019).

Postnatal maternal care has been shown to have profound effects on later-life behaviour for the offspring of numerous species (Curley & Champagne, 2016; Moore, Whiteman, & Martin, 2019). It has been shown that these alterations in later-life behaviour correspond to alterations in different neuroendocrine and neurotransmitter systems. These include the hypothalamic-pituitary-adrenal axis (Meaney, 2001), the hypothalamic-pituitary-thyroid axis (Hellstrom, Dhir, Diorio, & Meaney, 2012), the hypothalamic-pituitary-gonadal axis (Cameron, 2011), the oxytocinergic system (Champagne, Diorio, Sharma, & Meaney, 2001), the serotonergic system, (Courtiol, Wilson, Shah, Sullivan, & Teixeira, 2018) and the dopaminergic system (Peña, Neugut, Calarco, & Champagne, 2014). These systems are known to interact with each other or act separately to modulate a myriad of animal behaviours.

For rats, postnatal maternal care includes nesting and keeping the rat pups warm, retrieval of pups to the nest, nursing, and licking individual pups. The biological mechanisms underlying developmental programming of maternal care have been mainly studied in rats and mice; therefore, this chapter will primarily describe findings from the rodent model. However, there are likely evolutionarily conserved features that apply to other species, including humans (Beery & Francis, 2011; McGowan, 2015; McGowan et al., 2009).

1.1.2 Underlying Epigenetic Processes and Gene Regulation

Studies conducted within the past two decades have suggested that maternal care received can alter the expression of key genes through changes in their epigenetic regulation. The first study to allude to epigenetics as a potential biological mechanism for individual differences in animal behaviour used embryo transfers and cross-fostering of distinct inbred mouse strains. They reported that the mouse offspring behaviour more closely resembled their postnatal foster mother than the typical characteristics of their genetic strain (Francis, Szegda, Campbell, Martin, & Insel, 2003). The term “epigenetics” has had several definitions throughout the history of the field (Haig, 2012); for the purposes of clarity, I define epigenetics as a set of molecular mechanisms that stably regulate gene expression, or if a gene is expressed at all, in a given cell

type without changing the DNA sequence itself that can be inherited between daughter cells. This includes the interactions between DNA methylation, histone modifications, and noncoding RNAs with transcription factors that promote or repress gene transcription (Aristizabal et al., 2019). Most studies that have linked maternal care to epigenetic mechanisms, especially DNA methylation, do not detail their interactions with transcription factors and therefore those studies would be considered associative. The following studies have attempted to make these essential connections and have increased our understanding of the developmental programming of maternal care. It is noted that these studies to date primarily focus on the mechanisms underlying maternal care received and later-life stress reactivity and alterations in the hypothalamic-pituitary-adrenal (HPA) axis that controls the endocrine response (corticosterone in rodents) to stressors.

The pioneering work in elucidating these mechanisms as they relate to the effects of maternal care started with Weaver and colleagues' (2004) studies on the glucocorticoid receptor gene. There are natural variations between rat mothers in maternal care provisioning towards their offspring. The offspring that received high levels of maternal care were less reactive to stressors both behaviourally and physiologically than offspring that received low levels of maternal care (Caldji, Diorio, & Meaney, 2000; Francis, Diorio, Liu, & Meaney, 1999; Meaney, 2001; Menard, Champagne, & Meaney, 2004; Weaver et al., 2004). This reduction in stress reactivity was associated with higher expression of the glucocorticoid receptor gene in the hippocampus (Hellstrom et al., 2012; Liu et al., 1997; Weaver et al., 2004), which is linked to a faster negative feedback response from the HPA axis to stressors. In other words, rat offspring that received high levels of maternal care returned to homeostasis from environmental stressors earlier than rat offspring that received low levels of maternal care. In addition, the increase in glucocorticoid receptor transcript abundance was associated with a decrease in DNA methylation in a regulatory region of the gene that contains a binding motif to the transcription factor Nerve Growth Factor-Induced Protein A (NGFI-A) (Weaver et al., 2004). DNA methylation in regulatory regions typically represses gene transcription, and the researchers found that DNA methylation in this specific region of the glucocorticoid receptor gene blocks the binding of NGFI-A to the DNA, preventing transcription (Weaver et al., 2004, 2007). Conversely, NGFI-A binding to the glucocorticoid receptor gene is associated with DNA demethylation and an increase in glucocorticoid receptor transcript abundance (Weaver et al., 2007).

Another well-studied example is the regulation of corticotropin releasing factor (CRF) in the hypothalamus of rat pups that received augmented levels of maternal care early in life (Avishai-Eliner, Eghbal-Ahmadi, Tabachnik, Brunson, & Baram, 2001; Korosi et al., 2010; Molet et al., 2017). Brief separations (around 15 minutes) of the pups from the mother (termed 'handling') within the first postnatal week can increase maternal care provisioning after the pups are reunited with the mother (Lee & Williams, 1974; Reis et al., 2014) and have widespread effects on later-life phenotype (Pryce & Feldon, 2003). Offspring that went through the handling procedure had decreased transcript abundance of CRF, which is a neuropeptide that elicits the HPA axis response to stressors (Avishai-Eliner et al., 2001; Fenoglio, 2006; Korosi et al., 2010). The researchers also found that this decrease in CRF transcript abundance is associated with increased binding of the transcription factor Neuron-Restrictive Silencer Factor (NRSF), associated with transcriptional repression, in pup offspring and persisted into adulthood (Korosi et al., 2010; Molet et al., 2017). At adulthood, there were fewer CRF-producing neurons in the hypothalamus and the offspring had a milder HPA axis response to stressors (Korosi et al., 2010; Plotsky & Meaney, 1993).

Finally, early-life adversity by prolonged separations from the mother has been shown to alter the epigenetic regulation of arginine vasopressin (AVP) in the hypothalamus in mouse offspring (Murgatroyd et al., 2009). While brief separations from the mother can reduce stress reactivity of the offspring, prolonged separations (ranging between 3-24 hours) can increase stress reactivity due to the early-life stress of separation and concurrent decrease in maternal care received (Lehmann & Feldon, 2000; von Poser Toigo et al., 2012). In this study, the researchers found that mouse offspring that went through the prolonged separations had increased transcript abundance of Avp, another neuropeptide that elicits the HPA axis response to stressors, throughout life and increased stress reactivity in adulthood (Murgatroyd et al., 2009). In addition, this increase in Avp transcript abundance was associated with a decrease in DNA methylation in an enhancer region for AVP (Murgatroyd et al., 2009). There was also reduced binding of the transcription factor Methyl-CpG-binding protein 2 (MeCP2), associated with transcriptional repression, in this enhancer region (Murgatroyd et al., 2009). The changes in MeCP2 binding preceded the changes in DNA methylation, suggesting that the decreases in DNA methylation were a consequence of the reduced MeCP2 binding (Murgatroyd et al., 2009).

1.1.3 Oxytocinergic and Dopaminergic Systems

The studies described above suggest a link between maternal care received and later-life stress reactivity in the offspring through epigenetic regulation of key genes involved in the HPA axis and alter the endocrine response to stressors. Some studies have also suggested a link between maternal care received and behavioural and physiological changes involving the oxytocinergic and dopaminergic systems; however less is known about the underlying mechanisms involved in these alterations.

Oxytocin (OXT) is a neuropeptide produced in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and acts primarily on oxytocin receptors (OXTRs) ubiquitously expressed within the brain (Figure 1.1) and in certain areas of the peripheral body. The oxytocinergic system is involved in several complex behaviours including stress, social behaviour, and maternal care provisioning (Carter, 2003; Stoop, 2012). There can also be natural OXT production and release in response to tactile stimuli and warmth (Uvnäs-Moberg, 1998; Walker, Trotter, Swaney, Marshall, & Mcglone, 2017), which are prominent features of maternal care received in rats. The empirical work to directly connect maternal care received and alterations in the oxytocinergic system continues to be an intense area of investigation. Some studies have found that female rat offspring that receive high levels of maternal care have higher transcript abundance of Oxtr within the medial preoptic area of the hypothalamus (MPOA; Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; Champagne, Diorio, Sharma, & Meaney, 2001; Francis, Champagne, & Meaney, 2000; Francis, Young, Meaney, & Insel, 2002). However, there is little consensus on which regulatory regions of the OXTR gene are most sensitive to changes to maternal care received. One study showed that estrogen administration increases OXTR density in the MPOA in female offspring with high levels of maternal care only (Champagne et al., 2001), but the association with DNA methylation changes in the estrogen response elements within the OXTR gene is lacking (Beery et al., 2016).

The literature on the link between maternal care received and OXT production is more inconsistent. Some studies suggest an increase (Barrett, Arambula, & Young, 2015; Toepfer et al., 2017) in oxytocinergic activity while other studies show a decrease (Todeschin et al., 2009; Vogel Ciernia et al., 2018; Winkelmann-Duarte et al., 2007) in Oxt transcript abundance or oxytocin-producing neurons in response to higher levels of maternal care received or early-life

handling in rodent offspring. These studies primarily focus on the tactile stimuli the pups receive with high levels of maternal licking.

Pup OXT levels can also increase in response to thermotactile contact with the mother (Kojima, Stewart, Demas, & Alberts, 2012) and is essential for the development of thermal-seeking huddling behaviour (Kojima et al., 2012; Kojima & Alberts, 2011). Concurrently, OXT knockout mouse pups have impaired thermoregulation to cold stressors and form less cohesive huddles with other mouse pups (Harshaw, Leffel, & Alberts, 2018). Given the links between maternal thermotactile contact and pup thermoregulation (behaviourally and physiologically) with oxytocin, the role of warmth and temperature changes may be an important factor to consider.

Dopamine is a neurotransmitter that is synthesized in the midbrain and has multiple projections throughout the brain (Figure 1.1). The mesocorticolimbic projections specifically synthesizes dopamine in the ventral tegmental area (VTA) and projects to the basal ganglia, including the nucleus accumbens (NAcc), and to the prefrontal cortex. Dopamine acts on multiple receptors (DRD1, DRD2, DRD3, DRD4, DRD5) to initiate and refine behaviours related to attention, behavioural flexibility, sensitivity to rewarding stimuli, and maternal care provisioning among other complex behavioural phenotypes (Arias-Carrián, Stamelou, Murillo-Rodríguez, Menéndez-Gonzlez, & Pöppel, 2010; Nieoullon, 2002; Wise, 2004). These phenotypes are highly dependent on the brain area involved and the receptors that are being activated as well as interactions between different brain areas. Dopamine can be released in response to rewarding stimuli, which would include tactile stimuli (Maruyama, Shimoju, Ohkubo, Maruyama, & Kurosawa, 2012).

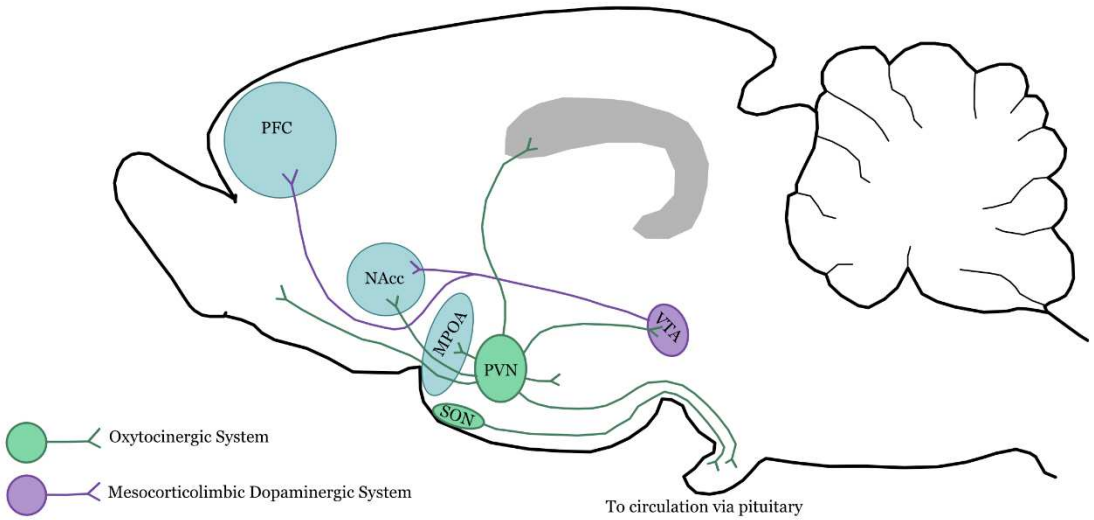

Figure 1.1 The oxytocinergic and mesocorticolimbic dopaminergic systems in the rat brain. Oxytocin is synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus and is released within the brain, including the medial preoptic area (MPOA), and into circulation by the median eminence and pituitary gland. The mesocorticolimbic dopaminergic system originates from dopamine-synthesizing neurons in the ventral tegmental area (VTA) and projects to the basal ganglia in the limbic system, including the nucleus accumbens (NAcc), and to the prefrontal cortex (PFC).

Studies examining natural variations in maternal care received have found that female rat offspring that receive high levels of maternal care have increased numbers of dopamine-synthesizing neurons in the VTA and increased transcript abundance of dopamine receptors (Drd1, Drd2, Drd3) in the nucleus accumbens at weaning (Peña, Neugut, Calarco, & Champagne, 2014). In addition, rat offspring with early-life maternal and sibling deprivation by artificial rearing have increased sensitivity to natural and drug rewards (Lomanowska, Rana, McCutcheon, Parker, & Wainwright, 2006), are more impulsive (Lovic, Keen, Fletcher, & Fleming, 2011), are slower to shift strategies in cognitive tasks (Lovic & Fleming, 2004) and are less attentive to pup stimuli later in life (Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Palombo, Nowoslawski, & Fleming, 2010) with corresponding changes in the dopaminergic system (Afonso, King, Novakov, Burton, & Fleming, 2011). Providing supplemental maternal licking-like tactile stimulation partially mitigates most of these effects (Afonso et al., 2011; Gonzalez et al., 2001; Lovic & Fleming, 2004; Lovic et al., 2011; Palombo et al., 2010), which suggests that maternal licking is a prominent factor influencing these alterations in the mesocorticolimbic dopaminergic system. However, the underlying changes in the pup brain that cascade to later-life behavioural phenotypes is not well understood.

While female rat offspring that receive high levels of maternal care have an increased number of tyrosine hydroxylase (rate-limiting enzyme that synthesizes dopamine) positive neurons in the VTA, this did not correspond to changes in DNA methylation in regulatory regions of the tyrosine hydroxylase gene (Peña et al., 2014). Studies that have investigated epigenetic regulation of the dopamine receptors in response to maternal care received are also limited; one study using a maternal deprivation procedure found a decrease in dopamine receptor 2 (Drd2) transcript abundance but this did not correspond to changes in DNA methylation in regulatory regions of the DRD2 gene (Zhu et al., 2010).

Conversely, one study found that the effects of artificial rearing on DRD2 density in the nucleus accumbens is dependent on rat offspring genotype in the DRD2 gene (Lovic et al., 2013). These gene x environment interactions related to the dopaminergic system have been found with other studies, especially with human cohorts where genetic variation between individuals is well-documented (Chen et al., 2011; Jönsson et al., 1999; Nikitopoulos et al., 2014; van der Meer et al., 2017). However, mechanistic studies linking dopamine-related genotypes and early-life maternal care received with persistent changes in the dopaminergic system in the brain can only be accomplished in animal models.

1.2 Variations within the Maternal Brain Correspond to Variations in Later-Life Maternal Care Provisioning

1.2.1 Changes in the Brain Prime New Mothers to Care for Offspring

Following parturition of offspring, rat mothers begin to perform caregiving behaviour towards their pups almost immediately. During gestation, changes in circulating progesterone and estrogen levels can lead to downstream alterations in the prolactin and oxytocinergic systems in the brain that prepare the mother to care for her offspring (Bridges, 1984, 2015; Rosenblatt, Mayer, & Giordano, 1988). Other studies in rats have shown that there is also a non-hormonal basis for the onset and maintenance of maternal behaviour, primarily involving neurotransmitters such as serotonin and dopamine (Bridges, 2015). This preparation to care for offspring can lead to considerable differences in transcript profiles between the maternal brain and the virgin female brain (Akbari et al., 2013; Ray et al., 2016).

1.2.2 Intergenerational Maternal Care Provisioning

Though the underlying biological processes during the preparation of caregiving for offspring would be similar between mothers, the resultant behavioural repertoire of rodent maternal care shows considerable variation. There is variation across mothers in in aggression and protective behaviours (Bosch, 2013), nest quality (Ugarte, Eguibar, Cortés, León-Chávez, & Melo, 2011), and pup-directed behaviour including retrieval latency and the licking of individual pups (Brown, Mathieson, Stapleton, & Neumann, 1999; Champagne et al., 2003; Moore, Wong, Daum, & Leclair, 1997). Some of these differences can be attributed to underlying genetic background (Bosch, 2013; Brown et al., 1999; Moore et al., 1997; Ugarte et al., 2011), but a

portion of variation is also either non-genomic (Champagne et al., 2003) or derives from an interaction between genes and environmental influences (Fleming & Kraemer, 2019).

One intriguing observation is that rat mothers that received low levels of maternal licking and grooming as pups tend to provide low levels of these same behaviors towards their pups, whereas the opposite is the case with mothers that received high levels of maternal care (Champagne et al., 2003; Francis et al., 1999). Cross-fostering experiments suggest that the postnatal maternal care received contributes to this intergenerational transmission of maternal care rather than genetic variation alone (Francis et al., 1999). Other studies show that female offspring with early-life adversity by maternal deprivation or maternal maltreatment provide lower levels of maternal care to their pups than the unmanipulated group (Gonzalez et al., 2001; Palombo et al., 2010; Roth, Lubin, Funk, & Sweatt, 2009), which can be transmitted to the following generation (Gonzalez et al., 2001). The biological mechanisms underlying this transmission of maternal care are only starting to be elucidated, but epigenetic mechanisms appear to be involved (Champagne, 2008; Keller, Doherty, & Roth, 2019; Stolzenberg & Champagne, 2016).

Two major systems that are altered in the rat maternal brain during the gestation and lactation period are the oxytocinergic (hormonal) and dopaminergic (non-hormonal) systems. Given that these systems are altered with differences in maternal care received (Section 1.1.3), they could also be involved in the transmission of maternal care across generations (Figure 1.2). This also suggests that altering these systems can disrupt the trajectory of intergenerational transmission of maternal care provisioning. While oxytocin and dopamine can affect maternal behaviour within numerous brain areas, there are a small number of critical brain areas that could be important for studying the intergenerational transmission of maternal care.

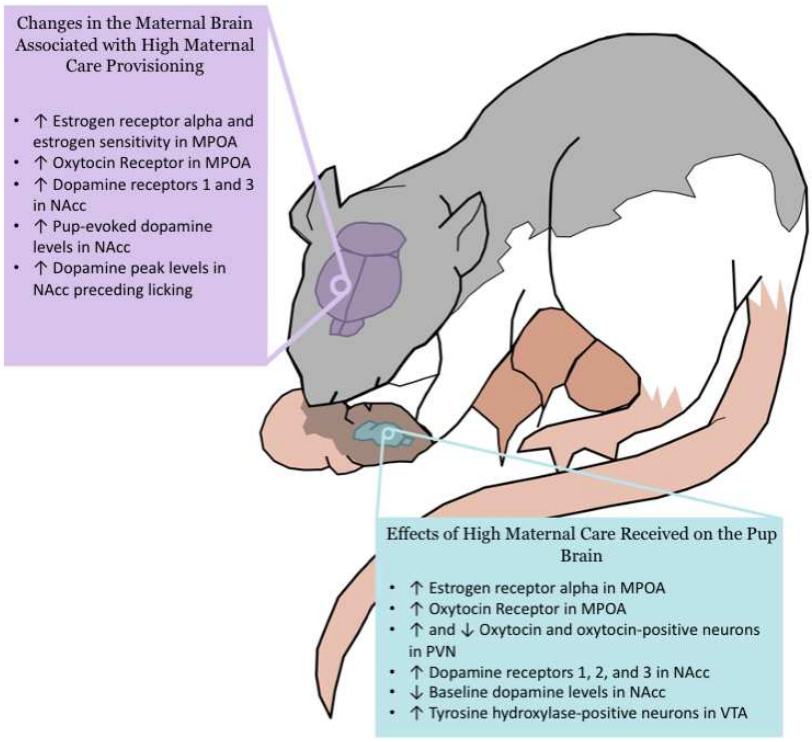

Figure 1.2 Summary of changes in the maternal brain associated with high maternal care provisioning and effects of high maternal care received on the pup brain. The comparable alterations in the oxytocinergic and dopaminergic systems suggest that these two systems are important for transmitting maternal care provisioning across generations of female offspring.

1.2.3 Oxytocinergic and Dopaminergic Systems

The role of oxytocin was initially discovered with studies observing the effects of pituitary extracts on uterine contractions and parturition (Blanks & Thornton, 2003; Dale, 1906) and the letdown of milk with lactation (Schafer & MacKenzie, 1911). There is now extensive evidence that OXT within the brain is involved with the onset of other maternal behaviours, including aggression towards intruders, pup retrieval, and pup licking (Bosch, 2013; Marlin, Mitre, D'Amour, Chao, & Froemke, 2015; Peterson, Mason, Barakat, & Pedersen, 1991), but not necessarily for the maintenance of these behaviours (Numan, 2006). The MPOA has been implicated as an essential brain area for the onset of maternal behaviour via oxytocinergic signaling. Rat mothers with knife cuts that disrupted MPOA circuitry, or received administration of oxytocin antagonists to the MPOA, show impaired or elimination of maternal behaviours (Numan & Callahan, 1980; Pedersen, Caldwell, Walker, Ayers, & Mason, 1994). In addition,

there is some evidence that estrogen receptor alpha can promote transcription of OXTR (Fleming, Spencer, Safe, & Bazer, 2006). Specifically downregulating the expression in estrogen receptors in the MPOA can also impair maternal behaviour in mice (Ogawa et al., 1998; Ribeiro et al., 2012). Taken together, these studies suggest that the density of estrogen receptors and estrogen sensitivity in the MPOA may be an important component in the development of the oxytocinergic system that underlie maternal behaviour.

The MPOA is also sensitive to differences in early-life maternal care received. Female rat offspring that receive high levels of maternal care have increased transcript abundance of estrogen receptor alpha in the MPOA (Champagne et al., 2006; Champagne, Weaver, Diorio, Sharma, & Meaney, 2003), with an associated decrease of DNA methylation in the promoter region of the estrogen receptor alpha gene and increased binding of the transcription factor Stat5b (Champagne et al., 2006). These changes in the estrogen receptor alpha gene preceded increases in Oxtr transcript abundance (Pena, Neugut, & Champagne, 2013). One caveat is that studies that manipulate early-life OXT levels also show alterations in estrogen receptor alpha (Perry, Paramadilok, & Cushing, 2009; Pournajafi-Nazarloo, Carr, Papademeteriou, Schmidt, & Cushing, 2007; Yamamoto, Carter, & Cushing, 2006) and OXTR (Bowen, Carson, Spiro, Arnold, & McGregor, 2011; Kenkel et al., 2019) levels in the brain, including the MPOA, during development and as adults. It is unknown if these mechanisms are connected temporally or act separately to change the functioning of the oxytocinergic system in the MPOA of the maternal brain.

The dopaminergic system in the maternal brain is involved with attention and salience of pup stimuli as well as retrieving and licking pups (Rincón-Cortés & Grace, 2020). The dopaminergic system is also involved in sensitizing virgin female rats to foster pups and is a central component to the “learned” aspects of maternal care provisioning (Parada, King, Li, & Fleming, 2008). The nucleus accumbens (NAcc) within the basal ganglia has been implicated as an important brain area for this “maternal memory” and variations in licking provisioning between rat mothers (Afonso, Grella, Chatterjee, & Fleming, 2008; Champagne et al., 2004; Hansen, Bergvall, & Nyiredi, 1993; Keer & Stern, 1999; Parada et al., 2008). More specifically, the shell region of the NAcc appears to be more involved in maternal care provisioning than the core region (Afonso et al., 2008; Champagne et al., 2004; Li & Fleming, 2003a, 2003b; Parada et al., 2008). Infusions of selective DRD1 agonists and antagonists in the NAcc of postpartum female rats have been

shown to facilitate and impair the onset of maternal behaviour, respectively (Numan et al., 2005; Stolzenberg et al., 2007, 2010). Infusions of selective DRD2 antagonists in the NAcc shell region of postpartum female rats also delay onset of maternal behaviour and rats with infusions of DRD1/DRD2 antagonists show the longest delay in expressing maternal behaviour (Parada et al., 2008). Infusions of DRD2 antagonists can also disrupt the retention of maternal behaviours (Byrnes, Rigero, & Bridges, 2002). These pharmacological studies also suggest that blocking dopamine receptors in the NAcc does not fully eliminate maternal behaviour; rather, licking and retrieval behaviour are impaired but other maternal behaviours (i.e., nursing) can be enhanced (Keer & Stern, 1999; Stern & Taylor, 1991). This suggests that the NAcc shell region is selectively involved in some aspects of maternal behaviour but not others. However, the role of the NAcc in the onset of maternal care provisioning and consolidation of maternal memory makes it an important brain region to study for the intergenerational transmission of maternal care.

Studies using the artificial rearing paradigm have shown that the functioning of the nucleus accumbens is affected by maternal and sibling deprivation and can impair maternal care provisioning (Afonso et al., 2011; Gonzalez et al., 2001). Overall, artificially reared virgin female rats have increased baseline levels of dopamine in the NAcc shell region, but a blunted peak in dopamine levels in response to pup stimuli (Afonso et al., 2011). This smaller peak in dopamine levels or smaller differential between baseline and pup-evoked dopamine levels could cause these artificially reared rats to be less attentive to pup cues. In addition, rat mothers that provide high levels of licking towards their pups have a higher peak in dopamine levels preceding licking bouts of pups than rat mothers that provide low levels of licking, and this peak in dopamine corresponds to the bout duration of pup licking (Champagne et al., 2004). The rat mothers that provide high levels of licking towards their pups also have higher density of DRD1 and DRD3 (but not DRD2) in the NAcc shell region (Champagne et al., 2004). However, similar to the effects of maternal care received reviewed in Section 1.1.3, there is limited evidence that there are differences in DNA methylation and other epigenetic mechanisms in dopamine-related genes in relation to intergenerational maternal care provisioning (Stolzenberg & Champagne, 2016). In addition, there are studies that suggest offspring genotype in dopamine-related genes affects mothering in human cohorts (Fleming & Kraemer, 2019; Mileva-Seitz et al., 2012) and could possibly interact with early-life experiences in rats (Lovic et al., 2013). Interestingly,

Lovic and colleagues found a DRD2 genotype x artificial rearing interaction in rats for DRD2 density in the shell region of the NAcc specifically, and not the core region of the NAcc or caudate nucleus in the basal ganglia (Lovic et al., 2013). Another potential hypothesis is that the changes in the MPOA oxytocinergic system (via estrogen receptor alpha) can lead to downstream changes in the NAcc dopaminergic system, as there is some evidence that the oxytocinergic and dopaminergic systems interact with each other in the maternal brain (D'Cunha, King, Fleming, & Lévy, 2011; Peña & Champagne, 2015; Shahrokh, Zhang, Diorio, Gratton, & Meaney, 2010; Stolzenberg & Champagne, 2016). This would imply that the effects of early-life maternal care received on the dopaminergic system in the maternal brain is dependent on changes in the oxytocinergic system.

1.3 Paradigms to Manipulate or Assess Maternal Care Received

1.3.1 Neonatal Handling and Maternal Separation

In the 1950's, researchers observed that separating rat pups from their mothers for brief periods of time (up to 15 minutes) every day before weaning led to attenuated stress reactivity (also interpreted as "emotionality") in these rats at adulthood as compared to unmanipulated litters. The effects of this brief separation on later-life stress reactivity and other behaviours were replicable across laboratories and considered robust. Another manipulation group involved separating pups from their mother for prolonged periods of time (3+ hours), termed maternal separation, and was used to simulate early-life adversity. Maternal separation was a common comparison group in neonatal handling studies. However, the effects of maternal separation are variable and less robust, possibly due to the wide range of protocols of early-life separation that are categorized under the umbrella term "maternal separation" (Lehmann & Feldon, 2000). Nevertheless, these studies were an important starting point to understand how early-life environments alter later-life behaviour.

Seymour Levine initially observed that pups that were briefly separated from their mothers, both with and without receiving a shock stressor, had more positive later-life outcomes than the litters that were not separated from their mothers (Levine, Chevalier, & Korchin, 1956). Subsequent studies have shown that neonatal handling can attenuate stress reactivity and the endocrine response to stressors (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Levine, Haltmeyer, Karas, & Denenberg, 1967; Meerlo, Horvath, Nagy, Bohus, & Koolhaas, 1999), affect learning

and memory in a task-specific manner (Kosten, Kim, & Lee, 2012; Kosten, Lee, & Kim, 2007; Pryce, Bettschen, Nanz-Bahr, & Feldon, 2003), reduce social and sexual behaviours (Aguilar, Caramés, & Espinet, 2009; Raineki, Lutz, Sebben, Ribeiro, & Lucion, 2013), and increase palatable food consumption (Silveira et al., 2004, 2008). It has also been shown that neonatal handling can mitigate the effects of prenatal stress (Lemaire, Lamarque, Le Moal, Piazza, & Abrous, 2006; Wakshlak & Marta, 1990). While handling has been shown to alter the trajectory of development of different later-life behaviours, not all outcomes are considered positive (Raineki, Lucion, & Weinberg, 2014), but could be considered adaptive to the early-life experience.

From the earliest studies, relevant environmental variables were identified for neonatal handling to exert its effects, namely tactile stimulation and the cooling of pups, and it was most effective during the first week of life (Russell, 1971). One explanation that attempted to integrate these variables was the maternal mediation hypothesis (Smotherman & Bell, 1980). The hypothesis states that pups that experience heat loss will solicit maternal retrieval through ultrasonic vocalizations (Blumberg, Efimova, & Alberts, 1992; Hofer, 1996) and result in more maternal care following the reunion of the rat mother (Bell, Nitschke, Gorry, & Zachman, 1971; D'Amato et al., 2005; Villescas, Bell, Wright, & Kufner, 1977). In addition, the variables relevant for handling could be proxies of the amount of maternal contact the pups receive (Russell, 1971). The cooling of pups could be a proxy of maternal absence for long periods of time, and tactile stimulation could be a proxy of maternal licking received.

The biological mechanisms underlying the effects of neonatal handling have also been proposed. Handling has been shown to alter stress-related gene expression in the brain of adult rats (Avishai-Eliner et al., 2001; Caldji, Francis, et al., 2000; Plotsky & Meaney, 1993), most notably an increase of glucocorticoid receptor transcript abundance in the hippocampus (Meaney, Aitken, Viau, Sharma, & Sarrieau, 1989). Since the release of thyroid hormones is important to induce thermogenesis in rat pups (Steele & Wekstein, 1972), it was proposed that the surges in thyroid hormone levels also caused the increase in glucocorticoid receptor transcript abundance (Meaney, Aitken, & Sapolsky, 1987). Injections of rat pups with Propylthiouracil, a drug that inhibits thyroid hormone synthesis, appear to reverse the effects of neonatal handling (Meaney et al., 1987). Further experiments suggested that increased serotonin signaling was also involved in the effects of neonatal handling (Meaney et al., 2000; Smythe, Rowe, & Meaney, 1994), with a

downstream increase in cyclic adenosine 3,5-monophosphate (cAMP)-inducible transcription factors including NGFI-A (Meaney et al., 2000). Administration of Propylthiouracil has been shown to decrease serotonin turnover (Meaney et al., 2000) but the underlying mechanisms are unknown.

While neonatal handling was a commonly-used paradigm to study the effects of early-life experiences, one challenging methodological issue was designing an appropriate control group (Pryce & Feldon, 2003). It is unknown how much stimulation a pre-weanling rat needs for optimal development in an artificial laboratory environment, and it could be argued that the unhandled group is *understimulated* and therefore an experimental group in itself (Pryce & Feldon, 2003). Thus, an animal facility reared group, which included regular cage changes with brief separations of the litter, has been used as an alternative control group, but differences in animal husbandry practices between institutions led to concerns of replicability across studies (Pryce & Feldon, 2003).

1.3.2 Natural Variations in Maternal Care

Another paradigm used to investigate the effects of early-life experiences on adult phenotype was to observe maternal care received directly, and correspond maternal care received to offspring later-life behaviour. As discussed in Section 1.2.2, natural variations in maternal care provisioning exist between rat mothers. Many studies that have investigated the role of maternal care received on later-life behaviour in rats measure maternal licking/grooming with arched back nursing as a proxy of high or low maternal care received. The litters are then categorized into high or low maternal licking/grooming if they are above or below one standard deviation from the average, respectively.

Maternal licking is a major source of tactile stimulation input for the rat pup and there are similarities between the effects of high maternal licking received and the effects of neonatal handling. Like neonatal handling, rats that received high levels of maternal licking have attenuated stress reactivity and endocrine response to stressors (Barha, Pawluski, & Galea, 2007; Weaver et al., 2004), increased glucocorticoid receptor transcript abundance in the hippocampus (Weaver et al., 2004), alterations in learning and memory in a task-specific manner (Liu, Diorio, Day, Francis, & Meaney, 2000), and reduced sexual behaviours and fecundity in females (Cameron, Fish, & Meaney, 2008) than rats that receive low levels of maternal licking. Several

of these studies also provided evidence that epigenetic mechanisms mediate the developmental programming of maternal care (Sections 1.1.2, 1.1.3, and 1.2.3). It was proposed that the epigenetic regulation in glucocorticoid receptor specifically was due to increased thyroid hormone and serotonin signaling in rats that received high levels of maternal licking (Hellstrom et al., 2012), which was also reported in neonatal handling studies. More specifically, the study by Hellstrom and colleagues suggests that the tactile stimulation derived from maternal licking could metabolize the thyroid hormone thyroxine (T4) to triiodothyronine (T3) (Hellstrom et al., 2012). It was concluded that the higher levels of T3 would increase serotonin turnover in the brain and trigger a signaling cascade to increase binding of NGFI-A to the glucocorticoid receptor promoter region (Hellstrom et al., 2012). However, there is still no known mechanism that directly links T3 with increased serotonin turnover.

More recent studies have reported that natural variations in maternal care provisioning exist between individual pups within a litter and this can correspond to differences in later-life behaviour between siblings (Cavigelli, Ragan, Barrett, & Michael, 2010). This includes stress reactivity (Pan, Fleming, Lawson, Jenkins, & McGowan, 2014; Pan et al., 2018; Ragan, Loken, Stifter, & Cavigelli, 2012; Ragan, Harding, & Lonstein, 2016), social behaviour (van Hasselt et al., 2012), maternal behaviour in sensitized virgin female rats (Ragan et al., 2016), and glucocorticoid receptor transcript abundance (Pan et al., 2014; van Hasselt et al., 2012) and DNA methylation (Pan et al., 2014).

While these observational studies have been seminal in understanding the biological mechanisms underlying developmental programming of maternal care, they are also correlational and therefore cannot account for all confounding factors. It is important that these studies are paired with experiments involving direct manipulations of the pups (Hellstrom et al., 2012). Neonatal handling could be re-purposed to manipulate specific factors involved in maternal care (e.g., tactile stimulation) and complement the observational studies on the effects of maternal care received on offspring later-life behaviour.

1.3.3 Challenges and Limitations of Studying the Link Between Maternal Care Received and Offspring Later-Life Behaviour

While the studies described above have contributed substantial knowledge to understanding developmental programming and the underlying biological mechanisms, there are challenges and

limitations in the field that may elude the discovery of other potential underlying mechanisms. The challenges and limitations described below are mainly derived from primarily examining the amount of licking the pups receive, and attributing this factor to most changes in later-life outcomes. Indeed, expanding the search for other relevant factors could help illuminate the underlying mechanisms between maternal care received and alterations in the oxytocinergic and dopaminergic systems. Moreover, this search may elucidate the biological mechanisms underlying the intergenerational transmission of maternal care provisioning.

One major challenge in the field is that maternal care is a complex phenotype that involves multiple potential variables that could independently or interactively affect later-life offspring phenotype. It is, therefore, difficult to ascertain which variables may be most relevant to the developmental programming of maternal care. For studies that only study licking, it may not be sufficient to observe the quantity of licking received; in these studies, the quality of licking received and the fragmentation or stability of maternal care may also be important outcome variables to consider (Baram et al., 2012; Ivy, Brunson, Sandman, & Baram, 2008). Concurrently, in neonatal handling studies, separating the litter can cause stress to the mother. and the glucocorticoids released can be transferred to the pups by the milk (Yeh, 1984). A series of studies found that offspring of mothers who received moderate levels of glucocorticoids via the drinking water can also show attenuated stress reactivity at adulthood (Casolini et al., 1997; Catalani et al., 2000; Catalani, Alemà, Cinque, Zuena, & Casolini, 2011), but the mother also provides more licking to the offspring (Casolini et al., 2007; Catalani et al., 2011). These studies suggest that multiple variables may contribute either separately or synergistically to later-life offspring phenotype. In addition, studies that investigate the intergenerational transmission of maternal care provisioning also indicate that multiple early-life variables can be transmitted across generations (Fleming et al., 2002). Experiments that study these variables separately are important to understand if the underlying mechanisms converge on one biological pathway or can affect separate pathways.

Another challenge in the field will be finding evolutionarily conserved biological mechanisms between rats and other species. While there is extensive evidence that early-life environments affect epigenetic mechanisms in the brains of multiple species, including humans (McGowan et al., 2009, 2008), pup licking is a species-specific maternal behaviour in rodents. Therefore, it is unclear if pup licking, or if the maternal contact during pup nursing, would closely resemble the

effects of human thermotactile touch, for example. It is even less obvious how pup licking may translate to other non-mammalian species that do not perceive non-noxious tactile stimuli, but also display maternal effects (Moore et al., 2019). Although the maternal behaviours transmitted across generations are species-specific, there may be a set of common biological mechanisms that researchers can derive from a comparative species approach. This challenge will also be important to address studies focused on social interventions in human medicine.

Finally, one additional limitation is that the studies described above, and others that simulate early-life adversity and maternal maltreatment, reflect the extremes in maternal licking received and early-life environments. While these factors alone can affect offspring phenotype, this does not reflect the early-life experience of most rats, and only studying the outliers could wash out other important contributing factors. Observational studies that measure more subtle inter-individual differences in maternal licking received within a litter found that pups that received more licking are more active, and make more perioral contact with their mother, as compared to their siblings that receive less licking (Pan et al., 2018; Ragan et al., 2012). These studies suggest that the pups themselves may have an active role in soliciting more or less maternal care. Observational studies within human families suggest similar findings, where different temperaments between siblings could cause differences in parent-child interactions (Jenkins, McGowan, & Knafo-Noam, 2016). These intrinsic differences between individuals could also contribute to later-life offspring phenotype and would, therefore, be important to study. It would also be critical to examine inter-individual differences in response to early-life maternal care received, as many of the previous studies in the field derive their conclusions from group averages.

1.4 Other Important Factors Involved in the Link Between Early-Life Maternal Care Received and Later-Life Phenotype

1.4.1 Interactions with Early-Life Temperature Exposure

As described in Section 1.3.1, one relevant factor identified in the neonatal handling studies is the cooling of the pups by the ambient room temperature exposure during separation. Separating the pups in thermoneutral conditions has previously been shown to reduce or eliminate the effects of handling on later-life phenotype (Hutchings, 1965; Schaefer, 1968). In addition, housing the litter in a room kept at 4°C without any other manipulations of the pups has also

been shown to affect later-life offspring phenotype (Bhatnagar & Meaney, 1995; Schaefer, Weingarten, & Towne, 1962; Schaefer, 1968). The maternal mediation hypothesis proposed that the cooling of the pups was only critical to solicit maternal care, but cooler ambient temperatures can elicit other physiological changes. The physiological effects of ambient temperature changes during development are also relevant to a wide range of other species (Gilbert, 2005, 2012).

Upon exposure to cooler temperatures, rats and other species will produce thyroid hormones via the hypothalamic-pituitary-thyroid axis. The two main thyroid hormones are thyroxine (T4) and triiodothyronine (T3). Triiodothyronine will activate non-shivering thermogenesis by increasing transcription of thermogenic genes in brown adipose tissue (Silva, 1995), which is one of the few mechanisms poikilothermic rat pups use to regulate their temperature in the first postnatal week (Blumberg & Sokoloff, 1998). Concurrently, T3 can promote transcription of other genes in other tissues by binding to thyroid hormone receptors, which acts as transcription factors that binds to thyroid hormone responsive elements on the DNA. Maintaining normal levels of thyroid hormones is essential for optimal brain development (Gilbert & Lasley, 2013; Oppenheimer & Schwartz, 1997). In addition, the effects of neonatal handling and maternal care received have been proposed to be mediated by increases in thyroid hormones resulting from both the cooling of pups during separation and tactile stimulation derived from maternal licking (Sections 1.3.1 and 1.3.2).

However, the effects of early-life ambient temperature exposure and licking-like tactile stimulation on the transcription of thyroid hormone-responsive genes in the brain has not been studied. Interestingly, thyroid hormone has been shown to promote transcription of DNA methyltransferase 3a (DNMT3A), a *de novo* DNA methyltransferase that catalyzes methyl groups to DNA (Kyono et al., 2016). This suggests an additional biological mechanism for the effects of neonatal handling and maternal care received. In addition, thyroid hormone is one regulator of OXT transcription (Adan, Cox, Beischlag, & Burbach, 1993; Adan, Cox, Van Kats, & Burbach, 1992; Dellovade, Zhu, & Pfaff, 1999). If an increase in thyroid hormones from exposure to cooler ambient temperatures can also increase Oxt transcript abundance early in life, this could alter the oxytocinergic system in the maternal brain later in life and increase maternal care provisioning (Section 1.2.3).

1.4.2 Interactions with Offspring Genotype and Gene x Environment Interactions

As mentioned in Sections 1.3.2 and 1.3.3, individual pups within a litter can receive different amounts of maternal licking and this could be due to intrinsic differences between the pups in soliciting maternal care. One underlying variable could be the genotype of the offspring. Genetic differences may affect the amount of maternal care an individual pup receives (Ashbrook, Sharmin, & Hager, 2017; Ashbrook, Gini, & Hager, 2015; Pan et al., 2018), and genetic differences could affect how an individual pup responds to maternal care received later in life.

One potential hypothesis underlying the interactions between the genotype of the offspring and their early-life environments is that some individuals are more sensitive to their early-life environments than others (Pluess, 2015). In other words, some individuals can suffer or thrive depending on their early-life environment, while other individuals are unresponsive to their early-life environment. These individual differences in environmental sensitivity have been associated with genetic variation in serotonin and dopamine-related genes (Nikitopoulos et al., 2014; Pluess, 2015) and appear in populations of multiple species including humans and rats (Wolf, Van Doorn, & Weissing, 2008).

Some studies have reported that gene x environment interactions are involved in the effects of early-life environments on later-life dopaminergic system functioning and dopamine-related behaviour (Sections 1.1.3 and 1.2.3), especially in human cohorts. The limited data on rats have reported similar findings (Lovic et al., 2013). Therefore, it is possible that the effects of maternal licking received on later-life dopaminergic phenotype, including the intergenerational transmission of maternal care, would be dependent on the offspring's genotype in dopamine-related genes. In addition, the effects of gene x environment interactions may involve a different underlying biological mechanism other than DNA methylation at regulatory regions of key dopamine-related genes. Some genetic variants do not affect protein function but can affect the transcript abundance of a gene. As gene transcription is dependent on the environment of the cell and the organism, the effects of certain genetic variants may not be apparent unless there are differences in the environment. However, one important consideration is that any genetic variant that is found to be involved in a gene x environment interaction may not be the causal variant; therefore, it is important to also understand how specific genetic variants could affect transcription of the gene.

1.5 Thesis Objective and Hypotheses

The **objective** of this thesis was to characterize the effects of factors beyond maternal licking received on later-life maternal care provisioning in female rat offspring. I focused on female rats because the mothers are typically the sole providers of care in this species. In addition, many previous studies that investigated the developmental programming of maternal care have focused on male rats or mice (Molet et al., 2017; Murgatroyd et al., 2009; Weaver et al., 2004). This thesis encompasses two complementary projects that investigate the role of early-life temperature exposure and offspring genotype on the oxytocinergic and dopaminergic systems, respectively.

For the first project, I exposed all-female litters to either ambient room temperature (19-22°C) or nest temperature (33-35°C) and provided half the siblings in each temperature group with supplemental tactile stimulation with soft paintbrushes by using a neonatal handling protocol. I assessed thyroid hormone receptor signaling, oxytocin transcript abundance, and the underlying changes in epigenetic mechanisms in the paraventricular nucleus of the hypothalamus at the first postnatal week (Chapter 2). I also measured intergenerational maternal licking provisioning in the female rat offspring at adulthood (Chapter 3). I **hypothesized** that early-life repeated room temperature exposure and supplemental tactile stimulation would synergistically increase thyroid hormone receptor signaling and Oxt transcript abundance and alter DNA methylation levels in the week-old pup brain. In addition, these changes in early-life Oxt transcript abundance would correspond to alterations in intergenerational maternal care provisioning at adulthood.

For the second project, I observed natural variations of maternal licking received between siblings in all-female litters and assessed later-life dopamine-related phenotypes (Chapter 4) and intergenerational maternal licking provisioning (Chapter 5). I also assessed naturally occurring genetic polymorphisms in dopamine-related genes and dopamine levels in select areas in both the adult female virgin and maternal brains. I **hypothesized** that that the relationship between inter-individual maternal care received and later-life dopamine-related behaviour and intergenerational maternal licking provisioning would be dependent on offspring genotype. In addition, this relationship would be mediated by differences in dopaminergic activity in the brain.

Chapter 2
Variations in Early-Life Temperature Exposure and Supplemental Tactile Stimulation on Thyroid Hormone Signaling in Female Rat Pups

This chapter is adapted from:

Lauby, S.C. & McGowan, P.O. (in press). Early-life Temperature Exposure Affects Thyroid Hormone Receptor Signaling and Epigenetic Regulation of the Paraventricular Nucleus in Female Rat Pups. *Proceedings of the Royal Society B*.

Contributions:

SCL and POM designed the study. SCL performed the rat pup manipulations, all downstream molecular work, and analyzed the data. POM supervised the research.

2 Variations in Early-Life Temperature Exposure and Supplemental Tactile Stimulation on Thyroid Hormone Signaling in Female Rat Pups

2.1 Introduction

Maternal care in early life, typically assessed by licking in rodents, has a profound influence on neurodevelopmental trajectories in offspring and their later-life behaviour (Champagne, 2018; McGowan & Roth, 2015). Early-life maternal separation, which disrupts maternal-pup contact, or natural variations in maternal care persistently alter transcript abundance of corticotropin releasing factor (Crf) (Korosi et al., 2010), arginine vasopressin (Avp; Murgatroyd et al., 2009), and the glucocorticoid receptor (Weaver et al., 2004, 2007) in the brain. These changes in transcript abundance have been linked to persistent epigenetic modifications of DNA and histones that alter the binding of transcription factors to DNA. In addition, other maternal factors may play a role in later-life offspring phenotypes either by themselves or via interactions with licking (Champagne, 2018; Russell, 1971). The exposure of pups to lower ambient (room) temperatures as a result of brief disruptions in mother-pup contact has been proposed to be an important component involved in the reduction in stress response following early-life handling (Hutchings, 1967; Russell, 1971; Schaefer et al., 1962). Pups have an inefficient thermoregulatory system (Blumberg & Sokoloff, 1998) and are dependent on non-shivering thermogenesis, huddling with siblings, and proximal contact with the rat mother (Bautista, García-Torres, Prager, Hudson, & Rödel, 2010; Blumberg & Sokoloff, 1998; Leon, Croskerry, & Smith, 1978) to maintain body temperature. Licking-like tactile stimulation has also been shown to reduce the body temperature of rat pups (Sullivan, Shokrai, & Leon, 1988).

Variations in both ambient temperature and licking alter thyroid hormone physiology. Room temperature exposure can induce release of thyroid hormones to evoke non-shivering thermogenesis in brown adipose tissue. In addition, there is some evidence that acute licking-like tactile stimulation can induce conversion of the thyroid hormone thyroxine to triiodothyronine (T3) and lead to downstream DNA demethylation of the glucocorticoid receptor promoter in the hippocampus (Hellstrom et al., 2012).

Thyroid hormones, especially T3, play important roles in neurodevelopment by regulating gene transcription through thyroid hormone receptor (Bernal, Guadaño-Ferraz, & Morte, 2003). In the

developing mouse brain, transcription of DNA methyltransferase 3a (Dnmt3a), an enzyme that catalyzes de novo DNA methylation modifications, can occur through the binding of liganded thyroid hormone receptor to thyroid hormone response elements (TREs) in intragenic regions of Dnmt3a (Kyono et al., 2016). Interestingly, mouse pups that receive higher levels of maternal care also show increased DNA methyltransferase 3a transcript abundance in the hippocampus at postnatal day 7 (Bedrosian, Quayle, Novaresi, & Gage, 2018). Thyroid hormone is also a regulator of oxytocin (Oxt; Adan, Cox, Van Kats, & Burbach, 1992), a neuropeptide produced in the paraventricular nucleus (PVN) of the hypothalamus and involved with stress attenuation, among other phenotypes. Previous work has demonstrated that thyroid hormone and other receptors can bind to the composite hormone response element (CHRE) in the promoter region of Oxt to activate transcription (Adan, Cox, Beischlag, & Burbach, 1993; Adan et al., 1992; Dellovade, Zhu, & Pfaff, 1999). Genetic deletion of this region abolishes transcription, indicating the CHRE is required for proper regulatory control of Oxt (Fields & Gainer, 2015). A CpG dinucleotide flanks the 5' end of the CHRE but its potential role in the epigenetic regulation of Oxt in the PVN has not been examined. Previous work in humans has investigated DNA methylation in the entire Oxt promoter region, including the CHRE (Haas et al., 2016; Toepfer et al., 2019), and the enhancer in the Oxt/Avp intergenic region (King et al., 2017). However, because peripheral tissues were examined in these studies, it is not known whether DNA methylation at this locus is involved in the regulation of Oxt transcript abundance in the brain.

In this study, we investigated the effects and interactions of variations in early-life temperature exposure and licking-like tactile stimulation on thyroid hormone receptor signaling in female rat pups. We focused on females in this study because there are possible sex differences in pup thermoregulation (Blumberg & Stolba, 1996) and maternal care received (Moore & Morelli, 1979). In addition, the increase in estrogen during sexual differentiation in the male pup brain can interact with thyroid hormone for gene transcription (Vasudevan, Ogawa, & Pfaff, 2002).

We measured levels of circulating T3 and transcript abundance in the PVN of thyroid hormone receptors and the thyroid hormone-responsive genes Dnmt3a and Oxt. We hypothesized that early-life room temperature and tactile stimulation would synergistically increase T3 levels as well as increase Dnmt3a and Oxt transcript abundance via changes in thyroid hormone receptor signaling. We moreover predicted that the increase in Oxt transcript abundance would correspond to altered DNA methylation at the CHRE locus and differential thyroid hormone

receptor binding. We also predicted that transcript abundance of other genes not directly regulated by thyroid hormone binding, including other DNA methyltransferases, Crf and Avp, would not show a similar pattern, and that Crf and Avp transcript abundance would be reduced with supplemental tactile stimulation.

2.2 Methods

2.2.1 Rat Breeding

Seven-week-old female (n = 28) and male (n = 16) Long-Evans rats were obtained from Charles River Laboratories (Kingston, NY, USA). They were housed in same-sex pairs on a 12:12 hour light-dark cycle (lights on at 7:00) with *ad libitum* access to standard chow diet and water. For breeding, one male was housed with two females for one week. Females were then housed separately and weighed weekly throughout pregnancy. All animal procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

Females were checked for parturition starting three weeks after breeding at 9:00 and 17:00. Postnatal day (PND) 0 was determined if the birth occurred between 9:00 and 17:00 or if pups were found at 9:00 but have not nursed yet. Pups found at 9:00 with a milk band were considered PND 1. At PND 1, litters were culled to four to six female pups and individually weighed. Paper towel strips were provided to all litters as nesting material. All litters in this study were derived from primiparous rat mothers. A total of 154 female rat pups were used for this study. One group of female rat pups was assessed for transcript abundance and DNA methylation and a separate group of female rat pups was assessed for triiodothyronine levels and chromatin immunoprecipitation enrichment.

2.2.2 Postnatal Manipulations

See Figure 2.1 for timeline of the experimental design between and within litters. From PND 2 to 7, nineteen whole litters were separated from their mother for approximately 25 minutes per day during the light phase (9:00 – 13:00) and placed in a small cage lined with corn cob bedding. Nine litters were placed in a cage warmed with a heating pad (set for 33-35° C; “nest temperature” condition) and ten litters were exposed to room temperature without extra heat (19-

22° C; “repeated room temperature” condition). A total of 51 pups were in the nest temperature condition and 54 pups were in the repeated room temperature condition.

During the daily 25-minute separation, two to three female rat pups within a litter received supplemental tactile stimulation with a camel hair paintbrush (Craftsmart) for 15 consecutive minutes (“Stimulated” condition; n = 78) while the remaining pups were left undisturbed (“Nonstimulated” condition; n = 76). Within the nest temperature condition, 26 pups were in the stimulated condition and 25 pups were in the nonstimulated condition. Within the repeated room temperature condition, 27 pups were in the stimulated condition and 27 pups were in the nonstimulated condition. Pups received the supplemental tactile stimulation on the dorsal region of their body at a rate of approximately two strokes per second. The same pups received supplemental tactile stimulation between days. All pups were individually weighed daily and interscapular temperature was measured with an infrared thermometer (VWR) before and after the tactile stimulation period from PND 3 to 7. From PND 2 to 6, female rat pups were individually marked using odorless and tasteless food colouring (Club House, London, Ontario, Canada) to distinguish between siblings. This method to identify individual siblings has been implemented in previous work by our laboratory, and there have been no effects of the food colouring on maternal care (Pan et al., 2014).

To investigate whether the changes in the temperature exposure and tactile stimulation condition occurred over multiple separations in week-old pups, nine litters were separated once at PND 7 and two to three female rat pups within a litter received supplemental tactile stimulation. These litters were acutely exposed to room temperature without extra heat (“acute room temperature” condition). A total of 49 pups were in the acute room temperature condition; 25 pups were in the stimulated condition and 24 pups were in the nonstimulated condition. All pups were individually weighed and interscapular temperature was measured with an infrared thermometer (VWR) before and after the tactile stimulation period.

The interscapular temperature change (before the tactile stimulation period minus after the tactile stimulation period) was calculated for all groups at PND 7 to verify the room temperature exposure conditions had reduced pup temperature while the nest temperature exposure condition kept a stable pup temperature during the separation period.

At PND 7, all female rat pups were sacrificed following the tactile stimulation period and blood and brain were collected. One stimulated and one nonstimulated sibling were decapitated at a time. To examine if the time elapsed since the tactile stimulation period would affect the physiology of the pups, we noted the order pups were sacrificed for use as a control variable. Brains were flash frozen in isopentane and kept on dry ice. Whole blood samples were kept on ice to coagulate order at least 30 minutes before being centrifuged at 4000 x g at 4° C for 30 minutes. The serum was transferred to a new tube and the cell pellet was discarded. Both serum and brain were stored at -80° C. One to two pups per stimulation group per litter were used for all downstream molecular analyses.

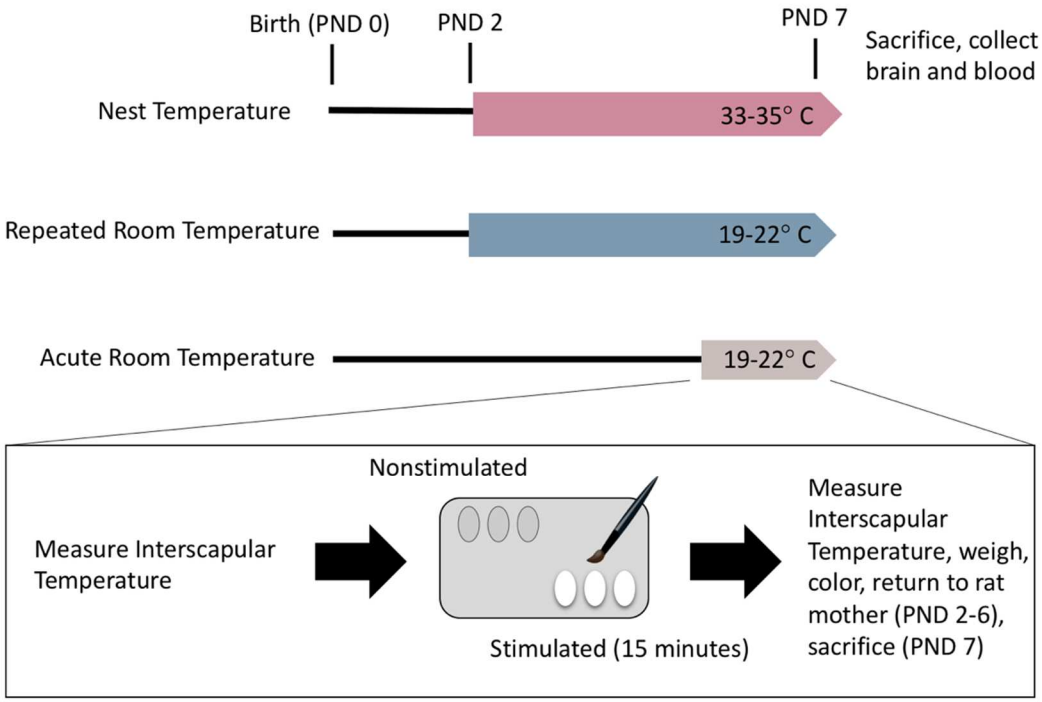

Figure 2.1 Schematic diagram of the experimental design between litters and within litters of female rat pups.

2.2.3 Maternal Care Observations

From PND 2 to 6, each litter was video recorded for one hour two times during the light phase (13:00-14:00, 17:00-18:00) and three times during the dark phase (21:00-22:22, 1:00-2:00, 5:00-6:00). These videos were coded with Observer XT 10.5 (Noldus) for maternal behaviour by five

coders with high inter-rater reliability (≥ 90%). Nursing, licking, nest-building, and other self-directed behaviours were scored every three minutes using an ethogram based on previous literature (Champagne, Francis, Mar, & Meaney, 2003). A total of 100 observations per day per mother were coded and each behaviour was represented as a percentage of the frequency of behaviour coded over total observations multiplied by 100. Total nursing was calculated as a sum of low crouch, high crouch, and supine nursing observations. Total licking was calculated as a sum of anogenital and body licking observations.

Two litters from the nest temperature condition had missing maternal care recordings at PND 2 and one litter from the repeated room temperature condition had missing maternal care recordings from PND 2-3 due to technical errors.

2.2.4 Serum Total Triiodothyronine Enzyme-Linked Immunosorbent Assay (ELISA)

The active thyroid hormone, triiodothyronine (T3), was measured in the pup serum (n = 5-7 per group) using an ELISA (MP Biomedicals Inc., USA) following the manufacturer's instructions. For each pup sample, technical duplicates were measured when possible and 50 µl of serum per well was used. To keep all samples within the linear phase of the standard curve, the serum was diluted 1:1 with the "0" standard. Each plate was run and normalized with a control sample with a known concentration of total T3 (Control Set I, Tri-level (for Steroid and Thyroid Hormones); MP Biomedicals Inc., USA). Concentration of total T3 was determined using a 4-point logistic curve using an online software (https://elisaanalysis.com/app) and multiplied by two to account for the dilution factor.

2.2.5 Transcript Abundance with Quantitative Polymerase Chain Reaction (qPCR)

Postnatal day 7 pup brains (n = 5-6 per group) were cryosectioned with 50 µm slices using a Leica CM3050S cryostat. The paraventricular nucleus of the hypothalamus (PVN) (-1.40 to -2.00 mm Bregma) was microdissected using an atlas for the developing rat brain (George Paxinos, Tork, L.H., & Valentino, 1990) and a supplementary atlas for the PND 7 rat brain (Khazipov et al., 2015). RNA was extracted using a RNeasy Micro Kit (Qiagen) following the manufacturer's instructions. Concentration and purity of RNA was assessed using a spectrophotometer (Nanodrop ND-2000C, Thermo Scientific). Up to 1 µg of RNA was

converted to cDNA (Applied Biosystems High Capacity cDNA Conversion Kit) and was diluted to 5 ng/µl assuming 100% conversion efficiency.

Transcript abundance of candidate genes were assessed using StepOne Plus real-time PCR software with Fast SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using technical triplicates. Specifically, I analyzed arginine vasopressin (Avp), corticotropin releasing factor (Crf), DNA Methyltransferase 1 (Dnmt1), DNA Methyltransferase 3a (Dnmt3a), DNA Methyltransferase 3b (Dnmt3b), oxytocin (Oxt), thyroid hormone receptor α1 (Thra1), and thyroid hormone receptor β (Thrb). Each plate was run and corrected with one randomly assigned cDNA sample that was measured on all plates. All transcripts were normalized to the GEOmean of the Actin-β (Actb) and Ubiquitin C (Ubc) transcript and relative quantification was calculated by the ΔCT method. Table 2.1 displays the custom-made primer sets created from Primer-BLAST software (National Center for Biotechnology Information) and previous literature (Abuaish, Spinieli, & McGowan, 2018; Matsuzaki et al., 2015).

Table 2.1 List of primers and sequences used for qPCR, bisulfite sequencing, and ChIP.

Gene	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	Reference
qPCR			
Actb	ACCCGCGAGTACAACCTTCTTG	TATCGTCATCCATGGCGAACTGG	
Avp	CATCCGACATGGAGCTGAGAC	CCACGCAGCTCTCATCGCT	
Crf	TTCCTGTTGCTGTGAGCTTG	TCACCTTCCACCTTCTGAGG	(Abuaish et al., 2018)
Dnmt1	ACCTACCACGCCGACAT	AGGTCCTCTCCGTACTCCA	
Dnmt3a	ACGCCAAAGAAGTGTCTGCT	CTTTGCCCTGCTTTATGGAG	
Dnmt3b	GATGTGACACCTAAGAGCAGCAGTAC	CAAACTCCTTGTCATCCTGATACTCA	
Oxt	TGCGCAAGTGTCTTCCCTGCG	AGCCATCCGGGCTACAGCAGA	(Matsuzaki et al., 2015)
Thra1	AAGCCAAGCAAGGTGGAGTGTG	ATCTGGTGACCTGGCACTGTTC	
Thrb	AGCTCTGGCATTCCCTTATTCA	ATCCGTGGTTTCCCTCTCCT	
Ubc	CACCAAGAAGGTCAAACAGGAA	AAGACACCTCCCCATCAAACC	
Bisulfite Pyrosequencing			
Oxt (Outer)	AGTTTTATTTTGAGGTATTGGATTTTATG	CACACTATTTAAAAACAAACCCTTCATT	
Oxt (Semi-Nested)		Biotin-ACTATTTAAAAACAAACCCTTCATTTACA	
Oxt (Sequencing)	TTGAGTTTTAGGTTATTAGTTG		
ChIP			
Dnmt3a (+30.3 kbp TRE)	GGAAGTCAGATGAGGTCACGG	CCAGAGCCAGAGCAGTTACTA	
Dnmt3a (+49.3 kbp TRE)	CACCAACTTCCTCCAGGGTTA	GCCCTTGACGGGTTATTCTT	
Oxt (CHRE)	CTCCAGGTCATTAGCTGAGGC	TGCATGACTGGTCACAGCAG	

Actb: Actin beta, **Avp**: Arginine vasopressin, **Crf**: Corticotropin releasing factor, **Dnmt1**: DNA methyltransferase 1, **Dnmt3a**: DNA methyltransferase 3a, **Dnmt3b**: DNA methyltransferase 3b, **Oxt**: Oxytocin, **Thra1**: Thyroid hormone receptor alpha-1, **Thrb**: Thyroid hormone receptor beta, **Ubc**: Ubiquitin C, **CHRE**: Composite hormone response element, **TRE**: Thyroid hormone response element

2.2.6 DNA Methylation Analysis of the Oxytocin Promoter

DNA from six PVN from each supplemental tactile stimulation group in the repeated room temperature condition and nest temperature condition (total n = 24) was extracted using the Masterpure Complete DNA/RNA Extraction kit (Epicentre) and 300 ng of DNA was used for bisulfite conversion using the Epitect Fast Bisulfite Conversion kit (Qiagen) following the manufacturer's instructions. Semi-nested PCR was performed with custom-made primers created from the Pyromark Q-CpG 1.0.9 software (Table 2.1) and targeted one CpG site flanking the oxytocin composite hormone response element in the promoter region (chr3:123106520; rn6). The biotinylated amplicons were verified with gel electrophoresis and extracted with the MinElute Gel Extraction Kit (Qiagen). Pyrosequencing was done using a Pyromark Q106 ID pyrosequencer with technical triplicates and CpG methylation levels were calculated using the Pyromark Q-CpG 1.0.9 software.

2.2.7 Chromatin Immunoprecipitation (ChIP) with qPCR for DNA Methyltransferase 3a and Oxytocin

Two PVN from the same litter and supplemental tactile stimulation group were pooled (total n = 3 for repeated room temperature and n = 4 for nest temperature) for chromatin immunoprecipitation (ChIP) based on the protocol from Stefanelli and colleagues (Stefanelli et al., 2018). Two batches were done with 1-2 pooled PVN samples per temperature condition per batch.

Pooled PVN tissue samples were crosslinked with 1% formaldehyde (Sigma-Aldrich) for 10 minutes at 26° C. The samples were quenched with 1.25 M Glycine and left at room temperature for 5 minutes. The PVN tissue was pelleted using a centrifuge at 21,100 xg for 30 seconds before five washes with ice-cold PBS and a protease inhibitor cocktail (Roche) dissolved in PBS. The PVN tissue was homogenized with SDS lysis buffer (0.25 M Sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES (pH 6.5), 5 mM $MgCl_2$, 0.5% Triton X-100) and centrifuged at 4,700 xg to pellet the cell nuclei. The SDS lysis buffer was removed and a salt buffer (50 mM NaCl, 10 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 1 mM $CaCl_2$) was added prior to sonication on the lowest power setting (Output power: 3-4 Watts; 3x for 10 sec on, 30 sec off; Fisher Scientific Sonic Dismembrator Model 100). The samples were incubated with 150 units micrococcal nuclease (Cell Signaling) at 37° C for 10 minutes before quenching with 5 µl 0.5 M EDTA and placed on

ice. 10% SDS was added to each sample before being centrifuged at 17,000 xg for 5 minutes, aliquoted, and diluted 4x with a ChIP dilution buffer. Each ChIP aliquot contained 20 µl of Millipore Protein G magnetic beads and 10 µg of thyroid hormone receptor α/β (Thermo-Fisher Scientific, cat no. MA1-215) or 2 µg of H3K27ac (Abcam, cat no. ab177178) antibody and incubated at 4° C overnight. The following day, the beads were pelleted using a magnetic separator and washed with ice-cold low-salt, high-salt, LiCl (Millipore), and Tris-EDTA buffers. Crosslinks were reversed for ChIP aliquots and input chromatin samples using 10 µg of proteinase K in Tris-EDTA buffer (with 1% SDS) at 65° C for at least 2 hours before purification using a PCR clean-up kit (BioBasic).

Primers for the Oxt composite hormone response element (CHRE) were created with Primer-BLAST software (Table 2.1). Primers for the Dnmt3a 30.3 kbp and 49.3 kbp thyroid hormone response element (TRE) were created with Primer-BLAST software using the rat homologous sequences from previous literature using mouse (Kyono et al., 2016; Table 2.1). Enrichment was measured for each gene locus using qPCR for the ChIP DNA and input chromatin samples with technical triplicates. The enrichment for thyroid hormone receptors and H3K27ac were normalized and calculated as a relative percentage of input chromatin.

2.2.8 Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corporation). To examine the effects of temperature condition on maternal care received within the first postnatal week, a repeated-measures 3 (Nest Temperature, Repeated Room Temperature, and Acute Room Temperature) x 5 (Postnatal days 2-6) linear mixed model was used to correct for missing datapoints and random factors. To examine the effects of room temperature exposure and tactile stimulation on female pup interscapular temperature change, serum T3 concentration, and transcript abundance, a 3 (Nest Temperature, Repeated Room Temperature and Acute Room Temperature) x 2 (Stimulated and Nonstimulated) general linear model was used to compare manipulation groups and their interactions. As there was a main effect of sacrifice order on Thra1 and Dnmt1 transcript abundance, a linear mixed model was used with order of sacrifice as a random factor. Significant effects of temperature condition were followed with a post-hoc test using Fisher's Least Significant Differences. Significant effects of tactile stimulation or a significant temperature condition x tactile stimulation interaction were followed with a post-hoc test within each

temperature condition. To examine the effects of room temperature exposure and tactile stimulation on DNA methylation of the Oxt promoter, a 2 (Nest Temperature and Repeated Room Temperature) x 2 (Stimulated and Nonstimulated) general linear model was used to compare manipulation groups and their interactions. To examine the effects of room temperature exposure on thyroid hormone receptor and H3K27ac enrichment on Dnmt3a and Oxt, a one-way (Nest Temperature and Repeated Room Temperature) linear mixed model was used with batch as a random factor. All effects were considered statistically significant at $p \leq 0.05$ and marginally significant at $p \leq 0.10$.

2.3 Results

2.3.1 Postnatal Day 7 Pup Characteristics and Maternal Care Received

There was a main effect of temperature exposure on interscapular temperature change at PND 7 ($F_{(2, 139)} = 91.777$, $p < 0.001$). Female rat pups in the nest temperature condition maintained a relatively stable interscapular temperature. In contrast, pups in both the acute and repeated room temperature conditions showed a significant reduction in interscapular temperature during the separation period. This reduction in temperature was greater for pups exposed to repeated room temperature compared to the pups in the acute room temperature condition (Figure 2.2A).

There was no main effect of temperature exposure on total licking received ($F_{(2,25.383)} = 1.498$, $p = 0.243$; Figure 2.2B) and total nursing received ($F_{(2,25.340)} = 1.276$, $p = 0.296$) from the rat mother during the first postnatal week. However, total nursing significantly declined over the first postnatal week ($F_{(2,24.461)} = 9.160$, $p < 0.001$) and there was a significant temperature exposure x postnatal day interaction ($F_{(2,24.438)} = 2.480$, $p = 0.040$; Figure 2.2C). Rat mothers with litters in the repeated room temperature condition and nest temperature condition provided significantly more nursing than mothers with litters in the acute room temperature condition at PND 4.

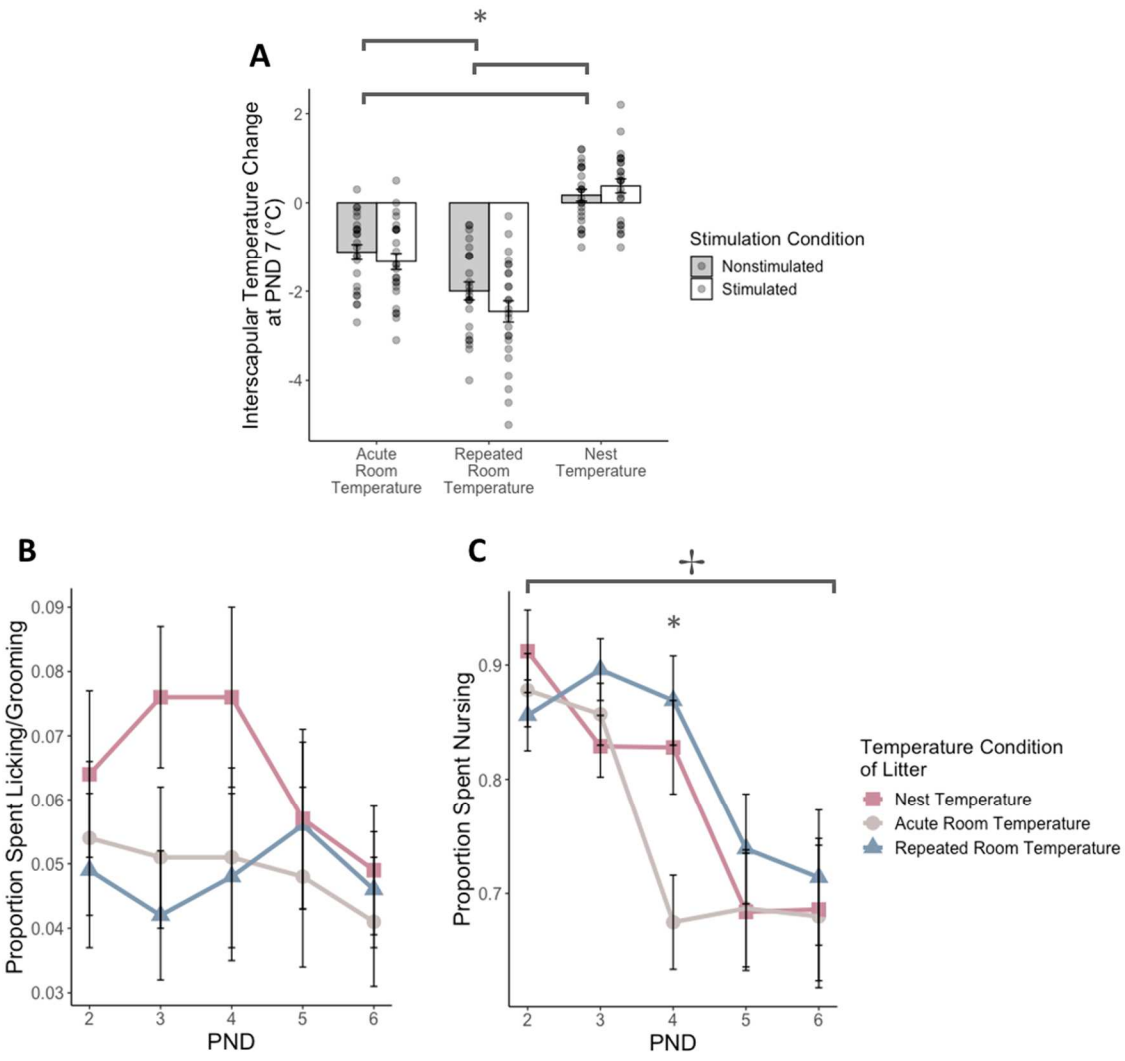

Figure 2.2 Early-life temperature exposure significantly affected temperature change in week-old female pups with minor differences in maternal care received. (A) Female rat pups exposed to repeated room temperature had a significantly more pronounced reduction in body temperature compared to pups exposed to acute room temperature. Both groups had a more pronounced reduction in body temperature than pups exposed to nest temperature. (B) Litters in the different temperature conditions received similar amounts of maternal licking during the first postnatal week. (C) Nursing decreased during the first postnatal week and the litters in the acute room temperature condition receiving less nursing at postnatal day (PND) 4 than litters exposed to repeated room temperature or nest temperature. Barplots are shown with mean +/- SEM and line graphs are shown with estimated marginal means +/- SEM. ∗ $p < 0.05$ main effect of temperature condition, ✢ $p < 0.05$ main effect of postnatal day.

2.3.2 Circulating Total Triiodothyronine Levels

There was a main effect of temperature exposure on total T3 levels in the PND 7 rat pup serum ($F_{(2,42)} = 35.141$, $p < 0.001$; Figure 2.3A). Female rat pups in the repeated room temperature condition had significantly lower levels of total T3 than pups in the nest temperature condition and acute room temperature condition (Post-hoc p's < 0.001). In addition, pups in the acute room temperature condition had higher levels of total T3 than pups in the nest temperature condition (Post-hoc $p < 0.001$). There was no main effect of supplemental tactile stimulation and no interaction (p's > 0.1) on serum total T3 levels.

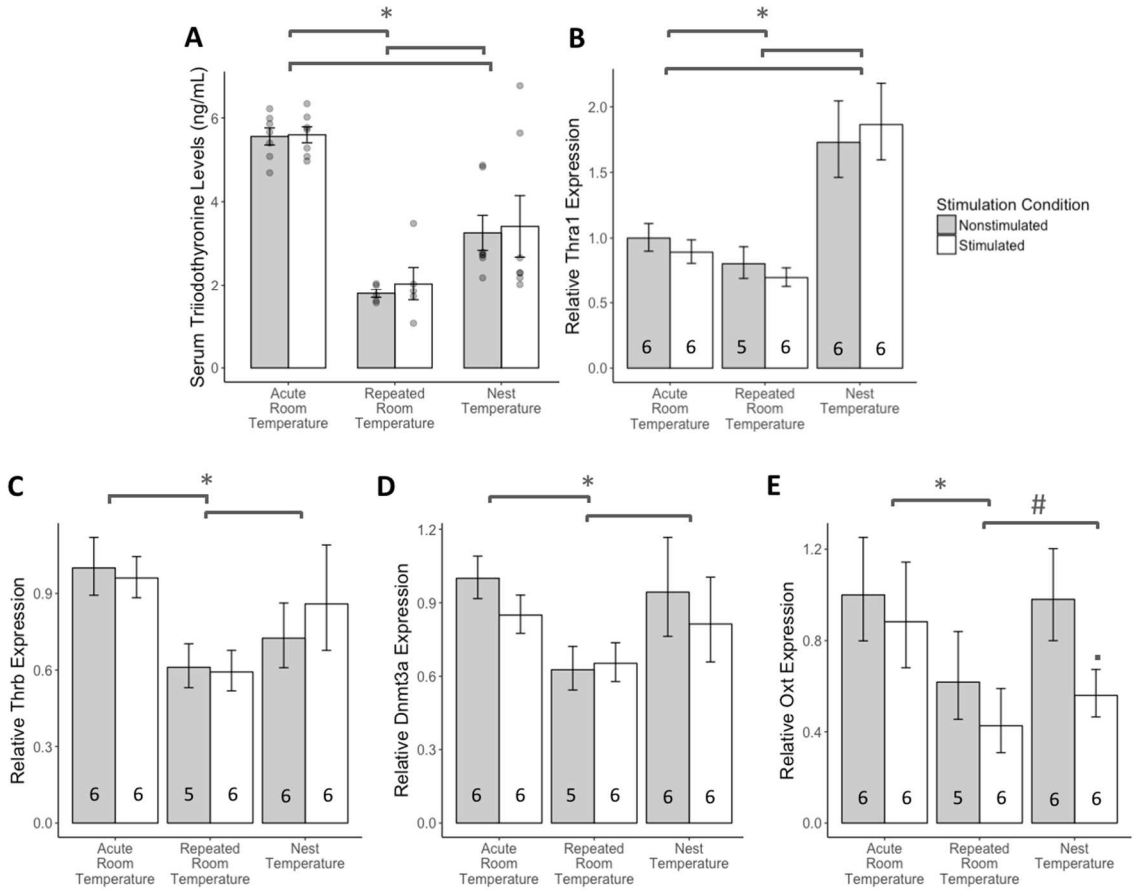

Figure 2.3 Repeated room temperature exposure decreased thyroid hormone activity with minimal effects of supplemental tactile stimulation. (A) Pups with repeated room temperature exposure had a significant decrease in circulating total triiodothyronine levels, (B) thyroid hormone receptor alpha 1 (Thra1) transcript abundance, (C) thyroid hormone receptor beta (Thrb) transcript abundance (D) DNA methyltransferase 3a (Dnmt3a) transcript abundance (E)

and a marginal effect on oxytocin (Oxt) transcript abundance than rat pups exposed to nest temperature. Pups with acute exposure to room temperature had significant increases in circulating total triiodothyronine levels and thyroid-related transcript abundance than pups with repeated room temperature exposure. (E) Pups provided supplemental tactile stimulation had decreased Oxt transcript abundance in the nest temperature condition. The serum triiodothyronine barplot is displayed with mean +/- SEM. The qPCR barplots are displayed as fold changes relative to the pups handled once and nonstimulated +/- SEM (recalculated as $2^{+/-SEM}$ to fit the logarithmic scale). ∗ $p < 0.05$, # $p < 0.10$ main effect of temperature condition; ▪ $p < 0.05$ main effect of tactile stimulation condition.

2.3.3 Transcript Abundance

Female rat pups with repeated room temperature exposure showed reduced thyroid hormone receptor signaling-related transcript abundance and rat pups with supplemental tactile stimulation showed reduced Oxt transcript abundance. There were main effects of temperature exposure on Thra1 ($F_{(2, 28.755)} = 113.774$, $p < 0.001$; Figure 2.3B), Thrb ($F_{(2, 29)} = 13.233$, $p < 0.001$; Figure 2.3C), Dnmt3a ($F_{(2, 29)} = 8.481$, $p = 0.001$; Figure 2.3D), and Oxt ($F_{(2, 29)} = 3.794$, $p = 0.034$; Figure 2.3E) transcript abundance. Specifically, pups in the repeated room temperature condition had a significant reduction of Thra1 (Post-hoc $p < 0.001$), Thrb (Post-hoc $p < 0.001$), Dnmt3a (Post-hoc $p = 0.001$), and Oxt (Post-hoc $p = 0.008$) transcript abundance compared to pups in the acute room temperature condition. Pups in the repeated room temperature condition also had a significant reduction of Thra1 (Post-hoc $p < 0.001$), Thrb (Post-hoc $p = 0.011$), Dnmt3a (Post-hoc $p = 0.003$), and a marginal reduction in Oxt (Post-hoc $p = 0.092$) transcript abundance compared to pups in the nest temperature condition. In addition, pups in the acute room temperature condition had a significant reduction of Thra1 (Post-hoc $p < 0.001$) and a significant increase of Thrb (Post-hoc $p = 0.018$) transcript abundance compared to the nest temperature condition.

There was a marginal effect of supplemental tactile stimulation on Oxt transcript abundance ($F_{(1, 29)} = 3.890$, $p = 0.058$). Pups with supplemental tactile stimulation had a decrease in Oxt transcript abundance if they were in the nest temperature condition ($F_{(1,10)} = 7.011$, $p = 0.024$; Figure 2.3E). There were no main effects of temperature or tactile stimulation on Avp (Figure

2.4A) and Crf (Figure 2.4B) transcript abundance and no interactions in any of the genes measured (p's > 0.1).

There was also a main effect of temperature exposure on Dnmt1 transcript abundance ($F_{(2, 28.757)} = 9.233$, $p = 0.001$; Figure 2.4C). Female rat pups with repeated room temperature exposure had a significant reduction in Dnmt1 transcript abundance relative to pups in the acute room temperature condition (Post-hoc $p = 0.015$) and pups in the nest temperature condition (Post-hoc $p < 0.001$). There was a marginal reduction in Dnmt1 among pups in the acute room temperature condition compared to pups in the nest temperature condition (Post-hoc $p = 0.087$). There were no main effects of temperature or tactile stimulation on Dnmt3b transcript abundance (Figure 2.4D).

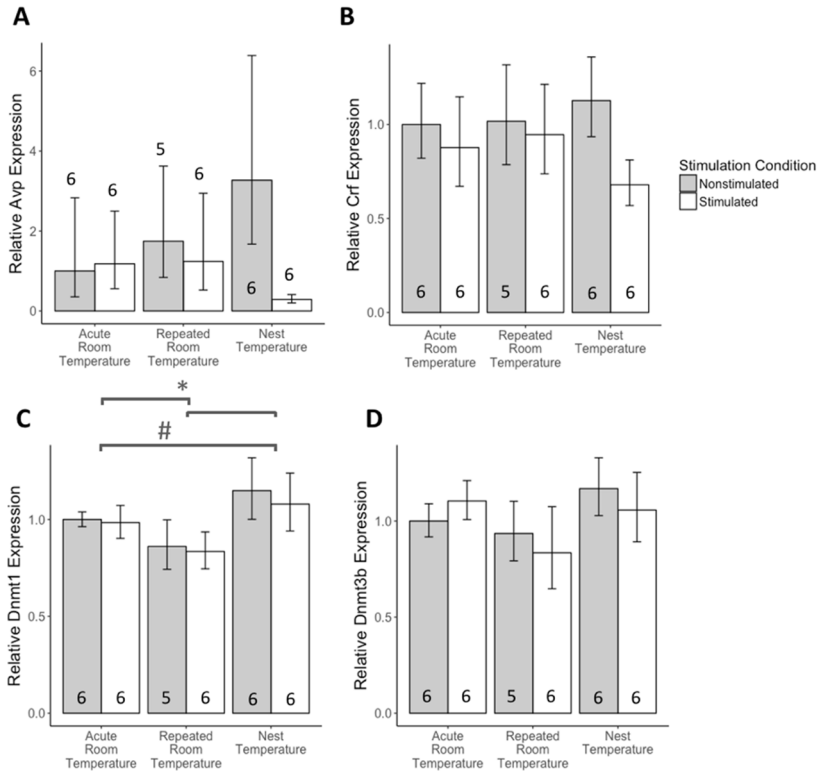

Figure 2.4 Early-life temperature exposure affected DNA methyltransferase 1 (Dnmt1) transcript abundance and neither early-life temperature exposure nor supplemental tactile stimulation affected DNA methyltransferase 3b (Dnmt3b) and other stress-related neuropeptides in the paraventricular nucleus. (A) Female rat pups with repeated room temperature exposure had a significant decrease in Dnmt1 transcript abundance than rat pups acutely exposed to room

temperature and rat pups exposed to nest temperature. There are no main effects or interaction of temperature exposure and supplemental tactile stimulation on (B) Dnmt3b, (C) arginine vasopressin (Avp) or (D) corticotropin releasing factor (Crf) transcript abundance. The qPCR barplots are displayed as fold changes relative to the pups handled once and nonstimulated +/- SEM (recalculated as $2^{+/-SEM}$ to fit the logarithmic scale). ∗ $p < 0.05$, # $p < 0.10$ main effect of temperature condition.

2.3.4 Oxytocin DNA Methylation Levels at the Composite Hormone Response Element

To investigate the long-term changes of room temperature exposure and supplemental tactile stimulation, we restricted the analysis on DNA methylation levels to the rat pups with repeated early-life separations. Female rat pups with repeated room temperature exposure had increased DNA methylation levels flanking the composite hormone response element (CHRE) in the Oxt promoter compared to pups in the nest temperature condition ($F_{(1,20)} = 5.256$, $p = 0.033$; Figure 2.5). There was no main effect of supplemental tactile stimulation and no interaction (p's > 0.1) on DNA methylation levels.

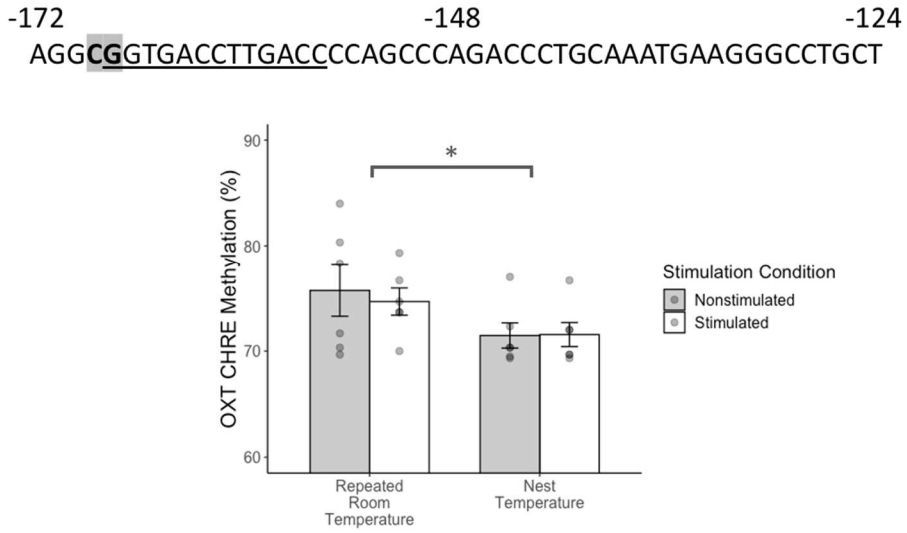

Figure 2.5 DNA methylation at the CpG site (highlighted) flanking the composite hormone response element in the oxytocin promoter (underlined) increased in female rat pups with repeated room temperature exposure. Barplot is displayed with mean +/- SEM. ∗ $p < 0.05$ main effect of temperature condition.

2.3.5 Chromatin Immunoprecipitation Enrichment of Thyroid Hormone Receptor and H3K27ac

The Oxt CHRE in the promoter region and two thyroid response elements (TRE) within the Dnmt3a gene (Figure 2.6A) were analyzed for thyroid hormone receptor binding and H3K27ac levels. Female rat pups in the repeated room temperature condition had a significant increase in thyroid hormone receptor enrichment at the Oxt CHRE relative to pups in the nest temperature condition ($F_{(1,4.015)} = 17.811$, $p = 0.013$; Figure 2.6B). There were no effects of repeated temperature at the Dnmt3a +30.3 kbp TRE ($F_{(1,4.046)} = 0.022$, $p = 0.889$) or +49.3 kbp TRE ($F_{(1,4.031)} = 0.234$, $p = 0.654$). Likewise, there were no effects of temperature on H3K27ac enrichment in any gene loci tested (p's > 0.1; Figure 2.6C).

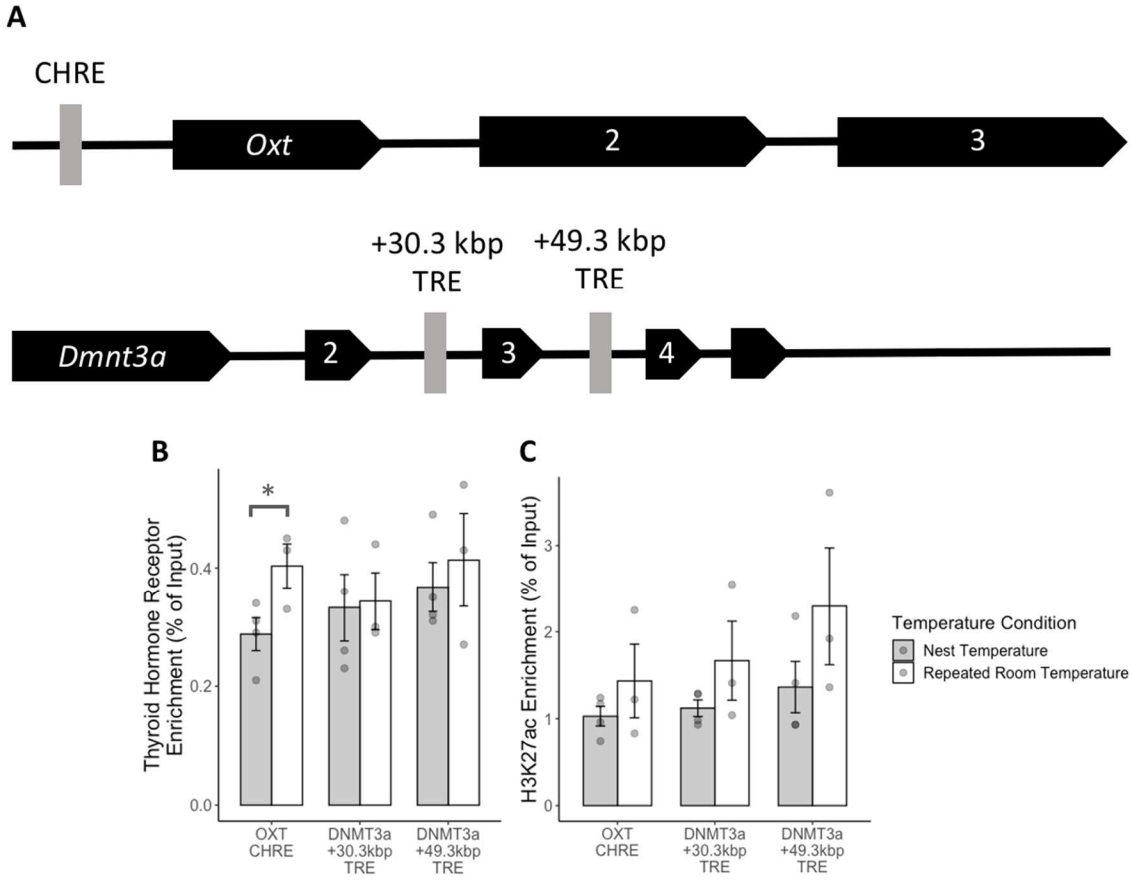

Figure 2.6 Early-life temperature exposure affected thyroid hormone receptor binding at the oxytocin composite hormone response element (CHRE) and did not change histone levels of H3K27ac in the paraventricular nucleus. (A) Schematic diagram of regulatory gene loci tested for Oxt and Dnmt3a. (B) Female rat pups with repeated room temperature exposure had

significant increases in thyroid hormone receptor enrichment at the Oxt CHRE but not at the Dnmt3a thyroid hormone response elements (TRE) with (C) no significant changes in H3K27ac enrichment in any gene loci. Barplots are displayed with mean +/- SEM. ∗ $p < 0.05$ main effect of temperature condition.

2.4 Discussion

In this study, we investigated how two factors rat pups commonly experience in the early-life maternal environment, variations in temperature exposure and licking-like tactile stimulation, would affect neurodevelopment by changes in thyroid hormone receptor signaling at the neonate stage. This is the first study to our knowledge to investigate the contribution of early-life room temperature exposure on epigenetic modifications in the brains of rat pups. We found that female rat pups subjected to repeated room temperature exposure in early-life showed a reduction in several measures of thyroid hormone receptor signaling relative to pups with early-life nest temperature exposure, including circulating triiodothyronine and transcript abundance in the PVN of thyroid hormone receptors and the thyroid hormone-responsive genes Dnmt3a and Oxt. These effects were associated with increased DNA methylation and thyroid receptor binding at the composite hormone response element in the Oxt promoter in room temperature-exposed pups. Female rat pups with acute room temperature exposure showed the highest levels of T3 and an increase in measures of thyroid hormone receptor signaling tested relative to rat pups with repeated room temperature exposure. There was no effect of supplemental tactile stimulation on most of the thyroid hormone receptor signaling measures and minor effects on transcript abundance of Oxt in the PVN. These findings indicate that early-life room temperature exposure, a proxy for reduced maternal contact, may influence offspring phenotype via changes in thyroid hormone receptor signaling and downstream DNA methylation modifications. In addition, our results suggest that the changes in Dnmt3a transcript levels and DNA methylation at the Oxt CHRE may occur as a consequence of alterations in thyroid hormone receptor signaling.

2.4.1 Effects on Thyroid Hormone Receptor Signaling

We predicted that room temperature exposure would increase circulating triiodothyronine levels in order to activate thermogenesis. However, we found decreased circulating triiodothyronine in response to repeated room temperature exposure, as well as decreased transcript abundance of thyroid hormone receptors and the thyroid hormone-responsive genes Dnmt3a and Oxt,

compared to pups with nest temperature exposure. Though the decrease in Oxt is a nonsignificant trend, it followed a general pattern of repression of thyroid hormone receptor signaling with an increase in DNA methylation in the Oxt promoter region. Female rat pups exposed to acute room temperature showed the predicted increase in these measures, demonstrating that a suppression in thyroid hormone receptor signaling occurs with repeated exposures to room temperature. We also observed minimal alterations in maternal care received between the different temperature exposure groups; therefore, the data suggest that these changes in the rat pups are more likely due to the temperature manipulations directly than to indirect changes in maternal care received.

Cold acclimatization in adult rats can decrease levels of thyroid hormone released in response to a cold stressor (Ibidapo & Sofola, 1989; Quintanar-Stephano, Quintanar-Stephano, & Castillo-Hernandez, 1991), possibly as thyroid hormone is no longer required to activate thermogenesis (Zaninovich, Raíces, Rebagliati, Ricci, & Hagmüller, 2002). Interestingly, rat pups exposed to repeated room temperature also showed a more pronounced decrease in interscapular temperature, the main site for thermogenesis, than rat pups with an acute exposure to room temperature during the separation period at PND 7. It is unknown if these changes in thyroid hormone physiology persist into adulthood and if they would affect other phenotypes. However, studies in mice show a potential link between nest quality, with lower quality nests associated with greater exposure of the pups to the ambient temperature, and increased metabolic rate, thermogenesis, and thyroxine levels at adulthood (Lacy, Lynch, & Lynch, 1978; St-Cyr, Abuaish, Welch, & McGowan, 2018). In addition, the downstream alterations in early-life Oxt transcript abundance could affect social interaction, response to stressors, and other behaviours at adulthood (Miller & Caldwell, 2015). Overall, these findings suggest that the effects of repeated room temperature exposure in early life may reflect a physiological adaptation to cold stressors in the rat pup. Future work is needed to elucidate the specific changes in thyroid hormone physiology that is underlying this adaptation.

We also found that repeated room temperature exposure was associated with decreased transcript abundance of Dnmt1, which has not been previously shown to be responsive to thyroid hormone. However, one study has shown Dnmt3a and Dnmt1 can cooperatively add de novo methyl groups to both strands of DNA (Fatemi, Hermann, Gowher, & Jeltsch, 2002). Incubation of a DNA fragment with Dnmt3a before Dnmt1 will stimulate DNA methylation modifications while

the inverse does not, suggesting that this relationship is mainly driven by changes in Dnmt3a (Fatemi et al., 2002), though follow-up studies have not been done to our knowledge. Therefore, it is possible that the changes in Dnmt1 transcript abundance in this study were a downstream effect of the changes in Dnmt3a transcript abundance.

We did not find effects of supplemental tactile stimulation on most of the thyroid hormone receptor signaling measures tested, which contrast from the study results by Hellstrom and colleagues (Hellstrom et al., 2012); however, their study used male rat pups as subjects with one instance of supplemental tactile stimulation for five minutes. It is possible that the effects of tactile stimulation on T3 levels are transient and the deiodinase activity decreased over prolonged periods of tactile stimulation in our study. It is also possible that female rat pups respond differently to tactile stimulation than male pups, given that males and females can respond differently to natural variations in maternal care received (Francis et al., 2002).

We found that supplemental tactile stimulation was associated with a trend in decreased Oxt transcript abundance, though this did not correspond to changes in DNA methylation in the CHRE. We also find similar but nonsignificant decreases in Crf and Avp transcript abundance. There is some evidence that tactile stimulation can increase oxytocinergic neuron activity (Barrett et al., 2015) and that variation in maternal care can be transmitted across generations through changes in the oxytocinergic system in female offspring (Champagne, Diorio, Sharma, & Meaney, 2001; Toepfer et al., 2017). However, other studies have shown that augmented maternal care and brief early-life separations decrease oxytocin transcript abundance and oxytocin-positive neurons in the hypothalamus (Vogel Ciernia et al., 2018; Winkelmann-Duarte et al., 2007). In addition, one study showed that the proximal thermotactile contact with the rat mother but not maternal licking increased oxytocin neuropeptide concentrations in the hypothalamus (Kojima et al., 2012). Therefore, the relationship between maternal care received and pup oxytocin appears to be complex, and temperature exposure may be one confounding factor in these studies.

2.4.2 Effects on DNA methylation and the Role of Thyroid Hormone Receptor

We hypothesized that changes in transcript abundance of Oxt and Dnmt3a would be mediated by differences in DNA methylation (in oxytocin) and thyroid hormone receptor binding. We found

that a decrease in Oxt transcript abundance in the repeated room temperature exposure condition corresponded to increased levels of DNA methylation and thyroid hormone receptor binding at the CHRE in the promoter region of oxytocin. There is some evidence that DNA methylation changes associated with variations in maternal care received occur as a consequence of differential transcription factor binding (Weaver et al., 2007). One possible explanation of our findings is that there is a repressive effect of unliganded thyroid hormone receptor on Oxt transcription. If unliganded by T3, thyroid hormone receptor can recruit repressors, including silencing mediator of thyroid and retinoic receptors (SMRT) and nuclear receptor corepressor 1 (NCOR1), and activate the histone deacetylase HDAC3 to silence gene transcription (Guenther, Barak, & Lazar, 2001; Wu & Koenig, 2000). HDAC3 appears to mainly affect H3K9ac levels (Bhaskara et al., 2010), which may explain the nonsignificant differences in H3K27ac observed in our study. However, no studies to our knowledge have found HDAC3 activity preceding DNA methyltransferase binding or DNA methylation modifications. These findings imply that higher DNA methylation at the oxytocin CHRE may occur as a consequence of unliganded thyroid hormone receptor binding, but future work is needed to elucidate this hypothesis more directly and which thyroid hormone isoforms are involved.

While repeated room temperature exposure decreased Dnmt3a transcript abundance, this was not associated with differences in thyroid hormone receptor binding in the regulatory TRE regions tested. We tested these two sites because of previous evidence of differential binding of thyroid hormone receptors in regulating Dnmt3a transcript in the developing mouse brain (Kyono et al., 2016). However, other TREs exist in the Dnmt3a gene and their responsiveness to thyroid hormone can be species specific (Kyono et al., 2016). Future studies are needed to verify which Dnmt3a TREs would be most relevant for the transcription of Dnmt3a in the neonatal rat brain. In addition, it is possible that our sample size and therefore statistical power was not large enough to detect differences in thyroid hormone receptor binding at the Dnmt3a TREs.

2.4.3 Limitations

We did not find differences in maternal care received throughout the first postnatal week between temperature groups, which contradicts previous literature on differences in maternal licking received immediately following reunion of the pups in neonatal handling studies (Smotherman & Bell, 1980; Villescas et al., 1977). While we did not observe long-term

differences in maternal care received between groups, maternal care received could still be a contributing factor to the thyroid hormone measures or transcript abundance of the neuropeptides tested in this study. It would be useful to observe the duration of maternal care received by live observations along with undisturbed video recordings in future work. In addition, it is possible that the all-female litters used in this study may not reflect typical maternal care patterns in mixed litters as male pups typically receive more licking from their mother (Moore & Morelli, 1979).

Another important caveat is that we assumed that the pups with nest temperature exposure and nonstimulated would be an unmanipulated control group. However, there could be other influential factors involved during the brief early-life separations that we did not consider. We would need to verify that the nest temperature exposed, nonstimulated group is an unmanipulated group by comparing to pups in an unhandled group that are sacrificed immediately after separation at PND 7. Similarly, the differences between the effects of acute room temperature exposure and repeated room temperature exposure on thyroid hormone receptor signaling could also be due to other influential factors related to multiple early-life separations. An acute nest temperature exposure group would have been useful to discern the effects of the acute temperature exposure from an acute separation from the mother more broadly.

Finally, although we observed statistically significant differences between temperature exposure groups on DNA methylation at the oxytocin CHRE, the differences are small (around 3.5%). However, a previous study in humans also reported small differences in DNA methylation at the oxytocin promoter region (Toepfer et al., 2019). Given that oxytocin is selectively expressed in neurons this may reflect differences in the number of oxytocin positive neurons in the PVN, as observed in other studies using neonatal handling (Baracz, Everett, Robinson, Campbell, & Cornish, 2020; Todeschin et al., 2009; Winkelmann-Duarte et al., 2007), and therefore could be biologically relevant. Unfortunately, we cannot infer the number of oxytocin positive neurons from qPCR data, but this would be important to verify in future studies.

2.4.4 Conclusions

Overall, our findings indicate that variations in early-life temperature exposure may affect DNA methylation levels indirectly by changes in DNA methyltransferases and directly by modifications at specific gene loci (oxytocin CHRE) as a consequence of differences in thyroid

hormone receptor signaling. There is accumulating evidence that the epigenome is dynamic and responsive to environmental exposures, but studies that have investigated the underlying mechanisms between environmental exposures and DNA methylation are limited. In addition, there is evidence that DNA methyltransferase recruitment is targeted based on transcriptional activity and transcription factor binding at specific gene loci (Aristizabal et al., 2019; Matthews & McGowan, 2019). Other studies have proposed that the effects of other steroid hormones are mediated by the their effects on DNA methylation modifications, either through differential steroid receptor binding or DNA methyltransferase activity (Crudo et al., 2013; Nugent et al., 2015), typically in light of transcriptional activation. Our findings support the hypothesis that steroid hormones can affect the epigenome through both steroid receptor binding and DNA methyltransferase activity as well as induce transcriptional repression.

More broadly, these findings also indicate the importance of variations in temperature exposure in a variety of models of early-life experience. Some studies separate rat pups at room temperature (e.g., Winkelmann-Duarte et al., 2007) or at thermoneutral conditions (e.g., Baracz et al., 2020) but do not consider the effects of temperature exposure on later-life phenotype. In addition, in human populations, preterm infants have challenges with thermoregulation similar to neonatal rat pups (Bissinger & Annibale, 2010). This population is also more likely to be diagnosed with congenital hypothyroidism than infants born at full term (Chung, 2019). Studies on the effects of temperature exposure on the human epigenome are currently limited (Xu et al., 2020) but would be important to consider in the future.

Chapter 3
Variations in Early-Life Temperature Exposure and Supplemental Tactile Stimulation on Intergenerational Maternal Care Provisioning

Contributions:

SCL and POM designed the study. SCL performed the rat pup manipulations, later-life maternal care provisioning observations, and analyzed the data. POM supervised the research.

3 Variations in Early-Life Temperature Exposure and Supplemental Tactile Stimulation on Intergenerational Maternal Care Provisioning

3.1 Introduction

The early-life maternal environment has a profound effect on offspring behaviour, including the transmission of maternal care across generations in the rat model (Champagne, Francis, Mar, & Meaney, 2003; Fleming et al., 2002; Francis, Diorio, Liu, & Meaney, 1999). Maternal care provisioning involves several neurotransmitter and hormonal systems, including dopamine (Rincón-Cortés & Grace, 2020) and oxytocin (Bridges, 2015; Pedersen, Caldwell, Peterson, Waker, & Mason, 1992), respectively. Alterations in the hormonal systems in the maternal brain are induced by fluctuations in circulating estrogen and progesterone during pregnancy and parturition (Bridges, 2015). More specifically, the oxytocinergic system in the maternal brain is responsive to changes in estrogen, which can promote transcription of oxytocin receptor and oxytocin by estrogen receptors alpha and beta, respectively (Fleming, Spencer, Safe, & Bazer, 2006; Patisaul, Scordalakes, Young, & Rissman, 2003; Shughrue, Dellovade, & Merchenthaler, 2002).

The oxytocinergic system is critical for the induction of maternal behaviour (Bridges, 2015; Pedersen et al., 1992), and is involved with maternal licking of pups (Pedersen & Boccia, 2003; Peterson, Mason, Barakat, & Pedersen, 1991), retrieval behaviour (Marlin et al., 2015), and the amount of time assuming a nursing posture over pups (Pedersen, Caldwell, Walker, Ayers, & Mason, 1994). In addition, previous work suggests that the oxytocinergic system is involved in the intergenerational transmission of maternal care (Pedersen & Boccia, 2002; Toepfer et al., 2017). Studies using natural variations in maternal care between litters in rats found that female rat offspring that receive high levels of licking/grooming have higher estrogen receptor alpha and oxytocin receptor transcript abundance in the medial preoptic area of the hypothalamus later in life than rat offspring that receive low levels of licking/grooming (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; Francis, Champagne, & Meaney, 2000; Pena, Neugut, & Champagne, 2013). The changes in estrogen receptor alpha transcript abundance corresponded to DNA methylation modifications on the Stat5b binding site in the estrogen receptor alpha gene (Champagne et al., 2006), but studies investigating DNA methylation modifications in the

oxytocin receptor gene have reported inconsistent findings (Beery et al., 2016). There is also some evidence that high levels of maternal licking received (assessed directly and indirectly) can affect oxytocinergic activity or the number of oxytocin positive neurons in the paraventricular nucleus of the hypothalamus in rodents, but some studies report an increase (Barrett et al., 2015; Lenz & Sengelaub, 2010) while others report a decrease (Vogel Ciernia et al., 2018; Winkelmann-Duarte et al., 2007) in these measures.

Previous work studying the biological mechanisms of the intergenerational transmission of rat maternal care have focused specifically on the transmission of maternal licking behaviour (Champagne et al., 2003; Francis et al., 1999; Meaney, 2001). In addition, licking-like supplemental tactile stimulation can partially mitigate the effects of early-life artificial rearing on later-life maternal care provisioning (Gonzalez et al., 2001; Melo et al., 2006; Novakov & Fleming, 2005; Palombo et al., 2010). However, there are other environmental factors than can be propagated across generations (Fleming et al., 2002). We have previously reported that female rat pups with early-life repeated room temperature exposure, a proxy of reduced maternal contact, have reduced oxytocin transcript abundance in the paraventricular nucleus (Chapter 2). This reduction corresponded to increased DNA methylation at a CpG site flanking the composite hormone response element in the oxytocin promoter, which can be bound by thyroid hormone receptor and estrogen receptor among other transcription factors (Chapter 2; Adan, Cox, Beischlag, & Burbach, 1993). Female rat pups with supplemental licking-like tactile stimulation also had a minor decrease in oxytocin transcript abundance with no changes in DNA methylation levels at the composite hormone response element (Chapter 2). As pharmacological manipulations of oxytocin early in life can have sustained effects on later-life behaviour, including maternal care provisioning (Bales et al., 2007; Miller & Caldwell, 2015; Pedersen & Boccia, 2002), it is possible that the biological effects of room temperature exposure would be stable throughout life and transmitted across generations by changes in maternal care provisioning. More specifically, mothers with room temperature exposure could have reduced thermotactile contact with their pups and a prolonged latency to retrieve their pups. These changes in maternal behaviours, mediated by reduced oxytocinergic activity, may cause the F_2 pups to maintain a lower body temperature.

In this study, we investigated the effects and interactions of variations in early-life temperature exposure and licking-like tactile stimulation on later-life maternal care provisioning in female rat

offspring. We measured different maternal behaviours following a short separation of the F_2 pups within the first postnatal week. We focused on licking, nursing and retrieval behaviour as the oxytocinergic system in the maternal brain is involved in these behaviours. We hypothesized that both early-life supplemental tactile stimulation and room temperature exposure would alter later-life maternal licking provisioning. We also predicted that rat mothers with early-life room temperature exposure would be slower to retrieve their pups and spend less time on the nest.

3.2 Methods

3.2.1 Rat Breeding

Seven-week-old female (n = 18) and male (n = 7) Long-Evans rats were obtained from Charles River Laboratories (Kingston, NY, USA). They were housed in same-sex pairs on a 12:12 hour light-dark cycle (lights on at 7:00) with *ad libitum* access to standard chow diet and water. For breeding, one male was housed with two females for one week. Females were then housed separately and weighed weekly throughout pregnancy. All animal procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

Females were checked for parturition starting three weeks after breeding at 9:00 and 17:00. Postnatal day (PND) 0 was determined if the birth occurred between 9:00 and 17:00 or if pups were found at 9:00 but have not nursed yet. Pups found at 9:00 with a milk band were considered PND 1. At PND 1, litters were culled to four to six female pups and individually weighed. Paper towel strips were provided to all litters as nesting material. A total of 90 female rat offspring were used for this study.

3.2.2 Postnatal Manipulations

See Chapter 2 Section 2.2.2.

There were seven litters (n = 36 pups) separated at room temperature (21-22°C), with 19 pups receiving supplemental tactile stimulation and 17 pups left undisturbed. There were seven litters (n = 34 pups) separated at nest temperature (33-35°C), with 16 pups receiving supplemental tactile stimulation and 18 pups left undisturbed. Instead of an acute room temperature condition, four litters (n = 20 pups) were left undisturbed throughout the first postnatal week ("unhandled"

condition). An unhandled condition is a common control group in neonatal handling studies (Pryce & Feldon, 2003).

At PND 8, all pups were individually earmarked and weighed. All female rat offspring were weaned at PND 21. For the litters in the room temperature and nest temperature conditions, one stimulated and one nonstimulated sibling were housed together in each cage whenever possible until adulthood.

3.2.3 Maternal Care Received Observations

A subset of litters was video recorded and coded with Observer XT 10.5 (Noldus) for maternal behaviours by four coders with high inter-rater reliability (≥ 90%), see Chapter 2 Section 2.2.3. Four litters with room temperature exposure, six litters with nest temperature exposure, and four unhandled litters were analyzed for maternal care received. One litter from the unhandled condition had missing maternal care recordings at PND 2.

3.2.4 Maternal Care Provisioning Observations

Since we expected that the maternal care provisioning of the unhandled female offspring would be similar to their mothers (Francis et al., 1999), only female rat offspring that were either separated at room temperature or nest temperature were tested for intergenerational maternal care provisioning. At adulthood (PND 80+), one stimulated and one nonstimulated sibling per litter (n = 7 per group) was housed with one male (n = 8) for a week for breeding. Females were then housed separately and weighed weekly throughout pregnancy. Females were checked for parturition starting three weeks after breeding at 9:00 and 17:00.

At PND 1, litters were culled to 2 to 4 males and 2 to 4 females with an even sex ratio when possible. One female from the nest temperature, nonstimulated condition and one female from the nest temperature, stimulated condition gave birth to fewer than two pups for one of the sexes; therefore, their first litter was culled and the female was re-mated. Two females from the nest temperature, nonstimulated condition were excluded after three unsuccessful breeding attempts. One mother from the nest temperature, stimulated condition was excluded after failing to lactate following parturition.

Between 8:30 to 12:00 on PNDs 2 to 6, the entire litter was separated from the mother for five minutes at room temperature. During the separation, the pups were weighed by sex but otherwise left undisturbed. The weights were calculated as a proxy for the body condition of the F_2 pups and possible differences in nursing behaviour from the F_1 mothers. The average weights per pup per sex were calculated for each litter. The whole litter was then placed on the opposite corner of the nest. Following reunion, the latency to retrieve the first pup was recorded and maternal behaviour was coded continuously for 15 minutes with Observer XT 11.5 (Noldus) by two coders with high inter-rater reliability (≥ 90%).

The maternal behaviours and self-directed behaviour coded are listed in Table 3.1. One behaviour, pup carrying, was added after the first two days of coding. Therefore, this behaviour was analyzed by calculating the average time spent pup carrying per day. All other behaviours coded were analyzed using total duration from PND 2 to 6. Maternal licking provisioning was analyzed with AG (ano-genital) and BD (body) licking duration combined (total licking duration). Nursing behaviour (low crouch and high crouch) was infrequent during the coding sessions and was summed with hovering to calculate the total time spent in the nest.

After 9:00 on PND 7, the mother was separated from the litter and sacrificed using CO_2 inhalation and brain and blood were collected for downstream molecular analysis. It was noted whether the mother was nursing at the time of separation. Brains were flash frozen in isopentane and kept on dry ice. Whole blood samples were mixed with 10% (by volume) 0.5 M EDTA and kept on dry ice. Both brain and whole blood were stored at -80°C.

Table 3.1 Ethogram for Intergenerational Maternal Care Provisioning Task.

	Behaviours	*Definitions*
Licking	**AG licking**	Ano-Genital licking. Quick and short licks that are close to the pup's genital region.
	BD licking	Body licking. These are long and slow licks across the whole body of the pup.
	Pup sniffing	Sniffing the pup without licking.
Nursing	**High crouch**	High arched back while nursing.
	Low crouch	Stomach is close to the ground while nursing over the pups.
	Hovering	Briefly arched over pups but not nursing.

Retrieval	**Retrieval**	The pup is carried to the nest (analyzed by latency to first retrieval).
	Pup Carrying	Retrieval that occurs after the pup is placed in the nest. The pup could be moved within the nest or carried outside of the nest (analyzed by average duration per day).
Self-Directed Behaviour	**Grooming**	Mother self-grooming.
	Feeding/Drinking	Grabbing food from the top of the cage or drinking water.
	Cage climbing/bar-biting	Climbing overhead cage bars or biting/chewing on the cage bars.

3.2.5 Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corporation). To examine the effects of variations in temperature exposure on maternal care received within the first postnatal week, a repeated-measures 3 (Nest Temperature, Room Temperature, and Unhandled) x 5 (Postnatal days 2-6) linear mixed model was used to correct for missing datapoints. Significant effects of variations in temperature exposure or a significant interaction with postnatal day were followed with a post-hoc test using Fisher's Least Significant Differences and re-analyzed with each postnatal day separately. To examine the effects of room temperature exposure and supplemental tactile stimulation on intergenerational maternal care provisioning within the first postnatal week, litter size, and sex ratio of the litter, a 2 (Nest Temperature and Room Temperature) x 2 (Stimulated and Nonstimulated) general linear model was used to compare manipulation groups and their interactions. Data from most observed behaviours (licking, pup sniffing, pup carrying, feeding/drinking, grooming, cage climbing/bar-biting) were $\log_{10}$-transformed before analysis to achieve normality. In addition, one extreme outlier (>3 SD from the mean) was removed from the pup carrying and cage climbing/bar-biting observations. To examine the effects of room temperature exposure and supplemental tactile stimulation on the F_2 pup weights (separated by sex) and retrieval latency within the first postnatal week, a repeated-measures 2 (Nest Temperature and Room Temperature) x 2 (Stimulated and Nonstimulated) x 5 (Postnatal Days 2-6) general linear model was used to compare manipulation groups and their interactions. All effects were considered statistically significant at $p \leq 0.05$ and marginally significant at $p \leq 0.10$.

3.3 Results

3.3.1 Maternal Care Received

There was a main effect of variations in temperature exposure on licking received ($F_{(2, 11.283)} = 4.192$, $p = 0.044$) from the rat mother within the first postnatal week. Unhandled litters received more licking than litters with room temperature exposure (Post-hoc $p = 0.013$). When analyzed separately by postnatal day, there was a main effect of variations in temperature exposure on licking received at PND 5 only (Figure 3.1A).

There was no main effect of variations in temperature exposure on nursing ($F_{(2, 11.021)} = 0.851$, $p = 0.453$) within the first postnatal week. However, nursing declined over the first postnatal week ($F_{(4, 11.080)} = 16.405$, $p < 0.001$) and there was a significant interaction of variations in temperature exposure x postnatal day ($F_{(8, 11.068)} = 3.403$, $p = 0.032$). Litters in the nest temperature condition received more nursing than litters in the room temperature condition at PND 5 (Figure 3.1B).

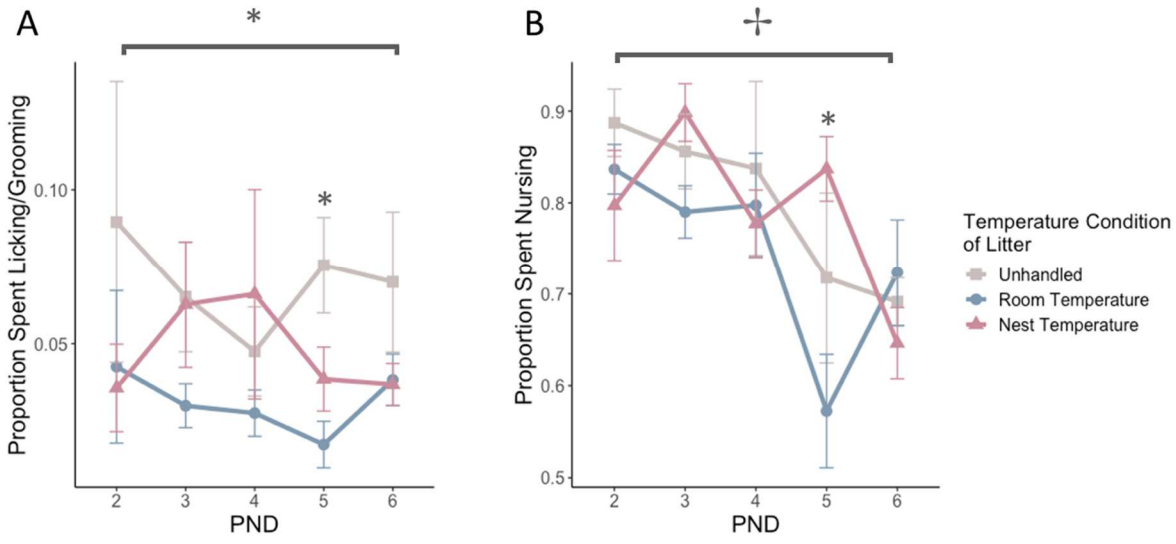

Figure 3.1 Litters with room temperature exposure had a minor reduction in maternal care received compared to litters with nest temperature exposure and unhandled litters. (A) Licking received was significantly reduced with litters separated at room temperature than unhandled litters. Separated by postnatal day (PND), the differences were only statistically significant at PND 5. (B) Nursing decreased over the first postnatal week and litters with room temperature

exposure received less nursing than litters with nest temperature exposure at PND 5. Line graphs are shown with mean +/- SEM. ∗ $p < 0.05$ main effect of variations in temperature exposure, ✢ $p < 0.05$ main effect of postnatal day.

3.3.2 Intergenerational Maternal Care Provisioning

3.3.2.1 F_2 Pup Characteristics

The initial litter sizes, sex ratio (female:male pups), and number of pups that died during the first postnatal week are displayed as Table 3.2. There were no main effects of variations in temperature exposure or supplemental tactile stimulation on litter size or sex ratio of the F_2 pups (all p's > 0.10). Four pups in total died over the first postnatal week; three of the F_2 pups were from litters with nest temperature, nonstimulated rat mothers and one F_2 pup was from a litter with a room temperature, stimulated rat mother.

All litters gained weight over the first postnatal week (females: $F_{(1, 21)} = 1085.191$, $p < 0.001$; males: $F_{(1, 21)} = 835.490$, $p < 0.001$). There were no main effects of temperature exposure or supplemental tactile stimulation on F_2 pup weights from PND 2 to 6 (all p's > 0.10) for female F_2 pups (Figure 3.2A) and male F_2 pups (Figure 3.2B).

Table 3.2 Initial litter size, sex ratio of pups born, and pups found dead during the first postnatal week for intergenerational maternal care provisioning testing.

	Early-Life Condition			
	Room Temperature		Nest Temperature	
	Nonstimulated	Stimulated	Nonstimulated	Stimulated
Initial Litter Size (Standard Error)	14.71 (1.64)	12.85 (1.62)	13.60 (1.08)	12.67 (1.69)
Female:Male Pup Ratio (Standard Error)	1.04 (0.09)	1.10 (0.28)	1.41 (0.66)	1.05 (0.22)
Number of Pups Found Dead Over First Postnatal Week		1 Female	2 Females, 1 Male	

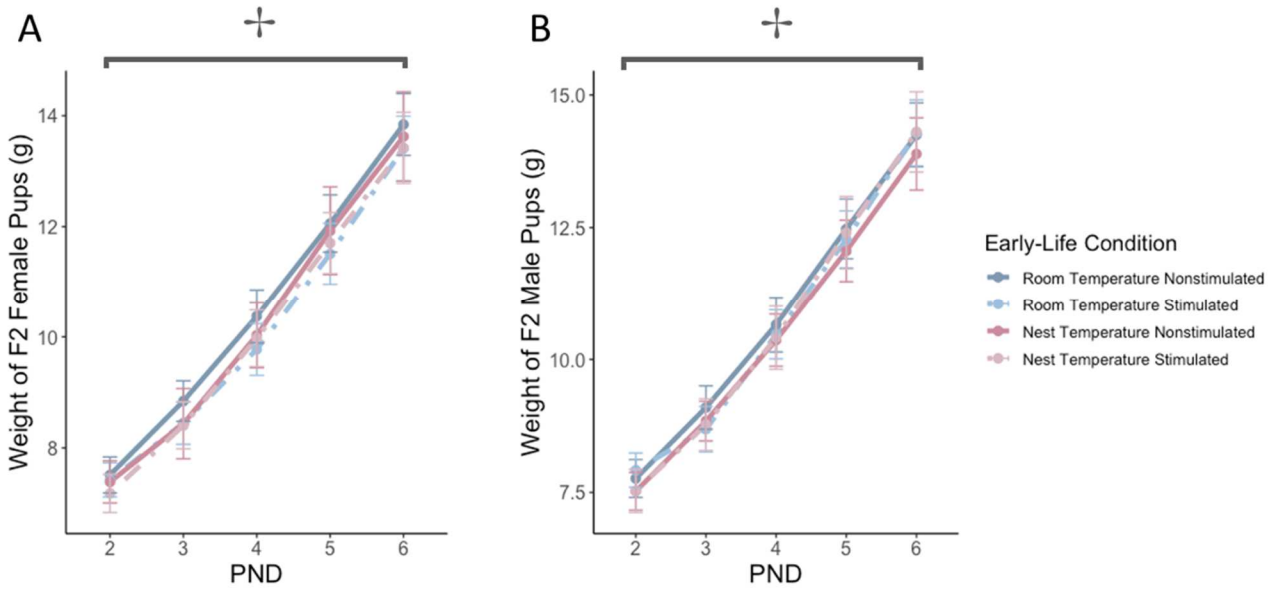

Figure 3.2 The F_2 pup weights over the first postnatal week were not significantly affected by their mothers' early-life condition. Although all F_2 pups gained weight throughout the first postnatal week, there were no significant differences in weights for (A) female pups or (B) male pups between their mothers' early-life condition. Line graphs are shown with mean +/- SEM. ✢ $p < 0.05$ main effect of postnatal day.

3.3.2.2 Maternal Licking Behaviour

There was a marginal effect of variations in temperature exposure on total licking provisioning ($F_{(1, 24)} = 3.566$, $p = 0.073$). Rat mothers with early-life room temperature exposure provided more licking to their offspring than mothers with early-life nest temperature exposure (Figure 3.3A). In addition, there was a main effect of supplemental tactile stimulation on total licking provisioning ($F_{(1, 24)} = 24.588$, $p < 0.001$). Rat mothers with early-life supplemental tactile stimulation provided more licking to their offspring than mothers without early-life supplemental tactile stimulation (Figure 3.3A). There was no significant interaction between variations in temperature exposure and supplemental tactile stimulation (($F_{(1, 24)} = 0.199$, $p = 0.660$). Though there were significant differences in maternal licking provisioning, there were no main effects of variations in temperature exposure or supplemental tactile stimulation on pup sniffing (all p's > 0.10; Figure 3.3B).

3.3.2.3 Time Spent on the Nest

There was no main effect of variations in temperature exposure on the duration of time spent on the nest ($F_{(1, 24)} = 1.835$, $p = 0.190$) but there was a marginal effect of supplemental tactile stimulation ($F_{(1, 24)} = 3.461$, $p = 0.077$) with no interaction ($F_{(1, 24)} = 0.249$, $p = 0.623$). Rat mothers with early-life supplemental tactile stimulation spent more time on the nest than mothers without early-life supplemental tactile stimulation (Figure 3.3C).

3.3.2.4 Maternal Retrieval and Pup Carrying Behaviour

There were no main effects of variations in temperature exposure or supplemental tactile stimulation on the latency to retrieve the first pup within the first postnatal week (all p's > 0.10). However, there was a main effect of temperature exposure on pup carrying behaviour ($F_{(1, 23)} = 5.547$, $p = 0.029$) and a main effect of supplemental tactile stimulation ($F_{(1, 23)} = 7.462$, $p = 0.013$) with no interaction ($F_{(1, 23)} = 1.403$, $p = 0.319$). Rat mothers with early-life nest temperature exposure performed more pup carrying per day than mothers with early-life room temperature exposure, and rat mothers without early-life supplemental tactile stimulation performed more pup carrying per day than mothers with early-life supplemental tactile stimulation (Figure 3.3D).

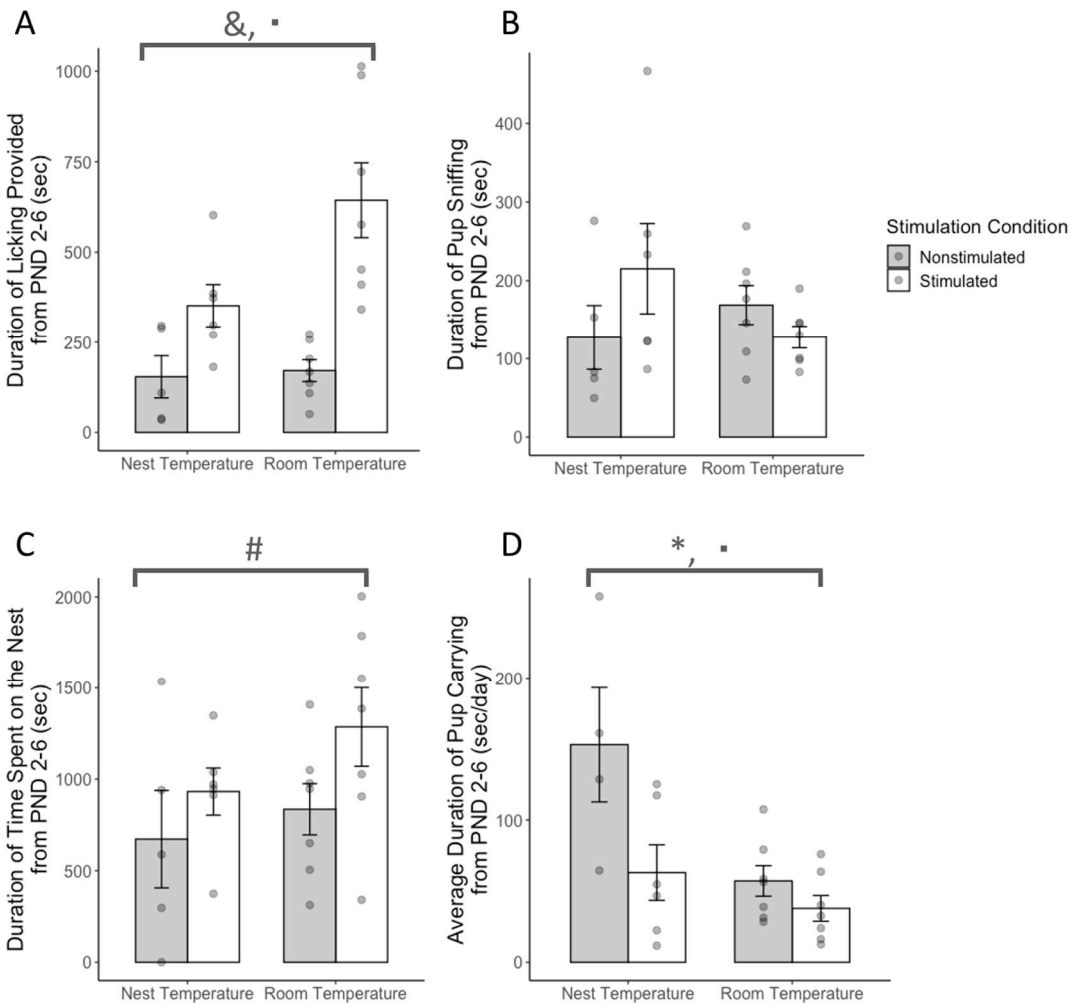

Figure 3.3 Both early-life temperature exposure and supplemental tactile stimulation affected maternal care provisioning. Rat mothers with early-life room temperature exposure and rat mothers with early-life tactile stimulation had higher (A) total licking provisioning without (B) any significant differences in pup sniffing between the early-life conditions. (C) Rat mothers with early-life supplemental tactile stimulation marginally spent more time on the nest. (D) Rat mothers with early-life nest temperature exposure and rat mothers without supplemental tactile stimulation had increased pup carrying behaviour. Barplots are shown with mean +/- SEM with datapoints from individual rat mothers. ∗ $p < 0.05$ main effect of temperature condition; & $p < 0.10$ main effects of temperature condition; ▪ $p < 0.05$ main effect of tactile stimulation condition; #$p < 0.10$ main effect of tactile stimulation condition.

3.3.2.5 Self-Directed Behaviours

There were no main effects of variations in temperature exposure or supplemental tactile stimulation on climbing or biting the cage bars, feeding, or self-grooming (all p's > 0.10).

3.4 Discussion

In this study, we investigated how two factors in the maternal environment rat pups commonly experience, variations in temperature exposure and licking-like tactile stimulation, affect maternal care provisioning to the next generation. This is the first study to our knowledge to investigate the effects of early-life room temperature exposure on intergenerational maternal care provisioning and if the effects of room temperature exposure could be transmitted across generations. We found that both early-life room temperature exposure and supplemental tactile stimulation increased later-life maternal licking provisioning without changes in pup sniffing behaviour. Although we did not find significant differences in early-life temperature exposure on the time spent on the nest or retrieval latency, mothers with early-life nest temperature exposure performed more pup carrying behaviour than mothers with early-life room temperature exposure. These findings show that early-life room temperature exposure can alter maternal care provisioning, though it is still unknown if early-life room temperature exposure can be transmitted across generations and how this could occur.

3.4.1 Effects of Maternal Care Received

We investigated the amount of maternal care received between litters separated at room temperature or nest temperature as well as litters that were undisturbed within the first postnatal week because early-life maternal care received can have a substantial impact on the amount of maternal care provisioning at adulthood (Champagne et al., 2003; Francis et al., 1999). In a subset of litters, we observed significant differences between variations in temperature exposure on maternal care received. However, there were no significant differences between maternal licking received between the litters with room temperature exposure and nest temperature exposure, and the differences in nursing was limited to postnatal day 5. Therefore, it is unlikely that the differences in maternal care provisioning between litters with variations in temperature exposure was due to differences in early-life maternal care received.

3.4.2 Effects on Maternal Licking Provisioning

We hypothesized that early-life variations in temperature exposure and supplemental tactile stimulation would alter later-life maternal licking provisioning. We found that rat mothers with early-life room temperature exposure marginally provided more licking to their pups than mothers with nest temperature exposure. This was an unexpected finding, given that our previous study showed that early-life repeated room temperature exposure decreased Oxt transcript abundance at postnatal day 7 with increased DNA methylation at the OXT composite hormone response element (Chapter 2) and developmental administration of oxytocin antagonists can decrease later-life maternal licking provisioning in female rats (Pedersen & Boccia, 2002) possibly by decreasing estrogen receptor density in the medial preoptic area (Perry et al., 2009; Yamamoto et al., 2006). However, the relationship between the dose of oxytocin administered early in life and later-life maternal care provisioning is not always linear (Bales et al., 2007) and both exogenous oxytocin and oxytocin antagonists can decrease estrogen sensitivity in sexual receptivity, likely by different biological pathways (Perry et al., 2009). Therefore, it is important to also investigate the changes in the oxytocinergic system, with Oxt and Oxtr transcript abundance and DNA methylation at the OXT composite hormone response element, in the maternal brains with different early-life temperature exposures.

In addition, we found that mothers with early-life supplemental tactile stimulation provided more licking to their pups. This is consistent with findings in the literature that show increased licking received or licking-like tactile stimulation received early in life could increase later-life maternal licking provisioning (Francis et al., 1999; Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001). Though our earlier work suggests that supplemental tactile stimulation decreases Oxt transcript abundance at PND 7, the effects were not consistent between temperature exposure groups (Chapter 2). It is also possible that the effects of supplemental tactile stimulation on maternal licking provisioning are mediated by changes in the dopaminergic system instead. The changes in the dopaminergic system in response to early-life maternal care or tactile stimulation received have been reported in previous work (Afonso et al., 2011; Peña et al., 2014). In addition, the dopaminergic and oxytocinergic systems in the maternal brain can interact with each other (Shahrokh et al., 2010; Strathearn, 2011). Future work will need to elucidate if changes in the oxytocinergic and dopaminergic systems in response to early-life temperature

exposure and supplemental tactile stimulation are converging to differences in maternal licking provisioning and the nature of these interactions.

3.4.3 Effects on Maternal Retrieval Behaviour

Though we did not find significant differences in latency to retrieve the first pup between early-life variations in temperature exposure or supplemental tactile stimulation, we did find that mothers with nest temperature exposure performed more pup carrying behaviour than mothers with room temperature exposure, mainly driven by the mothers without early-life supplemental tactile stimulation. Pup carrying, defined as subsequent retrieval of pups after they have been carried to the nest, has been previously described in studies of rodent mothers with septal lesions in the brain (Fleischer & Slotnick, 1978; Slotnick & Nigrosh, 1975). The lateral septum has connections from the medial preoptic area and bed nucleus of the stria terminalis, which are important for retrieval behaviour (Numan & Numan, 1996). Based on previous septal lesion experiments, it is possible that the pup carrying observed in this study may be disinhibited, repetitive retrieval behaviour (Fleischer & Slotnick, 1978). However, it is also possible that the pup carrying behaviour is a response to a suboptimal environment for the mothers with early-life nest temperature exposure. Early-life experiences can alter an individual's developmental trajectory to adapt to their predicted later-life environment; however, if the later-life environment does not match that prediction, then the individual can develop maladaptive phenotypes ("match-mismatch hypothesis"; Gluckman, Hanson, & Buklijas, 2010; Godfrey, Lillycrop, Burdge, Gluckman, & Hanson, 2007). In this study, all of the F_2 rat pups were separated at room temperature, regardless of the mothers' early-life temperature exposure condition. It would be important to further investigate whether returning the F_2 pups at nest temperature would reduce the pup carrying behaviour or if the mothers with early-life nest temperature exposure, especially without supplemental tactile stimulation, prefer establishing their nests in warmer areas of the cage than mothers with room temperature exposure.

3.4.4 Transmission of Room Temperature Exposure Across Generations

In this study, we did not directly find a mode of transmission of room temperature exposure across generations by either reduced time spent in the nest or prolonged latency to retrieve pups. However, there are other outcome measures not tested in this study but would be important for future studies. Besides testing for evidence for the match-mismatch hypothesis (discussed

above), investigating the cohesion of huddling between siblings in the litter (Harshaw & Alberts, 2012), directly monitoring the temperature of the pups throughout the day (Mumm, Kaul, Heldmaier, & Schmidt, 1989), and measuring circulating thyroid hormone levels of the rat mother and the F_2 pups (Chapter 2) could help elucidate possible mechanisms for the intergenerational transmission of room temperature exposure. In addition, it would be important to investigate the effects of the mother's early-life variations in temperature exposure on the F_2 pups' physiology and gene expression in the brain, especially with the female pups.

3.4.5 Limitations

One limitation is that we only measured intergenerational maternal care immediately after separating the pups. While this method has been used before to assess maternal care provisioning (Gonzalez et al., 2001; Palombo et al., 2010; Pan et al., 2014), maternal care provisioning naturally changes at different periods throughout the light-dark cycle (Champagne, Francis, et al., 2003). In addition, separating the pups themselves alters maternal care provisioning (Villescas et al., 1977). Assessing maternal care provisioning with noninvasive video recordings throughout the day could provide additional information on the generalizability of the effects of early-life variations in temperature exposure and tactile stimulation.

In addition, some of the female rat offspring were re-mated after giving birth to small litters and we assessed maternal care provisioning towards their second litter. Previous work that observed natural occurring variations in maternal care in rats indicate that maternal licking provisioning is stable across litters (Champagne et al., 2003) though maternal aggression may be heightened in multiparous rats (Nephew, Bridges, Lovelock, & Byrnes, 2009). However, there is also evidence that dopamine, prolactin, opiate receptors, and neuropeptide levels in the brains of multiparous rats are significantly different than primiparous rats (Bridges & Hammer, 1992; Felicio, Florio, Sider, Cruz-Casallas, & Bridges, 1996; Nephew et al., 2009). Therefore, it is unlikely that the parity status of the female rat offspring substantially contributed to the differences in intergenerational maternal care provisioning observed in this study, but it could affect oxytocin and oxytocin receptor transcript abundance in their brains.

3.4.6 Conclusions

Overall, these findings show that both early-life room temperature exposure and licking-like supplemental tactile stimulation can affect later-life maternal care provisioning to the next generation of rat offspring. This suggests that the effects of early-life room temperature exposure on the regulation of the oxytocin gene during development (Chapter 2) may have an impact on later-life behaviour. This could also suggest that differences in DNA methylation modifications on the oxytocin gene early in life may persist until adulthood and affect oxytocin transcript abundance and release from the paraventricular nucleus, but this would need to be verified in the virgin female brain and maternal brain. It would also be important to investigate the transcript abundance of estrogen receptor alpha and oxytocin receptor in the medial preoptic area of the maternal brain in response to early-life variations in temperature exposure and supplemental tactile stimulation. It may also be important to investigate dopamine levels or transcript abundance of dopamine receptors in the maternal brain in response to early-life supplemental tactile stimulation. In addition, the long-term effects of early-life room temperature exposure on behavioural phenotype suggest that early-life variations in temperature exposure, a proxy of maternal contact, as well as maternal licking may be important for the developmental programming of maternal care received on later-life behaviour.

Chapter 4
Inter-individual Maternal Licking Received and Dopamine Genotype Interactions on Dopaminergic Phenotypes in Virgin Female Rat Offspring

This chapter is adapted from:

Lauby, S.C., Chatterjee, D., Pan, P., McGowan, P.O., & Fleming, A.S. (2019). Inter-individual Maternal Care Received and Genotype Interactions Affect Dopaminergic Phenotypes in Female Rat Offspring. *Journal of Neuroendocrinology,* e12706. doi: 10.1111/jne.12706

Contributions:

SCL, PP, POM, and ASF designed the study. SCL and PP conducted the maternal care observations. SCL conducted the behavioral testing and genotyping. DC conducted the HPLC. SCL and ASF analyzed the data. POM and ASF supervised the research.

4 Inter-individual Maternal Licking Received and Dopamine Genotype Interactions on Dopaminergic Phenotypes in Virgin Female Rat Offspring

4.1 Introduction

The maternal environment can shape offspring behavior later in life, including how female offspring themselves provide maternal care (Fleming et al., 2002). This phenotypic plasticity could be an adaptive response to maximize offspring's chances of survival to environmental challenges (Cameron et al., 2008). The maternal brain involves coordination of several neural systems, including oxytocin and dopamine, to approach and respond to infant cues (Numan & Stolzenberg, 2008; Shahrokh et al., 2010; Strathearn, 2011). In rat models, disruption of maternal care through early-life isolation has been shown to affect offspring behavior in adulthood, including their maternal behavior and other dopamine-dependent behavior such as impulsive action, strategy shifting, and baseline reward sensitivity to drugs and sucrose (Baudin et al., 2012; Gonzalez et al., 2001; Lomanowska et al., 2006; Lovic & Fleming, 2004; Lovic, Keen, et al., 2011; McLean et al., 2010; Palombo et al., 2010). This impairment in adulthood by early isolation from the mother can be rescued with early-life supplemental tactile stimulation (Gonzalez et al., 2001; Lovic, Keen, et al., 2011).

Previous research has established that there is natural variation in how rodent mothers respond and care for their pups, measured by the total licking (LG) of the entire litter during the first postpartum week (Champagne et al., 2003). The quality of maternal care may also be determined by the average duration of licking per bout mothers provide to the entire litter. This has been shown in rat mothers who show natural variations in maternal care across time (Hellstrom et al., 2012; Pena et al., 2013), where high LG mothers show a higher duration of licking per bout rather than a higher frequency of licking bouts or a higher licking total. In addition, inducing disruption and fragmentation of maternal care by limiting nesting material produces anxiety- and depressive-like behavior in offspring even when the total duration of maternal LG is not affected (Baram et al., 2012; Ivy et al., 2008; Rice, Sandman, Lenjavi, & Baram, 2008).

Other studies, including our own, have demonstrated that mothers also show variation in their maternal licking to different pups within the litter (Cavigelli, Ragan, Barrett, & Michael, 2010; Pan, Fleming, Lawson, Jenkins, & McGowan, 2014a; Pan et al., 2018; Ragan, Loken, Stifter, &

Cavigelli, 2012; Ragan, Harding, & Lonstein, 2016; van Hasselt et al., 2012). We found that total inter-individual LG received is associated with differences in stress reactivity at adulthood (Pan et al., 2014, 2018). The effects of average licking bout length received by individual pups on their later-life behavior has not been explored.

However, the effects of early-life maternal care on later-life behavior could be influenced by offspring genotype. We recently reported that adult stress reactivity depends on an interaction between LG received and single nucleotide polymorphisms (SNPs) in genes coding for FK506-binding protein, glucocorticoid receptor and serotonin transporter (Pan et al., 2018). Gene x environment interactions are also known to exist for genes involved in dopamine signaling. For example, early-life adversity by artificial rearing increases dopamine receptor 2 (DRD2) expression in the nucleus accumbens shell of adult rats, but is dependent on the genotype for a DRD2 SNP (RS13448058) (Lovic et al., 2013). Studies in human populations have found gene x environment interactions with dopamine transporter (DAT), prenatal adversity and ADHD symptoms in children (Becker, El-Faddagh, Schmidt, Esser, & Laucht, 2008; van der Meer et al., 2017). Dopamine transporter genotype has been associated with its availability in human striatum to metabolize dopamine (Heinz et al., 2000), influencing the rate dopamine is cleared from the synapse. Like the dopamine transporter, catechol-O-methyltransferase (COMT) metabolizes dopamine and a common functional SNP is associated with its activity in the prefrontal cortex of humans (Chen et al., 2004), but gene x environment studies on these mechanisms have not been performed to our knowledge. In addition, we could not find studies on the effects of both DAT and COMT SNPs on phenotype in rat populations.

We have a limited understanding whether inter-individual licking received is associated with dopamine-dependent behavior and the extent to which genotypes involved in dopaminergic activity would moderate this relationship. Our goal is to understand if genetic variation would contribute to offspring adult behaviors and hence to maternal care transmission between generations. We used nulliparous female offspring to explore this question and assessed strategy shifting, impulsive action, and sucrose preference. Early-life maternal care received between litters has been demonstrated to affect dopamine-related phenotypes in female rat offspring (Peña et al., 2014). Strategy shifting assesses how quickly individuals switch strategies when a previous strategy no longer produces a reward. This task has been associated with medial prefrontal cortex functioning (Floresco, Block, & Tse, 2008). Both strategy shifting and

impulsive action have been shown to mediate early-life experiences on later-life provisioning of maternal care (Afonso, Sison, Lovic, & Fleming, 2007; Gonzalez, Jenkins, Steiner, & Fleming, 2012; Lovic, Palombo, & Fleming, 2011). Sucrose preference, typically used to measure anhedonia, naturally varies between individuals and correlates with dopamine receptor 2 function in the basal ganglia (Tõnissaar, Herm, Rinken, & Harro, 2006).

The purpose of this study was to investigate dopamine gene x maternal licking interactions on (a) strategy shifting, (b) impulsive action, (c) sucrose preference, and (d) baseline dopamine turnover in key brain areas of the dopamine system in female offspring. We measured inter-individual maternal licking within the first week of life and assessed adult female offspring behavior, dopamine and its metabolite levels, and variation in SNPs in the DRD2, DAT, and COMT genes.

We predicted that higher levels of inter-individual maternal licking received would facilitate strategy shifting and decrease impulsive action and sucrose preference. We hypothesized this would be mediated by differences in baseline dopamine turnover. In addition, we hypothesized both behavior and dopamine turnover would be moderated by genotype. The hypothesized moderated-mediation is visualized in Figure 4.1.

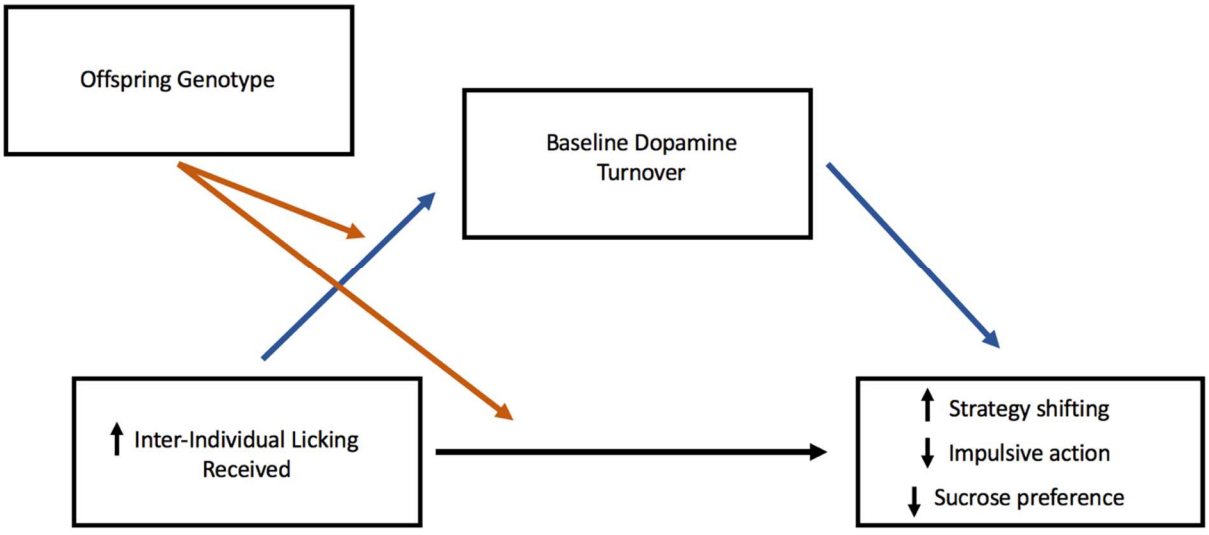

Figure 4.1 Hypothesized moderated-mediation between early-life licking received and dopamine-dependent behavioral outcomes. Baseline dopamine turnover would mediate the association and offspring genotype would moderate behavior and baseline dopamine turnover.

4.2 Methods

4.2.1 Rat Breeding

Seven-week-old female (n = 24) and male (n = 6) Long Evans rats were obtained from Charles River Laboratories. They were housed in same-sex pairs on a 12:12 hour light-dark cycle (lights on at 7:00) with *ad libitum* access to standard chow diet and water. For breeding, one male was housed with two females for one week. Females were then housed separately and weighed weekly throughout pregnancy. All animal procedures were approved by the Local Animal Care Committee at the University of Toronto in Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

Births were checked starting three weeks after breeding at 9:00 and 17:00. Postnatal day (PND) 0 was determined if the birth occurred between 9:00 and 17:00 or if pups were found at 9:00 but have not nursed yet. Pups found at 9:00 with a milk band were considered PND 1. At PND 1, litters were culled to five to six female pups and individually weighed. We focused on smaller litters in order to accurately measure maternal care received. Therefore, only female offspring were examined for this study to analyze a full range of maternal care within a litter. A total of 136 pups were assessed for maternal care.

4.2.2 Maternal Care Observations

At PND 1, 3, 5 and 7, maternal care was assessed as previously reported (Pan et al., 2014). From 10:00 to 17:00, litters were briefly separated from their mother (between 10-20 minutes) and individually marked using odorless and tasteless food coloring (Club House, London, Ontario, Canada) to distinguish between siblings. The entire litter was then placed in the opposite corner of the established nest and maternal behavior was observed for 30 minutes using Observer XT 11.5 (Noldus Information Technology, Wageningen, The Netherlands). To establish inter-rater reliabilities on behavioral observations, three researchers coded the same mothers with an experienced coder until high reliability (>90%) was consistently met.

The order pups were retrieved was recorded manually. Duration and frequency of anogenital licking and body licking were coded for individual pups, meaning each pup had designated keys in the Observer software. Hovering, nursing (blanket and arched-back), and nest-building were coded as a litter, since these behaviors typically involve whole litters. Self-directed behaviors

(feeding and self-grooming) by the mother were also coded. Cages were not changed throughout the maternal behavior observation period.

Total duration of licking (anogenital and body licking) and average duration of a lick bout (total duration of licking/number of licking bouts) across all four observation days for each pup were calculated as measures of maternal care. This was referred as “total licking duration” and “average licking duration”, respectively.

Female offspring were weaned at PND 22 and all offspring were pair-housed, the majority with siblings. Female offspring were weighed periodically until adulthood (PND 75).

4.2.3 Dopamine-Related Behaviour Tasks

A subset of PND 90+ female offspring (n = 55) were tested for either strategy shifting (n = 28) or a Differential Reinforcement of Low Rates (DRL-20 seconds) schedule (n = 27), followed by a sucrose preference task (n = 55). Other female offspring were assessed for distinct behavioral phenotypes in adulthood and will be reported elsewhere. We did not assess estrous cycle during these tasks; however, previous work has shown that variability across the cycle is not substantial enough to change the interpretability of several phenotypes (Beery, 2018). The testing occurred over two cohorts; the first cohort included one to two pups from 20 litters and the second cohort included five to six pups from four litters. There were no significant differences between cohorts in total litter size or any of the behavior measures (all p’s > 0.10) and were therefore analyzed together.

Sound-attenuating operant chambers (MED Associates, St. Albans, VT, USA) connected to a Dell desktop PC with MED-PC-IV software installed were used for the strategy shifting and DRL-20 tasks. Each operant chamber was equipped with a ventilation fan, two retractable levers, two stimulus lights above the levers, a house light, and a food magazine that dispensed plain 45 mg sucrose pellets (#F06233, Bio-Serv). Each animal was gradually reduced to 85-90% of their ad libitum feeding weight and handled daily (two minutes per day) six days before chamber acclimation. The day before chamber acclimation, offspring received 20 sucrose pellets in their home cages. All female offspring acclimated to the operant chamber for one day with exposure to the ventilation fan and house light for 30 minutes. Offspring completed an auto-shaping procedure for five days and went through a Fixed Ratio-1 schedule until they made over 100

lever presses over three consecutive days. To account for potential circadian rhythm effects, the two operant tasks were counter-balanced between the morning (9:00-11:00) and afternoon (15:00-17:00) over the two cohorts.

4.2.3.1 Strategy Shifting

Twenty-eight female offspring completed the strategy shifting task, a measure of behavioral flexibility, as described by Brady and Floresco (Brady & Floresco, 2015). To reduce the number of omitted responses, offspring were required to press a randomly extended lever within ten seconds at least 85 times out of 90 trials. After meeting this requirement, offspring were reinforced to press the lever on the illuminated side of the chamber (cued task) until they made 10 consecutive correct responses. The illuminated side was random between trials. Each day involved 150 trials and offspring were disqualified if criterion was not met after three days (450 trials). After reaching criterion on the cued task, offspring were reinforced to press a randomly assigned lever regardless of illuminated side (response task) until they made 10 consecutive correct responses. Trials to criterion on the response task, adjusting for the number of trials with no responses, was used as a measure of behavioral flexibility. Fewer trials reflected greater flexibility. Two offspring were disqualified for not reaching criterion in the cued task after 450 trials and one offspring was removed for completing the response task after 11 trials, leaving a total of 25 offspring reaching all criteria.

4.2.3.2 Differential Reinforcement of Low Rates (DRL-20)

Twenty-seven female offspring completed a Differential Reinforcement of Low Rates (DRL-20 seconds) schedule for 30 minutes over eighteen days as a measure of impulsive action (Dalley, Everitt, & Robbins, 2011). A DRL-20 schedule reinforced rats if they pressed a lever after 20 seconds has passed. Premature responses reset the timer with no reward dispensed. The first response in a session was always rewarded. Percent efficiency (number of rewarded presses / number of total presses * 100) was averaged over the last three days and used as a measure of impulsive action. Percent efficiency and average inter-response time were highly correlated in this task (Pearson's $r = 0.966$). Higher efficiency reflected lower impulsivity.

4.2.3.3 Sucrose Preference

All female offspring (n = 55) were individually housed and water-deprived for 10 hours before a two-choice bottle task. Tap water and tap water with 1% sucrose were randomly placed between offspring. Bottles were weighed before and after one hour in the dark phase (19:00 – 20:00). Nine offspring were excluded due to excessive liquid in the bedding, leaving a total of 46 offspring being analyzed. Sucrose preference (amount of sucrose water consumed / total liquid consumption * 100) was calculated and used as a measure of baseline reward sensitivity.

Forty-three offspring were sacrificed with CO_2 inhalation and decapitation following the sucrose preference task. Twelve offspring were sacrificed after further testing on intergenerational maternal care, which will be published in a subsequent report. Liver and whole brain tissue were collected and placed in dry ice or flash-frozen in isopentane, respectively. Tissue was stored in -80°C until further processing.

4.2.4 Genotyping

Liver DNA was extracted using an EZNA Tissue DNA Extraction kit (Omega Bio-Tek, Norcross, GA, USA) and assessed for single-nucleotide polymorphisms (SNPs) at two sites in the dopamine receptor 2 (DRD2) gene (RS107017253 and RS13448058), one site in the dopamine transporter (DAT) gene (RS13448119), and two sites in the catechol-o-methyltransferase (COMT) gene (RS107501401 and RS13451556). Polymerase Chain Reaction (PCR) was used with custom primer sets (Table 4.1) to amplify the region of interest. Target amplicons were verified with gel electrophoresis and the PCR products were purified using a QIAmp PCR Clean-Up kit (Qiagen, Hilden, Germany). Purified DNA (20 ng) was submitted for sanger sequencing (The Centre for Applied Genomics, Sickkids, Toronto, ON).

Table 4.1 List of PCR Primers for Genotyping.

Gene Name	SNP Accession	Primer	Sequence (5' to 3')
DRD2	RS107017253	Forward	CAACATCGAGTTCCGCAAGG
		Reverse	GCATCGAGCCAAGCTAACAC
	RS13448058	Forward	TGAGTGGGTGGACAAGTGA
		Reverse	TTTCAAGGCATGCTTCCTCT
DAT	RS13448119	Forward	CACTACTGCACCCCCAAATC
		Reverse	CTGACCAACTCCACCCTCAT
COMT	RS107501401	Forward	TGTTAAAACCCGTGTCTGCGG
		Reverse	AGTCCCAGTTCCGTGTTTGC
	RS13451556	Forward	CCACATGCTTCTCTAGGGCG
		Reverse	GCTGCTCCCTCTCACATACG

DRD2: Dopamine Receptor 2, **DAT**: Dopamine Transporter, **COMT**: Catechol-O-Methyltransferase

4.2.5 High Performance Liquid Chromatography

Forty-two female offspring brains were sliced and microdissected for specific brain areas in a Leica CM3050S cryostat. Medial prefrontal cortex (mPFC; +4.20mm to +2.70mm Bregma), nucleus accumbens core and shell (NAcc; +2.20mm to +1.20mm Bregma), medial preoptic area (MPOA; -0.30mm to -0.80mm Bregma), dorsal hippocampus (control brain region; -2.30mm to -3.30mm Bregma) and ventral tegmental area (VTA; -5.20mm to -5.60mm Bregma) were identified using an adult rat brain atlas (Paxinos & Watson, 1997). 5 µl of 1.0 M ascorbic acid was added to each sample to stabilize the neurotransmitters and were stored at -80°C.

To prepare the samples, the brain tissue was thawed on ice, suspended in 20 µl artificial cerebrospinal fluid (ACSF; Harvard Apparatus) and homogenized by four pulses of sonication (2 seconds per pulse). 2 µl of brain homogenate from each sample was analyzed for protein concentration using BioRad protein assay reagent (BioRad, Hercules, CA, USA). 1 µl of 0.2 M

perchloric acid per sample was added to the remaining homogenate and was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at -80°C.

Dopamine (DA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) concentrations were measured by high performance liquid chromatography (HPLC) as described by Chatterjee and Gerlai (Chatterjee & Gerlai, 2009). A BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) was used together with a Uniget C-18 reverse phase microbore column (#8949, BASi). The mobile phase contained a buffer [0.1 M monochloro acetic acid, 0.5 mM Na-EDTA, 0.15 g/L Na-octylsulfonate and 10 nM sodium chloride, pH 3.1], acetonitrile and tetrahydrofuran at a ratio of 94:3.5:0.7. The flow rate was 1.0 ml/min, and the working electrode (Uniget 3 mm glassy carbon, BAS P/N MF-1003) was set at 550 mV vs. Ag/Ag/Cl reference electrode. Detection gain was 1.0 nA, filter was 0.2 Hz, and detection limit was set at 20 nA. 5 µl of the sample supernatant was directly injected into the column. External standards for DA and DOPAC (Sigma) of known concentrations were used to quantify and identify peaks on the chromatogram. Under these parameters, the retention times for DA and DOPAC were approximately 3.7 minutes and 5.5 minutes, respectively.

DA and DOPAC concentrations were normalized against total protein concentration for each sample and expressed as ng/mg of protein. DOPAC divided by DA, or DOPAC/DA ratio, was calculated for baseline dopamine turnover, a measure of dopaminergic activity, in each brain area. We were unable to cryosection one brain due to extensive damage during collection and analyze two samples (one MPOA and one VTA) due to lost tissue.

4.2.6 Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corporation). To examine the relationship between maternal care and dopamine-related phenotypes, a Pearson correlation was used between total or average licking duration and the behavior measures and DOPAC/DA ratio in each brain area. In addition, to examine the relationship between behavior and dopamine turnover, a Pearson correlation was used between the behavior measures and DOPAC/DA ratio in each brain area. To examine the effects of genotype, a linear mixed model was used to compare offspring with each varying genotype to licking received and adult measures. Litter ID was used as a random effect to reduce between-litter effects (Wainwright, Leatherdale, & Dubin, 2007). Significant effects of genotype were followed with a post-hoc test using Fisher's Least

Significant Difference (LSD). To examine gene x environment interactions, a multiple regression was used with total lick duration or average lick duration (X_1) and each varying genotype (X_2; Model A) with an interaction term (X_1*X_2; Model B) for each behavior and brain area DA/DOPAC ratio:

Model A: $\hat{y} = b_0 + b_1X_1 + b_2X_2 + \varepsilon$

Model B: $\hat{y} = b_0 + b_1X_1 + b_2X_2 + b_3(X_1*X_2) + \varepsilon$

Regression models with a significant R^2 change when adding the interaction term were further analyzed with Hayes PROCESS module for SPSS (Version 3.1) using a simple moderation (Model 1; Hayes, 2013). PROCESS is a flexible modelling module that can conduct moderation analyses and probe conditional effects of a focal moderator. All effects were considered statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

4.3 Results

4.3.1 Correlations between Early-Life Licking Received, Baseline Dopamine Turnover and Behavior

Within litters, the two highest and two lowest licked pups consistently received high and low levels of licking, respectively, while the one to two remaining pups received mid-levels of licking. This was demonstrated with both total licking duration (Figure 4.2A) and average licking duration (Figure 4.2B). For the female offspring tested, both total licking duration (Figure 4.2C) and average licking duration (Figure 4.2D) showed a normal distribution. Total licking duration across all maternal care observation periods averaged 123.06 ± 8.43 seconds and ranged from 0 to 285.69 seconds. Average licking duration averaged 7.83 ± 0.47 seconds and ranged from 0 to 16.65 seconds per bout of individual licking.

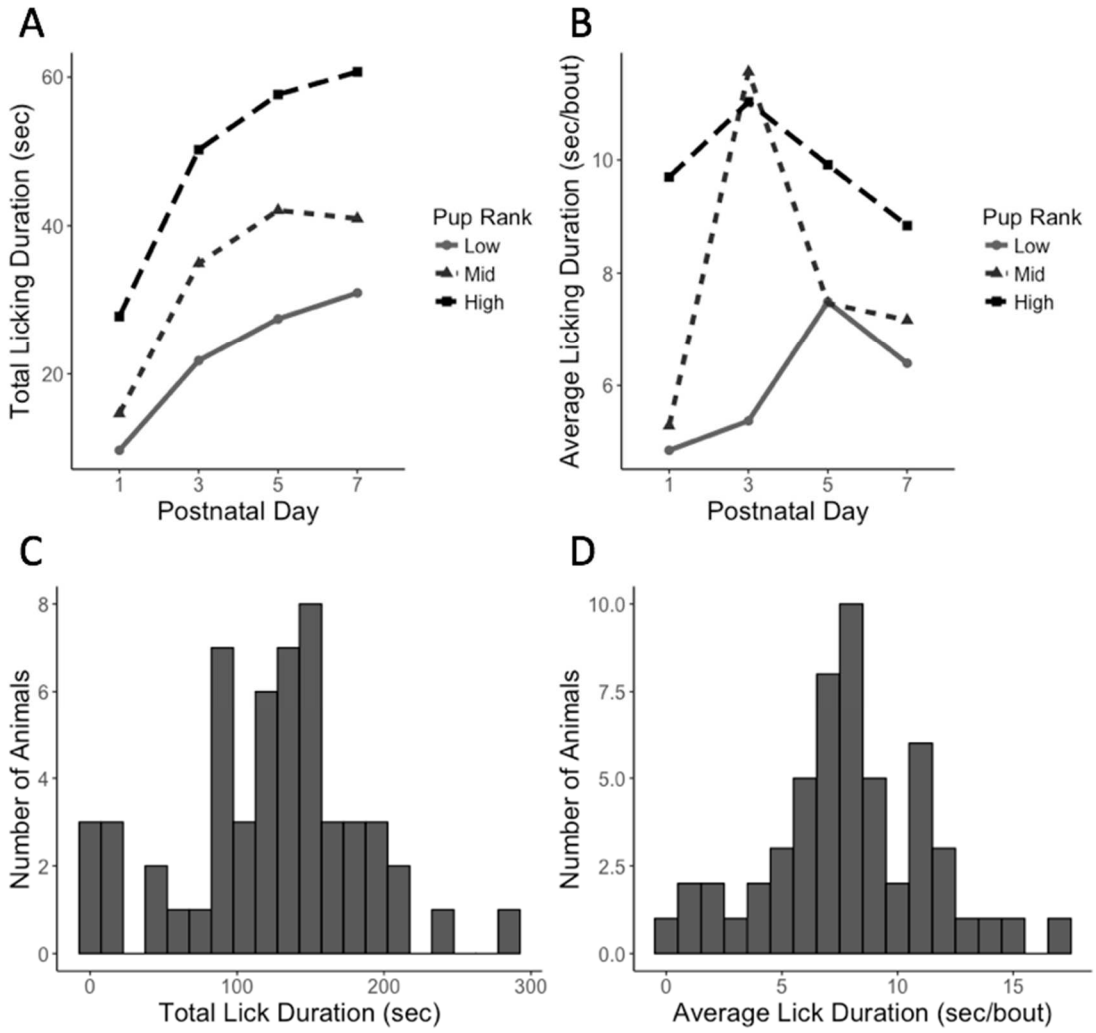

Figure 4.2 Maternal licking received by female pup offspring in the first week of life. For both (A) total licking duration and (B) average lick duration, the highest lowest licked pups in a litter consistently received high and low levels of licking, respectively, while the remaining pups received mid-levels of licking. (C) Total licking duration (15-second bins) and (D) average licking duration (1-second bins) across all observation days are normally distributed for the female offspring tested (n = 55).

Total licking duration positively correlated with baseline DOPAC/DA ratio in the nucleus accumbens (Pearson's r = 0.383, p = 0.012; Figure 4.3A) and was not associated with baseline DOPAC/DA ratio in other brain areas or any behavior measures. Average licking duration negatively correlated with sucrose preference (Pearson's r = -0.372, p = 0.012; Figure 4.3B) and

was not associated with trials to criterion in the strategy shifting task (Pearson's r = -0.276, p = 0.192), percent efficiency in the DRL-20 schedule (Pearson's r = -0.024, p = 0.907), or baseline DOPAC/DA ratio in any brain area.

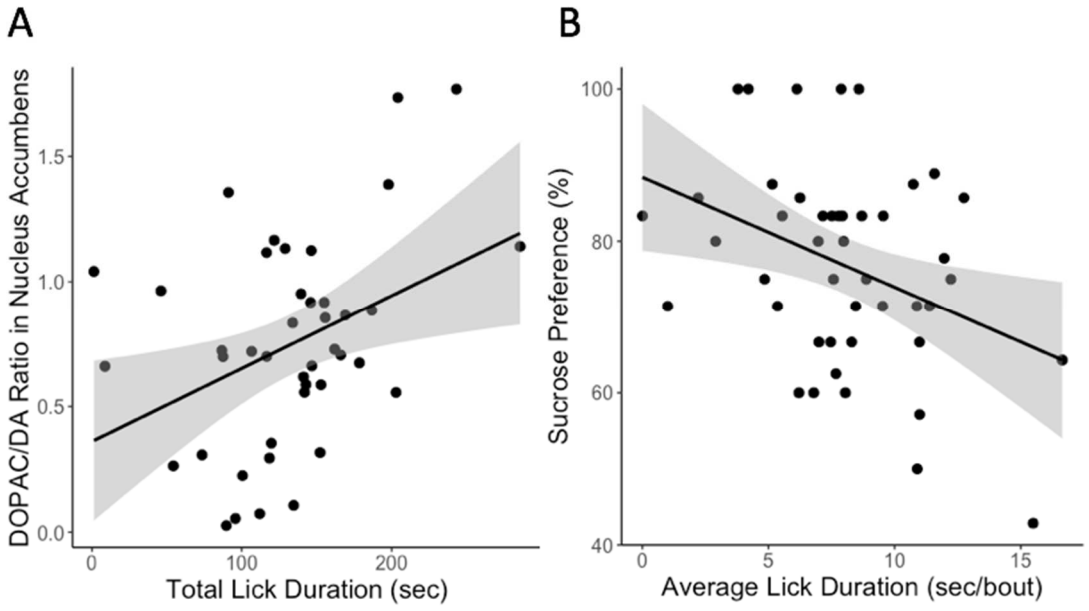

Figure 4.3 Maternal licking received correlated with some adult offspring outcomes. (A) Total licking duration positively correlated with baseline dopamine turnover in the nucleus accumbens. (B) Average licking duration negatively correlated with sucrose preference. Scatterplots are displayed with 95% confidence interval in gray.

4.3.2 Correlations between Behavior and Baseline Dopamine Turnover

Baseline DOPAC/DA ratio in the ventral tegmental area negatively correlated with percent efficiency in the DRL-20 schedule (Pearson's r = -0.534, p = 0.023; Figure 4.4A) and trials to criterion in the strategy shifting task (Pearson's r = -0.564, p = 0.015; Figure 4.4B). In addition, baseline DOPAC/DA ratio in the medial preoptic area negatively correlated with percent efficiency in the DRL-20 schedule (Pearson's r = -0.629, p = 0.005; Figure 4.4C). No other significant correlations were observed.

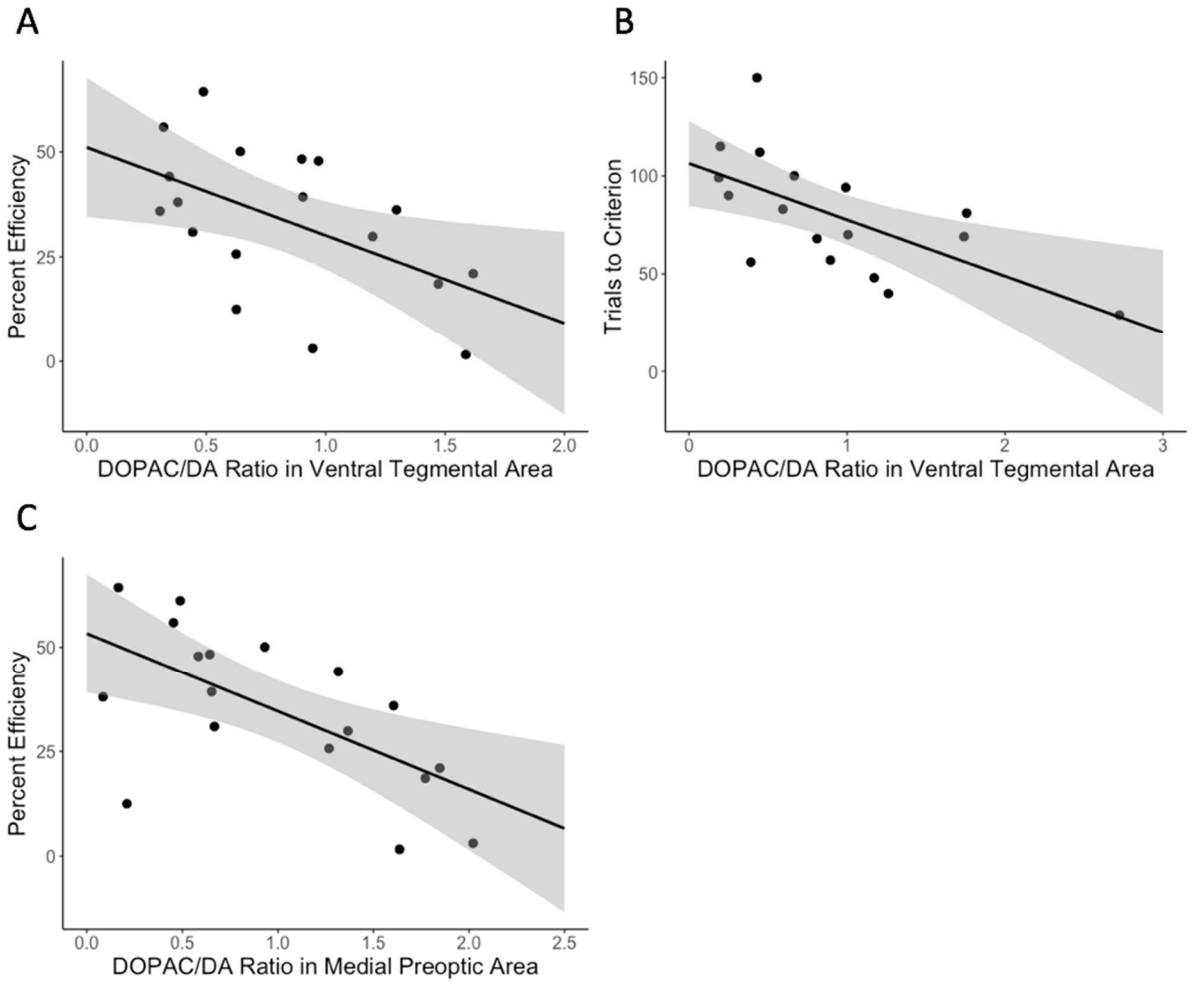

Figure 4.4 Baseline dopamine turnover in select brain areas correlated with performance in the DRL-20 and strategy shifting tasks. Baseline DOPAC/DA ratio in the ventral tegmental area negatively correlated with (A) percent efficiency in the DRL-20 schedule and (B) trials to criterion in the strategy shifting task. (C) Baseline DOPAC/DA ratio in the medial preoptic area negatively correlated with percent efficiency in the DRL-20 schedule. Scatterplots are displayed with 95% confidence intervals in gray.

4.3.3 Effects of Genotype

One site in the DRD2 gene (RS107017253) and the DAT gene (RS13448119) were polymorphic in female offspring. For DRD2, 45 offspring were A/A, 7 were A/G, and 3 were G/G. The A/G and G/G groups were combined for statistical analysis. For DAT, 20 offspring were A/A, 22 were A/G, and 13 were G/G. The other DRD2 site (RS13448058) exhibited low variation (52

G/G offspring and 3 A/G offspring) and the COMT sites exhibited no variation in female offspring.

4.3.3.1 DRD2 (RS107017253)

DRD2 genotype had a marginal effect on baseline DOPAC/DA ratio in the nucleus accumbens ($F_{(1,32.676)} = 3.965$, $p = 0.055$). Offspring with the A/A genotype had lower DOPAC/DA ratio than offspring with A/G or G/G genotypes. With the DRD2 genotype there were no significant differences in baseline DOPAC/DA ratio in other brain areas, any behavior measures, or licking received between female offspring.

4.3.3.2 DAT (RS13448119)

DAT genotype had no effect in any brain site on baseline DOPAC/DA ratio. However, DAT genotype was associated with significant differences in sucrose preference ($F_{(2,40.527)} = 3.804$, $p = 0.031$; Figure 4.5A). Heterozygous A/G offspring had the lowest sucrose preference, were comparable to offspring with the A/A genotype (Mean difference = -3.308 ± 4.364, LSD $p = 0.453$) and were significantly different from offspring with the G/G genotype (Mean difference = -12.797 ± 4.706, LSD $p = 0.010$). Offspring with the A/A genotype and G/G genotype were marginally different in sucrose preference (Mean difference = -9.489 ± 4.843, LSD $p = 0.057$).

In addition, we observed a marginal effect of DAT genotype on percent efficiency in the DRL-20 schedule ($F_{(2,21.133)} = 3.324$, $p = 0.056$; Figure 4.5B). Again, heterozygous A/G offspring had the lowest percent efficiency and were comparable to offspring with the A/A genotype. Offspring with the G/G genotype had the highest percent efficiency. There were no significant differences in trials to criterion in the strategy shifting task or licking received between female offspring with varying DAT genotype.

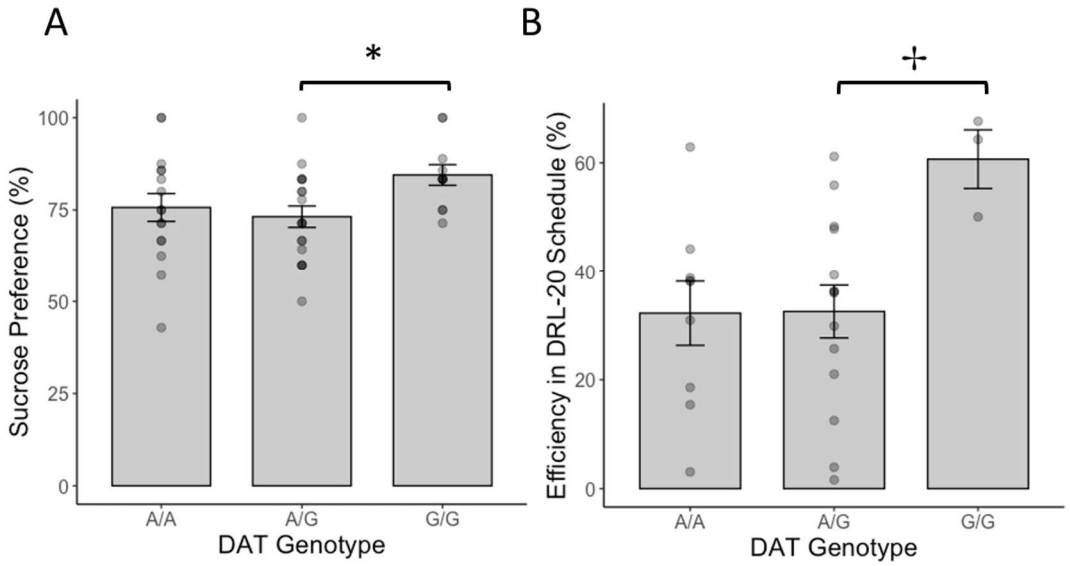

Figure 4.5 Dopamine Transporter genotype affected sucrose preference in adult offspring. (A) Heterozygotes had significantly reduced sucrose preference compared to homozygous G/G offspring and were similar to homozygous A/A offspring. A/A and G/G offspring were not significantly different from each other. (B) A similar pattern was observed for efficiency in the DRL-20 schedule but was not statistically significant. Bar plots are displayed with mean +/- standard error with individual datapoints. * Post-Hoc $p < 0.05$ and ✢ $p < 0.10$ with Fisher's Least Significant Difference.

4.3.4 Gene x Environment Interactions

Since female offspring with A/A and A/G DAT genotypes performed similarly in the behavior measures tested (all p's > 0.10), we grouped them together for multiple regression. We did not observe any total licking duration x genotype interactions and only report average licking duration x genotype interactions.

4.3.4.1 Strategy Shifting

DRD2 genotype interacted with average licking duration on performance in the strategy shifting task. Specifically, we found a significant R^2 change when the average licking x DRD2 interaction term was added to the regression model ($\Delta R^2 = 0.312$, $F_{(1,20)} = 10.312$, $p = 0.004$). Analysis with PROCESS also showed this interaction (Coefficient $\beta = 12.9739 \pm 4.0401$, $t_{(1,20)} = 3.2112$, p =

0.0044) and found a significant effect at the A/A genotype (Effect = -7.4747 ± 2.3497, t = -3.1811, p = 0.0047) but not the A/G + G/G genotypes (Effect = 5.4922 ± 3.2866, t = 1.6732, p = 0.1099). For female offspring with the A/A genotype, higher average licking duration was associated with fewer trials to criterion on the response task, corresponding to higher behavioral flexibility in adulthood (Figure 4.6A). We observed a marginal interaction with the DAT genotype ($\Delta R^2 = 0.128$, $F_{(1,20)} = 3.466$, $p = 0.077$) on the behavioral flexibility task.

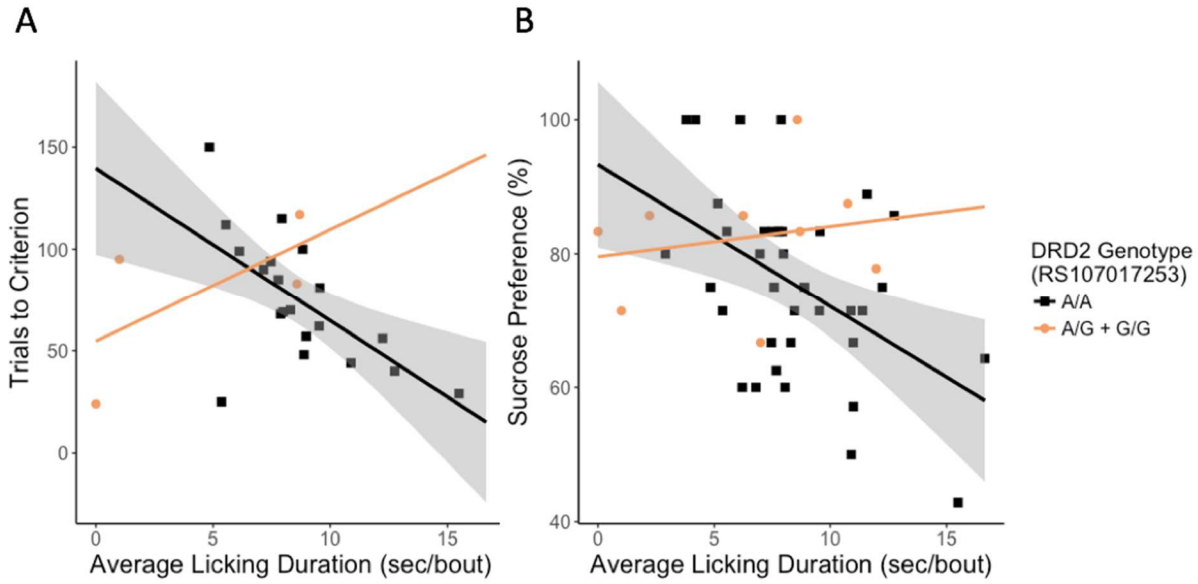

Figure 4.6 Association of average lick duration with strategy shifting and sucrose preference were moderated by dopamine receptor 2 genotype. Homozygous A/A offspring had (A) fewer trials to criterion in the strategy shifting task and (B) lower sucrose preference with more early-life licking received. Heterozygous and homozygous G/G offspring did not show an association with licking received. Scatterplots are displayed with linear regression lines for the A/A genotype (black) and A/G + G/G genotype (orange), with the 95% confidence interval for the A/A genotype in gray.

4.3.4.2 DRL-20

We did not observe an interaction with the DRD2 genotype ($\Delta R^2 = 0.045$, $F_{(1,22)} = 1.226$, $p = 0.280$) or the DAT genotype ($\Delta R^2 = 0.039$, $F_{(1,22)} = 1.266$, $p = 0.273$) with percent efficiency on the DRL-20 schedule.

4.3.4.3 Sucrose Preference

DRD2 genotype interacted with average licking duration on sucrose preference. Specifically, we found a significant R^2 change when the average licking x DRD2 interaction term was added to the regression model ($\Delta R^2 = 0.087$, $F_{(1,41)} = 4.687$, $p = 0.036$). Analysis with PROCESS also showed this interaction (Coefficient $\beta = 2.8812 \pm 1.39090$, $t_{(1, 41)} = 2.2011$, $p = 0.0334$) and we found a significant effect at the A/A genotype (Effect = -2.0189 ± 0.6488, t = -3.1118, p = 0.0034) but not the A/G or G/G genotypes (Effect = 0.8623 ± 1.1369, t = 0.7585, p = 0.4525). For female offspring with the A/A genotype, higher average licking duration was associated with lower sucrose preference, corresponding to lower baseline reward sensitivity in adulthood (Figure 4.6B). We did not observe an interaction with the DAT genotype ($\Delta R^2 = 0.030$, $F_{(1,41)} = 1.100$, $p = 0.300$).

4.3.4.4 Baseline Dopamine Turnover

We did not observe any interactions with the DRD2 genotype or DAT genotype for baseline DOPAC/DA ratio in any of the brain areas tested (Table 4.2).

Table 4.2 Results of Genotype x Average Lick Duration Multiple Regression Analysis.

Dependent Variable	Genotype	R^2 change	p-value*
Strategy Shifting (Trials to Criterion)	DRD2	**0.312**	**0.004**
	DAT	*0.128*	*0.077*
DRL-20 (Percent Efficiency in Last Three Sessions)	DRD2	0.045	0.280
	DAT	0.039	0.273
Sucrose Preference	DRD2	**0.087**	**0.036**
	DAT	0.020	0.300
Medial Prefrontal Cortex DOPAC/DA Ratio	DRD2	< 0.001	0.959
	DAT	0.045	0.181
Nucleus Accumbens DOPAC/DA Ratio	DRD2	0.031	0.228
	DAT	0.001	0.815

Medial Preoptic Area DOPAC/DA Ratio	DRD2	0.043	0.198
	DAT	0.005	0.665
Dorsal Hippocampus DOPAC/DA Ratio	DRD2	0.002	0.773
	DAT	0.009	0.544
Ventral Tegmental Area DOPAC/DA Ratio	DRD2	0.001	0.889
	DAT	0.028	0.298

***$p < 0.05$**, $p < 0.10$

4.4 Discussion

To our knowledge, this is the first study to investigate the effects of inter-individual maternal care on female offspring dopaminergic phenotypes and the moderating role of genotype. Our results show that a SNP in the DRD2 gene moderates the effect of average licking per bout received on two behavior measures, strategy shifting and sucrose preference, with a minimal role on baseline dopamine turnover. The updated moderation model is displayed in Figure 4.7. Specifically, early-life licking received affects female offspring carrying the A/A genotype and does not affect female offspring carrying the A/G or G/G genotypes. In addition, a SNP in the DAT gene, regardless of licking received, has an effect on sucrose preference and a marginal effect on impulsive action. This suggests that offspring's sensitivity to early-life environments can be influenced by genetic variation in dopamine-related genes. This idea has also been discussed in the context of sensory processing sensitivity in the human literature with serotonin- and dopamine-related genes (Chen et al., 2011). Sensory processing sensitivity is a stable trait within populations of multiple species that increases an individual's responsiveness to early-life environments (Pluess, 2015).

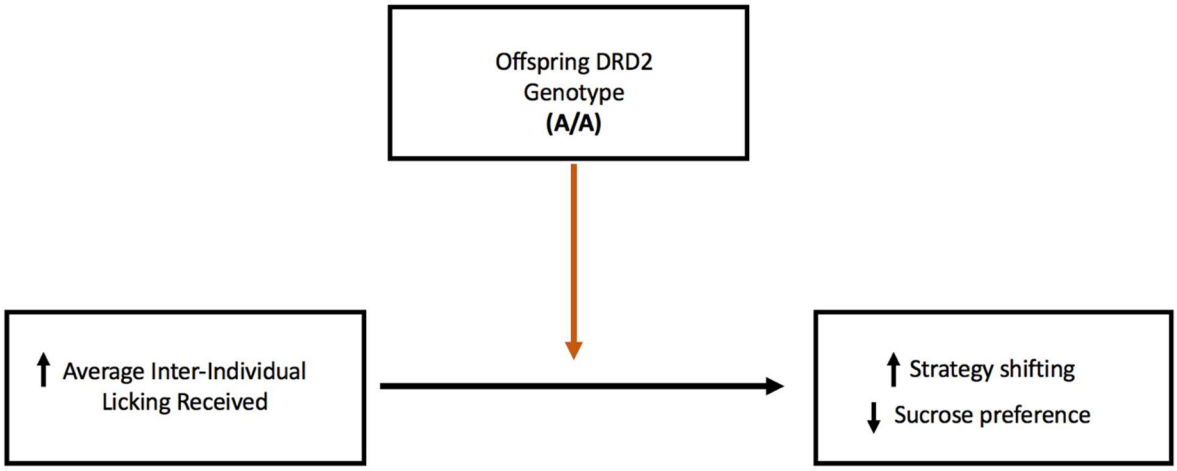

Figure 4.7 Updated moderation model between early-life licking received and dopamine-dependent behavioral outcomes. Offspring DRD2 genotype moderated the relationship between average lick received and later-life strategy shifting and sucrose preference.

4.4.1 Relationships Between Early-life Maternal Licking Measures and Phenotype

We examined two different methods to measure maternal licking, total duration and average duration per bout, to see if quality of maternal care plays a role in later-life offspring behavior and baseline dopamine turnover. We found each maternal care measure correlated with a different outcome; specifically, total licking duration positively correlated with nucleus accumbens dopamine turnover and average licking duration negatively correlated with sucrose preference. In addition, average licking duration showed stronger gene x environment interactions. Therefore, the average duration a mother licks a pup per bout may be a useful measure of inter-individual maternal care, similar to its use in whole-litter paradigms (Hellstrom et al., 2012; Pena et al., 2013), and can be a compliment measure to the total duration of licking received.

4.4.2 Relationships Between Behavior and Baseline Dopamine Turnover

We only found few correlations between the behavior measures and baseline dopamine turnover, which does not support our hypothesis that early-life licking effects on behavior are mediated by dopaminergic activity. However, baseline dopamine turnover does not necessarily predict stimulus-induced dopamine turnover. For example, a study that used maternal isolation found

that female offspring had a blunted stimulus-induced dopamine response to pups than undisturbed controls, even though their baseline dopamine levels were higher (Afonso et al., 2011). Investigating the relationships between inter-individual maternal licking received, behavior, and stimulus-induced dopamine turnover is a potential avenue for future work.

4.4.3 Effects of Genotype and Gene x Environment Interactions

Offspring with a heterozygous genotype for a SNP in the DAT gene had decreased sucrose preference and had a marginal decrease in DRL-20 efficiency. How this specific SNP affects dopamine transporter expression or function is unknown. While there is information on a DAT variable number tandem repeat and its effects on expression and behavior in human populations (Heinz et al., 2000), we did not find any studies of DAT variation with rat populations.

We found gene x environment interactions with a SNP in the DRD2 gene for strategy shifting and sucrose preference. DRD2 gene variation has been shown to interact with the effects of early-life environment on dopamine receptor 2 expression in Sprague-Dawley rats (Lovic et al., 2013), though the specific SNP in that study (RS13448058) showed low variation in our Long-Evans rat population (with three heterozygous offspring). Nevertheless, the pattern is similar: early-life events affect offspring with a certain genotype while others show a minimal association. We (Pan et al., 2018) also reported this pattern with SNPs in the FK506-binding protein, glucocorticoid receptor and serotonin transporter genes using rat siblings that received differential maternal licking. We currently do not know the specific transcriptional regulatory mechanisms involved in these interactions. In addition, one fundamental assumption of the moderation analysis is that there is a functional link between the DRD2 SNP and phenotype (Hayes, 2013).

Sucrose preference showed a significant negative correlation with average licking duration with all offspring, but the relationship was stronger for female offspring with the A/A DRD2 genotype. This relationship between sucrose preference and maternal care is consistent with previous findings on maternal isolation (Lomanowska et al., 2006); however, it is counter-intuitive if sucrose preference is viewed as a measure of depressive-like behavior. We tested sucrose preference after the other tasks in the operant chamber which used sucrose pellets as a reward. Familiarity with the taste of sucrose could help explain the phenotype this task measured, but further experiments are required to confirm this explanation. In addition, as we

only tested the female offspring once in the dark phase, we do not know if multiple testing would alter the results. However, a previous study demonstrated that sucrose preference scores are stable across days, especially during the dark phase (Tõnissaar et al., 2006).

Strategy shifting only showed a negative correlation with average licking duration for female offspring with the A/A DRD2 genotype. The relationship between strategy shifting and maternal care is consistent with previous findings on maternal separation and isolation (Baudin et al., 2012; Lovic & Fleming, 2004; McLean et al., 2010). These studies tested rats using an attentional set-shifting task, while we used a recently developed operant procedure to reduce training and extensive human handling during the task. The automated procedure would reduce individual variation from human error. The consistency indicates construct validity between the two testing paradigms and a robust relationship between early-life experiences and executive functioning. Testing the female offspring with a reversal task could provide additional insights regarding underlying neural circuitry, since strategy shifting and reversal learning involve different areas of the prefrontal cortex and can be dissociable (Floresco et al., 2008; Ghods-Sharifi, Haluk, & Floresco, 2008).

We did not find any relationships between maternal licking and impulsive action, which has been found with maternal isolation (Lovic, Keen, et al., 2011). Our data indicated that efficiency for a few female offspring did not increase from the first three sessions to the last three sessions of the DRL-20 schedule, indicating that the inability to learn the task in some subjects could be a confound. It is difficult to conclude whether these offspring were highly impulsive or did not learn the association required for the sucrose reward. In future studies, the inclusion of several sessions with DRL-5 and DRL-10 schedules may make the variations in DRL-20 performance easier to interpret.

4.4.4 Limitations

A caveat in this study is that the maternal care observations occurred immediately after handling the pups. Removing the pups from the nest was necessary to mark and differentiate siblings, but temperature changes in the pups and some tactile stimulation from the handler was unavoidable. Handling can induce changes in maternal care by increased solicitation from the pups (Bell et al., 1971), though it is unknown whether it can change maternal care between siblings. While our research group has used this paradigm in the past and found differences in female offspring

stress reactivity (Pan et al., 2014, 2018), inter-individual variation in maternal licking could be verified with an undisturbed observation period.

Another caveat is that our study design consisted of two cohorts and we analyzed as few as one to two female offspring from a litter. Therefore we did not analyze differences in behavior using within-litter pup rankings as we have done previously with a minimum of five female pups in a litter (Pan et al., 2014, 2018). Nevertheless, our correlational analysis of maternal licking identified findings that were in the direction we predicted from the literature and the interaction between average licking duration and DRD2 genotype was consistent between two different tasks. The external and internal replicability of our results provide evidence of a robust effect of maternal licking on dopamine-related behavior. However, a more comprehensive assessment of gene x environment interactions, with additional SNPs and behavior tasks, is needed to further support our interpretations.

In this study, we investigated the role of genotype in maternal care transmission of later-life dopamine-dependent behavior. Our findings suggest that the relationship between maternal licking and dopamine-related behavior depend on genotype for the DRD2 gene. These findings could help inform future work on the transmission of maternal care between generations. Previous work focusing on the maternal brain has highlighted the importance of experience and hormonal priming via DNA methylation of estrogen and oxytocin receptor genes (Beery et al., 2016; Stolzenberg & Champagne, 2016). Overall, our findings suggest that variation in the DRD2 gene, likely along with other genes, could influence intergenerational maternal care by altering the female offspring's sensitivity to the maternal environment.

Chapter 5
Inter-individual Maternal Licking Received and Dopamine Genotype Interactions on Intergenerational Maternal Care Provisioning

This chapter is adapted from:

Lauby, S.C., Ashbrook, D.G., Malik, H.R., Chatterjee, D., Pan, P., Fleming, A.S., & McGowan, P.O. (submitted). Genotype Effects and Gene x Environment Interactions on Inter-Individual Licking Received and Licking Provisioning in Female Rat Offspring. *BioRxiv.* doi: https://doi.org/10.1101/2019.12.29.890467

Contributions:

SCL, PP, ASF, and POM designed the study. SCL and PP conducted the maternal care received and maternal care provisioning observations. DGA ran the variant calling analysis from Long-Evans RNA-seq datasets and created the track for the UCSC genome browser. HRM did the brain cryosectioning and liver DNA extractions for genotyping. DC conducted the HPLC. SCL and HRM analyzed the data. ASF and POM supervised the research.

5 Inter-individual Maternal Licking Received and Dopamine Genotype Interactions on Intergenerational Maternal Care Provisioning

5.1 Introduction

The maternal environment plays a substantial role in influencing offspring phenotype in adulthood, including the intergenerational transmission of maternal care through the female lineage (Fleming et al., 2002). Both maternal care received and changes in the brain that occur when the mother is preparing for postnatal offspring care involve alterations in the dopaminergic (Champagne et al., 2004; Peña, Neugut, Calarco, & Champagne, 2014) and oxytocinergic systems (Beery, McEwen, MacIsaac, Francis, & Kobor, 2016; Francis, Young, Meaney, & Insel, 2002; Toepfer et al., 2017), as well as changes in estrogen signaling (Cameron et al., 2008; Champagne, 2008). Maternal care provisioning also involves neuropeptides aside from oxytocin, including prolactin and arginine vasopressin (Bridges, 2015). These mechanisms have been well-characterized in rodents and studies with humans show similar findings (Rosenblatt, 1994), suggesting that the factors involved in the intergenerational transmission of maternal care are evolutionarily conserved.

Rat pups with early-life adversity induced by maternal and sibling deprivation are less attentive to foster pups and their own offspring later in life than rat pups maternally reared (Gonzalez et al., 2001; Palombo et al., 2010). Longer durations of licking-like tactile stimulation provided during the deprivation period partially mitigates the later-life impairments in maternal care provisioning (Gonzalez et al., 2001; Palombo et al., 2010). In addition, naturally occurring variation between litters in maternal licking/grooming (LG) received can transmit between generations of offspring. High LG and low LG mothers (defined as +/- one standard deviation from the average) tend to produce female offspring that become high LG and low LG mothers to their offspring, respectively (Champagne, Francis, Mar, & Meaney, 2003; Francis, Diorio, Liu, & Meaney, 1999). This effect persists in cross-fostering experiments, suggesting a prominent role of postnatal maternal behaviour in the intergenerational transmission of maternal care (Francis et al., 1999).

Previous work on the intergenerational transmission of maternal care in rats has mainly focused on proxies of maternal neglect or the tail ends of the normal distribution of maternal care

received. We have more limited knowledge of factors that affect the intergenerational transmission of maternal care in the average rat mother. Seminal research examining natural variations in maternal care received between litters found that there is also substantial within-litter variation in later-life maternal LG provisioning (Champagne, Francis, Mar, & Meaney, 2003). In addition, studies with human cohorts in nonclinical populations have shown a modest relationship between parental care received and parental care provisioning (Van Ijzendoorn, 1992) and found that the genotype of the individual can interact with the early-life environment or directly affect maternal care provisioning (Fleming & Kraemer, 2019). Studies in mice also show an important role of offspring genotype in maternal care received (Ashbrook, Sharmin, & Hager, 2017; Ashbrook, Gini, & Hager, 2015).

Findings from our group and others have shown effects of early-life individual differences in maternal licking received on later life behaviour (Chapter 4; Pan, Fleming, Lawson, Jenkins, & McGowan, 2014; Pan et al., 2018; Ragan, Loken, Stifter, & Cavigelli, 2012; van Hasselt et al., 2012), including maternal retrieval behaviour in sensitized virgin female rat offspring (Ragan et al., 2016). We have also reported gene x environment interactions involved in some of these effects (Chapter 4; Pan et al., 2018). For example, we recently reported an interaction between a dopamine receptor 2 (DRD2) single nucleotide polymorphism (rs107017253) and early-life average licking duration per bout (a proxy of maternal care quality) on strategy shifting and sucrose preference, two dopamine-related phenotypes (Chapter 4). Executive function measured by strategy shifting performance has previously been shown to be related to maternal care provisioning in human cohorts (Gonzalez et al., 2012). However, studies on the effect of genotype in the Long-Evans rat model commonly used in these experiments have been limited by the lack of knowledge of genetic variation in this outbred strain. Although there are now data available on genetic variation within wild rat populations (Ness et al., 2012) and between inbred rat lines (Hermsen et al., 2015; STAR Consortium et al., 2008), no mapping has been carried out within the Long-Evans population.

The purpose of this study was to investigate the role of genotype and gene x environment interactions in the relationship between early-life maternal licking received and later-life licking provisioning, with a specific focus on the dopaminergic system of the maternal brain. We also aimed to investigate other genes relevant to maternal behaviour using a more systematic approach to identify and verify genetic variants in our Long-Evans rat population. We measured

inter-individual maternal licking received within the first week of life and assessed later-life female offspring maternal licking provisioning, maternal brain dopamine and its metabolite levels, and variation in single nucleotide polymorphisms (SNPs) in genes involved in dopamine, estrogen, and neuropeptide function. We predicted that higher early-life licking received would correspond to higher later-life licking provisioning in female F_1 offspring. In addition, we hypothesized that this relationship would be indirectly affected by dopaminergic activity in the maternal brain and interact with female F_1 offspring genotype in dopamine-related genes. The hypothesized moderated mediation model is displayed in Figure 5.1.

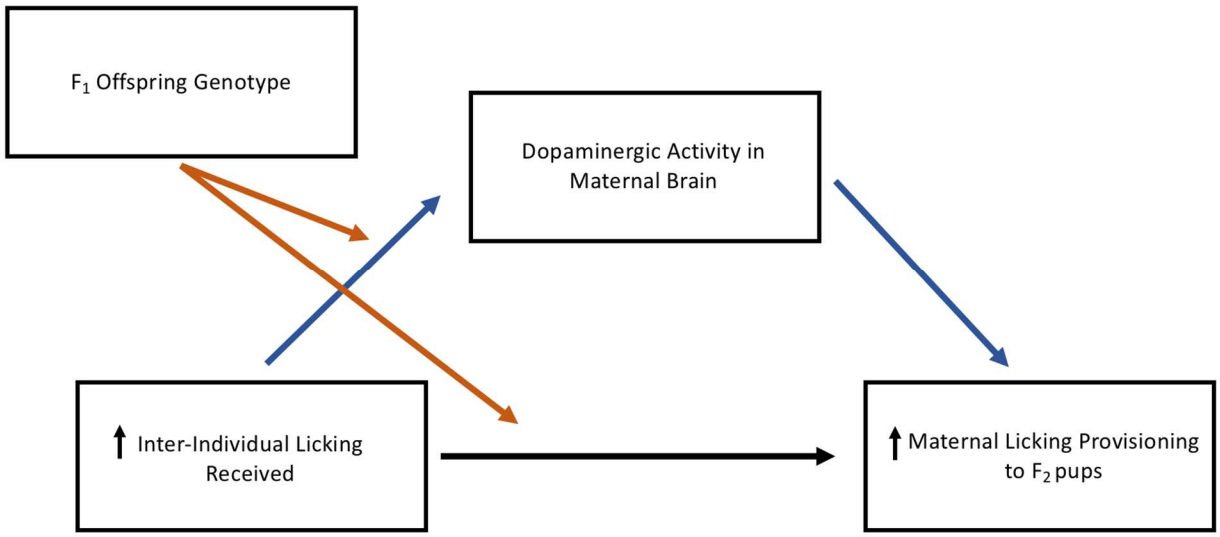

Figure 5.1 Hypothesized moderated mediation between early-life licking received and later-life licking provisioning. Inter-individual licking received early in life would positively associate with later-life maternal licking provisioning. Differences in dopaminergic activity in the maternal brain would account for this association and mediate the relationship between licking received and licking provisioning indirectly. Finally, offspring genotype in dopamine-related genes would interact with early-life inter-individual licking received and moderate later-life maternal licking provisioning and dopaminergic activity in the maternal brain.

5.2 Methods

5.2.1 Rat Breeding

Seven-week-old female (n = 24) and male (n = 6) Long-Evans rats were obtained from Charles River Laboratories. They were housed in same-sex pairs on a 12:12 hour light-dark cycle (lights

on at 7:00) with ad libitum access to standard chow diet and water. For breeding, one male was housed with two females for one week. Females were then housed separately and weighed weekly throughout pregnancy. The breeding males were used multiple times to produce four cohorts of litters in this study. All animal procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough and conformed to the guidelines of the Canadian Council on Animal Care.

The pregnant F_0 females were checked for parturition starting three weeks after breeding at 9:00 and 17:00. Postnatal day (PND) 0 was determined if the birth occurred between 9:00 and 17:00 or if pups were noted at 9:00 but have not nursed yet. Pups found at 9:00 with a visible milk band were determined to be PND 1. At PND 1, litters were culled to five to six female pups and individually weighed. We focused on smaller litters in order to accurately measure maternal care received. Therefore, male offspring were not examined for this study. A total of 136 F_1 female pups were assessed for maternal care received.

5.2.2 Maternal Care Received Observations

See Chapter 4, Section 4.2.2.

5.2.3 Maternal Care Provisioning Observations

A subset of adult F_1 female offspring (n = 54) were bred with sexually experienced males (n = 6) for one week. Twelve F_1 female offspring were previously tested in dopamine-related tasks (Chapter 4). Six females were unable to get pregnant after two rounds of breeding and two females failed to lactate following parturition, with a total of 46 F_1 rat mothers being used for intergenerational maternal care analysis. Births were checked starting three weeks after breeding at 9:00 and 17:00. At PND 1, cages were changed with no culling of pups. The litter sizes ranged from 4 to 18 pups. Litter size was not significantly correlated with maternal licking provisioning (Pearson's $r = 0.001$, $p = 0.994$).

From postnatal day 2 to 9, each litter was video recorded for one hour three times during the light phase (9:00-10:00, 13:00-14:00, 17:00-18:00) and three times during the dark phase (21:00-22:22, 1:00-2:00, 5:00-6:00) using security cameras connected to a DVR system (Swann Communications Ltd.). These videos were coded with Observer XT 10.5 (Noldus Information Technology) for maternal behaviour by four coders who were blind to the genotype of the F_1 rat

mother. Nursing, licking, nest-building, and other self-directed behaviours were scored every three minutes based on previous literature (Champagne, Francis, Mar, & Meaney, 2003). A total of 120 observations per F_1 mother per day were coded and maternal licking provisioning was represented as a percentage of the frequency of licking behaviour (body and anogenital combined) coded over total observations.

After 10:00 on PND 9 of the F_2 pups, the F_1 rat mother was separated from her pups and was sacrificed with CO_2 inhalation and decapitation. Liver and whole brain tissue were collected and placed in dry ice or flash-frozen in isopentane, respectively. Tissue was stored in -80°C until further processing.

5.2.4 High Performance Liquid Chromatography

Forty-six maternal F_1 rat brains were sliced and microdissected using a Leica CM3050S cryostat for high performance liquid chromatography (See Chapter 4, Section 4.2.5). Dopamine (DA) and 3, 4-dihydroxyphenylacetic acid (DOPAC) levels were normalized against total protein concentration for each sample. DOPAC divided by DA, or the DOPAC/DA ratio, was calculated as a measure of dopaminergic activity in each brain area. We were unable to analyze two prefrontal cortex samples, two nucleus accumbens samples, one medial preoptic area sample and three ventral tegmental area samples due to lost tissue during collection.

5.2.5 Genotyping

To identify variants which segregate within the Long-Evans population, a collaborator (Dr. David G. Ashbrook) took advantage of large amounts of RNA-seq data available online in the population. He identified 119 RNA-seq datasets on the Gene Expression Omnibus (GEO) which he could use for variant calling.

To validate variation in candidate SNPs, liver DNA from the F_1 rat mothers was extracted using an EZNA Tissue DNA extraction kit (Omega Bio-tek, Norcross, GA) and assessed for SNPs in genes relevant to maternal behaviour, broadly including dopamine-related, estrogen-related, and neuropeptide-related genes (Table 5.1). Purified DNA (35 ng/µl) was submitted for a multiplex assay at The Centre for Applied Genomics (SickKids, Toronto, Canada).

5.2.6 Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corporation). To examine the relationship between early-life maternal licking received and later-life maternal licking provisioning, a Pearson correlation was used between total or average licking duration received and the percentage of licking provisioning observed. In addition, to examine the relationship between later-life maternal licking provisioning and dopaminergic activity, a Pearson correlation was used between percentage of licking provisioning observed and the DOPAC/DA ratio in each brain area, with a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure to account for multiple analyses. Significant correlations with DOPAC/DA ratio in a brain area were followed up with Pearson's correlations with DOPAC and DA levels separately. To examine the effects of genotype, a one-way ANOVA was used to compare offspring with each varying genotype to early-life maternal licking received and later-life maternal licking provisioning with a FDR correction to account for multiple SNP analyses for each outcome licking measure. Significant effects of genotype were followed with a Tukey's post-hoc test. To examine gene x environment interactions and the mediating role of dopaminergic activity on later-life maternal licking provisioning, we used Hayes PROCESS module for SPSS (Version 3.2) using a simple moderation (Model 1) with a FDR correction to account for multiple analyses for each outcome dependent variable and a moderated mediation (Model 7; Hayes, 2013). A moderation tests for an interaction between an independent variable (X) and a moderator variable (W) on a dependent variable (Y) and a mediation tests for indirect associations of the independent (X) and dependent variable (Y) by a third causal variable (the mediator M_i). PROCESS is a flexible modelling module that can conduct moderation and mediation analyses using multiple regression and conduct post-hoc analyses of the conditional effects of a focal moderator and the indirect effects of a mediator.

The statistical model used for a simple moderation (Model 1) was:

Conditional direct effects of X on $Y = b_1 + b_3W$

The statistical model used for a moderated mediation (Model 7) was:

Conditional indirect effects of X on Y thorough $M_i = (a_{1i} + a_{3i}W)b_i$

Direct effect of X on $Y = c'$

Multi-categorical moderator variables (i.e., more than two genotypes) were analyzed using the indicator coding system. All effects were considered statistically significant if $p \leq 0.05$ and marginally significant if $p \leq 0.10$.

5.3 Results

5.3.1 Relationship between Early-Life Licking Received and Later-Life Licking Provisioning

Total licking duration received across all maternal care observation periods (PND 1, 3, 5, and 7) averaged 168.79 ± 18.6 seconds and ranged from 0 to 450.72 seconds (Figure 5.2A). Average licking duration per bout of licking received averaged 8.02 ± 0.56 seconds and ranged from 0 to 13.93 seconds (Figure 5.2B).

We first examined the relationship between early-life maternal care received and later-life maternal care provisioning. There were no direct associations between early-life licking received and later-life licking provisioning. Neither total licking received (Pearson's $r = -0.036$, $p = 0.812$; Figure 5.2C) nor average licking received (Pearson's $r = -0.027$, $p = 0.861$; Figure 5.2D) significantly correlated with later-life licking provisioning.

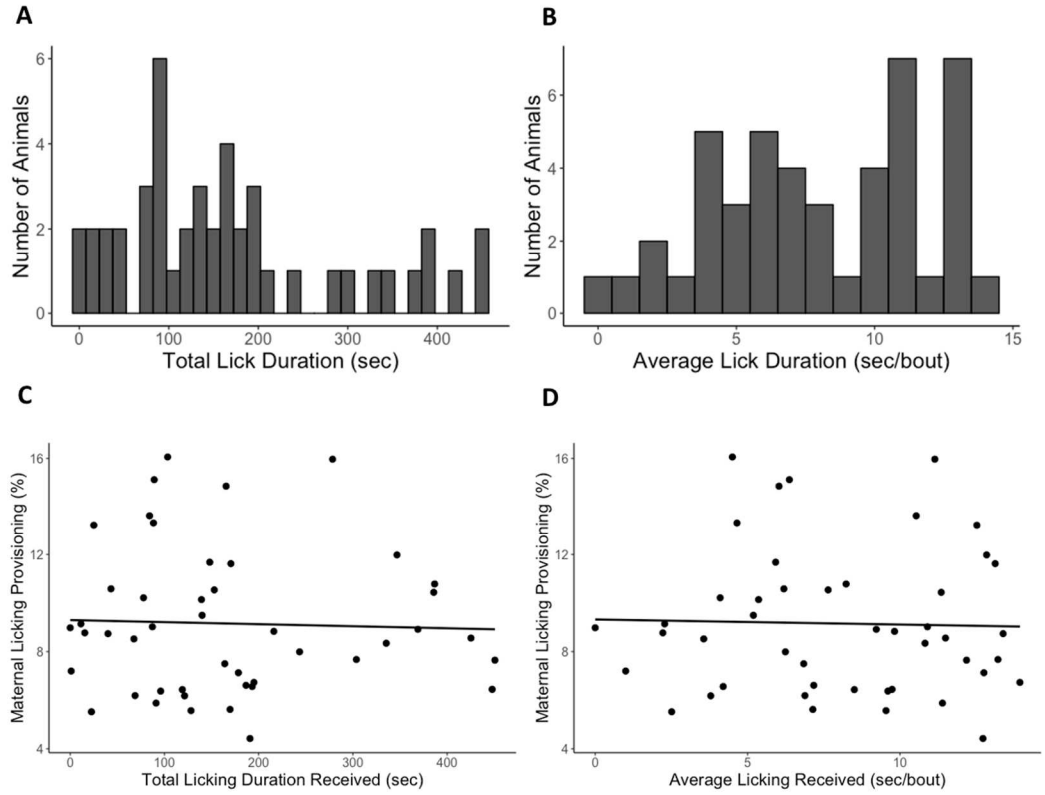

Figure 5.2 The distribution of maternal licking received within the first week of life and its direct association with later-life maternal licking provisioning. (A) Total licking duration (15-second bins) and (B) average licking duration per bout (1-second bins) received across all observation days (PND 1, 3, 5, and 7) for the female offspring tested (n = 46). There were no significant correlations between (C) total licking duration and (D) average licking duration per bout with later-life licking provisioning.

5.3.2 Relationship between Early-Life Licking Received, Later-Life Licking Provisioning and Dopaminergic Activity in the Maternal Brain

We then examined relationships between maternal care received and provisioning with DOPAC/DA ratio in the medial prefrontal cortex, nucleus accumbens, medial preoptic area, and ventral tegmental area in the F_1 maternal brain. There was a significant negative correlation between later-life licking provisioning and DOPAC/DA ratio in the nucleus accumbens (Pearson's r = -0.389, FDR adjusted p = 0.036; Figure 5.3A). Upon further analysis of DOPAC and DA levels separately, there was a significant positive correlation between later-life licking provisioning and DA levels (Pearson's r = 0.346, p = 0.021; Figure 5.3B) and no correlation with

DOPAC levels (Pearson's r = -0.060, p = 0.701; Figure 5.3C) in the nucleus accumbens. There were no other associations between later-life licking provisioning and DOPAC/DA ratio in any other brain regions.

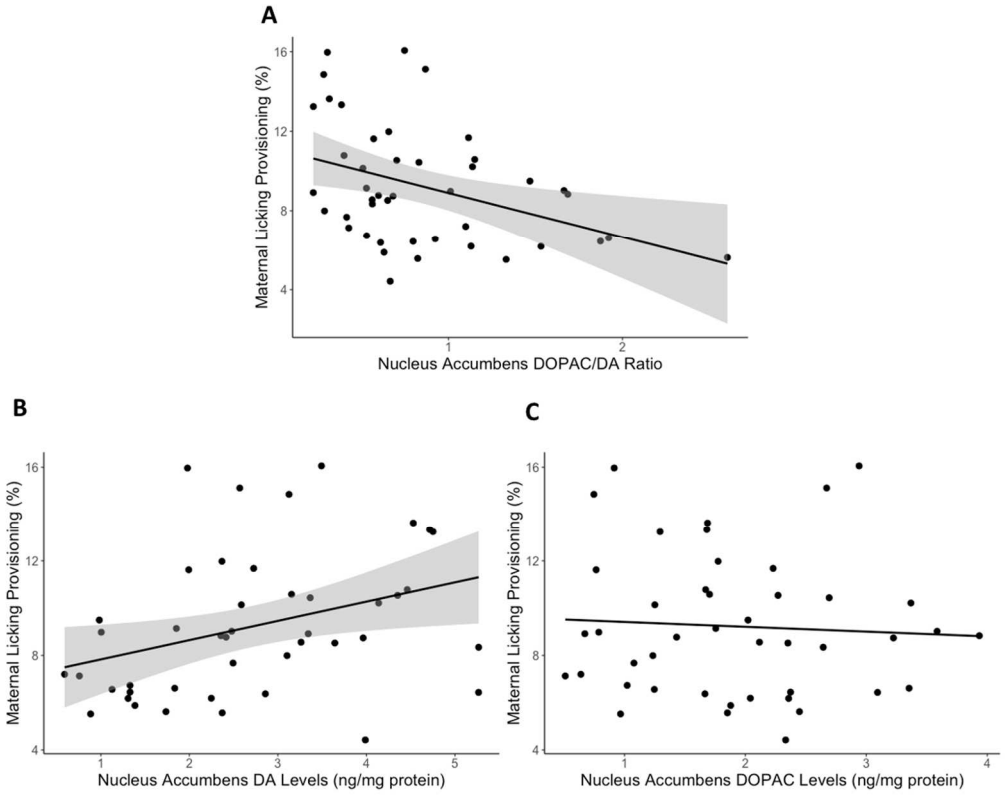

Figure 5.3 Dopaminergic activity in the nucleus accumbens of the maternal brain is associated with later-life licking provisioning. (A) The DOPAC/DA ratio in the nucleus accumbens was negatively correlated with later-life licking provisioning from postnatal day 2-9. Upon further analyses, the (B) dopamine (DA) levels in the nucleus accumbens was positively correlated with later-life licking provisioning and (C) there was no correlation with DOPAC levels in the nucleus accumbens. Scatterplots are displayed with 95% confidence interval in grey for the DOPAC/DA ratio and DA levels in the nucleus accumbens.

5.3.3 Effects of Genotype

Of the 16 variants in dopamine, estrogen, and neuropeptide-related genes, 11 showed variation in our animals (Table 5.1). These SNPs were in dopamine receptor 1 (DRD1; rs105271298), dopamine receptor 2 (DRD2; rs107017253), dopamine transporter (DAT; chr1:32354790 and

rs13448119), estrogen receptor alpha (ESR1; chr1:41325782 and chr1:41590586), estrogen receptor beta (ESR2; rs8170138, rs13448055, and rs106738562), aromatase (CYP19A1; chr8:58755644), and oxytocin receptor (OXTR; chr4:144398803). We were unable to find variation in the SNPs we tested in prolactin receptor (PRLR), arginine vasopressin (AVP) and arginine vasopressin receptor 1a (AVPR1A).

The one female offspring with the DRD1 A/A genotype was combined with the A/T genotype for analysis. In addition, since the three SNPs within ESR2 and the two SNPs within DAT were in complete linkage, only one SNP in each gene (rs8170138 and rs13448119, respectively) was used for analysis.

Table 5.1 List of candidate single nucleotide polymorphisms assessed for variation, information related to their location and function, and genotype frequencies in our Long-Evans rat population.

Gene	Coordinate	Location	Consequence	Accession Number	Genotype Frequencies
Dopamine-Related					
Dopamine Receptor 1 (DRD1)	chr17:11100256	upstream		rs105271298	**33 T/T, 12 T/A, 1 A/A**
Dopamine Receptor 2 (DRD2)	chr8:53736747	exon	synonymous	N/A	No Variation
	chr8:53742670	intron		rs107017253	**34 A/A, 12 A/G**
Dopamine Transporter (DAT)	chr1:32354790	exon	synonymous	N/A	**12 A/A, 26 A/G, 8 G/G***
	chr1:32362114	exon	synonymous	rs13448119	**13 G/G, 25 G/A, 8 A/A***
Estrogen-Related					
	chr1:41325782	exon	synonymous	N/A	**15 C/C, 31 C/T**

Estrogen Receptor Alpha (ESR1)	chr1:41590586	exon	synonymous	N/A	**31 C/C, 5 C/T, 10 T/T**
Estrogen Receptor Beta (ESR2)	chr6:99180312	exon	synonymous	rs8170138	**37 A/A, 9 A/G****
	chr6:99184395	exon	synonymous	rs13448055	**37 G/G, 9 G/A****
	chr6:99204426	exon	synonymous	rs106738562	**37 C/C, 9 C/T****
Aromatase (CYP19A1)	chr8:58755644	exon	synonymous	N/A	**13 T/T, 21 T/C, 12 C/C**
	chr8:58755702	exon	missense	rs197770329	No Variation
Neuropeptide-Related					
Prolactin Receptor (PRLR)	chr2:60285990	exon	synonymous	rs199216170	No Variation
Arginine Vasopressin (AVP)	chr3:123118892	intron		rs197835086	No Variation
Arginine Vasopressin Receptor 1a (AVPR1A)	chr7:67341790	intron		rs199089038	No Variation
Oxytocin Receptor (OXTR)	chr4:144398803	downstream		N/A	**23 A/A, 23 G/G**

,* SNPs are in linkage disequilibrium

There was a main effect of OXTR (chr4:144398803) genotype ($F_{(1,44)} = 9.14$, FDR adjusted $p = 0.032$) and a marginal effect of ESR2 (rs8170138) genotype ($F_{(1,44)} = 6.78$, FDR adjusted $p = 0.056$) and CYP19A1 (chr8:58755644) genotype ($F_{(2,43)} = 4.23$, FDR adjusted $p = 0.056$) on

average licking received. For OXTR, homozygous G/G female offspring received lower average licking per bout than homozygous A/A female rat offspring (Figure 5.4A). In addition, there was a main effect of ESR1 (chr1:41590586) genotype ($F_{(2,43)} = 9.54$, FDR adjusted $p = 0.002$) on later-life licking provisioning. Heterozygous C/T female rat offspring provided more licking than homozygous C/C (Tukey's post-hoc $p = 0.001$) and T/T (Tukey's post-hoc $p = 0.001$) female offspring (Figure 5.4B). There were no main effects of dopamine-related SNPs (DRD1, DRD2 or DAT) on early-life maternal licking received or provisioning.

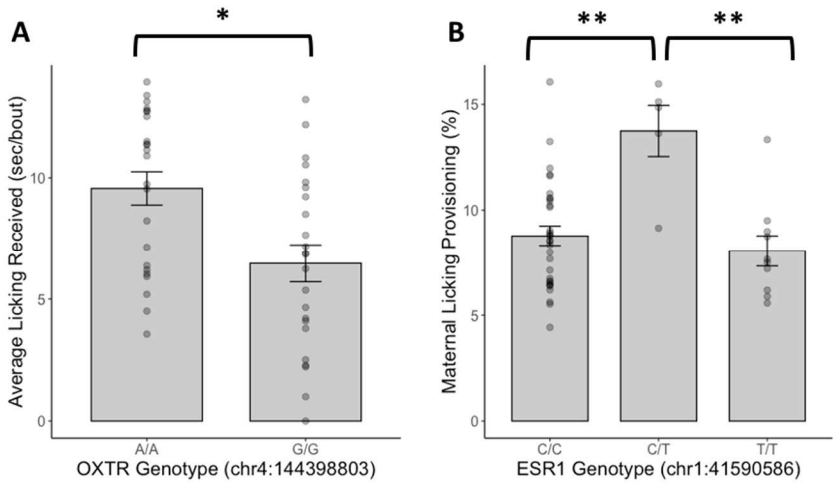

Figure 5.4 Offspring genotype within the oxytocin receptor and estrogen receptor alpha genes affects early-life licking received or later-life licking provisioning respectively. (A) Homozygous G/G female rat offspring for oxytocin receptor (OXTR; chr4:144398803) received lower average licking per bout than homozygous A/A female rat offspring and (B) heterozygous C/T female rat offspring for estrogen receptor alpha (ESR1; chr1:41590586) provided more licking than homozygous C/C and T/T female rat offspring. Barplots are displayed with mean +/- standard error with individual datapoints. * $p < 0.05$, ** $p = 0.001$ with Tukey's post-hoc tests.

5.3.4 Gene x Environment Interactions

We restricted our gene x environment analyses to dopamine-related SNPs (DRD1, DRD2, DAT) because we hypothesized that the indirect relationship between early-life licking received and later-life licking provisioning would be mediated by dopaminergic activity in the maternal brain. We also restricted our outcome measures to later-life licking provisioning and DA levels in the nucleus accumbens because this was the only dependent variable that significantly correlated

with later-life maternal licking provisioning. We analyzed both total licking received and average licking received as independent variables.

DRD2 (rs107017253) genotype interacted with average licking received on DA levels in the nucleus accumbens (Coefficient $\beta = -0.2643 \pm 0.093$, $t_{(1,42)} = -2.8366$, FDR adjusted $p = 0.0426$; Figure 5.5). Specifically, there was a significant effect for the A/G genotype (Effect = 0.230 ± 0.071, $t = 3.2363$, $p = 0.0024$) but not the A/A genotype (Effect = -0.0344 ± 0.060, $t = -0.5700$, $p = 0.5718$). No other gene x environment interactions were observed with total licking received or other dopamine-related SNPs. In addition, there were no significant gene x environment interactions on later-life licking provisioning.

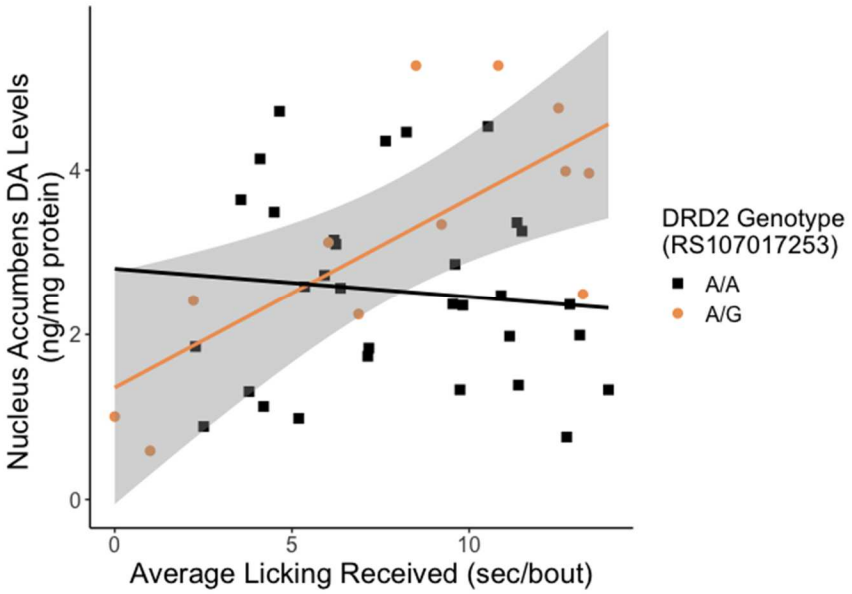

Figure 5.5 The association between average licking received and nucleus accumbens dopamine (DA) levels in the maternal brain is moderated by dopamine receptor 2 (DRD2) genotype. Heterozygous A/G female rat offspring had higher levels of DA in the nucleus accumbens with higher early-life average licking received. Homozygous A/A offspring do not show an association with early-life average licking received. Scatterplots are displayed with regression lines for A/A (black) and A/G (orange) with the 95% confidence interval for the A/G genotype in grey.

5.3.5 Moderated Mediation of Early-Life Licking Received on Later-Life Licking Provisioning

Based on the reported results, we conducted a moderated mediation analysis in PROCESS (Model 7) using average licking received as the independent variable, percent licking provisioning as the outcome dependent variable, DA levels in the nucleus accumbens as a mediator, and DRD2 (rs107017253) genotype as a moderator. Figure 5.6 displays the moderated mediation statistical model with all output coefficient β values. These β values also reflected previous analyses with a statistically significant DRD2 (rs107017253) genotype by average licking received interaction on DA levels in the nucleus accumbens as well as a statistically significant positive correlation between dopamine levels in the nucleus accumbens and maternal care provisioning.

We found an indirect moderation of DRD2 genotype between average licking received and later-life licking provisioning by DA levels in the nucleus accumbens. Specifically, there was a significant indirect effect for the A/G genotype (Effect = 0.2002, Bootstrap 95% CI [0.0185, 0.4063]) but not the A/A genotype (Effect = -0.0299 Bootstrap 95% CI [-0.1533, 0.0910]). This suggests that higher early-life average licking received was associated with higher DA levels in the nucleus accumbens of the maternal brain but only in female rat offspring with the A/G DRD2 genotype. Higher DA levels in the nucleus accumbens of the maternal brain of the female rat offspring with the A/G DRD2 genotype was then associated with higher later-life licking provisioning.

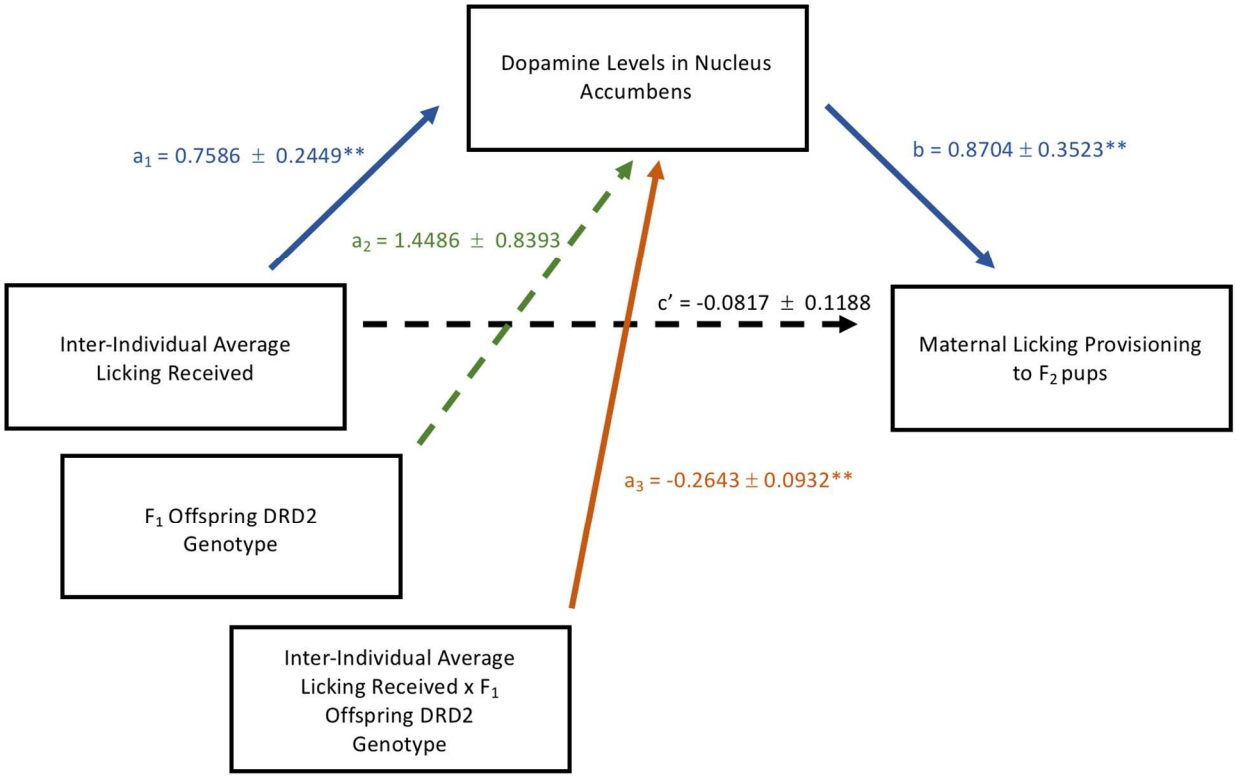

Figure 5.6 The moderated mediation statistical model (Model 7) analyzed with PROCESS with all output coefficient β values. There was a significant moderation of DRD2 (rs107017253) and average licking received (solid orange) on dopamine levels in the nucleus accumbens. Dopamine levels in the nucleus accumbens, in turn, significantly mediated (solid blue) the relationship between average licking received and maternal licking provisioning to the F_2 pups. ** $p < 0.05$

5.4 Discussion

In this study, we investigated the role of genotype in the transmission of inter-individual maternal care across generations of female rat offspring. To our knowledge, this is the first study that has examined the relationship between inter-individual maternal care received and maternal care provisioning to the next generation of offspring and the genetic factors that could influence this relationship. We found that the relationship between early-life licking received and later-life licking provisioning was indirectly affected by dopamine levels in the nucleus accumbens and dependent on DRD2 genotype. More specifically, female rat offspring with the A/G genotype showed a positive relationship between average licking received and dopamine levels in the nucleus accumbens of the maternal brain; there was no relationship with female rat offspring

with the A/A genotype. The higher DA levels in the nucleus accumbens corresponded with higher maternal licking provisioning from postnatal day 2-9. The updated moderated-mediation model is displayed in Figure 5.7. In addition, estrogen- and oxytocin-related SNPs affected both early-life licking received and later-life licking provisioning, suggesting that genotype can interact with the early-life maternal environment and influence later-life maternal care provisioning. Given that the early-life licking received we observed represented a continuous range of maternal care, these results suggest that genotype has an important role in the transmission of maternal care across generations in the average rat mother.

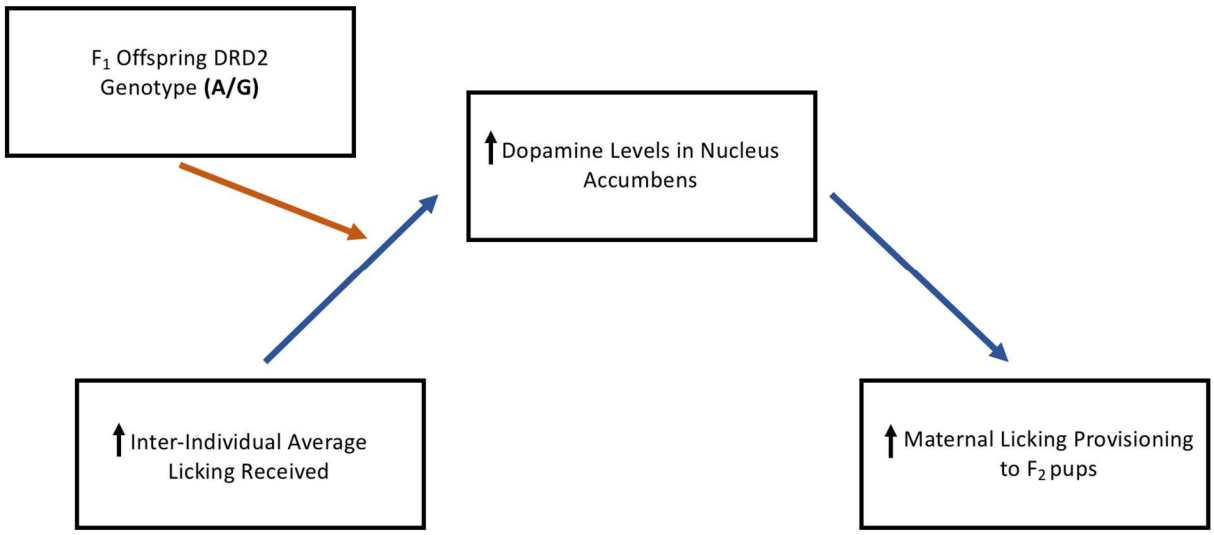

Figure 5.7 Updated moderated mediation model between early-life licking received and later-life licking provisioning. Offspring DRD2 genotype moderated the indirect relationship between early-life average licking received and later-life maternal licking provisioning by dopamine levels in the nucleus accumbens.

5.4.1 Relationship between Early-Life Licking Received and Later-Life Licking Provisioning

We first investigated the direct relationship between early-life licking received and later-life licking provisioning and did not find any correlation between the two measures. Previous work in rats has shown that inducing early-life adversity and, in studies observing natural variations in maternal care, the outliers of maternal care received (+/- one standard deviation) can transmit

across generations of offspring (Champagne et al., 2003; Francis et al., 1999; Gonzalez et al., 2001), demonstrating that early-life environmental influences are sufficient to alter maternal care to the next generation. However, there is substantial within-litter variation in the intergenerational transmission of maternal care, especially with female offspring born to mothers that provide average levels of LG (Champagne et al., 2003). Since we did not select for the tail ends of the normal distribution in inter-individual licking received across all litters for the maternal care provisioning observations, our findings may reflect the substantial variation in intergenerational maternal care transmission within the normal distribution of maternal care received.

5.4.2 Relationship between Later-Life Licking Provisioning and Dopaminergic Activity in the Maternal Brain

We found that the DOPAC/DA ratio (a measure of dopaminergic activity) in the nucleus accumbens was negatively associated with later-life licking provisioning, mainly due to the positive correlation with DA levels than a negative correlation with DOPAC levels. Previous studies have shown links between maternal care provisioning and dopaminergic activity in the nucleus accumbens, especially the nucleus accumbens shell (Afonso, Grella, Chatterjee, & Fleming, 2008; Afonso, King, Novakov, Burton, & Fleming, 2011; Champagne et al., 2004; Lee, Li, Watchus, & Fleming, 1999). The relationship between early-life licking received and later-life licking provisioning was also mediated by levels of dopamine in the nucleus accumbens in the maternal brain, suggesting that the nucleus accumbens may be an important site of intergenerational transmission of maternal care. Artificially reared female rat pups show later-life elevated basal dopamine levels and lower pup-evoked dopamine levels in the nucleus accumbens and a reduction in maternal care (Afonso et al., 2011; Gonzalez et al., 2001) than normal maternally reared offspring. Additional studies are needed to explore the potential role of the nucleus accumbens and its subregions in the transmission of the effects of maternal care received at the early-life pup stage.

5.4.3 Effects of Estrogen- and Oxytocin-Related SNPs on Early-Life Licking Received and Later-Life Licking Provisioning

Of sixteen variants of interest that we verified using massarray, five were not polymorphic. The two most likely reasons for this are that either a) the variant didn't exist in our population of

Long-Evans, either due to genetic drift, different suppliers, or simple change; or b) they were false positive calls, due to the greater difficulty in calling variants from RNA-seq data than from DNA-seq data. However, the majority of our call variants were confirmed (69%), and five are novel variants (83% of six novel variants probed). These findings show that this method is useful in understudied populations, which is important because a large amount of rat genetic variation is still undocumented.

We found that single nucleotide polymorphisms (SNPs) in candidate genes related to maternal care (oxytocin receptor and estrogen receptor alpha) affected early-life licking received or later-life licking provisioning. However, the effect of these individual SNPs on early-life licking received and later-life licking provisioning was minimal after accounting for multiple comparisons. In addition, we did not genotype the F_0 parent generation and therefore we were unable to account for the potential effects of the F_0 mothers' genotype on early-life licking received. Previous work in mice has demonstrated that the genotype of the offspring can affect the amount of care they receive, termed offspring genetic effects (Ashbrook et al., 2017; Ashbrook et al., 2015), which implies that the genetic profile of an individual can also affect its early-life experience and the individual is not simply a passive recipient of environmental exposures. Additional work is needed to determine biological consequences of these SNPs and it is likely that there are additive effects of different genotypes on maternal care received and provisioning that we were unable to elucidate in this study. The role of genotype in the male lineage may also be an important consideration when studying intergenerational transmission of maternal care, as the fathers provide half their genetic profile to the offspring and play an essential role in genomic imprinting (Wilkinson, Davies, & Isles, 2007).

5.4.4 Gene x Environment Interactions with DRD2 Genotype

We found a gene x environment interaction with average licking received and DRD2 genotype (rs107017253) on dopamine levels in the nucleus accumbens, which mediated the indirect association between early-life licking received and later-life licking provisioning. While a mediation analysis typically requires a significant direct association of the independent variable (early-life licking received) on the dependent outcome variable (maternal licking provisioning), we also hypothesized the sequence of causal variables *a priori* based on previous literature on the intergenerational transmission of maternal care. We previously reported similar interactions

between average licking received and the same DRD2 SNP in other dopamine-related behaviours (Chapter 4), but involving the A/A genotype, not A/G. It is possible that the nature of this interaction changes when the brain undergoes significant alterations in the dopaminergic system, such as when a female rat is pregnant and preparing for postnatal care of her offspring (Akbari et al., 2013; Byrnes, Byrnes, & Bridges, 2001). Identifying other biological mechanisms that underlie these gene x environment interactions, such as linkage disequilibrium and gene expression changes, could help elucidate this discrepancy especially since the female offspring with the A/G genotype are the minority population and therefore may be statistically underpowered in observational studies. Studies that investigate epigenetic mechanisms (e.g., DNA methylation) that would affect the expression of these genes would also be an important component in elucidating mechanisms underlying gene x environment interactions, as both maternal care received and the neural changes that prime mothers to provide care for offspring involve alterations in epigenetic mechanisms and gene expression (Beery et al., 2016; Cameron et al., 2008; Champagne, 2008; Stolzenberg, Stevens, & Rissman, 2014).

Studies of gene x environment interactions on maternal behaviour to date have focused on human cohorts. These studies have identified SNPs in oxytocin- and dopamine-related genes that interact with early-life experiences on different components of mothering behaviour (Fleming & Kraemer, 2019; Jonas et al., 2013; Mileva-Seitz et al., 2012; Mileva-Seitz et al., 2013; Cost et al., 2017) and that can be mediated by executive function (Cost et al., 2017) or depressive symptoms (Jonas et al., 2013). A strength of our study is application of statistical models typically used in human cohorts to rat populations, where causal biological mechanisms can be more readily examined (Jenkins et al., 2016). More specifically, we can look directly into the rat maternal brain for specific neurotransmitters, neuropeptides, and expression of relevant genes that would not be possible with human populations. This cross-species approach is important to refine hypotheses on factors important for maternal behaviour that are evolutionarily conserved.

Overall, this study suggests that offspring genotype for the dopamine receptor 2 gene may be an influential factor when assessing the intergenerational transmission of maternal care. More broadly, offspring genotype in dopamine-related genes may be a critical factor involved with the development of the dopaminergic system. While it has been shown in previous studies that early-life maternal licking received substantially alters the functioning of the dopaminergic system, the underlying biological mechanisms are not as well-understood as other later-life behavioural

phenotypes such as stress reactivity. Elucidating the functional consequences of the DRD2 SNP analyzed in this study will be important to understand the underlying biological mechanisms in the developmental programming of maternal care and its transmission across generations of female offspring.

Chapter 6
General Discussion

6 General Discussion

6.1 Overview of Results

This thesis describes findings on the intergenerational transmission of maternal care from two complementary projects that used either neonatal handling (Chapters 2 and 3) or an observational approach (Chapters 4 and 5). I found that early-life repeated room temperature exposure reduced thyroid hormone signaling measures as well as thyroid hormone responsive genes DNA methyltransferase 3a and oxytocin in week-old female rat pups (Chapter 2), along with increased later-life maternal licking provisioning to the next generation of rat pups (Chapter 3). In addition, I found that the effects of inter-individual maternal licking received on later-life performance in dopamine-related tasks in virgin female rats (Chapter 4) as well as dopamine levels in the nucleus accumbens (NAcc) of the maternal brain and, indirectly, maternal licking provisioning (Chapter 5) was dependent on the dopamine receptor 2 genotype (DRD2; rs107017253) of the female rat offspring. Overall, this thesis reports novel biological mechanisms underlying the developmental programming of maternal care that may be involved in the intergenerational transmission of maternal care in the rat model.

6.2 Implications of Early-Life Temperature Exposure

I found that pups with early-life repeated room temperature exposure had decreased thyroid hormone signaling compared to pups with early-life nest temperature exposure, and pups with acute room temperature exposure had increased thyroid hormone signaling (Chapter 2). In addition, early-life room temperature exposure appears to have long-term effects on later-life maternal care provisioning, especially maternal licking (Chapter 3). These findings show that early-life temperature exposure is a relevant variable involved in the developmental programming of maternal care.

Along with neonatal handling, early-life temperature exposure is a potential variable in multiple paradigms examining early-life environments on later-life phenotype. Previous studies in our lab that have observed within-litter variation in maternal care received have separated the pups at room temperature (Pan et al., 2014) or on a heating pad (Pan et al., 2018). The female rat offspring in the observational project were separated at room temperature (Chapters 4 and 5), which could possibly contribute to the intergenerational transmission of maternal care I

observed. However, since all the female offspring would have had early-life room temperature exposure, it is unlikely that this variable confounded the gene x environment interactions found in this project. Another example is the limited nesting paradigm that models low-resource settings (Walker et al., 2017) and also chronically exposes the rat pups to the ambient room temperature. One recent study has shown that litters with limited nesting material have reduced huddling cohesion and the pups have reduced thermogenic measures at PND 6 (Lapp, Mueller, & Moore, 2020), similar to the reduced thyroid hormone receptor signaling that I observed in Chapter 2. For future work in the field, early-life temperature exposure needs to be considered when examining the effects of the early-life maternal environment on later-life offspring phenotype.

Variations in temperature exposure are also a relevant abiotic factor for species in their natural habitats and the effects may be evolutionarily conserved. For avian species, lower ambient temperatures are associated with an increase in the amount of thyroxine the females deposit in the yolk of their eggs (Ruuskanen et al., 2016; Ruuskanen & Hsu, 2018). For several species of fish, turtles, and other reptiles, differences in early-life incubation temperature is important for sex determination, and this might be mediated by changes in DNA methylation levels at specific loci such as aromatase (Jonsson & Jonsson, 2019; Matsumoto, Buemio, Chu, Vafaee, & Crews, 2013; Matsumoto, Hannigan, & Crews, 2016). One recent study in zebra finches found that post-hatchling variations in ambient temperature exposure can alter DNA methylation levels globally and at specific loci in the blood (Sheldon, Schrey, Hurley, & Griffith, 2020). There is even some evidence that cold temperature exposure affects the epigenome in human infants and elderly adults, although the number and scope of these studies are currently very limited (Xu et al., 2020).

It is, however, unclear whether the handling manipulation would be applicable to rats in their natural environments. Rats in the wild typically rear their young in burrows (Calhoun, 1963) and the caging system used for laboratory rats does not mimic those conditions. It is possible that variations in temperature exposure could derive from environmental ambient temperatures or if a subordinate mother rat is away from her pups for long periods of time to gather resources (Calhoun, 1963). However, no studies to my knowledge have directly tested if either factor causes subsequent changes in the body temperature of the rat pups.

I did not find a synergistic effect of early-life temperature exposure and supplemental tactile stimulation either on week-old female pup phenotype or maternal care provisioning. Both early-life repeated room temperature exposure and supplemental tactile stimulation (marginally) decreased Oxt transcript abundance in the paraventricular nucleus of the week-old pups, but supplemental tactile stimulation did not affect DNA methylation levels at the composite hormone response element in the OXT gene (Chapter 2). It is also unlikely that the effects of supplemental tactile stimulation on Oxt transcript abundance were due to changes in thyroid hormone signaling. In addition, both early-life room temperature exposure and supplemental tactile stimulation increased later-life maternal licking provisioning, but the potential effects of supplemental tactile stimulation on the dopaminergic system may have also contributed to this finding (Chapter 3). Overall, it is likely that the effects of early-life temperature exposure and supplemental tactile stimulation affect different biological pathways that could converge on later-life maternal licking provisioning, but this needs to be verified in future work.

Unexpectedly, I found that the mothers with early-life nest temperature exposure had lower duration of maternal licking and increased pup carrying behaviour, but this could represent a mismatch of early-life temperature exposure and the later-life testing procedure with separating the F_2 pups at room temperature (Chapter 3). There is some previous work in rats that found if a female pup is scented with artificial lemon odour, that pup will become a mother that licks lemon-scented pups more than unscented pups later in life (Fleming et al., 2002), so it is possible that mothers with early-life nest temperature exposure are more likely to lick pups that are warm than pups that have been cooled. If I had placed the F_2 pups on a heating pad during the maternal care provisioning testing, I might have observed that the mothers with early-life nest temperature exposure provide more licking to the F_2 pups than mothers with early-life room temperature exposure. Another method to test the match-mismatch hypothesis is to place the maternal cage on a thermal gradient and observe where the mothers with different early-life temperature exposures prefer to keep their nests of pups. It is possible that the mothers with early-life nest temperature exposure would establish their nests in a warmer portion of the thermal gradient than mothers with early-life room temperature exposure. Investigating these findings in light of the match-mismatch hypothesis is important in order to accurately assess the effects of early-life temperature exposure on later-life maternal care provisioning and their adaptive features.

I also consistently found minimal effects of early-life temperature exposure and even neonatal handling on maternal care received throughout the day, in contrast to previous literature that reported increased maternal care following the reunion of pups after neonatal handling (Smotherman & Bell, 1980; Villescas et al., 1977) and long-term changes in the structure of maternal care in response to neonatal handling (Reis et al., 2014). However, a couple of studies have reported that prolonged maternal separation increases maternal care received upon reunion of the pups more than the brief neonatal handling procedure, possibly as a way to compensate for the longer separation time (Macrí, Mason, & Würbel, 2004), but prolonged maternal separation even with higher maternal care received does not attenuate stress reactivity later in life (Macrì, Chiarotti, & Würbel, 2008). The authors further argued that because litters with maternal separation received more maternal care upon reunion and yet showed higher stress reactivity than litters with neonatal handling, changes in maternal care received cannot be the sole mediator of the effects of neonatal handling and maternal separation (Macrì & Würbel, 2006). The early-life separations likely also have a direct, long-term effect on the pups.

6.3 Implications of Offspring Genotype and Gene x Environment Interactions

I found that the relationship between early-life average licking per bout received and strategy shifting, sucrose preference (Chapter 4), and maternal licking provisioning (Chapter 5) were dependent on DRD2 (rs107017253) genotype. Only one of these phenotypes (sucrose preference) showed a direct correlation between inter-individual licking received and later-life behavioural phenotype. These findings suggest that genetic variation between individuals can substantially influence responses to subtle variations in early-life maternal licking received. The effects of genetic variation between individuals could also explain why the studies on the effects of within-litter maternal care received does not always match the effects of between-litter maternal care received on later-life behaviour (sometimes with *opposing* trends; Ragan et al., 2012; Ragan et al., 2016; van Hasselt et al., 2012).

Another well-studied example of a genetic variant that confers differential sensitivity to the environment is a repeat polymorphic region within the promotor of the serotonin transporter (5HTT) gene. In human cohorts, individuals with the short form of the promotor region of 5HTT are more likely to report more depressive symptoms with multiple lifetime adversities than

individuals with the long form, who are less responsive to life adversities (Caspi et al., 2003; but also see Munafò, Durrant, Lewis, & Flint, 2009). Similar findings have been reported in non-human primates where early-life adversity interacts with the short form of the 5HTT promotor region with increased hypothalamic-pituitary-adrenal axis activity in response to stressors (Kraemer, Moore, Newman, Barr, & Schneider, 2008) as well as decreased serotoninergic activity in the brain (Bennett et al., 2002). One study in rats also found interactions with a serotonin transporter SNP with prenatal and postnatal stress on hypothalamic-pituitary-adrenal axis physiology and increased stress reactivity respectively (Belay et al., 2011). Though multiple genes may be involved in differential sensitivity to the environment, sensory processing sensitivity is considered a categorial phenotype (Aron, Aron, & Jagiellowicz, 2012; Greven et al., 2019). That is, individuals tend to acquire a set of traits together that make them non-sensitive, moderately sensitive, or highly sensitive to their environments. How these genetic variants contribute to sensory processing sensitivity is still poorly understood but a growing area of research.

With the neonatal handling project (Chapters 2 and 3), I provided 15 minutes of supplemental tactile stimulation to half the litters per day in the first postnatal week. This duration of supplemental tactile stimulation could represent an extreme in maternal care received and therefore was sufficient to alter later-life maternal care provisioning. In addition, my sample size (n = 5-7 per condition) would have been too small to detect substantial variation in DRD2 genotype in this study as only around 20% of the female rat offspring I did genotype were heterozygous or had the homozygous G/G allele. However, offspring genotype may have been an important consideration if I provided less supplemental tactile stimulation or studied the effects of different durations of supplemental tactile stimulation. In addition, it could explain a portion of individual differences in maternal licking provisioning I observed within each early-life condition (Chapter 3).

I also found that dopamine levels in the NAcc in the maternal brain mediated the indirect relationship between early-life maternal licking received and later-life maternal licking provisioning. However, the mechanism underlying the variation in dopamine levels is unknown and I did not measure DRD2 density or transcript abundance in these studies. In addition, I did not find any association of baseline dopamine levels or dopaminergic activity in the virgin female brain with the dopamine-related tasks. However, it is possible that if I measured

dopamine levels during the dopamine-related tasks using *in vivo* microdialysis or dopaminergic activity with voltammetry I could find a stronger association with the behaviours tested. In addition, these methods could also dissociate between the role of the nucleus accumbens shell from the nucleus accumbens core.

6.4 Limitations

One pervasive limitation shared by both projects is that I used different methods to assess maternal care received and maternal care provisioning. In the neonatal handling project, I assessed maternal care received by video recordings with instantaneous coding and assessed maternal care provisioning by live observation with continuous coding. The inverse occurred with the observational project. In previous work using natural variations in maternal licking between litters, high licking/grooming rat mothers display significantly higher levels of licking provisioning in both video recordings and live observations than low licking/grooming rat mothers (Champagne et al., 2003; Hellstrom et al., 2012; Pena et al., 2013). However, it is unknown if there is a direct positive correlation between maternal care provisioning coded in video recordings and live observations. Ideally, I would have assessed intergenerational maternal care provisioning using both video recordings and live observation in both projects.

The second limitation shared by both projects is that I only used female rat offspring and culled the male pups at postnatal day 1. While it made logical sense to focus on female offspring to study the intergenerational transmission of maternal care provisioning, the underlying biological mechanisms of developmental programming of maternal care discussed in this thesis may not be generalizable to both sexes. Moreover, normal mixed-sex litters would have more pups than what is feasible in female-only litters, which could have also affected thermoregulation of the pups in Chapters 2 and 3. Male pups could be less affected by early-life room temperature exposure as the developmental increase in estrogen receptor signaling during brain sexual differentiation could compete with thyroid hormone receptors on the OXT composite hormone response element (Adan et al., 1993; Dellovade et al., 1999). This competition between estrogen receptors and thyroid hormone receptors has also been shown to occur at regulatory transcription factor binding sites in other genes (Vasudevan et al., 2002). Therefore, studying sex differences would be important for future work on the effects of early-life variations in temperature exposure. On the other hand, a previous study that found DRD2 genotype (rs13448058) and

early-life adversity interactions on DRD2 density in the NAcc shell used only male rats (Lovic et al., 2013), which suggests that the gene x environment interactions observed in this thesis could be applicable to males.

6.5 Novel Biological Mechanisms Underlying the Developmental Programming of Maternal Care

I found that early-life repeated room temperature, a proxy of reduced maternal contact, could decrease transcript abundance of oxytocin (Oxt) and DNA methyltransferase 3a (Dnmt3a) and increase DNA methylation at the oxytocin composite hormone response element (CHRE) possibly as a consequence of reduced thyroid hormone receptor signaling (Chapter 2). Previous work has shown that the repressive actions of unliganded thyroid hormone receptor mediates the effects of hypothyroidism on neurodevelopment (Bernal, 2007; Hashimoto et al., 2001). While I only looked at two candidate genes (OXT and DNMT3A), there are numerous genes that are regulated by thyroid hormone receptor binding to its thyroid hormone response element (TRE; Grøntved et al., 2015).

Although the oxytocin CHRE has a CpG site flanking the binding motifs, the TRE binding motif in itself does not include a CpG di-nucleotide unlike some previously studied transcription factors like NGFI-A (McGowan et al., 2009; Weaver et al., 2004). It would be interesting to examine other genes with CpG sites in proximity to a TRE as higher DNA methylation levels in these sites may represent a "flag" to a larger repressive mechanism (including histone modifications) than causing the repression of transcription per se. Another possible but less likely explanation for these findings is that unliganded thyroid hormone receptor binding at the oxytocin CHRE prevented DNA demethylation flanking the CHRE. This alternative hypothesis cannot be ruled out until further *in vivo* longitudinal studies and *in vitro* functional studies are conducted. I would need to examine the typical ontology of DNA methylation levels at the oxytocin CHRE in the PVN in late prenatal and early postnatal life as this is the developmental period when oxytocin can be detected in the rat hypothalamus (Laurent, Hindelang, Klein, Stoeckel, & Felix, 1989). In addition, I would need to investigate if unliganded thyroid hormone can recruit DNA methyltransferase binding at specific loci with a proximity ligation assay (also see Nadeem et al., 2016) for thyroid hormone receptors and co-localization with the SMRT/NCOR1/HDAC3 corepressor complex and DNA methyltransferases.

Although early-life repeated room temperature exposure also decreased DNA methyltransferase 3a transcript abundance, it is unclear what the consequences would be for later-life phenotypes. Reduced Dnmt3a transcript may not necessarily correspond to reduced DNA methylation levels at all genes (OXT as one example). In addition, DNA methyltransferases are more likely to be recruited to specific loci than catalyzing DNA methylation at random sites (McGowan, 2015). There is, however, a link between high maternal care received, increased Dnmt3a transcript abundance, and higher DNA methylation levels at retrotransposons in mice (Bedrosian et al., 2018) which I did not investigate in this thesis but could be important to study in future work.

While I found that a SNP in the DRD2 gene (rs107017253) may confer sensitivity of early-life licking received on later-life dopamine-related phenotypes (Chapters 4 and 5) and dopamine levels in the NAcc in the maternal brain (Chapter 5), the functional significance of this SNP is still unknown. This implies that the gene x environment interactions with this DRD2 can only be considered associative. The study by Lovic and colleagues found DRD2 genotype (rs13448058) and early-life adversity interactions on DRD2 density in the NAcc shell in Sprague-Dawley rats (Lovic et al., 2013), but I found low variation with this SNP in our Long-Evans rat population (Chapter 4). However, it is plausible that both SNPs are in linkage disequilibrium to the causal variant and there are differences in haplotype blocks between different rat strains. This would also imply that neither SNP is the causal variant and I was unable to locate the causal variant with the limited existing information I had on genetic variation in the outbred Long-Evans population.

There are a couple of possible mechanisms where a genetic variant (SNP or Insertion/Deletion) could produce the gene x environment interactions observed in Chapters 4 and 5. One possible mechanism is that the genetic variant occurs within a regulatory site, such as an enhancer or repressor, or affects the binding motif of a transcription factor. Studies verifying the functional role of genetic variants can screen for candidates by *in silico* predictions and subsequent genetic manipulation experiments. One study in *Drosophila* demonstrated that a genetic variant on a predicted transcription factor binding site on one of the promotor regions of the foraging gene can induce differential histone methylation. Higher histone methylation corresponded to the rover foraging phenotype at adulthood (Anreiter, Kramer, & Sokolowski, 2017). Another possible mechanism is that a genetic variant can either create or eliminate a CpG di-nucleotide which would affect DNA methylation levels on that site. There is evidence from human cohorts

that a large portion of variation in DNA methylation patterns between individuals is due to the genetic variation between individuals (McRae et al., 2014) and not just environment alone.

The profound effects of maternal care received on later-life behaviour are likely due to the numerous variables in the maternal environment that alter different neuroendocrine and neurotransmitter systems in the brain. Based on the findings from this thesis, early-life temperature exposure could be primarily affecting the hypothalamic-pituitary-thyroid axis with downstream changes in the oxytocinergic system, while pup licking and licking-like tactile stimulation could be primarily affecting the dopaminergic system. While alterations on both the oxytocinergic and dopaminergic systems can converge on maternal care provisioning, oxytocinergic and dopaminergic signaling can affect different maternal behaviours at different periods throughout the gestational and lactation period. More specifically, oxytocin influences a large set of maternal behaviours such as nursing, pup retrieval, and pup licking, but seems to be only essential during the early postnatal period. Conversely, dopamine influences a subset of maternal behaviours, namely pup retrieval and pup licking, but is essential for the long-term maintenance of these behaviours. More notably, the oxytocinergic and dopaminergic systems influence different later-life behaviours in nonmaternal adults, where oxytocin is associated with social affiliation and stress reactivity and dopamine is associated with hedonic and learning processes. It will be essential to continue exploring other early-life factors in the maternal environment, their interactions with offspring genotype and epigenome, and their effects on other later-life behaviours for future work in this field.

6.6 Final Remarks

While this thesis focused on the biological mechanisms underlying developmental programming of maternal care, it is worth noting that investigating biological mechanisms alone cannot explain why an animal behaves in a certain manner. I would not have initially predicted that the mothers with early-life repeated room temperature exposure would have increased maternal licking provisioning based on the reduced Oxt transcript abundance with the week-old pups. However, given that the F_2 pups were also separated at room temperature, the match-mismatch hypothesis is worth exploring in future work. It would also be impossible to predict how an animal would behave by only identifying its genotype if the genotype confers differential sensitivity to the environment. How an animal behaves is still dependent on the environmental context they live

in. This consideration is essential in order to completely understand the adaptive significance of these biological mechanisms.

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