

Behavioural and neurobiological effects of early postnatal stress in mouse dams and their pups

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A thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in Nutrition and Food Biosciences

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Abstract

Background: Early life and postpartum represent periods of increased risk for the development of mental health disorders. Previous studies suggest that stress during these vulnerable periods can contribute to behavioural and neurobiological disruptions, yet the concurrent effects of stressors on the mother and offspring remain poorly understood. This study examined stress-induced disruptions in behaviour and physiological measures using postnatal maternal separation (MS) in both the dam and offspring. **Methods:** Eighteen female C57BL/6N mice were randomly assigned to a control or an MS condition, which consisted of daily 3-hour separations from postnatal day 2 (P2) to P14. Maternal care behaviour was assessed on P3 and P7. Depressive- and anxiety-like behaviour was examined on P22-23 in dams and on P42-43 in the offspring, with food reward-seeking also being evaluated in the latter. Tissue collection was conducted 24 hours after the last behavioural test, during which the medial prefrontal cortex and colon were collected from dams to assess pro-inflammatory cytokine expression, and the nucleus accumbens (NAc) from pups to examine mesolimbic-related markers. **Results:** MS disrupted components of maternal responsivity but had no effect on depressive- or anxiety-like behaviour or on cytokine expression in dams. In pups, MS promoted food reward seeking in both sexes but increased dopamine receptor 2 and neurotrophic receptor tyrosine kinase 2 expression in the NAc of males only, while not changing anxiety- or depressive-like behaviour. **Conclusion:** MS induced disruptions in select behavioural and physiological measures in both pups and dams, providing insight into the effects of stress during sensitive life periods.

Keywords: Anxiety, Depression, Early Life Stress, Inflammation, Maternal Separation, Mesolimbic System, Postpartum, Mouse Model, Reward

Acknowledgements

The completion of this thesis would not have been possible without the support and contributions of several individuals during my master's degree. I would first like to sincerely thank my supervisor, Dr. Marie-Claude Audet, for her support, expertise, and encouragement throughout every stage of this project. I am deeply grateful for the time and guidance she invested in shaping this thesis, and I feel very fortunate to have had the opportunity to learn under her mentorship throughout my MSc. I would also like to extend my gratitude to my thesis advisory committee members, Dr. Krista Power and Dr. Paul Albert, for their expertise, feedback and valuable contributions to this project. A special thank you to my project partner, Tamara Trowsse, for her commitment to this project from its early stages through to its completion, and for her support throughout the rewarding and challenging moments we shared. I also want to acknowledge the contributions of current and past members of the Audet Lab, Abigail Szczepanski, Ana Santos, Amy Coughlan, Elyse Hodel, Dan Sulatycky, Sobana Sriranjani, Maryam Sraidi and Dr. Caroline Wallace. Their support and collaboration had a meaningful impact on both this research and my master's experience. I would like to thank the Behavioural and Physiology Core and the Animal Care and Veterinary Service at the University of Ottawa for their assistance with animal handling and training, and for their expertise throughout this project. The completion of my master's research was supported by the Canadian Institutes of Health Research through the Canadian Graduate Scholarship - Master's as well as the Nutrition and Mental Health Scholarship (Master's) at the University of Ottawa.

Lastly, I wish to express my profound gratitude to my parents and my sisters, who have supported me throughout my educational endeavours and without their continued belief in me, this milestone would not have been possible.

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List of Abbreviations

ACTH	Adrenocorticotrophic hormone
BDNF	Brain-derived neurotrophic factor
BW	Body weight
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
Drd1	Dopamine receptor D1 (gene)
D1R	Dopamine receptor D1 (protein)
Drd2	Dopamine receptor D2 (gene)
D2R	Dopamine receptor D2 (protein)
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
E	Embryonic day
ELS	Early life stress
EPM	Elevated plus maze
GI	Gastrointestinal
HPA	Hypothalamic-pituitary-adrenal axis
IFN γ	Interferon gamma
IL	Interleukin
L-DOPA	Levodopa
LB	Limited bedding
LPS	Lipopolysaccharide
mPFC	Medial prefrontal cortex
MDD	Major depressive disorder
mRNA	Messenger ribonucleic acid
MS	Maternal separation
NAc	Nucleus accumbens
NPY	Neuropeptide Y
Ntrk2	Neurotrophic receptor tyrosine kinase 2
OCD	Obsessive compulsive disorder
OF	Open field
P	Postnatal day
PFC	Prefrontal cortex
PPA	Postpartum anxiety
PPD	Postpartum depression
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TNF- α	Tumour necrosis factor alpha
TrkB	Tropomyosin receptor kinase B

TST	Tail suspension test
VTA	Ventral tegmental area

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Figure 1: Experimental timeline

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Chapter 1: Background

The onset and development of depressive disorders have been suggested to result from a complex interplay of factors, including genetic predispositions, childhood adversity, and stressful life events, leading to impairments in the stress response, inflammatory processes, and the gut microbiota, among others (Remes et al., 2021; Medina-Rodriguez et al., 2023). Depressive disorders disproportionately affect women, with the risk of developing depression being twice as high in females compared to males (Salk et al., 2017). Women are at a particularly high risk of developing depression during the postpartum period, given that the hormonal, immune, and psychosocial changes occurring throughout pregnancy and after birth heighten vulnerability to mental health concerns (Brummelte & Galea, 2016; Zhao & Zhang, 2020). Postnatal stress, through its effects on the responsiveness of key stress response systems, is a prominent risk factor in the development of maternal mental health disturbances, with negative impacts on maternal care behaviours (Oyetunji & Chandra, 2020; Abrishamcar et al., 2024). Disruptions in caregiving, including diminished bonding, have been shown to contribute to the development of adverse physical and mental health outcomes in the offspring (Slomian, 2019), highlighting the need to better understand the effects of postnatal stress on both maternal mental health and long-term developmental trajectories.

In rodent models, maternal separation (MS) during the early postnatal period is an established paradigm for assessing the impact of postnatal stress in the dams and of early life adversity in the pups (Plotsky & Meaney, 1993). During this stressor, dams are typically separated from their pups for 3 hours daily from postnatal day 2 (P2) to P14, capturing the early developmental period in the offspring and the early postpartum period in the mother (Plotsky & Meaney, 1993; Weber et al., 2008). In rat dams, MS has been associated with increased anxiety-

and depressive-like behaviours, as well as deficits in maternal care, particularly in behaviours involving maternal responsiveness to pups (Bölükbas et al., 2020; Rombault et al., 2023; Zaccarelli-Magalhães et al., 2023). Similarly, MS-exposed mouse and rat offspring have been shown to exhibit increased anxiety- and depressive-like behaviour in adulthood, including impairments in motivation for rewarding stimuli, such as drugs and alcohol (Bian et al., 2015; Kesterling-Ferreira et al., 2021; Moffett et al., 2006). While the consequences of MS on affective and substance-seeking behaviours have been examined, the effect of the stressor on naturalistic reward seeking remains incompletely understood. Past studies have shown that MS increased intake of highly palatable food in rats (de Souza et al., 2018) and effort-based responding for a food reward in mice (Benoit et al., 2023). However, the effects of MS on palatable food-seeking in a risky environment have not yet been assessed.

Beyond the more studied hormonal alterations, there is increasing evidence that disturbances in inflammatory processes could be implicated in postpartum mental health disorders. In particular, postpartum depression (PPD) has been associated with increases in pro-inflammatory cytokines in the cerebrospinal fluid and the bloodstream (Sha et al., 2022; Boufidou et al., 2009; Achtyes et al., 2020), and women with inflammatory bowel disease, an illness characterized by increased levels of pro-inflammatory cytokines in the intestinal environment (Strober & Fuss, 2013), have been shown to be at higher risk of new-onset PPD (Vigod et al., 2019). However, despite postnatal stress being a known risk factor for PPD (Yim et al., 2015), little is known about how inflammation in the brain and the gastrointestinal (GI) tract could contribute to the pathogenesis of postpartum mental health disruptions. As such, examining pro-inflammatory markers in selected regions of the brain and GI tract following MS may provide novel insight into processes involved in the development of postpartum depressive

and anxiety-like behaviours. In the offspring, MS has been associated with sex-specific changes related to inflammatory, dopamine, and neurotrophic signalling in the nucleus accumbens (NAc) (Benoit et al., 2023; Wang et al., 2015; Ganguly et al., 2019), but relatively few studies have directly examined the expression of dopaminergic and neurotrophic receptors in the mesolimbic system in the context of risky reward seeking for palatable food.

This thesis examines the effects of MS in both the dam and the offspring. The first manuscript focuses on maternal care behaviour in the early postpartum period as well as on anxiety- and depressive-like behaviour and concurrent expression of pro-inflammatory cytokines in the brain and GI tract shortly after weaning. The second manuscript investigates the long-term effects of MS on food reward seeking in the context of risk, anxiety- and depressive-like behaviour, and dopamine and brain-derived neurotrophic factor (BDNF) receptor expression in the NAc in young adult offspring.

Chapter 2: Literature Review

2.1 Maternal Mental Health

2.1.1 Depression and anxiety during the postpartum period

Postpartum depression refers to major depressive episodes arising up to one year after giving birth (Stewart & Vigod, 2019). According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), major depressive disorder (MDD) is characterized by the presence of depressed mood or loss of pleasure/diminished interest in everyday activities, in addition to four other symptoms among weight loss or gain, sleeping too much or too little, fatigue, feelings of worthlessness or guilt, diminished ability to concentrate, psychomotor agitation or retardation, and recurrent thoughts of death (American Psychiatric Association, 2013). When occurring during the postpartum period, these symptoms may manifest as difficulty bonding with the baby and, in severe cases, thoughts related to self-harm or harm to the infant (Stewart & Vigod, 2019). Similarly, although postpartum anxiety (PPA) is not recognized as a specific disorder yet, anxiety in the postpartum period is described as the onset of an anxiety disorder within one year of birth (O'Hara & Wisner, 2013). There are several types of anxiety and anxiety-related disorders that may develop in the postpartum period, including generalized anxiety disorder, panic disorder, obsessive-compulsive disorder (OCD), and post-traumatic stress disorder (O'Hara & Wisner, 2014). Based on the DSM-5, anxiety and related disorders share several overlapping diagnostic criteria, including excessive fear, distress, and avoidant behaviours related to a perceived threat (Fairbrother et al., 2025). In a postpartum context, this may present as heightened fear about pregnancy, birth, or infant safety and include behaviours such as excessive monitoring, fear of harming the baby, and avoidance of situations related to caregiving (Somerville et al., 2015; Fairbrother et al., 2025). Both PPD and PPA are highly

prevalent in the Canadian population, with 23% of women reporting symptoms consistent with these mood disorders in the year after birth (Government of Canada, 2019). Importantly, PPD and PPA are commonly comorbid, with 8% of postpartum Canadian women having both (Government of Canada, 2019).

2.1.2 Impact of postpartum depression and anxiety on maternal outcomes

Maternal mental health concerns may have adverse effects on women's overall well-being in the short and long term. During the postpartum period specifically, depression has been linked to lower scores across multiple physical and mental health-related quality of life domains, including physical functioning, vitality, role limitation due to physical/emotional health, bodily pain, and social function (Da Costa et al., 2006). Concerningly, suicide is one of the leading causes of death during the perinatal period in Canada, with the largest number of suicides occurring within the last trimester of pregnancy and the last quarter of the first postpartum year, potentially highlighting periods of increased vulnerability (Grigoriadis et al., 2017). Beyond the short-term effects on maternal health, longitudinal evidence suggests that postpartum mental health disorders are associated with worse maternal health outcomes beyond one year postpartum. In a cohort study, Abdollahi and Zarghami (2018) showed that women with PPD were more than twice as likely to continue to experience depressive symptoms and chronic health diseases (e.g., diabetes mellitus, hypertension, and heart disease) four years after the birth of their child. Beyond the detrimental effects on maternal mental health, stress during the postpartum period, as well as experiencing PPD and PPA, has been associated with negative effects on the health and development of the child, including physical growth, language development, and communication skills (Oyetunji & Chandra, 2020).

2.2 Vulnerability to Mental Health Disorders During the Postpartum Period

2.2.1 Risk factors for postpartum depression and anxiety

The development of PPD and PPA is thought to arise from multiple interacting factors. One of the strongest predictors of PPD is a prior history of mental illness. When assessing depression and anxiety scores at several time points throughout the perinatal period, including Weeks 12-14 and 29-30 of gestation and Weeks 4-6 postpartum, Jiménez-Barragán et al. (2024) found that prior history of depression or anxiety consistently predicted a positive screening across all time points. Furthermore, having a family history of psychiatric illness is another potent risk factor for the development of PPD. In a meta-analysis comprising 26 studies and 100,877 participants, it was found that women who had a family history of mental health disorders were more than twice as likely to develop PPD compared to women with no familial history (Zacher Kjeldsen et al., 2022), suggesting inherited risk may contribute to vulnerability to postpartum mental health disturbances. Perceived social support, which is one's appraisal of adequate emotional, instrumental, and informational support within their social network (Drageset, 2021), has consistently been linked to postpartum mental health (Cho et al., 2022; Agrawal et al., 2022; Ekpenyong & Munshitha, 2023), with low to moderate perceived social support being associated with increased risk of PPD (Cho et al., 2022). Support from one's partner is critical in this context, with low marital satisfaction being a strong predictor of postpartum mental health concerns (Wei et al., 2025).

2.2.2 Stress in the postpartum period

Acute and chronic stressor exposure during the postnatal period has also been identified as a key contributor to the development of PPD and PPA. Acute stressors refer to single-time occurrences, for instance, the death of a loved one, traumatic birth experiences, or natural

disasters, while chronic stressors involve situations that persist over time, such as intimate partner violence, financial problems, or living in an environment that is not safe (Hammen et al., 2005). Reid and Taylor (2015) found that acute and chronic stressors that occur during the perinatal period independently increase PPD risk even when prior depression history as well as social and demographic factors are accounted for, highlighting perinatal stress as a strong predictor of postpartum mental health concerns. Psychosocial stress in particular is a consistently reported factor in the development of PPD (Yim et al., 2015), with stressors related to caregiving and interpersonal relationships, including childcare stress, decreased social support, marital relationships, and marital status, found to be associated with disorder development (Beck et al., 2001; Ojomo et al., 2025). Using a network analysis, which examines how strongly individual symptoms are related to one another (Borsboom, 2021), Wang et al. (2024) found that postpartum-specific stressors such as lack of time for oneself, dissatisfaction with physical appearance after childbirth, and irregular patterns of the baby's sleep were closely interconnected with core depressive symptoms, including feeling sad or miserable and crying related to being unhappy. Notably, studies of incarcerated women separated from their children showed higher rates of PPD and feelings of emptiness and sadness that were specifically related to being apart from their child (Chambers et al., 2009; Chawla et al., 2024; Kramer et al., 2025), indicating that maternal-child separation may be a stressful experience for mothers, with an impact on their mental health. Similarly, mothers who had their child removed from their custody showed higher levels of anxiety, depression, and substance use disorders, and visited their physician more often for mental health concerns compared to mothers who had experienced the death of their child (Wall-Wieler et al., 2018). Although custody removal-related mental health concerns may not solely be attributed to the child being removed from the home, these findings highlight the

profound impact that mother-child separation disruptions may have. These connections between stressful experiences and mental health symptoms suggest that PPD and PPA may be in part related to the presence of certain stressors that are unique to this period.

2.3 Adaptations to the Postpartum Period

2.3.1 Behavioural and cognitive adaptations to the postpartum period

The transition following childbirth is characterized by major behavioural and cognitive changes that support the maternal caregiving role. When a baby is born, mothers experience heightened preoccupation, exhibiting behaviours such as checking and worrying about the baby's well-being (Leckman et al., 2004). In the postpartum period, this is a normal adaptive response that enables the mother to attend to the infant's physiological and psychological needs, with preoccupation peaking after birth and decreasing over subsequent months (Kim, 2016).

However, when this vigilance becomes excessive and disproportionate to risk, this may become maladaptive and contribute to PPA, specifically postpartum OCD (Ferra et al., 2024).

Conversely, heightened vigilance may represent a manifestation of postpartum OCD, as part of the disorder's clinical presentation (Hudepohl et al., 2022). Women experiencing postpartum OCD often report obsessions related specifically to the well-being of their infant, such as aggressive intrusive thoughts (e.g., dropping the baby, suffocation) and obsessions about germs or contamination in the baby's environment (Ferra et al., 2024). These intrusive thoughts often lead to compensatory behaviours, including checking, seeking reassurance, and avoidance, maintaining the anxiety, which in turn compromises maternal well-being (Ali, 2018; Ferra et al., 2024). Another important adaptation that occurs in the postpartum period involves changes in the mother's reward circuits, which become more sensitive to infant-related cues, thereby increasing motivation for caregiving (Kim, 2016; Rincón-Cortés & Grace, 2020). Disruptions in this regard

may be associated with difficulties in responding to infant needs, which may negatively affect the mother-child bond as well as maternal and offspring outcomes (Saharoy et al., 2023).

2.3.2 Physiological adaptations during the postpartum period

The perinatal period involves several physiological adaptations that support fetal development and prepare the mother for the transition from pregnancy to birth and subsequent caregiving. While the etiology of PPA and PPD is not yet fully understood, it has been observed that interruptions or alterations in the normal trajectory of hormonal, neurobiological, and immune adaptations during this period may contribute to the increased vulnerability of women to mental health disorders during pregnancy and postpartum (Schiller et al., 2015; Abou-Saleh et al., 1998; Skrundz et al., 2011; Levin & Ein-Dor, 2023; Boufidou et al., 2009; Sha et al., 2022). A major contributor to the development of postpartum mental health disorders is the rise and decline of sex hormones in the perinatal period, especially estradiol and progesterone, which increase drastically during pregnancy, followed by a sharp decline following the birth of the child (Ducik et al., 2024). In human and animal studies, it has been demonstrated that the withdrawal of these hormones following birth is related to anxiety- and depressive-like behaviours in rodents and anxiety and depressive symptoms in humans (Zhang et al., 2016; Schiller et al., 2015). Importantly, evidence suggests that these associations are not explained solely by the magnitude of hormone withdrawal contributing to mental health concerns but rather reflect increased vulnerability to the normal fluctuations of sex hormones in the transition from pregnancy to the postpartum period (Schiller et al., 2015; Bloch et al., 2000; Brummelte & Galea, 2016; Eisenlohr-Moul et al., 2023). One potential mechanism underlying this sensitivity is the influence of fluctuations in estradiol and progesterone on neurosteroid synthesis, neural responsivity, and the metabolism of neurotransmitter precursors; deviations from their normal

trajectories are therefore thought to contribute to vulnerability to postpartum mental health disorders (Levin & Ein-Dor, 2023).

In addition to sex hormones, disruptions in postpartum adaptations related to stress responsivity may represent another key pathway by which postpartum mental illnesses develop. Notably, during the peripartum period, particularly late pregnancy and into lactation, hypothalamic-pituitary-adrenal (HPA) axis responsiveness is suppressed to support fetal development, as high maternal corticosteroid levels can be harmful (Hillner et al., 2012). Generally, high stress responsivity has been identified as a risk factor in mental health disturbances, including depressive and anxiety disorders (Kiecolt-Glaser et al., 2020). However, in the context of postpartum mental health disorders, it has also been demonstrated that decreased cortisol is associated with the onset of PPD, potentially reflecting a dysfunction of HPA axis regulation throughout the peripartum period (Dickens & Pawluski, 2018). Hormones associated with caregiving behaviours, including oxytocin and prolactin, are increased during the postpartum period and dampen the response of corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus, thereby supporting maternal care behaviours while limiting the risk of stress-related mental health disorders such as anxiety and depression (Slattery & Neumann, 2008). Consistent with this, lower levels of prolactin were associated with an increased incidence of PPD (Abou-Saleh et al., 1998), while low levels of oxytocin mid-gestation predicted depression development within the 2 weeks following birth (Skrundz et al., 2011). Furthermore, a rat model showed that downregulation of prolactin receptors led to higher stress-induced adrenocorticotrophic hormone (ACTH) levels and increased anxiety-like behaviours in lactating dams (Torner et al., 2002), suggesting a relationship between these stress-regulation processes and postpartum anxiety-related outcomes.

Another physiological system implicated in the etiology of postpartum mental health disorders is the immune system. Gestation and the postpartum period are characterized by changes in immune function that support fetal development and parturition, with these systems gradually returning to baseline following birth (Abu-Raya et al., 2020). Changes in pro-inflammatory cytokines, both central and peripheral, may influence behaviour by altering inflammatory signalling pathways in the brain, leading to subsequent changes in neurotransmitter, neuropeptide, and growth factor levels and function (Felger & Lotrich, 2013). Disruptions in normal immune adaptation in the perinatal period may represent a mechanism underlying the development of postpartum mental health disorders. Several studies have found that exaggerated levels of pro-inflammatory cytokines during gestation and postnatally are associated with postpartum mental health concerns. Plasma levels of interleukin (IL)-1 β and IL-6 were positively associated with depressive symptom severity on the Edinburgh Postnatal Depression scale at each trimester of pregnancy and at postpartum (Sha et al., 2022). Consistent with these findings, Achtyes et al. (2020) reported that increased plasma IL-6 and IL-8 levels measured at 8 weeks postpartum were associated with more severe depressive symptoms. Similarly, Boufidou et al. (2009) found that levels of IL-6 and tumour necrosis factor (TNF)- α in the cerebrospinal fluid at delivery were positively associated with depressive symptom severity at 6 weeks postpartum, providing indirect evidence of a relationship between central immune processes and postpartum maternal mental health. While circulating pro-inflammatory cytokines have been associated with PPD, little is known about the involvement of inflammatory factors in postpartum mental health in brain regions implicated in emotional regulation, such as the prefrontal cortex (PFC) (Dixon et al., 2017), although in the general population there is evidence that IL-1 β , IL-6, and TNF- α are elevated in the PFC of individuals with mental health disorders

who died by suicide (Pandey et al., 2012). It is also unclear how stress during the postnatal period, a known risk factor in postpartum mental health disorders (Yim et al., 2015), may affect neuroinflammatory processes involved in depressive and anxiety symptoms.

Additionally, there is evidence that bidirectional communication between the gut and the brain is involved in the pathophysiology of mental health disorders (Cryan et al., 2019). One of the pathways by which this may occur is the disruption of inflammatory immune signalling between the gut and the brain due to increased permeability of the intestinal barrier, allowing the passage of bacterial fragments into circulation, and/or elevated inflammatory activity at the intestinal barrier (Cryan et al., 2019; Maes et al., 2008). In relation to stress, chronic social defeat in mice results in more social avoidance behaviour, increased intestinal permeability, and elevated levels of pro-inflammatory interferon-gamma (IFN γ)-producing cells, while decreasing levels of anti-inflammatory IL-4-producing cells in the colon (Russo et al., 2023), linking stress-induced colonic inflammation and barrier permeability to behavioural disruptions. Chronic social defeat in mice also downregulated the tight junction protein claudin 5 in the NAc, thereby allowing the passage of IL-6 at that site and promoting depressive-like behaviour (Ménard et al., 2017). Importantly, neuroinflammation elicited by the bacterial endotoxin lipopolysaccharide (LPS) has also been related to anxiety- and depressive-like behavioural changes (Gao et al., 2025; Zhang et al., 2024; He et al., 2025). Taken together, these studies using rodent models suggest that stressors may simultaneously disrupt permeability of the gut and brain barriers and promote neuroinflammation, potentially contributing to behavioural alterations. However, it is not well understood whether gut inflammation may be related to the development of postpartum mental health disorders. Limited epidemiological evidence suggests that women with inflammatory bowel disease, characterized by inflammation of the GI tract in part driven by

increased cytokines (Neurath, 2014), had a 20% higher risk of developing new-onset mental illness during the postpartum period compared to women without the disease (Vigod et al., 2019). Collectively, these findings highlight the need for further investigation into the contribution of inflammatory processes in the gut-brain axis to stress-related behavioural disruptions in the postpartum period.

2.4 Maternal Separation as a Model of Postnatal Maternal Stress

2.4.1 The maternal separation paradigm

In rodent models, MS is an established paradigm for assessing the impact of early postnatal stress in both pups and dams (Plotsky & Meaney, 1993). In the classic stressor paradigm, pups are separated from the dams daily for 3 hours between P2 and P14, encompassing the early developmental period in the offspring and the mother's early postpartum period. The MS paradigm has traditionally been used as a model of early life stress (ELS) in pups, but increasing evidence in mice and rats suggests it may also serve as a stressor for dams (Bölükbas et al., 2020; Rombaut et al., 2023). In the postpartum context, MS has been shown to elicit depressive- and anxiety-like behaviours in mouse and rat dams, demonstrating that the impacts of the stressor extend beyond those observed in the offspring (Mundorf et al., 2022). Importantly, multiple variations of the MS stressor exist, which involve differences in the duration of separation sessions, the number of days during which the stressor is performed, and the setup of the separation itself (e.g., pups separated as a litter versus individually, use of new cages versus home cages, separation in a different space versus remaining in the same housing room). These variations in how the stressor is administered have resulted in differences in the behavioural and physiological effects of MS in mice (Tractenberg et al., 2016), especially in relation to maternal caregiving. Notably, shorter separations are associated with increased

grooming and licking of the pups, whereas separations longer than 3 hours per day are associated with decreased grooming and licking (Rombaut et al., 2023).

2.4.2 Physiological consequences of maternal separation in dams

At the neurobiological level, MS in dams has been associated with reduced expression of genes implicated in stress regulation, structural changes in the brain, and disruptions in HPA axis regulation. Among these changes, rat dams exposed to MS had downregulated expression of *Morcl* in the medial PFC (mPFC) (Bölükbas et al., 2020), a gene previously identified as an epigenetic brain marker of ELS in adult rats and associated with MDD (Nieratschker et al., 2014). Exposure to MS in rat dams also increased oxytocin receptor binding in the prelimbic cortex and the medial preoptic area (Demarchi et al., 2023), a key region implicated in parental behaviour, potentially suggesting physiological adaptations related to caregiving in response to the MS stressor (Champagne et al., 2001; Hidema et al., 2024; Demarchi et al., 2023). Prolonged daily MS also increased corticotropin-releasing hormone (CRH) and reduced neuropeptide Y (NPY) in the hypothalamus of rat dams (Maniam & Morris, 2010). Increased hypothalamic CRH expression is suggestive of HPA axis hyperactivity, which is commonly observed in both anxiety and depressive disorders (Stanton et al., 2023; Iob et al., 2020; Keck & Holsboer, 2001), while NPY reductions in both humans and animal models have been associated with depressive- and anxiety-like behaviours (Redrobe et al., 2002; Cohen et al., 2012). Using a paradigm that combines limited nesting with MS, a recent study by O'Chan et al. (2026) in C57BL/6J mice showed that this stressor reduced transcriptional changes in the dorsal hippocampal formation, which they identified as a region supporting the development of cognitive adaptations to motherhood, particularly in relation to long-term potentiation, dopaminergic synapses, and oxytocin signalling. Together, these findings demonstrate that postnatal stress, especially in the

form of MS, may disrupt neurobiological changes associated with motherhood. Despite evidence suggesting that MS disrupts neurobiological adaptations associated with the postpartum period, it is not known whether MS results in changes in pro-inflammatory cytokines in the brain and GI tract.

2.4.3 Behavioural consequences of maternal separation in dams

In addition to physiological effects, MS has been reported to alter behaviour and maternal care. Studies in rats have found that MS increased learned helplessness and behavioural despair in the dams, reflective of depressive-like behaviours (Boccia et al., 2007; Bousalham et al., 2013). Notably, these effects appear to be specific to prolonged MS sessions (3 hours per day or more), whereas studies employing brief separation paradigms (approximately 15 minutes per day) more frequently report null findings (Mundorf et al., 2022). In contrast, data on anxiety-like behaviour are limited to studies predominantly conducted in rats and are highly variable, with both significant and null effects being observed (Bousalham et al., 2013; Bölükbas et al., 2020; Stevenson et al., 2009; Maniam & Morris, 2010). This may be attributed to variations in stressor protocols, as well as to other factors such as rodent strain and whether the dam was primiparous or multiparous, which have been shown to vary across studies examining these effects (Aguggia et al., 2019; Mundorf et al., 2022). One of the most consistently reported behavioural consequences of MS is alterations in maternal care behaviours, although the direction of these changes varies across studies. Multiple studies examining maternal care in mice and rats following daily separations of 3 or more hours have found increases in arched-back nursing, licking, and time spent in the nest (Baracz et al., 2020; de Almeida Magalhães et al., 2018; Maniam & Morris, 2010; Zimmerberg et al., 2003), although reductions in maternal caregiving behaviours have also been reported using this same paradigm (Rombaut et al., 2023; Zaccarelli-

Magalhães et al., 2023; Boccia et al., 2007; Bailoo et al., 2014). Given variability in rodent strain, separation duration, postnatal days included in the separation period, and the day testing was conducted, differences in maternal care behaviours across studies may reflect methodological variation in how the stressor was implemented.

2.5 Early Life Stress and Developmental Trajectories

2.5.1 Exposure to stressors during early life and health outcomes

Early life adversity is a term encompassing stressful events before age 18 (Centers for Disease Control and Prevention, 2024). These experiences can be related to childhood maltreatment, including physical, sexual, and/or emotional abuse and/or neglect, witnessing domestic violence, as well as other circumstances such as war, accidents, and bullying (World Health Organization, 2024). The developmental origins of health and disease hypothesis suggests that early life exposures play a key role in the development of chronic health conditions, including physical problems such as cardiovascular disease, asthma, and certain types of cancers as well as mental disorders, including depression and anxiety (Centers for Disease Control and Prevention, 2024; Daníelsdóttir et al., 2024; Dube et al., 2009; Nelson et al., 2020). Exposure to ELS is highly prevalent in the Canadian population, with a large national cohort study reporting that approximately 61.6% of participants experienced at least one adverse childhood experience (Joshi et al., 2021). Notably, a dose-response relationship has been noted between the number of these experiences and health outcomes, where exposure to a higher number of adverse events during childhood was associated with a greater risk of health concerns, including severe obesity, depressive disorders, suicide attempts, ischemic heart disease, and cancer (Felitti et al., 1998).

2.5.2 Early life biological development and vulnerability to stress

Early life is a critical period in the development of several key biological systems during which the brain, immune system, HPA axis, and gut-brain axis undergo rapid maturation (Shonkoff, 2000; Jena et al., 2020; Palma et al., 2026). In this period, extrinsic factors, including environmental and social influences such as the physical environment, parenting, caregiving experiences, and socioeconomic status, may exert profound effects on development (Wallerich et al., 2023). The influence of these factors may, at least in part, be attributed to developmental plasticity, which refers to the ability of a genotype to produce various phenotypes by adapting to environmental conditions (Bateson et al., 2004). While plasticity enables adaptation to one's environment, it can also increase vulnerability to adverse experiences, exerting lasting effects on the development of biological systems (Bateson et al., 2004; Smith & Pollak, 2020). This vulnerability is particularly important given the extensive neurodevelopment occurring in early life, including brain regions implicated in emotional regulation and the stress response, such as the PFC, hippocampus, and amygdala (Leisman et al., 2025). For instance, it has been shown that healthy young adults with a history of early life emotional abuse had reduced functional connectivity between the PFC and the amygdala, suggesting lasting alterations in brain function resulting from ELS (Fan et al., 2014). Early life stress also has enduring effects on brain regions involved in reward processing, with adolescents who reported adverse experiences in childhood showing hypoactivity in the NAc, which was associated with greater depression scores (Goff et al., 2013). Exposure to childhood maltreatment has also been associated with smaller hippocampal volume in adulthood (Riem et al., 2015). As reduced volume of the hippocampus has been related to increased vulnerability to multiple mental health disorders, including MDD, bipolar disorder, and schizophrenia (Brosch et al., 2022), these findings suggest that there may

be structural changes induced by ELS that may contribute to the development of later mental health disturbances. Childhood trauma exposure has been associated with HPA axis activation in some individuals and a blunted response in others, indicating an overall pattern of abnormal cortisol regulation resulting from traumatic experiences in childhood while the HPA axis is still developing (Cross et al., 2017; Juruena et al., 2021). Additionally, evidence suggests that individuals with MDD and a history of ELS have higher serum cytokine levels, particularly IL-6, compared with controls and those with MDD without a history of ELS (Gill et al., 2020; Pedrotti Moreira et al., 2018). In individuals with MDD, it has also been shown that childhood sexual abuse was associated with higher serum levels of IL-6 and TNF- α , whereas this was not observed with recent events or perceived stress (Grosse et al., 2016). Collectively, these studies indicate that immune-related changes as a result of ELS may be one of the mechanisms contributing to the development of mental health disorders, at least depression.

2.5.3 Early life stress and the development of mental health disorders

Childhood adversity has been consistently positioned as a significant risk factor in the development of mental health disorders, including MDD, anxiety disorders, and substance use disorders, with maladaptive family functioning such as abuse or neglect being the most significant predictor (Kessler et al., 2010). In the case of depressive disorders, individuals who had experienced ELS were approximately 2.5 times more likely to develop MDD before the age of 18 compared to those who had not (LeMoult et al., 2020). Importantly, individuals with MDD who reported a history of childhood trauma were 43% less likely to remit or experience improvement in their symptoms (Nanni et al., 2012). Similarly, ELS increases the risk for anxiety disorders, particularly sexual and physical abuse (Lindert et al., 2014). Early life stress has likewise been linked to substance use disorders, in a dose-response manner, where exposure

to 4 or more adverse childhood experiences was linked to a 7.2-fold increase in the risk for alcohol use disorder and a 4.5-fold increase in the risk for illicit drug use (Anda et al., 2006). Disruptions in motivated behaviour and in the ability to experience pleasure, characteristic of reward-related behaviour, are an overlapping feature of multiple mental health disorders, including depression, anxiety, and substance use disorders (Scholz et al., 2026; Meyer et al., 2016), and have been observed in the context of ELS (Eck et al., 2020; Birn et al., 2017). Evidence from both human and rodent studies suggests that the increased vulnerability to adverse mental health outcomes may be attributable, at least partly, to the converging effects of ELS on neurodevelopment, HPA axis function, neurotransmitter systems, and immune responses (Speranza et al., 2024). Together, these neurobiological and immune changes can result in impairments in goal-directed behaviour and decision-making, which are also a shared characteristic of multiple mental health disorders (Puetz & McCrory, 2015; Epstein & Silbersweig, 2016). Consistent with this, adults who had previously experienced childhood maltreatment had higher anhedonia and depression scores, perceived rewarding cues as less positive, and had a dampened response to reward cues in the left globus pallidus, linking ELS to long-term alterations in reward-related neural activity (Dillon et al., 2009). Birn et al. (2017) also found that ELS altered the valuation of risk compared to a reward in a gambling task, where adult individuals who had experienced high levels of childhood stress made more impulsive decisions, poorer quality decisions, and had more difficulty adjusting their decisions based on feedback given to them by the test, suggesting that ELS may disrupt reward-related decision-making and the adaptability of these processes. However, despite the well-established association between mental health disorders and stress in early life, the mechanisms underlying

these disorders remain incompletely understood. As such, animal models, including MS, provide an opportunity to investigate the behavioural and neurobiological consequences of ELS.

2.6 Maternal Separation as a Model of Early Life Stress

2.6.1 Maternal separation in pups: influence of maternal care

As previously described, MS is an established paradigm used to examine the behavioural and physiological consequences of repeated separation sessions between the dams and their pups in early life on offspring development (Plotsky & Meaney, 1993). The MS stressor is hypothesized to be stressful for the pups, largely due to the temporary absence of maternal stimuli (e.g., tactile stimulation, scent) and care, as well as to alterations in maternal care behaviour following separation episodes (Meaney et al., 2001; Rombaut et al., 2023). In general, maternal care behaviours such as licking/grooming and arched-back nursing are essential for pup development (Meaney et al., 2001). Studies examining variations in maternal care in rats without a stressor have found that different behavioural phenotypes can emerge depending on the level of care provided (Liu et al., 1997; Caldji et al., 1998). For instance, Liu et al. (1997) found that offspring that had received lower levels of grooming during the first 10 days of life showed higher plasma levels of ACTH and corticosterone in response to a restraint stressor and higher CRH mRNA expression in the paraventricular nucleus relative to high-grooming dams, suggesting that maternal care during early life may play a role in the responsivity of the HPA axis. Further supporting this, Caldji et al. (1998) found that offspring exposed to low licking, grooming, and arched-back nursing had increased levels of CRH in the locus coeruleus and reduced exploratory behaviour in the open field test relative to offspring that received high levels of these maternal care behaviours. Research examining maternal care behaviour and offspring outcomes within the same MS paradigm remains limited. When both have been measured, MS in

the form of 3 hours a day from P2 to P21 in C57BL/6J mice resulted in impairments in maternal care behaviour relative to shorter bouts of separation or no stressor (Rombaut et al., 2023), while decreasing affiliative behaviour in adult offspring (Rombaut et al., 2023). Using a similar paradigm consisting of MS and a limited bedding and nesting environment, simulating the effects of both separation and resource scarcity in BALB/cJ mice, Orso et al. (2020) found alterations in caregiving in stressed dams relative to non-stressed controls, as well as pronounced anxiety-like behaviours and changes in HPA axis markers in the adult offspring. Taken together, this evidence suggests that disruption in maternal caregiving following MS could play a role in shaping offspring behavioural and physiological measures, although causation cannot be inferred from these findings.

2.6.2 Physiological consequences of maternal separation in pups

Maternal separation has been shown to induce changes in several physiological measures in the offspring, including those related to the HPA axis, neuroinflammation, and mesolimbic system function. In relation to the HPA axis, elevated hypothalamic CRF levels in response to a stressor and at baseline (Plotsky & Meaney, 1993), higher basal plasma ACTH, but a blunted response to a stressor in adulthood (Daniels et al., 2004; Plotsky et al., 2005), as well as higher basal serum corticosterone levels (Spirka et al., 2020; Roque et al., 2014) have been observed in MS rat offspring, the latter having been linked to many mental health disorders such as depression, anxiety, OCD, and bipolar disorder (George et al., 2025). Alongside HPA axis dysregulation, MS in mice and rats has been shown to alter the expression of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α , with effects differing based on the conditions in which they were assessed (e.g., basal versus stress-induced) and on sex (Dutcher et al., 2020; Dong et al., 2024). Jahng et al. (2010) demonstrated that in addition to exhibiting higher basal

and stress-induced corticosterone levels, adult rat offspring exposed to MS had lower c-Fos expression in the NAc and midbrain dopamine neurons and downregulated tyrosine hydroxylase mRNA in the ventral tegmental area and substantia nigra in response to a stressor, demonstrating that HPA axis dysregulation following MS may be accompanied by changes in mesolimbic reward circuit functioning. Alterations in the brainstem dopaminergic system have also been observed in the context of MS, with findings from de Souza et al. (2018) in Wistar rats showing elevated brainstem dopamine 1 (D1R) and dopamine 2 (D2R) receptor expression only in males, even if food intake was higher in both sexes. Collectively, these findings indicate that MS induces long-term alterations in dopaminergic reward-related circuits in multiple brain regions, with sex being an important factor influencing these changes. Relatedly, male-specific reductions resulting from corticosterone injections have been reported in rats in the expression of the BDNF receptor, tropomyosin receptor kinase B (TrkB) (Hill et al., 2014), with TrkB signalling in the NAc being shown to contribute to the maintenance of reward-seeking behaviour, particularly in the context of drug seeking (Bahi et al., 2008; Graham et al., 2009). Despite evidence suggesting that MS affects components of the mesolimbic system in a sex-dependent manner, its effects on dopamine receptor and TrkB expression within the NAc, and the associated behavioural consequences for reward-seeking in anxiogenic conditions remain understudied.

2.6.3 Behavioural consequences of maternal separation in pups

The behavioural effects of the MS stressor in adult offspring are variable, with studies reporting inconsistent findings regarding their presence and directionality. A systematic review conducted by Tractenberg et al. (2016) examined 94 mouse studies utilizing MS and showed heterogeneous effects in anxiety- and depressive-like behaviour in C57BL/6 mice. This review identified several methodological factors that may have contributed to the variability in the

reported behavioural effects, including separation duration, strain, type of control group used (i.e., standard rearing, non-handled, early handling), and housing conditions, suggesting that methodological differences across studies may have driven divergent findings (Tractenberg et al., 2016). Notably, the genus of rodents (e.g., mouse or rat) may be particularly important in influencing how they respond, physiologically and behaviourally, to the MS stressor. For instance, Wistar and Sprague-Dawley rats are more sensitive to the anxiety-like effects of the stressor than C56BL/6 and BALB/c mice (Wang et al., 2020), whereas cognitive flexibility is more impaired by the stressor in mice (Ou-Yang et al., 2022), suggesting that genus may be a key contributor to the variability in behavioural responses to MS.

The effects of MS on anxiety-like behaviours in both mice and rats are particularly inconsistent, with increases observed in some studies (Cevik et al., 2023; Kestering-Ferreira et al., 2021) and null effects reported in others (Zoicas & Neumann, 2016; Wang et al., 2011; Bian et al., 2015). Depressive-like effects of MS have also been reported in both mice and rats (Bian et al., 2015; Wei et al., 2023; Takabayashi, 2025), alongside others observing no change in depressive-like behaviour (Millstein & Holmes, 2007; Savignac et al., 2011). In contrast to the more extensively studied depressive- and anxiety-like behaviour, outcomes related to reward, particularly in the context of natural rewards such as highly palatable food, remain incompletely understood. Importantly, depressive-like behaviours and reward seeking can be interrelated, as anhedonia, the inability to experience pleasure or reward, is a core symptom of MDD (American Psychiatric Association, 2013). For instance, the sucrose preference test, in which a rodent's preference to consume sucrose is examined, is considered an assessment of anhedonia, with lower sucrose intake being associated with a reduced ability to experience pleasure (Liu et al., 2018). Reductions in sucrose preference in the context of MS have been reported in multiple

studies in mice and rats (Novick et al., 2019; Amiri et al., 2016; Hui et al., 2011; Sadeghi et al., 2016), and while this test provides valuable insight into anhedonia and responsiveness to a reward, additional behavioural paradigms are necessary to distinguish depressive-like behaviour from other aspects of reward processing. Research examining reward-seeking behaviour following MS in mice has found that the stressor increased motivation to seek ethanol on a progressive ratio schedule (Bertagna et al., 2021) and increased ethanol preference when given a choice between ethanol and water (Rodolpho et al., 2025; Portero-Tresserra et al., 2018). The effects of MS on certain aspects of reward seeking may be sex-specific, with stressed C57BL/6J males showing higher ethanol intake (Talani et al., 2025). Furthermore, MS rats that consumed more ethanol had higher corticosterone levels in response to a startle stressor and spent less time spent in the open arms of the elevated plus maze (Huot et al., 2001), suggesting that MS-related changes in reward seeking may, in part, be driven by HPA axis dysregulation and heightened anxiety. Parallels from the drug- and ethanol-reward literature can be drawn to naturalistic rewards, such as highly palatable food, given that MS also increases intake in stressed rat offspring, irrespective of sex (de Souza et al., 2018). In examining food motivation, Benoit et al. (2023) found that MS increased motivation to obtain a highly palatable food reward when the required effort was high (i.e., more lever presses) in C3H/HeN mice but not in C57BL/6J mice. Transcriptional analysis also revealed that MS induced NAc gene expression changes only in male C3H/HeN mice, involving glutamatergic and GABAergic signalling pathways, with L-DOPA, the precursor of dopamine, predicted to be the most significant upstream regulator (Benoit et al., 2023).

2.6.4 Sex differences in early life stress and maternal separation

The extent to which the behavioural and physiological effects of MS differ between females and males is incompletely understood. The study of sex differences in the context of mental health disorders is highly important due to differences in prevalence, with internalizing disorders such as anxiety, depression, and OCD being more common in females while externalizing disorders, including attention deficit hyperactivity disorder, are more common in males (Kayrouz et al., 2025). Notably, sex differences in stress reactivity have been demonstrated using fMRI, which showed that during a stressor, women had greater activation in limbic and striatal regions, whereas men had higher activation in the PFC (Goldfarb et al., 2019). Sex differences in the stress response are thought to occur in part due to the influence of sex chromosomes and gonadal factors. A study conducted by Barko et al. (2026) demonstrated that sex chromosomes were the primary contributors to stress-induced transcriptional changes in the NAc, whereas in the PFC, both sex chromosomes and gonadal type contributed, suggesting that the influence of sex chromosomes and gonadal factors on stress responsivity may differ by brain region. A meta-analysis of 212 animal studies investigating the effects of experimental models of ELS in offspring showed that anxiety-like behaviour, memory alterations, and decreased social behaviours were more consistently observed in males exposed to ELS than in females (Bonapersona et al., 2019). Similarly, male-specific changes resulting from MS have been observed in mice and rats within neurobiological pathways involved in reward processing, including decreased glutamatergic transmission in NAc medium spiny neurons (Talani et al., 2025), increased D1R and D2R expression in the brainstem (de Souza et al., 2018), and transcriptional changes in the NAc (Benoit et al., 2023). Taken together, these findings suggest

that sex is a key factor influencing behaviour and physiological measures following ELS exposure.

2.7 Literature Gaps

Overall, few studies have simultaneously examined the effects of MS on dams and their pups, and little is known about how the stressor may differentially affect maternal and offspring outcomes. In dams, pro-inflammatory cytokines in the brain and GI tract following MS exposure and whether these can be associated with anxiety- and depressive-like behaviour have not been examined. In pups, it has not been assessed whether MS results in sex-specific differences in motivation for a food reward in a risky environment, as well as in the expression of dopamine and neurotrophic factor receptors in the NAc.

2.8 Research Questions, Objectives and Hypotheses

This thesis addresses these knowledge gaps through two manuscripts. Chapter 3 examines the behavioural alterations and inflammatory changes in the brain and colon resulting from MS in dams during the postpartum period, whereas Chapter 4 addresses the long-term behavioural and neurobiological consequences of early life exposure to the same stressor in young adult offspring.

Chapter 3 addresses three objectives related to the effects of MS in dams. The first objective assessed whether the stressor induced alterations in maternal care during the early postpartum period and whether these effects were consistent across time points. The second objective determined whether MS-induced anxiety- and depressive-like behaviours were present shortly after weaning. Finally, a third objective quantified the expression of IL-6, IL-1 β , and TNF- α in the mPFC and colon to examine if the behavioural alterations at weaning were associated with changes in pro-inflammatory cytokines.

Consistent with previous evidence, it was hypothesized that dams would exhibit disruptions in maternal care behaviours compared with non-separated controls (Alves et al., 2020; Maniam & Morris, 2010; Rombaut et al., 2023). The stressor was also anticipated to increase depressive-like behaviours (Boccia et al., 2007; Bousalham et al., 2013), but not anxiety-like behaviours, given the inconsistent findings regarding anxiety-related outcomes in postpartum dams (Bousalham et al., 2013; Bölükbas et al., 2020; Stevenson et al., 2009; Maniam & Morris, 2010). Although the effects of MS on pro-inflammatory cytokines have not previously been examined in dams, it was expected that it would increase the expression of IL-6, IL-1 β , and TNF- α in the mPFC and colon, based on evidence linking circulating inflammation to PPD (Achtyes et al., 2020; Boufidou et al., 2009; Sha et al., 2022), IBD to PPD (Vigod et al., 2019), and behavioural alterations following chronic stressors to gut and brain inflammatory and barrier disruptions (Audet et al., 2011; Nie et al., 2018; Russo et al., 2023; Ménard et al., 2017; Doney et al., 2023).

Chapter 4 addresses three objectives on the effects of MS in pups. The first objective assessed whether MS altered the motivation to obtain a food reward within a risky environment, as well as depressive- and anxiety-like behaviour in young adulthood. The second objective examined whether behavioural alterations were accompanied by changes in the expression of dopamine (D1R, D2R) and BDNF (TrkB) receptors. The third objective examined the sex-specific effects of MS on these behavioural and physiological outcomes.

Previous reports suggest that MS alters anxiety- (Cevik et al., 2023; Kestering-Ferreria et al., 2021) and depressive-like (Bian et al., 2015; Wei et al., 2023; Takabayashi et al., 2025) behaviours as well as increases reward-seeking in the context of drugs and palatable food in adulthood (Bertagna et al., 2021; Rodolpho et al., 2025; Portero-Trasserra et al., 2018; Benoit et

al., 2023; de Souza et al., 2018). As such, it was expected that offspring would exhibit changes in anxiety- and depressive-like behaviour in young adulthood and an increase in the motivation for a food reward in a risky environment. Relatedly, MS has been associated with changes in markers related to dopamine and BDNF in the NAc (Wang et al., 2015; Meaney et al., 2002; Romano-López et al., 2016; Sasagawa et al., 2017), and therefore it was anticipated that stressor exposure would result in alterations in D1R, D2R, and TrkB in the NAc of young adult offspring. Finally, given that MS has been shown to have sex-specific effects on both behavioural and physiological measures (Wigger & Neumann, 1999; Dong et al., 2024; de Souza et al., 2018; Hill et al., 2014), it was hypothesized that affective and reward-related behaviour as well as expression of D1R, D2R, and TrkB in the NAc would be differentially affected by the stressor.

Chapter 3: Could postpartum behavioural disturbances resulting from postnatal stress be related to inflammatory changes in the gut-brain axis?

Full-Length Research Report

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Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

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3.1 Abstract

Background: Stressors experienced during the postpartum period may increase vulnerability to mental health disorders, including postpartum depression and anxiety. Previous studies suggest that stress-induced inflammation in the brain and gastrointestinal system contributes to behavioural and neurobiological disruptions, although this remains understudied in the postpartum context. This study examined the effects of maternal separation (MS), an established model of postnatal stress in dams, on maternal care, depressive- and anxiety-like behaviours, as well as on changes in pro-inflammatory cytokines in the brain and colon. **Methods:** Female C57BL/6N mice were randomly assigned to a control or an MS condition that consisted of 3-hour separation sessions from postnatal days (P) 2 to 14. Maternal care behaviour was assessed on P3 and P7. Anxiety- and depressive-like behaviour was assessed on P22 and P23, followed by collection of the medial prefrontal cortex (mPFC) and colon on P24 to determine mRNA expression of interleukin (IL)-6, IL-1 β , and tumour necrosis factor (TNF)- α . **Results:** Exposure to MS altered maternal care behaviour, with differences observed on P3 and P7. Although behaviours and pro-inflammatory cytokines assessed in the brain and gut shortly after weaning remained unchanged after MS, TNF- α expression in the mPFC was positively correlated with grooming time in the splash test. **Conclusion:** These findings suggest that MS induces a transient change in caregiving behaviours during the early postpartum period, with no lasting changes in behavioural or pro-inflammatory cytokine expression after weaning.

Keywords: Anxiety, Depression, Gut-Brain Axis, Inflammation, Maternal Care, Maternal Separation, Mouse Model, Postpartum

3.2 Introduction

Postpartum depression and anxiety are prevalent mental health conditions in the Canadian population, with 23% of women reporting symptoms consistent with either of these disorders in the year after birth and 8% of women experiencing comorbid symptoms (Government of Canada, 2019). Both can disrupt the mother-infant relationship, with harmful consequences for the mother and the child (Stewart & Vigod, 2019; Somerville et al., 2015; Fairbrother et al., 2025), highlighting the need to advance our understanding of the mechanisms that may contribute to their pathophysiology. The postpartum period is characterized by extensive behavioural and physiological adaptations that support the caregiving role and child development (Kim, 2016; Rincón-Cortés & Grace, 2020; Levin & Ein-Dor, 2023). Alongside the dysregulations in the normal fluctuations of sex hormones and/or changes in hypothalamic-pituitary-adrenal axis function that may occur during the perinatal period (Hillerer et al., 2012; Schiller et al., 2015; Levin & Ein-Dor, 2023), psychosocial stressors have been consistently reported as a major risk factor in both postpartum depression and anxiety development (Reid & Taylor, 2015; Yim et al., 2015). To examine how stressors experienced during the period following parturition may contribute to the development of these disorders, animal models including maternal separation (MS), which can act both as an early life stressor for the offspring and as a postnatal stressor for the dam, are being used (Plotsky & Meaney, 1993).

Studies using MS in rodents have reported alterations in maternal care behaviours, such as arched-back nursing, grooming, licking, and pup retrieval (Baracz et al., 2020; de Almeida Magalhães et al., 2018; Rombaut et al., 2023) as well as increases in depressive- and anxiety-like behaviours in stressed dams (Boccia et al., 2007; Bousalham et al., 2013; Maniam & Morris et al., 2010). However, comparatively little is known about the physiological mechanisms

underlying these postpartum behavioural phenotypes. Elevated levels of pro-inflammatory cytokines in the plasma and cerebrospinal fluid of women have been associated with the severity of postpartum depressive symptoms (Sha et al., 2022; Achtyes et al., 2020; Boufidou et al., 2009), suggesting that disturbances in inflammatory processes, both in circulation and the central nervous system, could be implicated in postpartum mental health disorders. Women with inflammatory bowel disease, which involves increased pro-inflammatory cytokines in the gastrointestinal tract (Strober & Fuss, 2011), are at a higher risk of a new-onset mental health disorder during the postpartum period (Vigod et al., 2019), raising the possibility that intestinal inflammatory disturbances may also contribute to postpartum mental health disorders. Supporting this, perturbations to the signalling of inflammatory molecules between the intestinal environment and the brain are increasingly recognized as an important factor in the pathogenesis of mental health disorders (Cryan et al., 2019). In non-postpartum contexts, chronic stressors in mice increased barrier permeability and pro-inflammatory factors in selected intestinal and brain regions, contributing to behavioural impairments (Russo et al., 2023; Nie et al., 2018; Ménard et al., 2017; Doney et al., 2023; Szyszkowicz et al., 2017). However, whether these processes are disrupted in the context of postpartum mental health disturbances remains unknown.

Here, we examined the effects of repeated postnatal stress in the form of MS on maternal caregiving during the early postpartum period and on anxiety- and depressive-like behaviours shortly after weaning. To determine whether behavioural alterations were accompanied by inflammatory changes in the gut-brain axis, the mRNA expression of pro-inflammatory cytokines in the medial prefrontal cortex (mPFC) and colon was quantified.

3.3. Methods

3.3.1 Animals

Eighteen female and nine male C57BL/6N mice, aged 6 to 8 weeks at their arrival, were used for breeding purposes (Charles River Laboratories, Montréal, Canada). All mice were housed in 27 cm x 21 cm x 14 cm polypropylene cages with a cardboard house, a cotton nestlet, and standard bedding, and maintained on a 12-h light-dark cycle, with the light on from 0700 to 1900 h, at 23°C and 63% humidity, with food (Envigo, 2014 Teklad global 14% protein rodent maintenance diet) and water provided ad libitum. The University of Ottawa's Animal Care Committee approved all experimental procedures (#4170), in compliance with the Canadian Council on Animal Care guidelines.

3.3.2 Experimental timeline

The study consisted of two experimental groups, one that experienced MS ($n = 9$) and another that remained undisturbed under standard housing conditions ($n = 9$; Control). After their arrival, mice were housed individually and habituated to their new housing conditions for at least a week. Once this period had elapsed, soiled bedding from a male's cage was placed in the cages of two females for 48 hours to induce estrus, after which the two females and the male were placed in the male's home cage for mating. Upon the detection of a copulation plug, indicating Embryonic Day 0.5 (E0.5), female mice were returned to their home cages. Following the birth of the pups on postnatal day 0 (P0), litters were randomly assigned to the MS or the Control groups. The MS stressor was conducted from P2 to P14, after which all litters remained in standard housing conditions until weaning on P21. Behavioural testing (splash, open field, elevated plus maze, and tail suspension tests) was completed on P22 and P23. Mice were

ethanized on P24, approximately 24 hours after the last behavioural test, for the collection of the mPFC and colon.

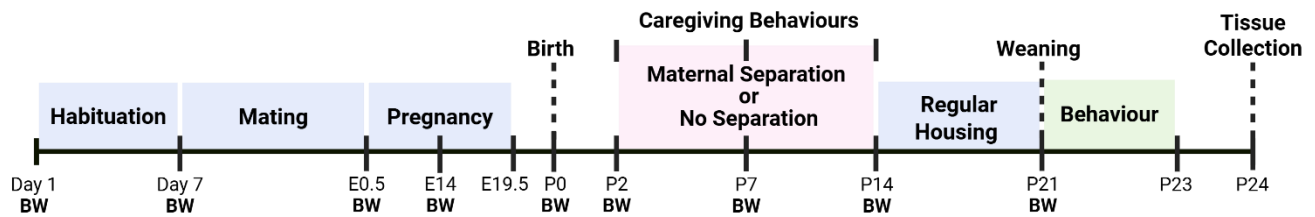


Figure 1: Timeline depicting the sequence of experimental manipulations from mice arrival to post-behavioural sample collection. BW: body weight; P: postnatal day, E: embryonic day.

3.3.3 Maternal separation procedures

Half of the litters underwent MS from P2 to P14, with the dams and their pups being separated for 3 hours each day (9:00 am to 12:00 pm), as described previously (Lippmann et al., 2007; Plotsky & Meaney, 1993). Briefly, pups were placed together in a new cage containing fresh bedding, a nestlet, and a cardboard house within the same housing room, with a 30°C heating pad placed underneath the cage to reduce the risk of hypothermia. Dams were also placed in a new cage containing fresh bedding, a nestlet, a cardboard house, water, and chow, and transported to a different, empty housing room. After each daily MS session, pups were reunited with their dam in their regular home cage until the next session. The other half of the litters remained in standard housing conditions, where dams and their pups always remained in their home cage in the housing room and were only handled for the purpose of recording body weight or weekly scheduled home cage changes.

3.3.4 Body weight measurements

The body weight of the dams was assessed at birth (P0), before the stressor (P2), mid-stressor (P7), at the end of the stressor (P14), and at weaning (P21) to examine the weight trajectory of the dams throughout different phases of the postpartum period. The percentage

change in weight from P2 to P14 was also calculated by dividing the difference between the two values by the body weight at P2 and multiplying by 100.

3.3.5 Behavioural testing

Maternal care behaviour was assessed on P3 and P7. The elevated plus maze and open field tests were administered on P22 to assess anxiety-like behaviours, while the splash and tail suspension tests were performed on P23 to assess depressive-like behaviours, following an order from least stressful to most stressful. Prior to behavioural testing, mice were habituated for approximately one hour in the room where the test would occur.

3.3.5.1 Maternal care behaviour

Assessment of maternal care behaviour occurred approximately 2 hours after the termination of the MS stressor or at equivalent times for non-stressed mice. Following habituation to the testing room, dams were placed in a new cage in a room different from the one where testing was conducted, while their pups remained in the home cage, which was placed in a soundproof box. After 5 minutes, dams were reunited with their pups in their home cage, and their behaviour was recorded on video for another 5 minutes. The latency to first sniff the pups, the time spent sniffing and carrying pups, and the time spent moving nesting material were manually scored by a trained observer unaware of the experimental conditions. A second observer, also blinded to the experimental conditions, scored 25% of the videos to assess interrater reliability. The total time invested in maternal care behaviour was determined by combining the time spent sniffing and carrying pups and moving nesting material.

3.3.5.2 Elevated plus maze

The elevated plus maze was completed on P22 to assess time mice spent in the open (6 cm × 75 cm) and closed (6 cm × 75 cm × 20 cm) perpendicular arms of a black opaque acrylic

plastic apparatus raised 74 cm above the floor. More time spent in the open arms is suggestive of decreased anxiety-like behaviour (Komada et al., 2008; Walf & Frye, 2007). Mice were placed facing the intersection of the open and closed arms and allowed to explore the space for 5 minutes. The test was conducted under room lighting set at approximately 300 lux, and the apparatus was cleaned with a Quato solution between trials. A video-tracking software (EthoVision XT, Noldus) was used to determine the time spent in the open and closed arms and the total distance travelled.

3.3.5.3 Open field test

The open field test was also conducted on P22, at least two hours after the elevated plus maze, to examine the time mice spent at the periphery compared to the center of a 45 cm x 45 cm x 45 cm opaque white acrylic plastic apparatus. Increased time spent in the center is suggestive of decreased anxiety-like behaviour (Seibenhener & Wooten, 2015). Mice were placed in the bottom-right corner of the apparatus and allowed to explore for 10 minutes. The test was completed under approximately 300 lux of light, and the apparatus was cleaned between trials with a Quato solution. Video tracking software (EthoVision XT, Noldus) was used to determine the number of times the mouse entered and the time spent in the center (15 cm x 15 cm) and the corners (10 cm x 10 cm) of the open field, as well as the total distance travelled.

3.3.5.4 Splash test

The splash test was conducted on P23 to examine grooming behaviours (Isingrini et al., 2010). Reduced time spent grooming is reflective of a decreased motivation for self-care behaviours, as seen in human depression (Cathomas et al., 2015), whereas increased grooming time is indicative of hyperarousal (Kalueff et al., 2016). Mice were sprayed twice on their dorsal coat with a 10% sucrose solution, placed in an empty housing cage, and grooming behaviours

were videotaped for 10 minutes. The test was conducted in a room with lighting at approximately 530 lux, using a new cage for each trial. Videos were scored manually by a trained observer unaware of experimental conditions to determine the total time the mouse spent grooming, the time spent in grooming sessions lasting over 3 seconds (termed sustained), and the total number of grooming sessions. An independent observer blinded to the experimental conditions scored 25% of videos to assess interrater reliability.

3.3.5.5 Tail suspension test

The tail suspension test was performed last on P23, at least two hours after the splash test, to examine how long a mouse remains immobile in an inescapable situation. Increased immobility time is thought to be an indicator of passive coping responses to stress, a symptom found in human depression (Cryan et al., 2005; Steru et al., 1985). The tail suspension test was conducted using an apparatus that includes an interface cabinet, user interface software, and load-cell amplifier hardware (Tail Suspension Test Cubicle [SOF-821], Med Associates Inc). Mice were attached to an aluminum bar at the end of their tail with surgical tape to prevent them from escaping the suspended position. Testing was conducted under approximately 100 lux of light, and the apparatus was cleaned with a Quato solution between trials. The mice remained attached to the aluminum bar for 6 minutes, and their movements were recorded. The aluminum bar was attached to a strain gauge, and data were recorded by software (Tail Suspension SOF-821), allowing examination of immobility time.

3.3.6 Brain and intestinal tissue collection

On P24, approximately twenty-four hours after the last behavioural test, mice were euthanized using rapid decapitation. Immediately afterward, whole brains were removed and placed on a cold stainless-steel matrix (2.5 cm x 3.75 cm x 2.0 cm, with slots spaced at

approximately 500 μm) on an ice plate. The brain was sectioned coronally to collect the mPFC, which was placed in nuclease-free tubes positioned on dry ice and subsequently stored at -80°C . In parallel, the GI tract was removed from the abdominal cavity and placed on a nuclease-free surface on top of an ice plate. The whole colon was dissected and placed in nuclease-free tubes on dry ice, and then stored at -80°C .

3.3.7 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from homogenized mPFC and colon samples using the manufacturer's instructions (EZ-10 Spin Column Total RNA Miniprep Kit, Bio Basic Inc). RNA quality was assessed in a representative subset of samples from each group using the Agilent Fragment Analyzer (Stem Core, University of Ottawa). All evaluated samples demonstrated high RNA quality, with an average RNA integrity number score of 9.8. Concentration and purity were then assessed using a NanoDropTM One Spectrophotometer (Thermo Fisher Scientific). Only RNA samples with 260/280 ratios between 1.80 and 2.20 were included. The total RNA was converted into complementary DNA (cDNA) using iScriptTM Reverse Transcription Supermix (BioRad) and a T100 Thermal Cycler (BioRad). cDNA samples were analyzed by RT-qPCR with SsoAdvanced Universal SYBR Green Supermix (BioRad) and a CFX96 Touch Real-Time PCR Detection System (BioRad). Samples were analyzed in triplicate, and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as a reference gene. Fold changes in the mRNA expression of genes of interest in the mPFC and colon were determined using the $-2^{\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001, 2008), relative to the non-stressed group. Primer sequences are included in Supplementary Table 1.

3.3.8 Statistical analyses

Statistical analyses were conducted in R (version 4.5.0), and graphs were plotted in GraphPad Prism (version 10.6.0). All data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test. Potential outliers were assessed using the robust regression and outlier removal (ROUT) method ($Q = 1\%$), but none were identified in either the behavioural or RT-qPCR data. A limited number of samples were excluded from analyses due to either technical difficulties with the equipment used for behavioural testing or poor RNA concentrations and/or purity, and thus the sample size and degrees of freedom may differ between outcomes. A repeated two-way analysis of variance (ANOVA) with Stressor (Control versus MS) as a between-group factor and Time (P0, P2, P7, P14, P21) as the within-group factor was used to compare body weights across postpartum time points. When a significant main effect or interaction was detected, follow-up analyses were conducted using Bonferroni's multiple comparisons test. Differences between the MS and Control groups in all other outcomes were assessed using independent t-tests for normally distributed data, Mann-Whitney U tests for data that did not meet the assumption of normality, or Welch's t-tests for data that did not have the same variance. Spearman correlation analyses were conducted to examine associations between behavioural and biological measures. The alpha level for significance was 0.05 for all analyses.

3.4 Results

3.4.1 Body weight

As seen in Figure 2A, changes in body weight across the postpartum period were comparable in MS and non-stressed dams (Time: $F(3.08, 49.26) = 56.19$, $p < 0.0001$; Stressor and Stressor $\times$ Time: $p > 0.05$). Follow-up comparisons indicate a significant increase in body weight between P0 and P14 ($p < 0.0001$), followed by a decrease from P14 to P21 ($p < 0.0001$), approximately returning to levels observed on P0 ($p > 0.05$) irrespective of the stressor condition. The weight gained between P2 and P14, encompassing the duration of the MS stressor, did not differ between stressed and non-stressed dams (Figure 2B; $p > 0.05$).

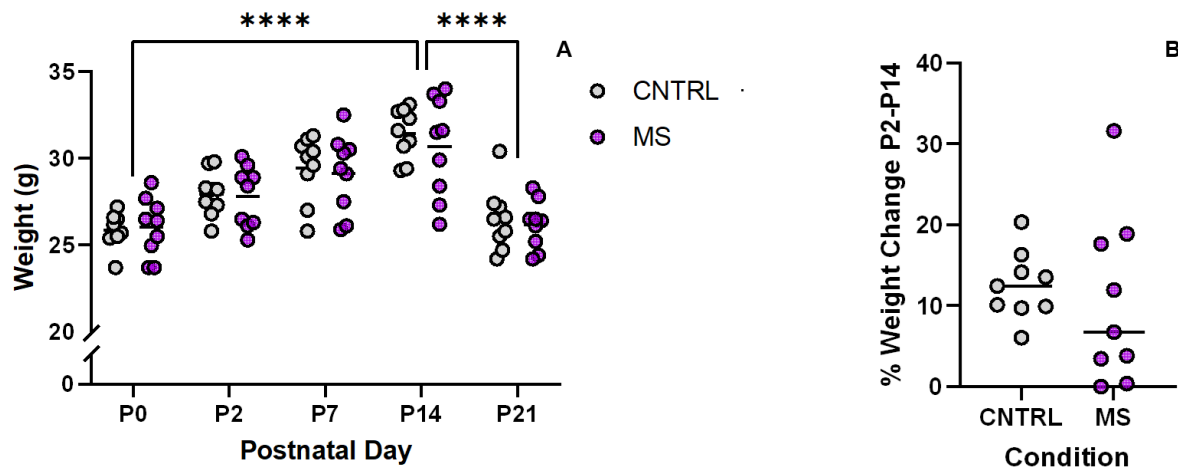


Figure 2: Body weight assessed in non-stressed control (CNTRL) and maternally separated dams (MS). Measures include body weight at time points during the postpartum period, including postnatal day 0 (P0), 2 (P2), 7 (P7), 14 (P14), and 21 (P21) (A), and the percentage change in body weight across the stressor period from P2 to P14 (B). Dots represent individual dams, and horizontal lines represent the median. CNTRL ($n = 9$), MS ($n = 9$). **** $p < 0.0001$ between postnatal days.

3.4.2 Maternal care behaviour

On P3, MS dams took more time to go sniff their pups after a 5-minute separation than non-stressed dams (Figure 3A; $t(10) = 2.828$, $p = 0.018$), an effect that was no longer apparent on P7 (Figure 3E; $p > 0.05$). In contrast, the reductions in the time carrying pups (Figure 3G; $U = 8.500$, $p = 0.020$) and in overall maternal care (Figure 3H; $U = 12.000$, $p = 0.072$) in MS dams were observed on P7 but not on P3 (Figures 3C, D; p 's > 0.05). The time spent sniffing the pups did not differ between MS and non-stressed dams at P3 and P7 (Figures 3B, F; p 's > 0.05).

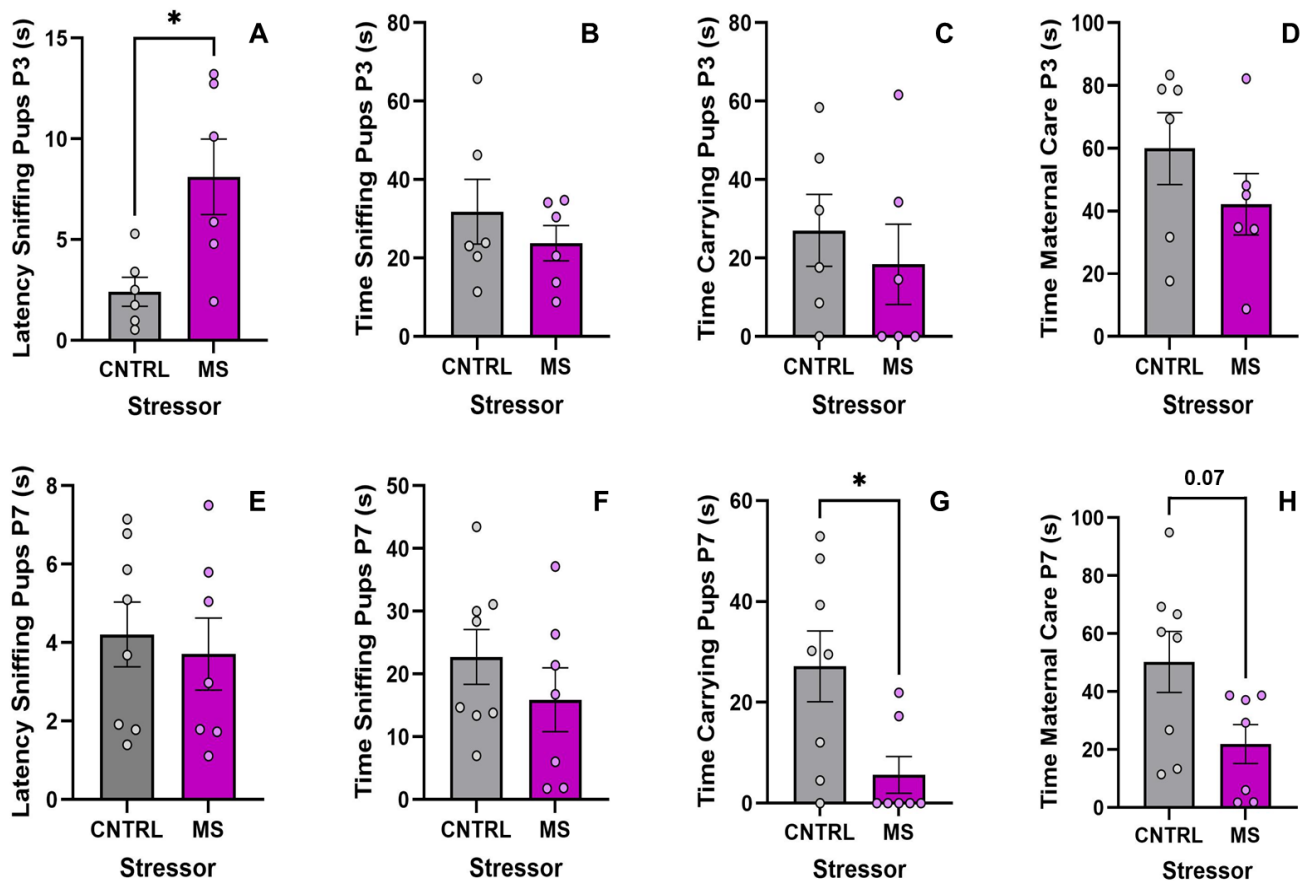


Figure 3: Maternal care behaviours assessed on postnatal days 3 (P3) and 7 (P7) in non-stressed control (CNTRL) and maternally separated (MS) dams upon reunion with their pups following a 5-minute separation session. Behaviours include the latency to first sniff their pups (A, E), the total time spent sniffing their pups (B, F), the total time carrying their pups (C, G), and the total time engaged in maternal care behaviours (D, H). Dots represent individual dams and bar plots,

and error bars represent means $\pm$ SEM. P3: CNTRL ($n = 6$), MS ($n = 6$); P7: CNTRL ($n = 8$), MS ($n = 7$). * $p < 0.05$ relative to CNTRL.

3.4.3 Anxiety-like behaviours

No differences were detected between the MS and non-stressed dams in both tests assessing anxiety-like behaviours. As shown in Figure 4A-C, the time spent in the open arms and closed arms, as well as the total distance moved by the mouse in the elevated plus maze, were similar across groups (p 's > 0.05). Likewise, the time spent in the center and in the corners in the open field, as well as the total distance travelled throughout the arena were unaffected by the MS stressor (Figure 4D-F; p 's > 0.05).

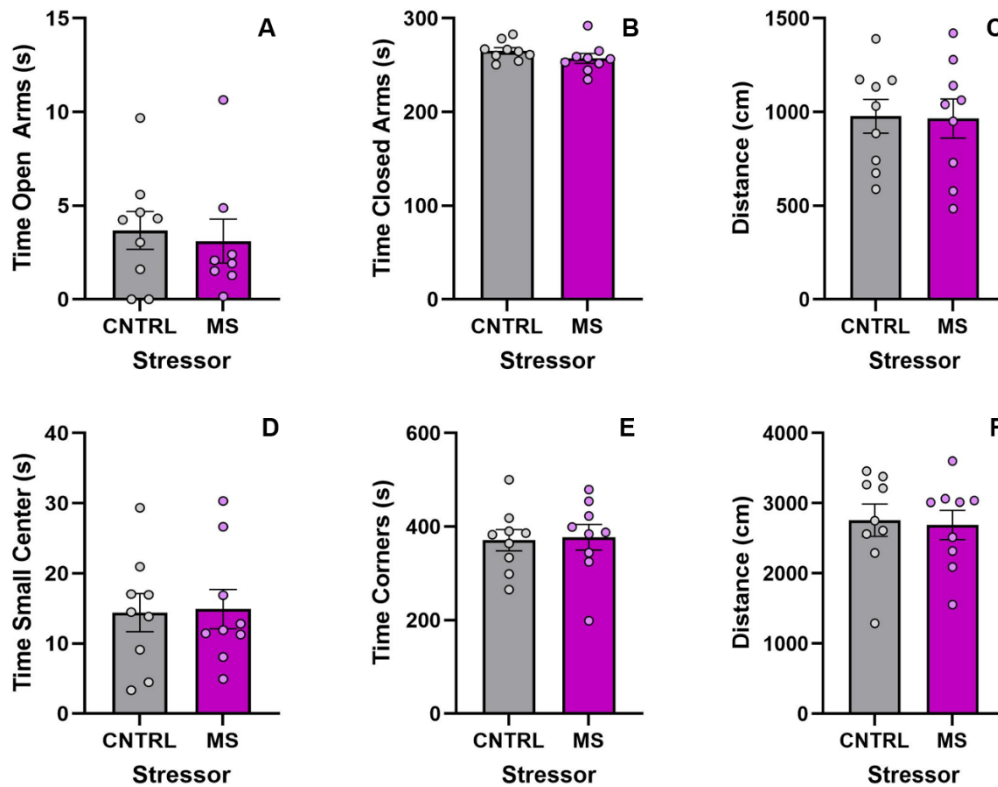


Figure 4: Anxiety-like behaviours were assessed on P22. time in the open arms (A), time spent in the closed arms (B), and the total distance travelled (C) in the elevated plus maze as well as time spent in the center (D), time spent in the corners (E), and total distance travelled (F) in the open field test as well as in non-stressed control (CNTRL) and maternally separated (MS) dams. Dots represent individual dams, and bar plots and error bars represent means $\pm$ SEM. CNTRL ($n = 9$), MS ($n = 9$).

3.4.4 Depressive-like behaviours

In the splash test, the two groups did not differ in the number of grooming bouts initiated and the total time spent grooming (Figures 5A, B; p 's > 0.05). However, MS dams tended to spend more time in sustained grooming bouts, defined as grooming episodes lasting longer than 3 seconds (Figure 5C; $t(16) = 1.947$, $p = 0.0693$). The total time spent immobile during the tail suspension test did not differ between MS dams and non-stressed dams (Figure 5D, $p > 0.05$).

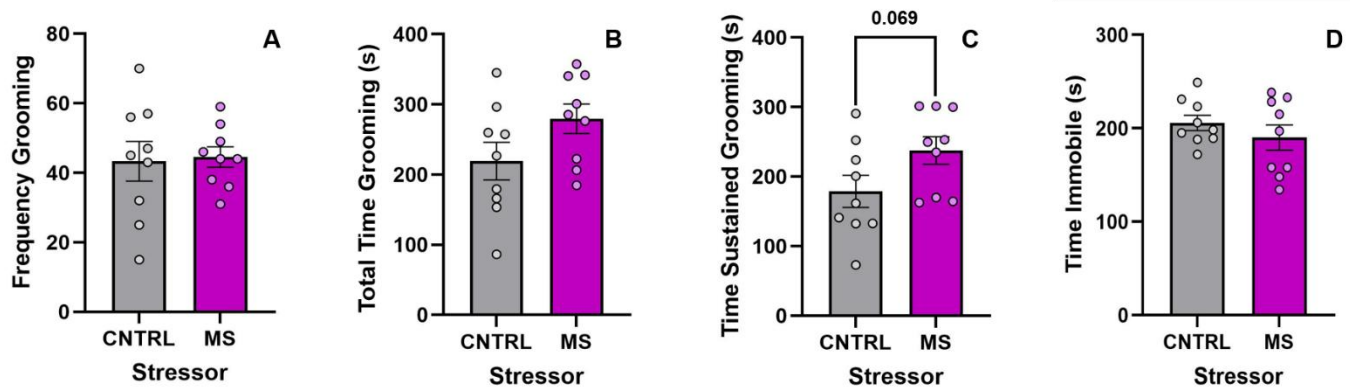


Figure 5: Depressive-like behaviours were assessed on P23. Frequency of grooming episodes (A), total time spent grooming (B), and time engaged in grooming episodes longer than 3 seconds (C) in the splash test, as well as immobility time in the tail suspension test (D), in non-stressed control (CNTRL) and maternally separated (MS) dams. Dots represent individual dams, and bar plots and error bars represent means $\pm$ SEM. CNTRL ($n = 9$), MS ($n = 9$).

3.4.5 Gene expression of pro-inflammatory cytokines in the medial prefrontal cortex and the colon

No differences were observed between the MS and non-stressed groups in the mRNA expression of the pro-inflammatory cytokines IL-6 and TNF- α in the mPFC (Figure 6A, B; p 's > 0.05). In the colon, the expression of IL-6 and TNF- α was not altered by the MS stressor (Figure 6C, D; $p > 0.05$), but MS dams showed a non-significant tendency towards having a lower IL-1 β expression (Figure 6E; $t(14) = 1.849$, $p = 0.0858$).

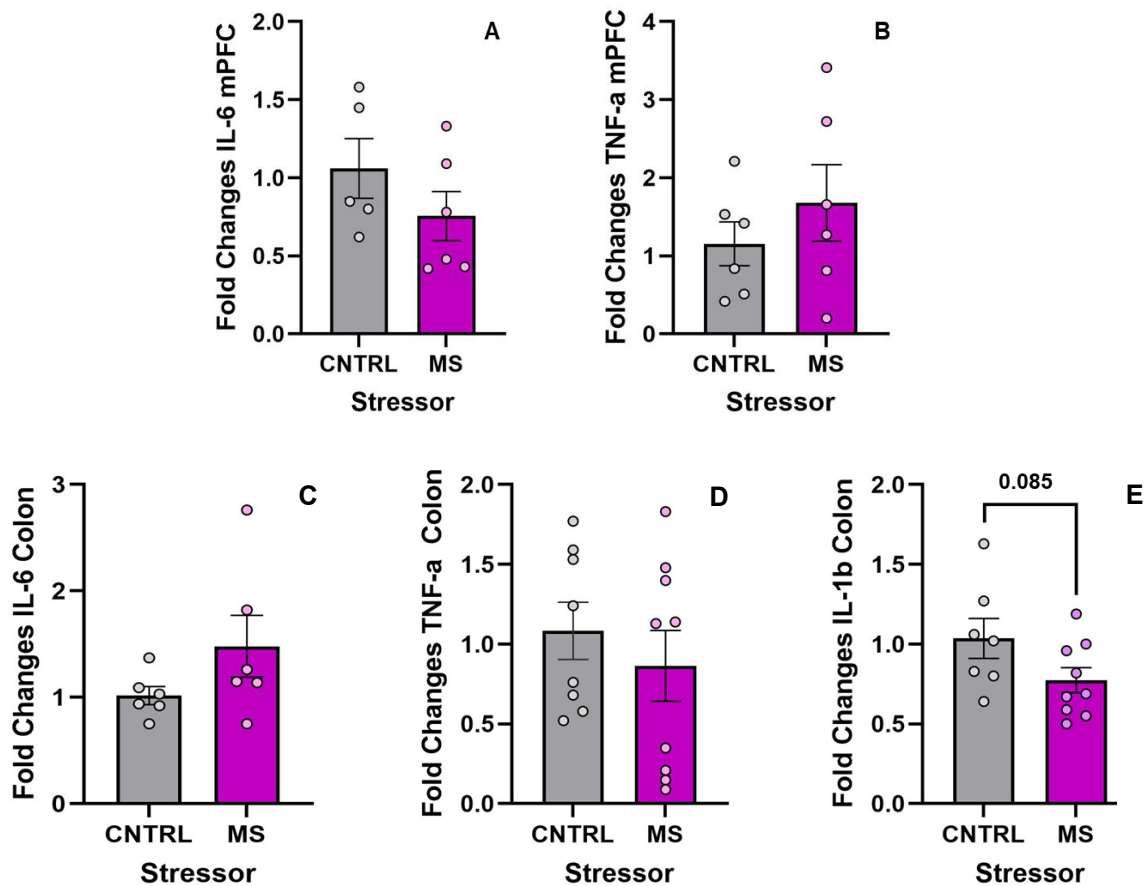


Figure 6: Fold changes in the mRNA expression of interleukin (IL)-6 (A) and tumour necrosis factor (TNF)- α (B) in the medial prefrontal cortex (mPFC) and of IL-6 (C), TNF- α (D), and IL-1 β (E) in the colon of non-stressed control (CNTRL) and maternally separated (MS) dams. Dots represent individual dams, and bar plots and error bars represent means $\pm$ SEM. mPFC: CNTRL ($n = 5-6$), MS ($n = 6$); Colon: IL-6: CNTRL ($n = 6-8$), MS ($n = 6-9$).

3.4.6 Correlations between behaviour and pro-inflammatory cytokine expression in the colon and mPFC

To determine whether behavioural measures were associated with prefrontal and colonic pro-inflammatory cytokine expression among the full sample of dams (including all mice from the MS and Control groups), Spearman correlations were used to assess relationships among behavioural measures in the tail suspension, splash, open field, and elevated plus maze tests, and fold changes in the mRNA expression for IL-6, TNF- α , and IL-1 β in the colon and mPFC. As depicted in Figure 7, strong positive correlations were observed between the expression of TNF-

α in the mPFC and the time spent in total and sustained grooming ($r = 0.72, p = 0.007$ and $r = 0.73, p = 0.006$, respectively). No other statistically significant correlations between behavioural and mRNA expression measures were identified (all p -values > 0.05).

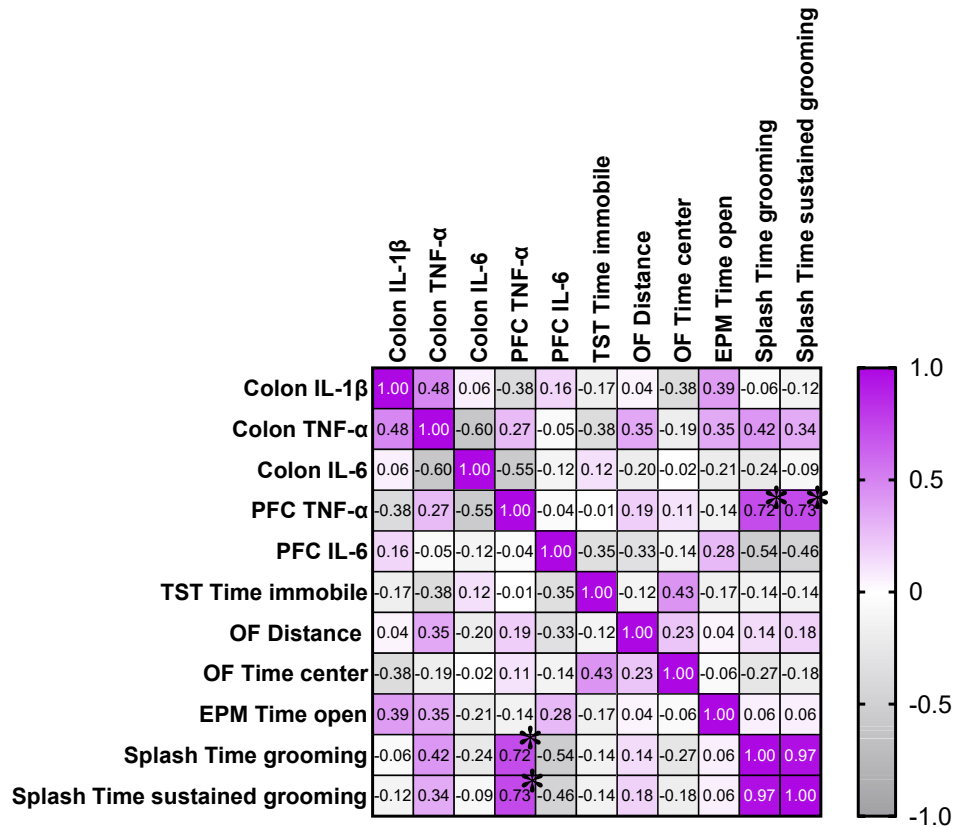


Figure 7: Correlations between behaviours in the tail suspension (TST), open field (OF), elevated plus maze (EPM), and splash tests and fold changes in the mRNA expression of interleukin (IL)-6, tumour necrosis factor (TNF)- α , and IL-1 β in the colon and medial prefrontal cortex (mPFC). * $p = 0.007$ for total grooming time, $p = 0.006$ for sustained grooming

3.5. Discussion

Stress during the postnatal period is a major risk factor in the development of postpartum mental health disorders (Reid & Taylor, 2015; Yim et al., 2015), yet the potential contribution of stress-induced inflammatory activation in the brain and GI tract remains understudied, despite evidence showing the involvement of gut and brain pro-inflammatory increases and barrier disruptions in the development of behavioural disturbances following chronic stressors in non-postpartum contexts (Audet et al., 2011; Nie et al., 2018; Russo et al., 2023; Ménard et al., 2017; Doney et al., 2023). The present study examined whether MS impacted maternal care behaviour as well as anxiety- and depressive-like behaviour and whether these were accompanied by differences in the expression of pro-inflammatory cytokines in the mPFC and the colon. Our findings demonstrate that MS resulted in impairments in maternal care behaviours that differed across the postpartum period, with MS dams taking more time to approach their pups on P3 and less time physically moving them on P7 relative to non-stressed dams. Although anxiety- and depressive-like behaviours and pro-inflammatory cytokines assessed in the mPFC and colon shortly after weaning remained unchanged by MS, higher prefrontal TNF- α was associated with increased grooming time in the splash test.

Maternal care findings suggest that daily separation in the early postpartum period in mice does not result in a general disruption of maternal care behaviour, but rather that specific components of caregiving may differ in their susceptibility to MS at different stages of the postpartum period, in agreement with previous reports (Orso et al., 2018). Generally, disruptions in maternal caregiving behaviours are among the most consistently reported findings in dams following repeated separation from their pups, although the directionality of these changes vary across studies, with both increases and reductions being reported in mice and rats (Mundorf et

al., 2022). Consistent with the increased latency to approach pups observed at P3 in our study, Aggugia et al. (2013), Demarchi et al. (2023), and Maghami et al. (2018) reported that MS-exposed rat dams took more time to retrieve the first pup in the pup retrieval test on P3, supporting the finding that maternal responsiveness towards the pups is altered by MS in the early postpartum period. Consistent with evidence that MS disrupts the initiation of maternal care behaviours beyond P3, Rombaut et al. (2023) found that the time to initiate engagement in maternal behaviour was delayed throughout the postpartum period (P2-P14) in C57BL/6J mice. Notably, studies using rats have also observed increases in maternal care, with MS promoting licking, grooming, and arched-back nursing (de Almeida Magalhães et al., 2018; Maniam & Morris, 2010; Baracz et al., 2020). While it is not entirely clear what may be driving these differences, variations in the duration, location, and postnatal timing of observation sessions used to examine maternal behaviour may, in part, contribute to inconsistencies reported across studies, making it challenging to compare these outcomes directly. Similar to our finding that maternal care impairments vary within the first postnatal week, stressed mouse dams engaged less in maternal care on P2 to P4 but more on P6 and P7 relative to controls (Orso et al., 2018), suggesting that MS may disrupt the normal trajectory of maternal care behaviour. With regard to the temporal variation in maternal care impairments from P3 to P7, it is important to consider that maternal behaviour in rodents is highly organized, with certain behaviours, such as nursing and nesting, varying with the developmental stage of the pups (Weber et al., 2008). As maternal care behaviour adapts to the developmental needs of the pups (Olazábal et al., 2013), the effects of stress on caregiving may differ depending on the postpartum stage at which the behaviour is assessed.

In contrast to the effects observed on maternal care, depressive- and anxiety-like behaviours were not affected by MS, at least when examined shortly after weaning, indicating that the behavioural consequences of MS in the dams may not persist beyond the early postpartum period. These findings are in line with other studies in mice and rats that did not observe alterations in anxiety- (Bölükbas et al., 2020; Stevenson et al., 2009) and depressive-like behaviours (Wu et al., 2021; Aguggia et al., 2013) as a result of MS following weaning, although other studies have reported such effects (Bousalham et al., 2013; Boccia et al., 2007; Maghami et al., 2018). Again, methodological differences may, in part, account for the variability observed in depressive- and anxiety-like behaviours in MS dams across studies, including variation in the length of daily separation, the number of days on which the stressor occurs, the rodent genus and/or strain used, and the type of behavioural test (Alves et al., 2020). It is also possible that MS alone does not result in lasting behavioural alterations but instead increases vulnerability to subsequent stressor exposure. A study conducted by Wu et al. (2021) did not observe depressive- and anxiety-like behaviours at weaning with MS alone, but upon a lipopolysaccharide (LPS) injection as a second “hit”, elevations in depressive- and anxiety-like behaviour were apparent, potentially pointing towards enhanced stress reactivity in the dams rather than lasting overt behavioural effects. Although there is limited evidence on the timing of mental health symptoms during the postpartum period, women appear to be most susceptible to developing depressive and anxiety disorders during the first 6 weeks after birth (Kettunen et al., 2014), with symptom onset in the first 8 weeks being associated with more severe depressive symptoms (Putnam et al., 2017), suggesting that mental health symptoms may be most pronounced during the early postnatal period.

Similar to the behavioural outcomes, MS did not alter the expression of IL-6, IL-1 β , or TNF- α in the mPFC or colon. To our knowledge, changes in pro-inflammatory cytokines following MS in the brain and colon have not been examined previously. Pro-inflammatory cytokine elevations in the cerebrospinal fluid and the plasma have been reported in women with PPD (Sha et al., 2022; Achtyes et al., 2020; Boufidou et al., 2009), but whether postnatal stressors promote these effects remains incompletely understood. The lack of alterations in prefrontal and colonic IL-6, IL-1 β , and TNF- α after weaning is consistent with the absence of anxiety- or depressive-like effects at that time, indicating that MS may not induce prolonged behavioural or physiological consequences in the dam. Given that tissue collection occurred on P24, 10 days following the end of the stressor and three days after weaning had been completed, it cannot be excluded that changes in pro-inflammatory cytokines occurred earlier in the postpartum period but were no longer detectable at that time.

Interestingly, despite the lack of persistent behavioural effects, MS dams exhibited a non-significant trend toward spending more time in grooming episodes of 3 seconds or greater, despite no change in grooming time overall, suggesting that the stressor may have exerted subtle effects on the structure of grooming behaviour. Increases in grooming after acute stressor exposure have been reported in several studies (Stone et al., 2003; Jia et al., 2023; Zhang et al., 2011), which is in line with one of the proposed roles of grooming being an adaptive displacement behaviour involved in de-arousal (Kalueff et al., 2016). While the effects of acute stressors on grooming have been well-characterized in non-postpartum contexts, comparatively less is known regarding the impact of stressors during the postnatal period on these behaviours. Using a chronic social defeat stressor, Denmark et al. (2010) found that measures capturing grooming overall, such as the total duration of grooming and number of grooming bouts, were

not affected by the stressor, but differences were observed in the patterning of grooming, suggesting that chronic stress may affect certain qualities of grooming behaviour rather than its overall presentation. While our study did not evaluate the microstructure of grooming, which consists of a repeated chain of stereotyped movements organized in distinct phases (Kalueff et al., 2016), the trend toward the stressed group spending more time in grooming episodes of 3 seconds or more, despite no overall difference in grooming time, may similarly reflect changes in specific grooming characteristics in the context of MS. Alteration in grooming patterns may be suggestive of stress-related anxiety-like behaviour, as grooming microstructure is disrupted by anxiogenic agents and normalized by anxiolytic compounds (Kalueff & Tuohimaa, 2005).

The strong positive association between prefrontal TNF- α and grooming time measures suggests a potential role for TNF- α in this brain region in modulating grooming behaviours, irrespective of stressor exposure. While the exact mechanisms by which TNF- α could potentially contribute to grooming alterations remain unclear, transgenic mice with a central nervous system-specific overexpression of TNF- α showed increased grooming behaviour, and this was accompanied by reduced immunoreactivity of tyrosine hydroxylase (Aloe & Fiore, 1997), which may suggest the involvement of dopamine in this behavioural phenotype. This is consistent with the observation that drug-induced antagonism of TNF- α in depressed patients results in greater motivation and willingness to exert effort for a reward (Treadway et al., 2024). Given that grooming is in part regulated by the striatum and limbic circuitry involved in motivation, affective state, and motor sequencing (Kalueff et al., 2016), evidence suggesting that TNF- α is implicated in effort-based motivation may further support our finding that TNF- α influences grooming behaviour.

A limitation of this study is that the assessment of depressive- and anxiety-like behaviours was conducted shortly after weaning, approximately 10 days following the completion of the stressor, by which time the postpartum period could have concluded. This was done primarily to avoid introducing the potential confound of additional prolonged separations from the litter during behavioural testing but limited the ability to observe postpartum-specific depressive- and anxiety-like effects. As such, it cannot be excluded that changes in these behaviours may have been present throughout the early postpartum period but had resolved by the time behavioural testing was conducted. Likewise, tissue collection occurred on P24, and it may also be possible that pro-inflammatory cytokine transcription was no longer affected beyond the early postpartum period. Furthermore, maternal care behaviours were evaluated only at P3 and P7 following a 5-minute reunion, thereby limiting the assessment of dynamic changes in maternal caregiving throughout the postpartum period. While this allows observation of acute reunion behaviours, it may not be representative of broader disruptions in maternal care behaviour observed in other studies using longer recordings (Rombaut et al., 2023). Another consideration is that dams were singly housed during the habituation and gestational periods and post-weaning, which in itself can promote anxiety-like behaviour, elevated corticosterone levels, and altered maternal care behaviour (Khayat et al., 2025; McDonald et al., 2023). While single housing during gestation is standard procedure to accurately determine the date and time of parturition as well as the identification of dam-pup units, it cannot be excluded that social isolation during pregnancy may have introduced an additional source of stress for the dams.

3.6 Conclusion

Overall, MS had selective effects on parameters of maternal caregiving that varied by the postnatal day assessed, suggesting dynamic changes in disruptions to maternal care behaviours in

the early postpartum period. Changes in anxiety- and depressive-like behaviours and in pro-inflammatory cytokine expression in the mPFC and colon were not observed, which may be reflective of the methodological implementation of the MS stressor or may be due to transient effects not observed due to the use of a single endpoint. Future studies should examine the temporal progression of maternal care behaviours throughout the postpartum period and employ multiple cohorts to assess whether there are transient changes in affective behaviours and pro-inflammatory cytokine expression in the brain and colon.

Author contributions

VM and MCA designed the experiment. VM and TT conducted the experiment and performed the behavioural, molecular (RT-qPCR), and statistical analyses. VM and MCA interpreted the data and wrote the manuscript, which will be edited by all authors before submission.

Declaration of competing interest

All authors declare that the research work was conducted in the absence of any personal, professional, or financial relationships that could be construed as a conflict of interest.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council (NSERC #RGPIN-2016-06146 Discovery Grant to MCA). The Canadian Institutes for Health Research (Canada Graduate Scholarship – Master's Program to VM), NSERC (Undergraduate Student Research Award to TT), and the Parviz Sabour MSc Scholarship in Nutrition and Mental Health (MSc Scholarship to VM) also supported this research. We would like to thank the Animal Care and Veterinary Service and the Animal Behaviour and Physiology Core at the University of Ottawa.

Chapter 4: Early-life adversity induces changes in palatable food-seeking behaviours and sex-specific changes in the mesolimbic system

Full-Length Research Report

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Conflict of Interest Statement: All authors declare that there are no conflicts of interest.

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4.1 Abstract

Background: Stressors experienced during early life may disrupt the developmental trajectory of several systems, including the hypothalamic-pituitary-adrenal axis and the mesolimbic system, and contribute to mental health disorder vulnerability. Using maternal separation (MS), this study examined the long-term behavioural and mesolimbic consequences of early life stress, as well as sex differences in these effects. **Methods:** C57BL/6N litters were assigned to a Control or an MS condition, which consisted of daily 3-hour separations between dams and their pups from postnatal day 2 (P2) to P14. Food reward-related motivation as well as depressive- and anxiety-like behaviours were assessed on P42 and P43 in female and male offspring, and the nucleus accumbens (NAc) was collected on P44 to determine the expression of dopamine receptor D1 (*Drd1*), *Drd2*, and neurotrophic receptor tyrosine kinase 2 (*NTrk2*). **Results:** Males gained more weight and consumed more food than females post-weaning, irrespective of the stressor condition. MS increased approaches to and time spent with a palatable food reward within an anxiogenic environment in both sexes, whereas the stressor upregulated the expression of *Drd2* and *NTrk2* in the NAc of male mice only. No differences were detected in anxiety- or depressive-like behaviours. **Conclusion:** These findings suggest that reward-related processes may be preferentially affected by MS and that its effects on the mesolimbic system are sex-specific.

Keywords: Dopamine Receptors, Early Life Stress, Mesolimbic System, Nucleus Accumbens, Palatable Food, Reward-Seeking, Sex Differences

4.2 Introduction

Early life stress (ELS) refers to stressful events occurring before the age of 18, including childhood maltreatment, physical, sexual, or emotional abuse, neglect, and witnessing domestic violence, among others (Centers for Disease Control and Prevention, 2024; World Health Organization, 2024). Early life exposure to stressors has been shown to promote the development of depressive, anxiety, and substance use disorders, particularly when the stressor arises from disrupted family functioning, such as abuse and neglect (Kessler et al., 2010). The long-term behavioural and physiological consequences of ELS are thought to result from its disruptive effects on the maturation of several key biological systems, including but not limited to the immune system, the hypothalamic-pituitary axis, the gut-brain axis, and the mesolimbic system (Jena et al., 2020; Nakama et al., 2023; Danese & Lewis, 2017; Agorastos et al., 2019).

Maternal separation (MS) is an established ELS paradigm used in rodents to model the experience of caregiver neglect (Plotsky & Meaney, 1993). Findings related to depressive- and anxiety-like behaviours in the context of MS are heterogeneous, likely attributable to methodological differences in the MS protocols across studies (Tractenberg et al., 2016), with some observing changes in behaviour (Cevik et al., 2023; Kesterling-Ferreira et al., 2021; Bian et al., 2015; Wei et al., 2023; Takabayashi, 2025), whereas others report null effects (Zoicas & Neumann, 2016; Wang et al., 2011; Millstein & Holmes, 2007; Savignac et al., 2011). Exposure to MS has also been shown to alter reward-related behaviours, particularly in the context of ethanol seeking, with the stressor increasing motivation for ethanol consumption (Bertagna et al., 2021), preference for the substance (Rodolpho et al., 2025; Portero-Tresserra et al., 2018) and its intake (Talani et al., 2025). However, whether MS influences motivation for naturalistic rewards, such as highly palatable food, remains understudied.

Limited evidence indicates that MS increases basal intake of highly palatable food in female and male rats (de Souza et al., 2018) and effort-based responding to obtain this type of food in both female and male mice (Benoit et al., 2023). Although these findings suggest that MS alters motivation for a food reward, potential sex differences in these effects remain poorly understood, and food reward seeking has yet to be assessed in an anxiogenic environment. Reward-related behaviour primarily recruits the mesolimbic dopamine system, composed of the ventral tegmental area and the nucleus accumbens (NAc) (Lewis et al., 2021). ELS in humans and in rodent models has been shown to alter the developmental trajectory of the mesolimbic system and interconnected brain regions, including the amygdala and prefrontal cortex (PFC), resulting in lasting changes in reward processing (Novick et al., 2019; Birnie et al., 2020). In particular, adult MS rodents exhibited lower brain-derived neurotrophic factor (BDNF) expression in the medial PFC, as well as reduced dopamine transporter levels, altered dendritic morphology, and decreased dopamine receptor expression in the NAc, with some effects differing between males and females (Wang et al., 2015; Meaney et al., 2002; Romano-López et al., 2016; Sasagawa et al., 2017).

In order to assess the effects of MS on motivation in the context of risk-taking, distinct from previous literature examining reward approach and effort-based responding for a food reward, our study utilized a modified open field task, where a highly palatable food was placed in the center of an open field, which is an anxiogenic environment for mice. We also quantified the expression of genes encoding the dopamine 1 receptor (*Drd1*), dopamine 2 receptor (*Drd2*), and neurotrophic receptor tyrosine kinase 2 (*Ntrk2*) in the NAc to assess whether reward-related behavioural impairments were accompanied by changes in the mesolimbic system. Depressive- and anxiety-like behaviour was also assessed to determine if MS was related to changes in non-

rewarded behaviours. Additionally, all measures were compared between males and females to determine whether the stressor had sex-specific effects on these outcomes.

4.3 Methods

4.3.1 Animals

Eighteen female and nine male C57BL/6N mice, aged 6 to 8 weeks at their arrival, were used for breeding purposes (Charles River Laboratories, Montréal, Canada). All mice were housed in 27 cm x 21 cm x 14 cm polypropylene cages with a cardboard house, a cotton nestlet, and standard bedding, and maintained on a 12-h light-dark cycle, with the light on from 0700 to 1900 h, at 23°C and 63% humidity, with food (Envigo, 2014 Teklad global 14% protein rodent maintenance diet) and water provided ad libitum. The University of Ottawa's Animal Care Committee approved all experimental procedures (#4170), in compliance with the Canadian Council on Animal Care guidelines.

4.3.2 Experimental timeline

The study consisted of two experimental groups, one that experienced MS and another that remained undisturbed under standard housing conditions (Control). After their arrival, mice were housed individually and habituated to their new housing conditions for at least a week. Once this period had elapsed, soiled bedding from a male's cage was placed in the cages of two females for 48 hours to induce estrus, after which the two females and the male were placed in the male's home cage for mating. Upon the detection of a copulation plug, indicating Embryonic Day 0.5 (E0.5), female mice were returned to their cages. Following the birth of the pups on postnatal day 0 (P0), mice were randomly assigned to the MS ($n = 28$) or the Control ($n = 23$) groups, with each experimental condition containing 9 litters. The MS stressor was conducted from P2 to P14, after which all litters remained in standard housing conditions until weaning on

P21. Body weight and food intake were assessed at different times pre- and post-weaning. Behavioural testing (elevated plus maze, modified open field, and tail suspension tests) was completed on P42 and P43. Mice were euthanized on P44, approximately 24 hours after the last behavioural test, for the collection of the NAc.

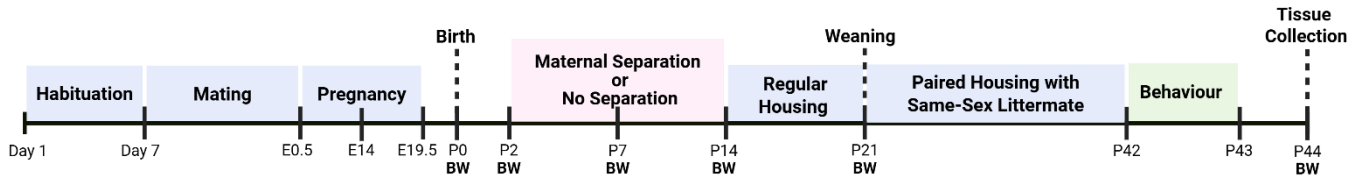


Figure 1: Timeline depicting the sequence of experimental manipulations from habituation to post-behavioural sample collection. BW: body weight; P: postnatal day, E: embryonic day

4.3.3 Maternal separation procedures

Half of the litters underwent MS from P2 to P14, with the dams and their pups being separated for 3 hours each day (9:00 am to 12:00 pm), as described previously (Lippmann et al., 2007; Plotsky & Meaney, 1993). Briefly, pups were placed together in a new cage containing fresh bedding, a nestlet and a cardboard house within the same housing room, with a 30 °C heating pad placed underneath the cage to reduce the risk of hypothermia. Dams were also placed in a new cage containing fresh bedding, a nestlet, a cardboard house, water, and chow, and transported to a different, empty housing room. After each daily MS session, pups were reunited with their dam in their regular home cage until the next session. The other half of the litters remained unmanipulated in standard housing conditions, except for body weight measurements or weekly scheduled home cage changes.

4.3.4 Body weight and food intake

Body weight was assessed on P2, P7, P21, and P44. The measurements for P2 and P7 were recorded without knowing sex, and the percentage change in weight across these time points was calculated by dividing the difference between the two values by the body weight at P2

and multiplying by 100. Food intake was measured over a 24-hour period between P39 and P40. In each home cage containing 2 same-sex littermates, 100 grams of chow (Envigo, 2014 Teklad global 14% protein rodent maintenance diet) was provided at approximately 0900 am on P39, with water provided ad libitum. At 0900 am on P40, the food was removed and weighed to determine the amount of chow consumed over a 24-hour period.

4.3.5 Behavioural testing

The elevated plus maze and modified open field tests were administered on P42 to assess anxiety-like behaviours, while the tail suspension test was performed on P43 to assess depressive-like behaviours, following an order from least stressful to most stressful. Prior to behavioural testing, mice were habituated for approximately one hour in the room where the test would occur.

4.3.5.1 Elevated plus maze

The elevated plus maze was completed on P42 to assess time mice spent in the open (6 cm × 75 cm) and closed (6 cm × 75 cm × 20 cm) perpendicular arms of a black, opaque acrylic plastic apparatus raised 74 cm above the floor. More time spent in the open arms is suggestive of decreased anxiety-like behaviour (Komada et al., 2008; Walf & Frye, 2007). Mice were placed facing the intersection of the open and closed arms and allowed to explore the space for 5 minutes. The test was conducted under room lighting set at approximately 300 lux, and the apparatus was cleaned with a Quato solution between trials. A video-tracking software (EthoVision XT, Noldus) was used to determine the time spent in the open and closed arms and the total distance travelled.

4.3.5.2 Modified open field test

The modified open field test was also conducted on P42, at least two hours after the elevated plus maze test, to examine the behaviour of mice in the presence of a food reward rich in sugars and fats (Hershey's Chipits Reese's Peanut Butter Baking Chips) placed in a metal dish positioned in the center of a 45 cm x 45 cm x 45 cm opaque white acrylic plastic open field. As open spaces are anxiogenic for mice, the modified open field test examines whether they approach a highly palatable food despite the risk posed by the environment (Lockie et al., 2017). More time spent in the interaction zone, defined as the 10 cm x 10 cm space taken up by the food bowl, and more frequent approaches to this zone suggest increased reward-driven motivation (Lockie et al., 2017). To minimize novelty effects, mice were habituated to the food reward two days prior to testing, with approximately 10 peanut butter chips placed in each home cage on P40. Mice were placed in the bottom-right corner of the apparatus and allowed to explore for 10 minutes. The test was completed under approximately 300 lux of light, and the apparatus was cleaned between trials with a Quato solution. Video tracking software (EthoVision XT, Noldus) was used to determine the total time spent and the number of times the mouse entered the interaction zone (10 cm x 10 cm), as well as the total distance travelled within the 45 cm x 45 cm arena.

4.3.5.3 Tail suspension test

The tail suspension test was performed last on P43 to examine how long a mouse remains immobile in an inescapable situation. Increased immobility time is thought to be an indicator of passive coping responses to stress, a symptom found in human depression (Cryan et al., 2005; Steru et al., 1985). The tail suspension test was conducted using an apparatus that includes an interface cabinet, user interface software, and load-cell amplifier hardware (Tail Suspension Test

Cubicle [SOF-821], Med Associates Inc). Mice were attached to an aluminum bar at the end of their tail with surgical tape to prevent them from escaping the suspended position. Testing was conducted under approximately 100 lux of light, and the apparatus was cleaned with a Quato solution between trials. The mice remained attached to the aluminum bar for 6 minutes, and their movements were recorded. The aluminum bar was attached to a strain gauge, and data were recorded by software (Tail Suspension SOF-821), allowing examination of immobility time.

4.3.6 Brain tissue collection

On P44, approximately twenty-four hours after the last behavioural test, mice were euthanized using rapid decapitation. Immediately afterward, whole brains were removed and placed on a cold stainless-steel matrix (2.5 cm x 3.75 cm x 2.0 cm, with slots spaced at approximately 500 μ m) on an ice plate. The brain was sectioned coronally to collect the NAc, which was placed in nuclease-free tubes positioned on dry ice and subsequently stored at -80°C.

4.3.7 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from homogenized NAc samples using the manufacturer's instructions (EZ-10 Spin Column Total RNA Miniprep Kit, Bio Basic Inc). RNA quality was assessed in a representative subset of samples from each group using the Agilent Fragment Analyzer (Stem Core, University of Ottawa). All evaluated samples demonstrated high RNA quality, with an average RNA integrity number score of 9.8. Concentration and purity were then assessed using a NanoDropTM One Spectrophotometer (Thermo Fisher Scientific). Only RNA samples with 260/280 ratios between 1.80 and 2.20 were included. The total RNA was converted into complementary DNA (cDNA) using iScriptTM Reverse Transcription Supermix (BioRad) and a T100 Thermal Cycler (BioRad). cDNA samples were analyzed by RT-qPCR with SsoAdvanced Universal SYBR Green Supermix (BioRad) and a CFX96 Touch Real-Time PCR

Detection System (BioRad). Samples were analyzed in triplicate, and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a reference gene. Fold changes in the mRNA expression of genes of interest in the NAc were determined using the $-2^{\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001, 2008), relative to the non-stressed group of the same sex. Primer sequences are included in Supplementary Table 1.

4.3.8 Statistical analyses

Statistical analyses were conducted in R (version 4.5.0), and graphs were plotted in GraphPad Prism (version 10.6.0). All data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test. Outliers were assessed using the robust regression and outlier removal (ROUT) method ($Q = 1\%$), but none were identified. A limited number of samples were excluded from analyses due to either technical difficulties with the equipment used for behavioural testing or poor RNA concentrations and/or purity, and thus the sample size and degrees of freedom may differ between outcomes. Differences between the MS and Control groups in body weight changes from P2 to P7 (Control versus MS) and in gene expression at P44 (Control versus MS within each sex) were assessed using independent t-tests, as the data were normally distributed for each outcome. When examining the effects of the Stressor (MS versus Control) and Sex (Male versus Female) on body weight changes, food intake, and behavioural measures in the adult offspring, a series of two-way analyses of variance (ANOVA) was used. When significant interactions were identified between Stressor and Sex, follow-up analyses were conducted using Bonferroni's multiple comparisons tests to examine simple effects between groups. The alpha level for significance was 0.05 for all analyses.

4.4. Results

4.4.1 Body weight changes and food consumption

In the early postnatal period, the percentage of body weight gained by the pups between P2 and P7 did not differ between the MS and non-stressed groups (Figure 2A, $p > 0.05$).

However, when examining the percentage of weight gain from weaning at P21 to P44, there was a main effect of Sex, with males gaining more weight than females during this period, regardless of whether they had experienced MS (Figure 2B, $F_{(1,46)} = 32.36$, $p < 0.0001$). Males also consumed more food, irrespective of the stressor condition, when measured over 24 hours between P40 and P41 (Figure 2C, $F_{(1,21)} = 10.93$, $p = 0.0034$).

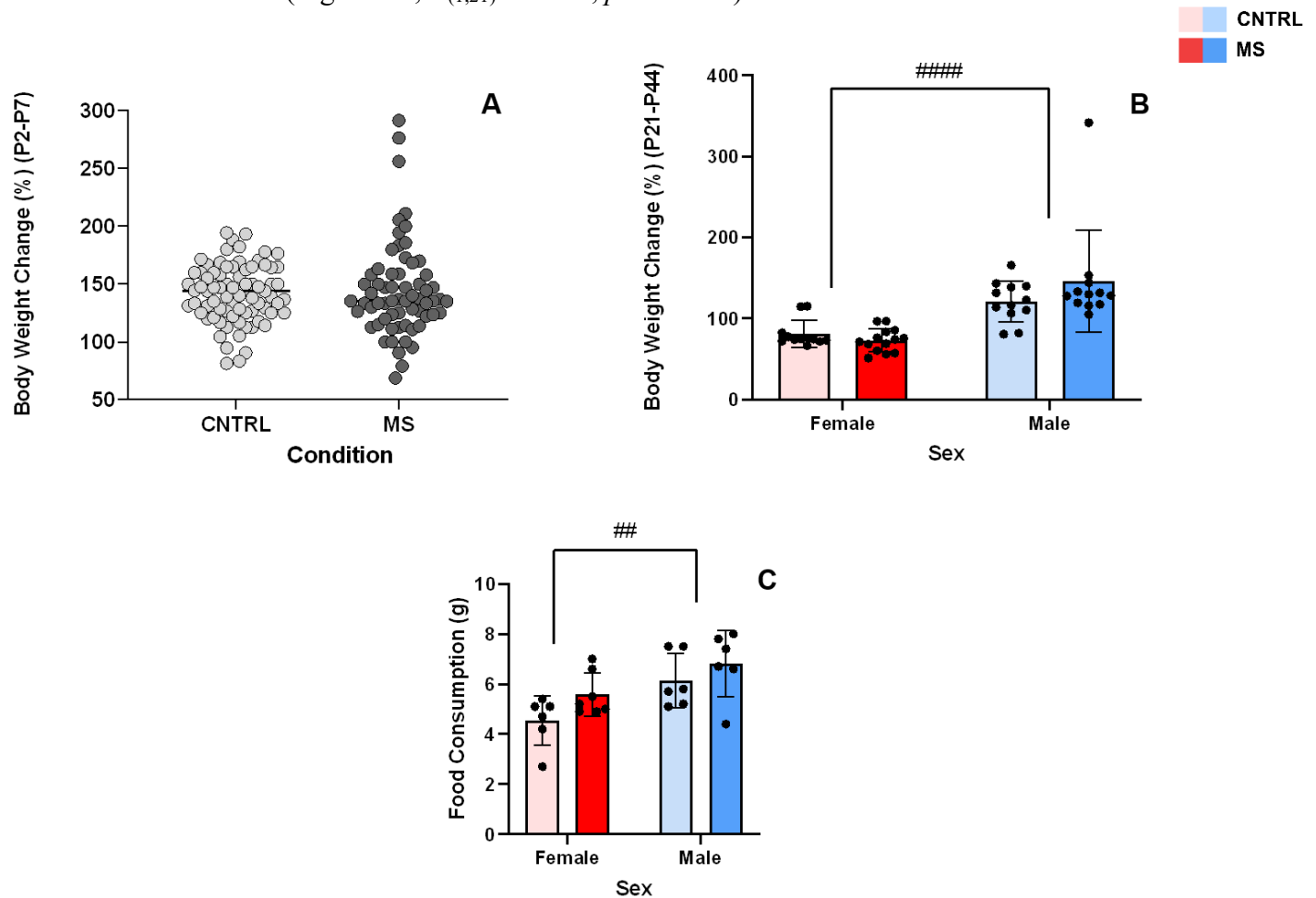


Figure 2: Percentages of body weight change (%) from postnatal day 2 (P2) to P7 (A) and from P21 to P44 (B), as well as food consumption in grams over 24 hours from P40 to P41 (C) in male and female offspring. Bars represent mean $\pm$ SEM. For body weight change, each dot represents

the recorded weight of an individual mouse (A, B). For food intake, each dot represents the food intake per cage, representing the intake of 2 same-sex siblings within the cage (C). Body weight P2-P7: CNTRL ($n = 24$); MS ($n = 26$). Body weight P21-P44: Female CNTRL ($n = 12$), Female MS ($n = 14$), Male CNTRL ($n = 12$), Male MS ($n = 12$). Food intake: Female CNTRL ($n = 6$), Female MS ($n = 7$), Male CNTRL ($n = 6$), Male MS ($n = 6$). $##p < 0.01$ and $####p < 0.0001$, relative to females (main effect of Sex).

4.4.3 Modified open field test

The MS stressor and sex had independent effects on the behaviours assessed in the modified open field test. As shown in Figure 3A and B, MS offspring spent more time with the food reward located in the center of the arena ($F_{(1,45)} = 12.01$, $p = 0.0012$) and approached the interaction zone, which is comprised of the area covered by the 10 x 10 cm food dish, more frequently ($F_{(1,46)} = 8.515$, $p = 0.0054$) compared to non-stressed controls, irrespective of their sex. As well, female offspring spent more time with the food reward (Figure 3A, $F_{(1,45)} = 7.781$, $p = 0.0077$) and travelled a greater distance in the arena (Figure 3C, $F_{(1,46)} = 18.43$, $p < 0.0001$) compared to males, irrespective of whether they experienced MS.

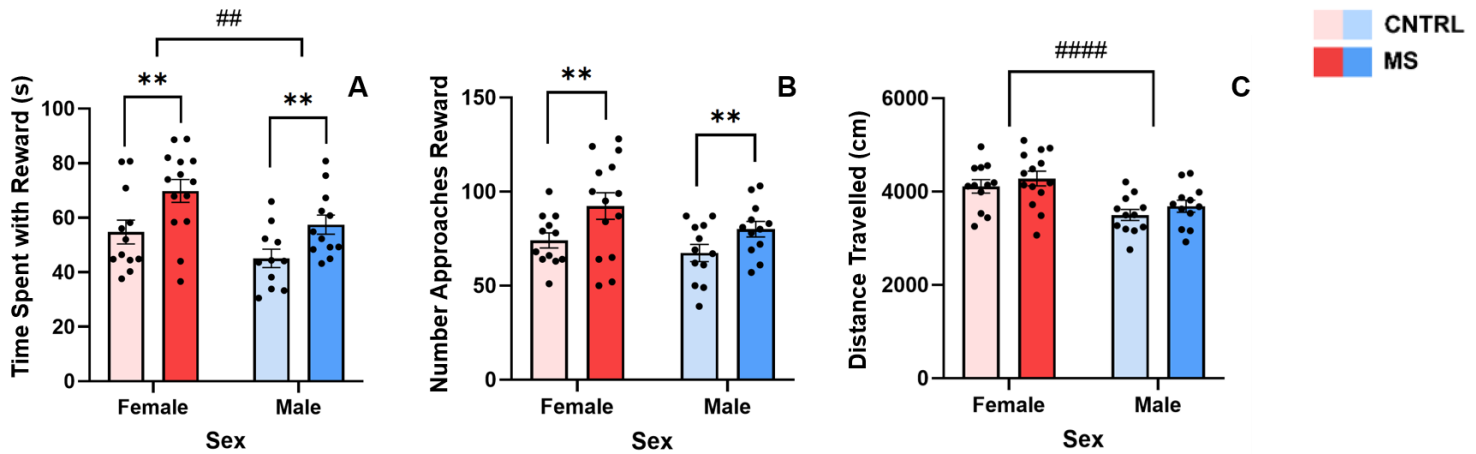


Figure 3: Time spent with the food reward (A), number of times reaching the food reward (B), and total distance travelled in the open field in centimetres (C) in the modified open field test in non-stressed control (CNTRL) and maternally separated (MS) pups. Dots represent individual mice, and bar plots and error bars represent means $\pm$ SEM. Female CNTRL ($n = 12$), Female MS ($n = 14$), Male CNTRL ($n = 12$), Male MS ($n = 12$). $**p < 0.01$, main effect of Stressor; $##p < 0.01$ and $####p < 0.0001$, main effect of Sex.

4.4.4 Other behaviours

As shown in Figure 4, the time spent in the open and closed arms and the distance travelled in the elevated plus maze did not differ between groups, with no main effect of Stressor or Sex or their interaction being identified as non-significant across all measures (Figure 4A-C, p 's > 0.05). Likewise, the time spent immobile in the tail suspension test was not affected by the MS stressor or sex, and no interaction between Stressor and Sex was observed (Figure 4D, p 's > 0.05).

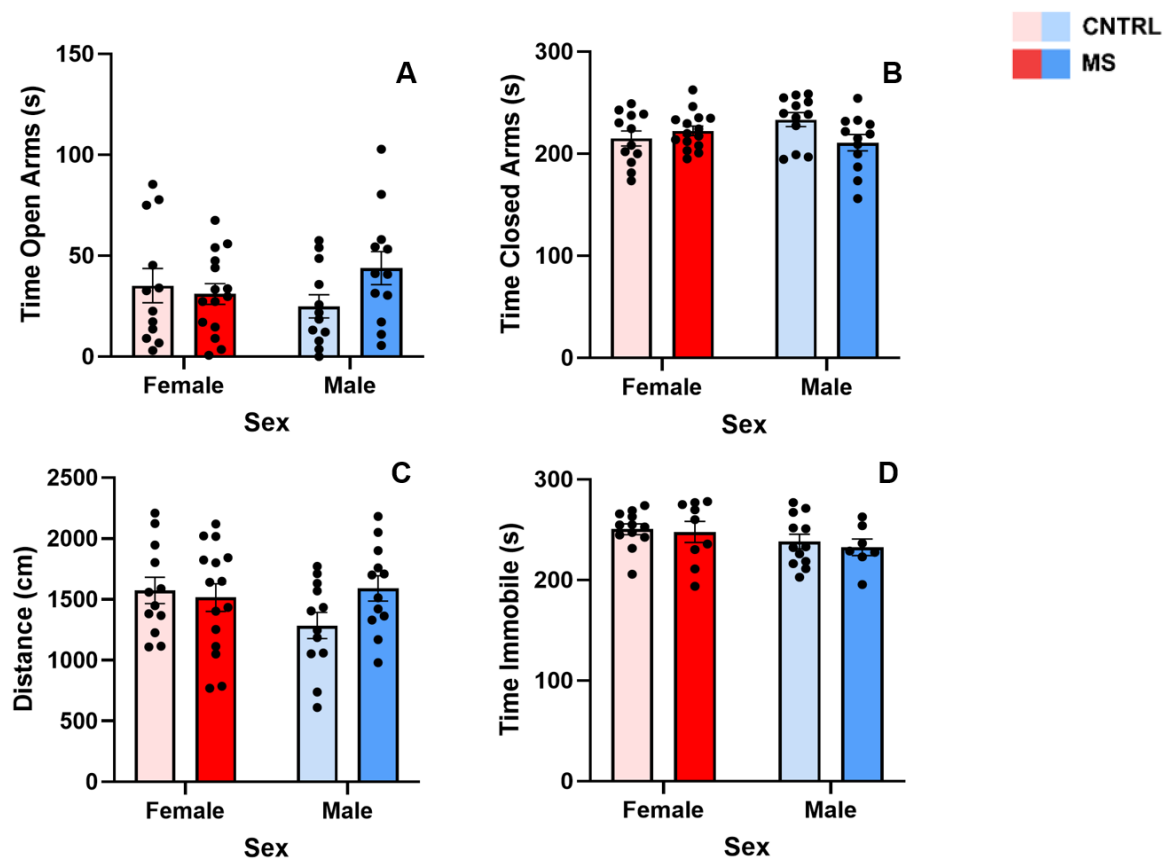


Figure 4: Time in the open arms (A), time in the closed arms (B), and total distance travelled (C) in the elevated plus maze as well as immobility time (D) in the tail suspension test in the non-stressed control (CNTRL) and maternally separated (MS) pups. Dots represent individual mice, and bar plots and error bars represent means ± SEM. Elevated plus maze: Female CNTRL ($n = 12$), Female MS ($n = 15$), Male CNTRL ($n = 12$), Male MS ($n = 12$). Tail suspension test: Female CNTRL ($n = 12$), Female MS ($n = 9$), Male CNTRL ($n = 12$), Male MS ($n = 7$).

4.4.5 Dopamine and BDNF receptors in the nucleus accumbens

The MS stressor had a significant effect on the mRNA expression of *Drd2* in the NAc in male offspring, with stressed males showing a higher expression relative to non-stressed controls (Figure 5A, $t(10) = 2.230$, $p = 0.0498$), while this effect was not observed in females (Figure 5D, $p > 0.05$). Similarly, stressed male pups exhibited a higher expression of *Ntrk2* in the NAc compared to male offspring that were not stressed (Figure 5C, $t(10) = 2.381$, $p = 0.0386$), an effect that was not observed in females (Figure 5F, $p > 0.05$). Finally, the expression of *Drd1* was not affected by MS in either female or male offspring (Figure 5B, E).

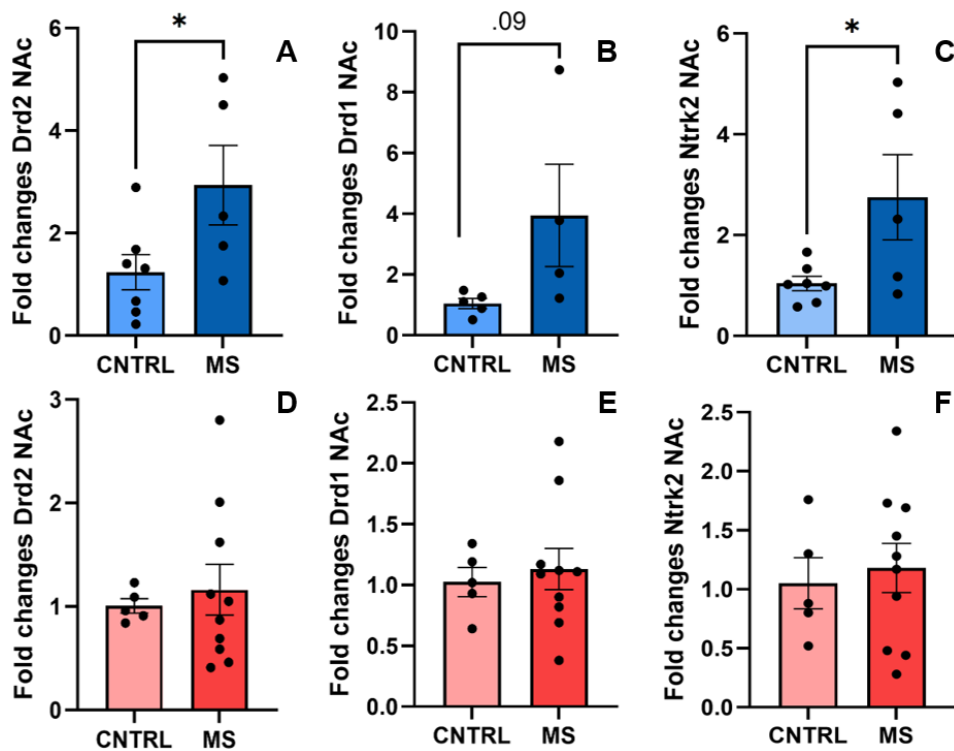


Figure 5: Fold changes in the mRNA expression of dopamine receptor 2 (*Drd2*) (A, D), dopamine receptor 1 (*Drd1*) (B, E) and neurotrophic receptor tyrosine kinase 2 (*Ntrk2*) (C, F) in the nucleus accumbens (NAc) of non-stressed control (CNTRL) and maternally separated (MS) pups. Dots represent individual mice, and bar plots and error bars represent means $\pm$ SEM. *Drd2*: ($n = 5-10$); *Drd1*: ($n = 4-8$) *Ntrk2*: ($n = 5-10$) * $p < 0.05$ compared to same-sex CNTRL.

4.5. Discussion

Early life exposure to stressors has been identified as a key risk factor in the development of substance use disorders (Kirsch & Lippard, 2022; Anda et al., 2006), with evidence suggesting that the increased vulnerability to these conditions could be in part due to stress-induced alterations in reward-related circuitry, particularly the mesolimbic system (Rodrigues et al., 2011). As an animal model of ELS, MS has been shown to induce persistent alterations in reward-seeking behaviours, commonly assessed using drug and alcohol paradigms (Bertagna et al., 2021; Rodolpho et al., 2025; Portero-Tresserra et al., 2018; Talani et al., 2025). However, the effects of MS on reward-seeking in the context of palatable food rewards and how these may differ by sex remain incompletely understood. To address this gap, we examined the impact of MS on the motivation to obtain a food reward in an anxiogenic environment, thereby assessing willingness to take risks to obtain rewards. We additionally assessed depressive- and anxiety-like behaviours and the expression of *Drd1*, *Drd2*, and *Ntrk2* in the NAc to determine whether changes in food reward motivation occurred alongside altered non-rewarded behaviour and mesolimbic dopaminergic and neurotrophic markers.

Our findings demonstrate that MS increased motivation toward a food reward in a risky environment in both females and males without alterations in depressive- and anxiety-like behaviours. In contrast, MS increased the expression of *Drd2* and *Ntrk2* in the NAc of males but not of females. The finding that MS increased motivation toward a highly palatable food reward is consistent with other studies that demonstrated increased palatable food intake in rats (de Souza et al., 2018) and increased motivation to obtain a food reward in female and male mice (Benoit et al., 2023) exposed to MS. Whereas Benoit et al. (2023) assessed effort-based motivation for a food reward on a progressive ratio schedule, to our knowledge, our study is the

first to assess food motivation in an anxiogenic environment in the context of MS. Although effort-based response differs from the risk of entering an anxiogenic open space in terms of the barrier to acquiring the reward (e.g., effort versus risk) (Bailey et al., 2016), these findings suggest that MS may affect cost-benefit decision-making regarding motivation by a highly palatable food reward. Disrupted risk-reward decision-making has also been observed in humans with a history of ELS, with individuals who experienced high levels of ELS showing impairments in a risk-reward gambling paradigm, making poorer-quality decisions, and exhibiting greater impulsivity (Birn et al., 2017). Together with our study results, these animal and human findings suggest that ELS may affect reward-related decision-making when risks are involved.

We also observed that females spent more time with the food reward, in line with other studies (Sinclair et al., 2017), without sex interacting with the stressor, indicating that exposure to MS did not result in sex-specific changes in motivation for the highly palatable food. Our findings are generally consistent with those of a recent meta-analysis by Dory et al. (2025) on post-weaning behaviours following MS exposure, which showed that while the stressor had a robust effect on palatable food intake, sex did not influence this effect. While our study examined the motivation for the food reward, this may suggest an increased hedonic valuation of palatable food irrespective of sex. In contrast to Dory et al. (2025), who observed increased regular chow intake as a result of MS exposure, we did not observe a main effect of stress, but males consumed more food than females. The observation that females travelled more in the open field does not, however, allow us to conclude that locomotion alone explained females' increased time with the food, since sex did not influence the number of food approaches. Furthermore, the absence of sex differences in the distance travelled in the elevated plus maze

suggests that this increased locomotion was specific to the modified open field test. It is also important to acknowledge that the modified open field test shares similarities with the novelty-suppressed feeding test (Bondoff et al., 1988; Santarelli et al., 2003), in which the latency to approach a chow food pellet placed in the center of an open field is measured in food-restricted rodents. As this test primarily assesses approach-avoidance conflict between a novel anxiogenic environment and the drive to consume food in a hungry state, a greater latency to approach the center of the open field despite physiological hunger is commonly interpreted as being representative of increased anxiety-like behaviour, as avoidance of the anxiogenic environment overrides the homeostatic drive to eat (Santarelli et al., 2003). Conversely, in the present study, mice were not food-restricted and did not have a physiological need to eat at the time of testing. Therefore, the increased food-approach behaviour in MS offspring may be more representative of increased reward-seeking or hedonic valuation of the food reward within the context of a novel anxiety-provoking environment.

Despite the robust effects of MS on motivation toward a food reward, no changes in depressive- and anxiety-like behaviours were apparent in the elevated plus maze and tail suspension tests, indicating that the behavioural effects of the MS stressor used in this study were specific to reward-related processes. Overall, similar to the literature pertaining to MS outcomes in dams, findings related to depressive- and anxiety-like effects in the offspring are highly heterogeneous. Our findings are comparable to other studies, with null effects reported following repeated 3- or more-hour separations in terms of anxiety- (Zoicas & Neumann, 2016; Wang et al., 2011; Bian et al., 2015) as well as depressive-like behaviour (Millstein & Holmes, 2007; Savignac et al., 2011). In contrast, several studies reported increased anxiety-like (Cevik et al., 2023; Kesterling-Ferreira et al., 2021) and depressive-like behaviour (Bian et al., 2015; Wei et al.,

2023; Takabayashi, 2025). While it is not possible to determine with certainty what underlies this variability, methodological differences likely contribute to these inconsistent findings. A systematic review by Tractenberg et al. (2016) compared behavioural and physiological outcomes across 96 MS mouse studies, highlighting the heterogeneity of the literature and showing that MS protocols differed in the chronicity/duration and contact of pups while separated (individual versus isolation from littermates), temperature control of pups when away from the dam, separation environment (home cage versus novel cage) and strain, among other variations. As such, methodological differences should be considered when comparing behavioural and physiological outcomes in studies. Interestingly, a study utilizing a highly similar protocol design to the one in this study, consisting of 3-hour separations from P1 and P14 in C57BL/6J mice, found that MS did not affect depressive- and anxiety-like behaviours in the elevated plus maze, splash, or open field tests but that the stressor increased ethanol intake on P28. Together, these findings suggest that under comparable conditions, MS may selectively affect reward-related behaviour without observable changes in anxiety- and depressive-like behaviours. Further reinforcing the reward-related behavioural findings of this study, selected mesolimbic markers, including *Drd2* and *NTrk2*, were upregulated in the NAc of stressed males, with no differences in *Drd1*. Our data are in alignment with those of Benoit et al. (2023), who observed that MS increased effort-based responding for a highly palatable food and that there were transcriptional changes associated with glutamatergic and GABAergic signalling pathways only in MS males, with computational analysis predicting L-DOPA as being the most significant upstream regulator related to these changes. Collectively, our findings and those of Benoit et al. (2023) provide converging evidence of the effects of MS on sex-specific dopamine-related

signalling in the NAc of males, as well as alterations in motivation for food reward irrespective of sex.

This pattern has also been observed in other brain regions, with dopamine receptors D1 and D2 being selectively upregulated in the brainstem of males separated in the dark cycle, suggesting a contribution of both sex and circadian rhythm to dopamine receptor alterations, while palatable food intake was higher in the stressed group independent of sex and separation timing (de Souza et al., 2018). The dopamine D2 receptor has been implicated in reward-related impulsive behaviour and in the regulation of feeding, and its disruption has been associated with compulsive eating (Kim et al., 2018; Baik, 2013). Given the observed upregulation of the D2 receptor in the current study, it is possible to think that this receptor may be a mesolimbic system alteration contributing to changes in motivation for a highly palatable food reward. It has also been shown that TrkB-BDNF expression in D2-expressing medium spiny neurons in the NAc contributes to stress susceptibility following chronic social defeat stress (Pagluisi et al., 2022). As such, the simultaneous upregulation of *Drd2* and *Ntrk2* may reflect an interconnected mechanism influenced by MS stress. Potentially, the lack of change in *Drd1* expression may indicate that the MS stressor's effects on the NAc are receptor-specific. Taken together, these findings demonstrate that MS during the early postnatal period induces sex-specific changes in components of the mesolimbic system.

A limitation of this study is that behavioural testing was conducted at a single time point on P42 and P43. Therefore, it cannot be excluded that alterations in depressive- and anxiety-like behaviour were transient but were no longer present at P42 or that they could have developed afterward during adulthood. The assessment of *Drd1*, *Drd2*, and *NTrk2* was also conducted based on tissue collected on P44, thereby preventing examination of dynamic changes in

behavioural and physiological alterations in the offspring. Additionally, only one test was utilized to assess motivation for a food reward. While our study introduces a novel aspect to the MS literature by using the modified open field test, it is not possible to determine whether the increased food reward approach is due to a general increase in reward responsivity to food or reflects altered valuation of risk versus the motivation to obtain the food. Comparing the modified open field test to another test that does not introduce a cost (e.g., effort or risk), as well as to general palatable food intake, would make it possible to distinguish whether the MS stressor results in global disruptions of motivation or whether this is a reflection of reward-motivation in the context of risk-taking.

4.6 Conclusion

Collectively, the findings of this study suggest that exposure to MS preferentially affected reward-driven food-approach behaviour in the modified open field task, with sex differences in certain parameters. Specifically, females travelled a greater distance and spent more time with the food reward compared to males, irrespective of the stressor condition. Sex differences were also observed in the expression of *Drd2* and *Ntrk2* in the NAc, with their expression being elevated in males relative to same-sex non-stressed controls, but no differences were observed between stressed and non-stressed females. Depressive- and anxiety-like behaviours were not observed at the time behavioural tests were conducted on P42 and P43, which may reflect the specific methodology used in this study or transient effects at earlier or later timepoints that were not captured by the timing of behavioural testing and endpoint used in this study. Future studies should use more than one behavioural test related to motivation for a food reward to further assess which dimension of reward-related behaviour is affected by the

stressor, and multiple cohorts to assess how the stressor's effect on behavioural and neurobiological outcomes may vary over time.

Author contributions

VM and MCA designed the experiment. VM and TT conducted the experiment and performed the behavioural, molecular (RT-qPCR), and statistical analyses. VM, TT, and MCA interpreted the data and wrote the manuscript, which will be edited and approved by all authors before its submission.

Declaration of competing interest

All authors declare that the research work was conducted in the absence of any personal, professional, or financial relationships that could be construed as a conflict of interest.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council (NSERC #RGPIN-2016-06146 Discovery Grant to MCA). The Canadian Institutes for Health Research (Canada Graduate Scholarship – Master's Program to VM), NSERC (Undergraduate Student Research Award to TT), and the Parviz Sabour MSc Scholarship in Nutrition and Mental Health (MSc Scholarship to VM) also supported this research. We would like to thank the Animal Care and Veterinary Service and the Animal Behaviour and Physiology Core at the University of Ottawa.

Chapter 5: General Discussion

5.1 Discussion

Stress during the early postnatal period, which is considered a sensitive window for both the offspring (early life) and the mother (postpartum), can have particularly potent effects on the development of mental health disorders due to the extensive behavioural and physiological adaptations that characterize these stages (Jamil et al., 2025; Pechtel & Pizzagalli, 2011; Numan & Woodside, 2010). ELS is a well-recognized risk factor in the development of several mental health disorders (Carr et al., 2013), with disruptions in family function such as abuse and neglect being most predictive of disorder development (Kessler et al., 2010). Likewise, psychosocial stress is a consistently reported predictor of mental health disturbances during the postnatal period, including PPD (Yim et al., 2015). Importantly, maternal mental health and child outcomes are interrelated, with PPD being related to disruptions in cognitive and emotional development in the child, and alterations in maternal caregiving thought to contribute to these effects (Slomian et al., 2019). Maternal separation is an animal stress model that simulates neglect in offspring and forced separation stress in dams. As such, MS captures the effects of stress during the vulnerable early life period in offspring and simultaneously the postpartum period for the mother. While the consequences of MS are often studied in terms of its outcomes in dams or pups independently, this thesis assessed the behavioural and physiological consequences of the stressor in both postpartum dams and their offspring during the early developmental period.

Overall, our findings show that MS produced selective and distinct behavioural and physiological disruptions in the dams and offspring. In postpartum dams, MS resulted in time-dependent maternal care alterations: those exposed to the stressor took longer to initiate maternal

behaviour on P3, while on P7 they spent less time transporting pups than non-stressed dams, indicating that impairments in caregiving were not consistent throughout the postpartum period. In contrast, MS did not induce lasting behavioural alterations in dams, as evidenced by the absence of depressive- and anxiety-like behaviours after weaning and by no changes in the expression of IL-6, IL-1 β , and TNF- α in the colon and mPFC. In young adult offspring, MS increased motivation to obtain a food reward in an anxiogenic context in both sexes and in males upregulated *Drd2* and *Ntrk2* expression in the NAc. Similar to the dams, no depressive- or anxiety-like effects were observed in MS offspring. One overarching conclusion from these data is that MS did not result in any generalized behavioural disruptions but instead induced subtle alterations in caregiving in dams during the postpartum period and pronounced changes in food-reward seeking in pups during young adulthood. Potentially, these findings may suggest that some behaviours may be more vulnerable to the MS stressor depending on the sensitive life stage during which it is experienced. In dams, a possible explanation for maternal care behaviours being affected is that the MS stressor specifically challenged their ability to interact with their pups, which is in line with other studies conducted in rats (de Almeida Magalhães et al., 2018; Maniam & Morris, 2010; Baracz et al., 2020), including those demonstrating a delay in initiating maternal care behaviour on P3 (Aggugia et al., 2013; Demarchi et al., 2023; Maghami et al., 2018). Maternal care behaviours in rodents are, in part, maintained by a range of cues from the pups, including ultrasonic vocalizations emitted by the pups and olfactory cues (Smotherman et al., 1978; McRae et al., 2023). Even outside the context of postpartum physiological adaptations, exposure to pups in virgin female rats induced maternal care behaviours such as crouching, licking, and grooming (Martín-Sánchez et al., 2015; Seip & Morell, 2008), suggesting that physical interaction with pups is a crucial factor in initiating and maintaining maternal care

behaviour, independently of hormonal, neurobiological, and immune changes normally occurring during the perinatal period that prime the maternal brain for caregiving. As such, the stressor-induced disruption of maternal care behaviour in the current study could reflect the impact of repeated interruptions to reciprocal interactions between the dam and her pups, which are important for maternal caregiving in the postpartum period. Parallels can be drawn with evidence from the human literature supporting the notion that stress may interrupt caregiving behaviours. For example, breastfeeding is a maternal behaviour that is highly sensitive to infant-related cues, in which the baby's saliva and suckling can influence breast milk production and composition, and alter maternal immune responses and hormones in response to infant cues (Guillén-Morales et al., 2025). It has been shown that higher levels of psychological distress are related to breastfeeding impairments, including delays in secretory activation (Nagel et al., 2021), linking stress to delays in maternal responsiveness.

Importantly, following weaning, there were no changes in anxiety- or depressive-like behaviours, potentially indicating that the effect of MS in dams is transient, primarily affecting maternal caregiving. However, these findings should be interpreted in the context of the experimental paradigm utilized in this study, given that the literature on depressive- and anxiety-like behaviour is heterogeneous and some studies in rats have reported these behaviours being present at weaning (Bousalham et al., 2013; Boccia et al., 2007; Maghami et al., 2018), with methodological differences potentially contributing to this variability (Mundorf et al., 2022). The lack of depressive- and anxiety-like behaviour after weaning may also help explain why no changes were observed in IL-6, IL-1 β , and TNF- α expression in the mPFC and colon. Given animal and human evidence that pro-inflammatory cytokines in the bloodstream and brain are associated with depressive- and anxiety-related outcomes, within and outside of the postpartum

context (Pandey et al., 2019; Sha et al., 2022; Achtyes et al., 2020; Boufidou et al., 2009), the absence of changes in these cytokines is in line with the lack of affective behavioural alterations after weaning. However, it cannot be excluded that there were transient changes in IL-6, IL-1 β , and TNF- α , and in depressive- and anxiety-like behaviours in stressed dams that were not captured during the behavioural tests and at the time of euthanasia.

In offspring, the MS stressor resulted in an increased motivation to obtain a food reward in an anxiogenic environment, without significant changes in anxiety- or depressive-like behaviour. Additionally, stressed males had increased *Drd2* and *Ntrk2* mRNA expression in the NAc relative to their non-stressed counterparts, whereas no differences were observed in females or in *Drd1* expression in the NAc. These findings suggest that the effect of MS on pups may be selective for specific behavioural processes and induce sex-specific changes in the NAc. Potentially, systems involved in motivation and reward are particularly susceptible to disruption by ELS. The mesolimbic system, including the ventral tegmental area (VTA) and NAc, and interconnected brain regions, including the amygdala and PFC, begin their development during gestation and continue their maturation from the early postnatal period and into adolescence (Birnie et al., 2020; Galvan, 2010). As this system is highly plastic throughout development, exposure to stress during this period may disrupt its maturation, resulting in lasting changes in reward processing (Novick et al., 2019). Animal and human studies have shown that ELS may influence developmental trajectories through changes in dopaminergic signalling, connectivity between components of the mesolimbic system, altered synaptic plasticity in key regions such as the NAc, and epigenetic modifications, among others (Birnie et al., 2020; Hanson et al., 2021). In our study, we observed MS-related physiological alterations only in males, suggesting that the neurobiological consequences of ELS may differ between sexes. While the precise

neurobiological mechanisms underlying these sex-specific responses remain unclear, they are thought to arise in part from interactions among gonadal hormones, connectivity, neurotransmitters, receptors, and regulatory factors within reward-related circuitry (Turner et al., 2025; Orsini et al., 2022). Evidence for sex-specific neurobiological responses to ELS has been demonstrated by other studies. In particular, when examining the transcriptional changes across several components of reward circuitry following an early life stressor consisting of MS and limited bedding, Peña et al. (2019) found that transcriptional changes in markers of plasticity were increased in the male NAc, but not the female NAc or VTA and PFC in either sex, suggesting some of the changes induced by ELS may be specific to sex and brain regions. Similarly, Benoit et al. (2023) reported transcriptional changes following MS in the NAc of male mice associated with glutamatergic and GABAergic signalling pathways, with the precursor of dopamine (L-DOPA) predicted to be the most significant upstream regulator. Collectively, these findings are consistent with the NAc being a region with increased vulnerability to the consequences of ELS.

The behavioural alterations observed following MS also provide important insight into the effects of ELS on reward processing. The modified open field, as described in Lockie et al. (2017), involves placing palatable food, in this case sweetened peanut butter chips, in the center of the arena of the open field test. As open spaces are anxiogenic for mice, this test creates an approach-avoidance conflict, assessing the motivation towards the food against the risk of entering an exposed environment. To our knowledge, this is the first study to examine the effects of MS on the motivation for a highly palatable food in an anxiogenic environment. This provides novel insight into how ELS influences reward-related decision-making in the context of a naturalistic reward. Previous studies have demonstrated that exposure to MS influences

responsiveness to food reward, particularly in the context of the sucrose preference test (Novick et al., 2019; Amiri et al., 2016; Hui et al., 2011; Sadeghi et al., 2016). However, while the sucrose preference test assesses the perceived hedonic value of the reward, it does not evaluate motivation to obtain a food reward when competing factors, such as effort or risk, are present. A study by Benoit et al. (2023) found that effort-based motivation is affected by MS, as evidenced by performance on the progressive ratio task, with increasing amounts of effort being required to obtain the food reward. It was demonstrated that mice exposed to MS had greater motivation than those not stressed to obtain highly palatable milk when high effort was required (Benoit et al., 2023). Interestingly, these effects were specific to C3H/HeN mice but were not observed in C57BL/6J mice, pointing to strain-specific differences in susceptibility to reward-related behavioural changes following MS. Our study extends the literature by demonstrating that MS also alters reward-seeking behaviour for food under conditions of approach-avoidance conflict, in which approaching the highly palatable food entails overcoming the risk of crossing into an open space. Together, findings on reward responsiveness, effort-based motivation, and reward seeking in the presence of risk show that MS alters multiple dimensions of food reward processing. In the context of our study, the selective effects on reward-related behaviour without any anxiety- or depressive-like behaviours being observed may suggest that these processes are preferentially affected by the MS stressor. It is also important to acknowledge that variations in experimental methodology across studies may result in a range of behavioural phenotypes due to differences in stress timing/duration, housing conditions, rodent strain used, and separation environment, among other factors (Tractenberg et al., 2016). Disruptions in risk-reward decision-making have also been observed in humans following exposure to ELS. Using a gambling task in which participants had to decide on different options with varying probabilities

of winning and losing, it was found that individuals with high levels of childhood stress made poorer quality choices by placing higher bets on choices with a low probability of winning and made their decision more quickly, suggesting disruptions in the evaluation of risk and a propensity toward impulsive behaviour (Birn et al., 2017). While the involvement of ELS in altering reward processing has been examined in the context of substance use disorders (Kirsch & Lippard, 2023), our findings, together with prior literature, suggest that these effects also extend to naturalistic rewards. Importantly, the behavioural consequences of ELS may also have implications for disorders involving disruptions in food-related behaviours such as eating disorders. Eating disorders such as anorexia nervosa, bulimia nervosa, and binge eating disorder have been associated with a history of ELS, including physical and emotional neglect (Ali & Turgoose, 2025; Amiri & Sabzehparvar, 2025). While it is not clear how ELS contributes to this process, changes in approach-avoidance processing of food stimuli have been shown in individuals with binge eating disorder (Leehr et al., 2016), highlighting that the modified open field task may have translational relevance to food-related psychopathology.

Although this study does not provide causal evidence that maternal care behaviour influences the relationship between MS and offspring outcomes, the concurrent effects of the stressor on caregiving behaviours in dams and reward-related outcomes in offspring suggest that these outcomes may be interrelated through disruptions in mother-offspring interactions. It has previously been shown that variations in key maternal care behaviours, including licking, grooming and arched-back nursing, resulted in differential offspring behavioural phenotypes and physiological measures, where low levels of maternal care were associated with increased HPA axis reactivity, including elevated CRH and ACTH in the paraventricular nucleus, and disturbances in behaviour such as reduced time spent in the center of an open field and increased

closed arm time in the elevated plus maze (Pedersen et al., 2011; Liu et al., 1997; Caldji et al., 1998). A study by Peña et al. (2014) demonstrated that natural variations in licking and grooming resulted in differences in the expression of *Drd1* and *Drd2* in the NAc, increased immunoreactivity of tyrosine hydroxylase in the VTA, and differences in the preference for highly palatable food in the offspring of high and low grooming dams, providing evidence that maternal care behaviour can influence the development of components of the mesolimbic dopamine system and reward-directed behaviour. It has also been demonstrated that gestational stress can alter maternal caregiving and subsequent offspring behaviour, in which previously high-grooming stressed dams exhibited maternal care behaviours similar to those of non-stressed and low-grooming stressed dams, with their offspring exhibiting reduced open-field exploration relative to high-grooming controls (Champagne & Meaney, 2006). Although the specific maternal care behaviours assessed differed from those used in our study, collectively these papers provide evidence that disrupted maternal care is a plausible mechanism by which offspring outcomes may be affected by the MS stressor.

One explanation for how maternal caregiving may influence reward-related outcomes in offspring has been proposed by Opendak et al. (2025), who suggested that early-life caregiving interactions are the primary source of reward appropriate to that developmental stage, through stimuli such as maternal milk, tactile stimulation, and odour, thereby promoting maturation and scaffolding of the mesolimbic system. As a result, disruptions in caregiving and, therefore, in access to stimuli that serve as sources of reward in early life may influence the developmental trajectory of the mesolimbic reward system, providing a potential explanation for how stress-induced disruptions in maternal behaviour may contribute to offspring reward-related outcomes. Although future studies are required to establish a causal relationship between maternal care

behaviour and reward-seeking in offspring following MS, our findings that these disruptions occur concurrently highlight the need for further investigation.

While this study provides novel insights into the effects of MS on the mother-offspring dyad, several limitations should be considered when interpreting these findings. One limitation is that behavioural tests and tissue collection occurred at a single time point. Given that early life and the postpartum period are inherently dynamic, characterized by behavioural and physiological adaptations, it is plausible that MS-related disruptions in affective behaviour and cytokine expression may not have been captured at the time points used in this study. This confirms that, in this context, these behaviours do not persist beyond the postpartum period in the dams and into young adulthood in the pups, but it is not possible to determine whether there were changes during the acute period following the stressor. In particular, for dams, post-weaning was selected as the endpoint to avoid confounding by additional separation periods in the MS group or by introducing separations in the control group. It has been shown that even short separation periods, such as 15 minutes per day, induce changes in both maternal and offspring behaviour (Rombaut et al., 2023). Consequently, it is not possible to know whether changes in depressive- and anxiety-like behaviour or in prefrontal and colonic pro-inflammatory cytokines occurred during the early postpartum period. This is important to consider in terms of translatability given that the risk of postpartum mental health disorders fluctuates throughout the postpartum period, with the first 6-8 weeks following childbirth emerging as a time of increased risk for postpartum depression and anxiety (Kettunen et al., 2014; Putnam et al., 2017). Potentially, this may be mitigated in future studies by using multiple cohorts, with behavioural testing and tissue collection occurring throughout early life and the postpartum period, thereby capturing the evolution of these measures across these sensitive periods. Another limitation of

this study is that the type of maternal care assessment used may not reflect overall maternal care behaviour. Our study used a modified version of the pup retrieval test, in which the dam was removed from the home cage for 5 minutes and then returned to the corner of the cage to examine how maternal care behaviour is affected upon reunion. This allows for the assessment of maternal responsiveness toward her litter following a removal, including behaviours such as latency to first sniff pups, total time spent sniffing, carrying behaviour, and passive contact with pups, among others. However, this test reflects the dam's acute response to a brief separation, not necessarily the overall patterns of maternal care behaviour. For instance, this test does not capture some of the maternal behaviours including licking/grooming and arched back nursing, which are known to contribute to the behavioural phenotype and physiological measures in pups (Pedersen et al., 2011; Liu et al., 1997; Caldji et al., 1998; Peña et al., 2014), and in MS studies examining stress-induced changes in maternal behaviour by using prolonged naturalistic observation have found that these behaviours are altered by the stressor (de Almeida Magalhães et al., 2018; Maniam & Morris, 2010; Baracz et al., 2020; Romabaut et al., 2023). As such, it is not possible to determine, within the context of our findings, whether acute responsiveness is specifically the maternal care behaviour disrupted by the MS stressor, or whether this represents one of several alterations in overall caregiving by dams. Future studies that combine a reunion task with prolonged observation of maternal behaviour would provide a more comprehensive understanding of the impacts of long MS on caregiving.

Finally, the modified open field task is a novel contribution of our study to the MS literature, showing that exposure to separation stress during early life may lead to changes in motivation towards a food reward in an anxiogenic environment. However, given that this test was not compared with other tests related to motivation for a highly palatable food, it cannot be

determined with confidence whether increased motivation to approach the food reward reflects altered naturalistic reward responsiveness, risk-taking, or decision-making in the context of an approach-avoidance conflict. It may therefore be beneficial to conduct the modified open field test alongside other behavioural tasks that examine different aspects of food motivation, such as the sucrose preference test or a progressive ratio task with palatable food.

5.2 General Conclusion

Taken together, this thesis demonstrated that MS, in the form of 3-hour daily separations from P2 to P14, differentially affects mothers and their offspring, with distinct behavioural and neurobiological consequences observed in both. The effects in dams were limited to selective disruptions to maternal caregiving, without long-term consequences for affective behaviour or pro-inflammatory cytokine expression in the mPFC and colon. Although MS did not alter cytokine expression in dams, the positive association between TNF- α in the mPFC and time spent grooming suggests a potential relationship between neuroinflammation and grooming phenotype. In contrast, the offspring showed lasting changes in reward-related behaviour and sex-specific changes in *Drd2* and *NTrk2* expression in the NAc. Examining both dams and pups within the same experiment provides a more comprehensive view of the effects of MS, given its simultaneous disruption of postnatal and early life environments and highlights that stress during sensitive windows may have different consequences depending on the context in which it occurs.

Appendix

Supplementary Table 1 – Primer Sequences

Gene	Sequence
<i>Mus Gapdh</i>	Forward: 5' - GGT CGG TGT GAA CGG ATT TG -3'
	Reverse: 5' - TGC CGT GAG TGG AGT CAT ACT G -3'
<i>Mus Il-6</i>	Forward: 5' - ACG GCC TTC CCT ACT TCA CA -3'
	Reverse: 5' - TGC CAT TGC ACA ACT CTT TTC TC -3'
<i>Mus Il-1β</i>	Forward: 5' - TGC CAC CTT TTG ACA GTG ATG -3'
	Reverse: 5' - GTG CTG CTG CGA GAT TTG AA -3'
<i>Mus Tnf-α</i>	Forward: 5' - CTC AGC CTC TTC TCA TTC CTG C -3'
	Reverse: 5' - GGC CAT AGA ACT GAT GAG AGG G -3'
<i>Mus Ntrk2</i>	Forward: 5' - CAA CCT GCG GCA CAT AAA TTT C -3'
	Reverse: 5' - CAG GAG CAC GTG AAC GGA TTA C -3'
<i>Mus Drd1</i>	Forward: 5' - CTG TCC CAG ATC GGG CAT TTG -3'
	Reverse: 5' - GTC ACT TTT CGG GGA TGC TG -3'
<i>Mus Drd2</i>	Forward: 5' - GAC ACC ACT CAA GGG CAA CT -3'
	Reverse: 5' - TCC ATT CTC CGC CTG TTC AC -3'

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