Method, degree and lineage of prenatal maternal stress: effects on inflammatory and stress marker profiles of reproductive tissues and pregnancy outcomes

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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University of Alberta

Abstract

Prenatal maternal stress (PNMS) is any type of stress that pregnant women and animals experience. It was reported that over 70% of pregnant women experience low to moderate stress, while 6% experience high stress. Growing evidence indicates that PNMS influences maternal and offspring health outcomes. PNMS alters the homeostatic milieu of pregnancy, impairs the maternal-fetal neuroendocrine axis, and increases systemic and gestational tissues' pro-inflammatory mediators. Such changes increase the risk of pregnancy complications, including preterm birth. Adversity during critical periods of embryonic and fetal development may alter the transcriptome and affect gene expression and phenotypes in adulthood. These stress-induced changes can be inherited through paternal and maternal lineages via transgenerational transmission of traits to the offspring and may cause low birth weight, cardio-metabolic dysfunction, cognitive, and behavioural adverse outcomes. However, the molecular mechanisms underlying stress-related adverse pregnancy outcomes and fetal programming are poorly understood.

In this dissertation, I investigated the effects of PNMS on reproductive tissues' inflammatory and endocrine programming of the offspring and the risk of preterm birth across generations. First, rats were subjected to preconceptional and gestational social isolation stress (SIS), and its effects were assessed across four generations. I found that SIS shortened F0 dams' pregnancy duration and reduced the birth weights of female and male offspring. In addition, SIS programmed the offspring's long-term metabolic outcomes and altered uterine inflammatory and stress markers, predominantly inducing an adaptive phenotype through the F1-F3 generations. Interestingly, the stress-induced changes were similar in the two stress paradigms used in the study, where the offspring exposed to ancestral stress (transgenerational group) or cumulative stress (multigenerational group) appeared to build resilience to counteract SIS.

Next, I showed that chronic variable stressors (CVS) - SIS and restraint stress - have immediate, long-term, and transgenerational effects on the offspring. F0 pregnant rats were subjected to CVS from gestational days (GD)12-18 while their F1-F3 offspring were undisturbed. The F1 female and male offspring gave rise to two cohorts: maternal transgenerational prenatal stress (MTPS) and paternal transgenerational prenatal stress (PTPS), respectively. The MTPS study revealed that transmission of

stress occurs through the maternal lineage, altering uterine markers of inflammation and stress through generations without inducing preterm birth or low birth weight. I also observed signs of adaptation to stress in the offspring's uterine biomarkers, suggesting that resilience is passed down to the offspring. Enriched environment intervention was implemented in the F1 generation from weaning until GD20 to dampen stress effects across generations. However, the enrichment intervention produced stress-like effects in the F1-F3 generations, possibly due to the involuntary and unexpected environmental change to a novel cage in which they were not programmed to live. This can be considered positive stress, in which novelty, larger spaces, and frequent exercise may be perceived as stress and cause uterine gene expression changes across generations. Still, it might not produce the adverse health consequences of chronic stress.

In the PTPS study, the effects of CVS were transmitted from F0-stressed mothers to sons and then to grandchildren through the paternal lineage. It was observed that stress altered uterine and testicular inflammatory and stress markers by primarily upregulating female gene expression and decreasing it in the male offspring. Yet, pregnancy lengths remained unchanged in the paternal lineage. CVS also programmed the F2 offspring to higher pre-pregnancy and gestational body weights in females and increased neonatal birth weights in males.

This work confirms that PNMS and resilience are passed on to later generations regardless of the stress type. Stress consistently altered inflammatory and metabolic markers in the reproductive tissues and blood of exposed mothers and their offspring - even in the F3 generation not directly exposed to the insult - suggesting genuine transgenerational inheritance. I also showed that enrichment therapy could be stressful for animals and that its outcomes vary based on the context of the analyzed variables. Additionally, I demonstrated through our three PNMS cohorts that generational programming of stress and housing types depend on the sex and generation of the offspring, stress method, degree, time of exposure, and animal individuality. Finally, I suggest that additional stress hits in future generations might exacerbate the prenatally programmed imbalance of uterine inflammatory and stress-related mediators, predisposing them to preterm birth and other pregnancy complications.

Preface

This thesis is an original work by Nayara Gabriela Antunes Lopes. The research conducted for this thesis forms part of a research collaboration led by Professor David M. Olson at the University of Alberta and Professor Gerlinde A.S. Metz at the University of Lethbridge. The studies were conducted in agreement with the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee (Protocols #1705 and 1715). All data are reported as results in this thesis.

Components of chapter four of this thesis have been published in the International Journal of Molecular Sciences as Lopes, N.A.; Falkenberg, E.A.; Wiley, C.; Patel, V.; Serrano-Lomelin, J.; Fang, X.; Weiler, A.M.; McCreary, J.K.; Metz, G.A.S.*; Olson, D.M.*. Social Isolation Stress Modulates Pregnancy Outcomes and the Inflammatory Profile of Rat Uterus. Int. J. Mol. Sci. 2022, 23, 6169.
Lopes, N.A, Fang, X. designed and planned the molecular experiments; Falkenberg, E.A, McCreary, J.K., Weiler, A.M. designed and planned the animal model, and collected tissues; Lopes, N.A, Wiley, C.; Patel, V. performed the experiments; Lopes, N.A. and Serrano-Lomelin, J. conducted the formal analysis; Lopes, N.A wrote the manuscript; Lopes, N.A., Serrano-Lomelin, J., Metz, G.A.S., and Olson, D.M. revised the manuscript; Metz, G.A.S., and Olson, D.M were the supervisory authors, conceptualized, and designed the study.

Components of chapter five of this thesis are included in a manuscript currently under review at the International Journal of Molecular Sciences. Lopes, N.A.; Ambeskovic, M.; King, S.E.; Faraji, J.; Soltanpour, N.; Falkenberg, E.A.; Scheidl, T.; Patel, M.; Fang, X.; Metz, G.A.S.*; Olson, D.M.*. Environmental enrichment promotes transgenerational programming of uterine inflammatory and stress markers comparable to gestational chronic variable stress.
Lopes, N.A. and Fang, X. designed and planned the molecular experiments; Ambeskovic, M., King, S.E.; Faraji, J., Soltanpour, N., Falkenberg, E.A., and Patel, M. designed and planned the animal model, and collected tissues; Lopes, N.A and Scheidl, T. performed the experiments; Lopes, N.A. conducted the formal analysis; Lopes, N.A wrote the manuscript; Lopes, N.A., Ambeskovic, M., King, S.E., Faraji, J., Soltanpour, N., Falkenberg, E.A., Scheidl, T., Patel, M., Fang, X., Metz, G.A.S.*, and Olson, D.M.* revised

the manuscript; Metz, G.A.S., and Olson, D.M were the supervisory authors, conceptualized, and designed the study.

Appendix A1 has been published as “Lopes, N.A. [1,2]; Falkenberg, E.A.[3]; Wiley, C.[1]; Patel, V. [1]; Serrano-Lomelin, J. [1]; Fang, X. [1]; Weiler, A.M. [3]; McCreary, J.K. [3]; Metz, G.A.S. [1,3*]; Olson, D.M. [1,2,3,4*]. Social Isolation Stress Modulates Pregnancy Outcomes and the Inflammatory Profile of Rat Uterus. Int. J. Mol. Sci. 2022, 23, 6169.” Lopes, N.A. summarized the data and designed the tables.

Dedication

For Lucas Costa,
who supported me from day one, guided me through any difficulties, and loved and cared for me.

Joshua 1:9 “Have I not commanded you? Be strong and courageous. Do not be afraid; do not be discouraged, for the LORD your God will be with you wherever you go.”

Acknowledgements

I am deeply grateful for everyone who accompanied me on this journey. First and foremost, I praise God, the Lord and Savior Jesus Christ, who has never let me down and gave me strength, guidance, and encouragement throughout all these years and during the construction of this thesis. I believe God has been communicating this thesis' findings to alert us that all we do in this life is reflected in the generations to come.

Six years ago, I was a clinical pharmacist at a Maternity Hospital and came across several preterm births. I was always intrigued on the reasoning behind such a high number of premature infants being born. At that time I started to research this topic and began looking into possible research programs to pursue. I was very blessed to find Dr. David Olson's website. I remember telling my husband that his research was super interesting, exciting, and a great fit for what I envisioned working on. I was astonished when Dave replied to my e-mail so quickly. He was extremely supportive and guided me through all the initial steps of moving to Canada and starting graduate school. He has helped shape my scientific development, building my independence and confidence as a scientist. Also, Dave has always been generous with his support and has become a great career mentor. I am so thankful for your belief in me and for accepting me to the Olson lab.

I am also very grateful for my co-supervisor Gerlinde Metz for her mentorship and contributions to these projects. I really appreciate all the hard work and effort you and your team did to benefit these projects. Gerlinde has been supporting and encouraging me throughout the years and making sure I would accomplish the success I always aimed for, even with challenges happening along the way. Thank you so much for letting me be part of your incredible team!

I have been privileged to be surrounded by outstanding scientists and friends in the Olson lab, Davidge's lab, Riddells's lab, Hemming's lab, and Stepheny (ha ha). My special thanks also go to Sheena (lab mom) and Kelycia, my labmates and dearest Canadian friends. They have supported and encouraged me throughout this whole program, and helped me with bench work, math, writing, practicing presentations, and even personal issues. You two have finished shaping the scientist I am today – immensely grateful. It was a pleasure having morning espresso with both of you. I had lots of fun and always learned much from your life experiences. Grad school would not have been the same without you there. I also want to thank Stepheny that has been my friend, advocate, and motivator. She is always optimistic, ready to help in every way, and one of the best scientists I have ever encountered. I really appreciate your support of my scientific work and career development.

I am very thankful to my committee members Dr. Meghan Riddell and Dr. Robin Clugston for their research guidance, support, mentorship, and understanding during these five years. I also want to thank Dr. Sloboda for being my external examiner and Dr. Paige Lacey my internal external examiner.
I want to thank my project students Camille Wiley, Vaishvi Patel, Taylor Scheidl, and Wendy Xu for their help with my research projects, commitment, and hard work. I have grown as a better mentor because of you. A special thank you to Dr. Anna Noga for assisting in preparing my manuscripts for publishing and keeping me on track: I have learned so much from you. Thank you so much! I am also really grateful to recent Olson lab members Carla Sosa and Tania Antunes for all your help during the last year.

I would like to acknowledge and thank the members of the Department of Physiology and Obstetrics and Gynecology at the University of Alberta for always being there to help their graduate students and providing excellent education and financial support. A special thanks to Dr. Funk, Ms. Kim Sawada, Ms. Donna Simpson, Ms. Audrey Lin, and Dr. Kassiri for their support. It was a pleasure being part of both departments and participating in all events you organized over the years, including volunteering in the Physiology Graduate Student Association. Also, the Canadian Institute of Health Research (CIHR) financial support during my degree and maternity leave is much appreciated.

Finally, I am enormously grateful for having my family by my side, ensuring I would never give up and cheering me up. I have no words to thank Lucas, my husband and biggest advocate, friend, companion, and fan. Thank you for being there when I cried and was scared, for being so generous and kind, for your endless support, for making me laugh and enjoy life together, and for endeavouring with me in Canada's winter wonderland. He does everything in his power to see me succeed and be happy. I am so grateful to my daughter Chloe for providing me with the best enrichment experience. I have been extremely at ease, resilient, persistent, and happy during this year beside you. You are the sunshine of my life and a great teacher already. A big thanks to my aunt Sonia, who did everything for me this year. She has taken care of Chloe during the past year, taught her Portuguese, cooked, cleaned the house, and much more to guarantee I finish my program on time. I am so grateful to my cousin Isabela for being so kind, ready to help and always listening to my complaints over the years.

Last but not least, I am grateful to all my YEG friends who have enjoyed life with me. You have supported me in my program, distracted me from my worries, and motivated me to continue strong. A special thanks to Flavinha Rago and Joao Felipe, my grad student friends, who shared all the struggles with me and spent hours revising my research presentations and abstracts over the years. My Ph.D program and this thesis would not have been possible, meaningful, and pleasant without all of you.

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Abbreviations

Activator protein-1 (AP-1)

Adrenocorticotropic hormone (ACTH)

Allostatic load (AL)

Analysis of variance (ANOVA)

Autonomous nervous system (ANS)

Central nervous system (CNS)

Chronic variable stress (CVS)

Complementary deoxyribonucleic acid (cDNA)

Corticosteroid-binding globulin (CBG)

Corticotropin-releasing hormone (CRH)

Corticotropin-releasing hormone receptor 1 (CRHR1)

Corticotropin-releasing hormone receptor 1 (CRHR2)

Cyclooxygenase-2 (COX-2)

Cyclophilin A (Peptidilprolyl Isomerase A or *Ppia*)

Damage-associated molecular patterns (DAMPs)

Developmental origins of health and disease (DOHaD)

Dihydroethidium (DHE)

Deoxyribonucleic acid methylation (DNAm)

Enzyme-linked immunosorbent assay (ELISA)

Ethylenediaminetetraacetic acid (EDTA)

Extracellular vesicles (EVs)

Filial (F)

Gestational days (GD)

Glucocorticoid receptors (GR)

Glucocorticoid response elements (GREs)

Glucocorticoids (GCs)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glycoprotein 130 (gp130)

Hydrochloric acid (HCl)

Hypothalamic paraventricular nucleus (PVN)

Hypothalamic-pituitary-adrenal (HPA)

Hypothalamic-pituitary-gonadal (HPG)

IL-1 receptor 1 (IL-1R1)

IL-1 receptor 2 (IL-1R2)

IL-1 receptor 3 (IL-1R3)

IL-1 receptor accessory protein (IL-1RAP)

IL-6 receptor (IL-6R)

Intergenerational (IG)

Interleukin-1 (IL-1)

Interleukin-1 receptor antagonist (IL-1Ra)

Interleukin-6 (IL-6)

Intracellular IL-1 receptor antagonist (icIL-1Ra)

Lactational day (LD)

Lipopolysaccharide (LPS)

Luteinizing hormone (LH)

Maternal immune activation (MIA)

Maternal transgenerational prenatal stress (MTPS)

Matrix metalloproteinases (MMPs)

Mean fluorescence intensity (MFI)

Membrane-bound glucocorticoid receptor (mGR)

Microribonucleic acids (miRNAs)

Mineralocorticoid receptors (MR)

Mineralocorticoids (MCs)

Mitogen-activated protein kinase (MAPK)

Multigenerational (MG)

Nitric oxide (NO)

NLR family pyrin domain containing 3 (NLRP3)

Non-coding ribonucleic acids (ncRNAs)

Nuclear factor kappa B (NF-κB)

Nuclear receptor subfamily 3 group c member 1 (NR3C1)

Nuclear receptor subfamily 3 group c member 2 (NR3C2)

Oxytocin receptor (OTR)

Paternal transgenerational prenatal stress (PTPS)

Pathogen-associated molecular patterns (PAMPs)

Pattern recognition receptors (PRRs)

Phenyl methane sulfonyl fluoride (PMSF)

Phosphate-buffered saline (PBS)

Postnatal day (P)

Prenatal maternal stress (PNMS)

Preterm premature rupture of membranes (pPROM)

Primordial germ cells (PGC)

Prostaglandins (PGs)

Quantitative real-time polymerase chain reaction (RT–qPCR)

Radio immunoprecipitation assay (RIPA)

Reactive oxygen species (ROS)

Ribonucleic acid (RNA)

Secreted IL-1 receptor antagonist (sIL-1Ra)

Social isolation stress (SIS)

Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Soluble IL-6 receptor-α (sIL-6Rα)

Standard error of the mean (SEM)

Superoxide dismutase (SOD)

Suppressor of cytokine signalling (SOCS)

Sympathetic nervous system (SNS)

Threshold cycle (Ct)

Toll-IL-1-receptor (TIR)

Toll-like receptors (TLR)

Transgenerational (TG)

Tumour necrosis factor-α (TNF-α)

Uterine activation proteins (UAPs)

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1)

11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2)

Chapter 1

General introduction

1.1 The exposome

The nature and nurture debate has long been known in the epidemiological and biomedical fields. Nature denotes the genes and hereditary factors that influence a person's characteristics, i.e., the genome, while nurture relates to environmental variables encountered after birth, i.e., the epigenome [1]. This historical debate seems irrelevant with the current knowledge of the significant contribution of environmental factors to the development of diseases and to shaping a person's phenotype.

To exemplify the complex interactions between the genome and environmental factors, Dr. Christopher Wild first introduced the exposome concept in 2005. The exposome refers to environmental exposures an individual experiences from the prenatal period onwards [2] (Figure 1.1). This concept is essential to address the global burden of diseases whereby environmental exposures play a central role in the development of chronic diseases.

Nongenetic factors cause most diseases and involve interactions between internal physiological processes and environmental factors [3]. The exposome concept was expanded in 2012 to include three categories of nongenetic exposures that overlap and are interconnected, including personal factors, external factors, and biological responses internal to the body [4] (Figure 1.1). Personal factors refer to environmental contaminants, diet, lifestyle factors like smoking and tobacco, infectious agents, medical interventions, and so on. External factors include psychological and mental stress, urban and rural environments, climate, social capital, etc. Lastly, the exposome considers internal processes in the body, such as inflammation, oxidative stress, ageing, metabolism, epigenetics, gene expression, etc. The interplay between internal and external factors may contribute to internal biological responses [4]. Therefore, the exposome has complemented the human genome concept, and the contribution of both is required to understand disease risk in humans [5].

Further amendments were made to the exposome concept to capture the accumulation of external influences on the genome throughout the entire lifespan [3]. They proposed that all human actions, including social interactions, nutrition, breathing, physical activities, and metabolic and cellular processes impose changes on our phenotype. This leads to continuous adaptation and maladaptation to external stimuli over a lifetime, highlighting the influence of cumulative biological responses. Behavioural

and endogenous processes were also incorporated into this broad concept to cover all the environmental influences humans are exposed.

Understanding the synergy between different domains and the genome could provide insights into mechanistic pathways and attribute cause and effect relationships to an exposure-disease association. That way, a better understanding of the environmental risk factors could significantly contribute to prevention strategies to mitigate the adverse effects of diseases.

Figure 1.1: The exposome: interactions between the genome and environmental factors.

Composite of all nongenetic environmental exposures an individual acquires from conception to death. The exposome describes the contributions of the personal, external, and biological response domains to health outcomes. Understanding the interconnection between the three domains helps to create an integrated and multifaceted system equivalent to the human genome and produces more comprehensive environmental exposure data. From Barcelona Institute for Global Health (ISGlobal), 2018 (https://www.isglobal.org/en_GB/-/el-exposoma-comprendiendo-el-efecto-del-entorno-en-nuestra-salud).

1.2 The stress response system and allostatic load

Stress is any internal or external physical or psychological stimulus that disrupts homeostasis and results in a biological response, a ubiquitous and essential process of life [6,7]. Homeostasis is defined as coordinated physiological reactions to establish a dynamic equilibrium of the internal systems to guarantee optimal activity [8]. A stress response is triggered when the body perceives an event as stress to return the body to homeostasis by enhancing cognitive and physical performance and ensuring survival [9]. The stress system consists of coordinated structures integrating various brain regions, neuroendocrine, and immune systems that detect and respond to real or potential threats [10,11]. These systems regulate the stress response through intricate mechanisms between cells, molecules, and receptors [12].

The concept of stress and homeostasis was expanded years later when the allostasis and allostatic load theory was introduced [13,14]. Allostasis is the body's response to stress to maintain stability [13]. The allostasis response is produced by the activation of the central stress response systems: the sympathetic-adreno-medullar axis, secreting epinephrine and norepinephrine, and the hypothalamic-pituitary-adrenal (HPA) axis, secreting glucocorticoids (GCs) [14]. It was proposed that the body continually adapts to external challenges to promote survival and reproduction rather than preserves a constant internal milieu to maintain homeostasis [13,15,16].

Although these physiological adaptations are ideal during acute conditions, they can lead to maladaptive stress responses over prolonged periods of stress [17]. The allostatic load describes the wear and tear on the body following chronic and cumulative exposure to insults over a lifetime [7,18]. Repeated stressful stimulus triggers cumulative allostatic responses by modulating stress mediators that do not completely return to baseline [18] (Figure 1.2). The body adapts to this new baseline, ultimately adjusting and creating a new set-point for future physiological equilibrium. The remaining difference between initial and new thresholds is described as allostatic load or cumulative burden of stress. When chronic stress persists and crosses the new threshold, the individual is susceptible to diseases [16,17] (figure 1.2).

Resilience has a foundation in early life, during which positive experiences build a healthy brain capable of supporting cognitive flexibility and coping with stressful stimuli [19]. The individual traits that

enable flexibility and resilience accumulate throughout life, even though the biological systems are most adaptable early in life. Repeated stressful stimuli over prolonged periods overload the stress system, disrupt the hippocampus and hypothalamus feedback inhibition systems, and increase the incidence of chronic diseases. Chronic stress combined with the individual inability to cope results in allostatic overload [14]. Part of the regulatory responses to ensure physiological equilibrium involve negative feedback mechanisms to contain and prevent an exacerbated stress response [18].

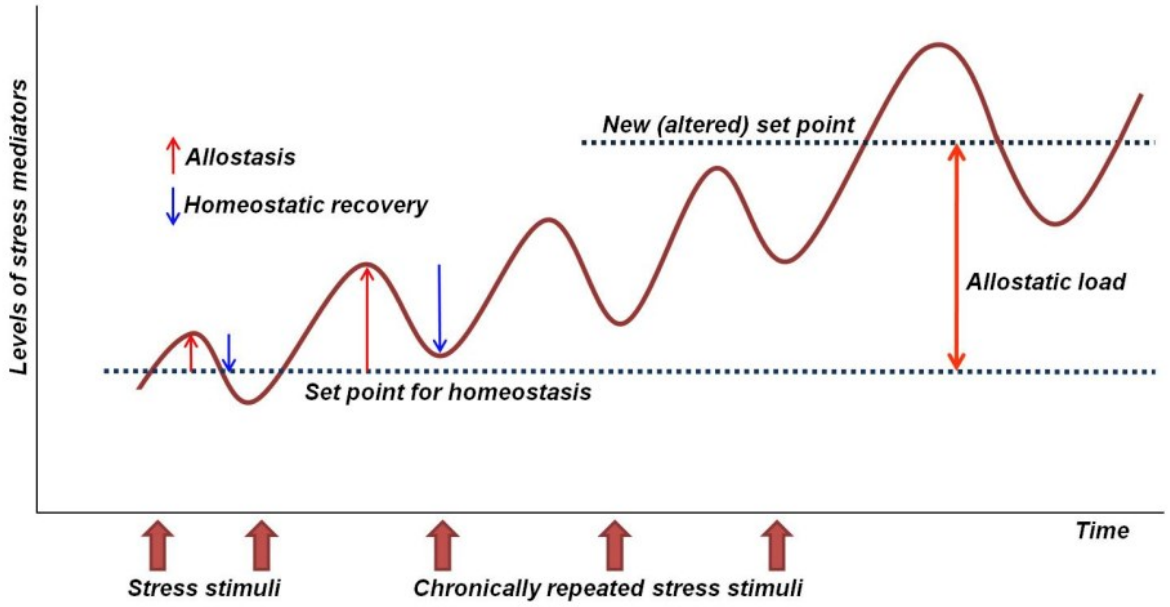

Figure 1.2: Effects of chronic stress on allostatic load.

Stressful stimuli trigger a stress response, where the sympathetic nervous system (SNS) and HPA axis are activated, release metabolic and stress hormones, and regulate the production of pro- and anti-inflammatory cytokines. With repeated stress, the body might not return to its homeostatic levels (blue arrow) and may require a more robust allostasis to return to baseline. A new set point for homeostasis is created to adapt to the anticipated chronic exposure to stress. The difference between the initial levels of stress mediators and the new set point represents the cumulative burden of stress or allostatic load. Changes in allostatic load can occur in stress mediators such as inflammatory cytokines, cortisol, insulin; or in non-communicable diseases such as hypertension, neurodegenerative disorders, obesity, among others. From ref [18]. The figure was created and published by BMB Reports

1.2.1 Overview of acute and chronic stress responses

The acute stress response is established following a perceived threat by quickly activating the hypothalamus and brain stem and stimulating the sympathetic-adreno-medullar axis and HPA axes' responses [20]. The short-term sympathetic-adreno-medullar axis response activates the sympathetic neurons of the autonomous nervous system (ANS) to produce catecholamines (epinephrine and norepinephrine) [20,21]. The catecholamines generate a series of events that promote energy mobilization, peripheral vasoconstriction, increased heart rate, the force of contraction and blood pressure. They also modulate immune responses, inflammation and reproduction [22]. In fact, acute stress has been associated with increased pro-inflammatory cytokines and chemokines in the plasma, creating a short-term inflammatory response [23,24].

The parasympathetic branch of the ANS reduces the stress response by returning the body to a state of relaxation [21]. As part of the parasympathetic division, the vagus nerve controls homeostatic mechanisms such as heart rate, visceral functions, glucose production, and immune responses [25]. The vagus nerve regulates the inflammatory reflex, consisting of the communication between the brain and immune system, upon activation by cytokines or pathogen-associated molecular patterns (PAMPs) [26].

Chronic stressors are pervasive and experienced frequently and over longer periods. Repeated hits cause an inadequate stress response where the sympathetic nervous system (SNS) is hyperactive and the HPA axis becomes dysregulated. This results in physical, psychological, and behavioural complications [27]. Long-term activation of both the SNS and HPA axis can have deleterious outcomes for the gastrointestinal tract, neuroendocrine, immune, reproductive, and cardiovascular systems [9]. Perceived chronic stress has also been linked with elevated inflammatory activity, which causes harmful long-term effects on health [28].

1.2.2 Regulation of the hypothalamic-pituitary-adrenal (HPA) axis stress response

The HPA axis is the central regulator of the stress response [29]. It consists of the hypothalamic paraventricular nucleus (PVN), the anterior lobe of the pituitary gland, and the adrenal gland. Neurons of

the PVN synthesize and secrete arginine vasopressin and corticotropin-releasing hormone (CRH), which play major roles in the stress response. CRH neurons are regulated by various neurotransmitters that exert either inhibitory effects (e.g., GABA and opioids) or excitatory effects (e.g., serotonin and NE) [30]. Upon activation, neurons in the PVN release CRH and AVP neuropeptides into the hypophysial portal circulation. CRH travels to the anterior pituitary and binds to its G-protein coupled receptor corticotropin-releasing hormone receptor 1 (CRHR1) in the corticotroph cells. This stimulates the production of proopiomelanocortin, which is then cleaved to adrenocorticotropic hormone (ACTH) and secreted into the bloodstream [31] (Figure 1.3). The ACTH binds to the melanocortin 2 receptors localized in the zona fasciculata of the adrenal glands. This interaction induces cholesterol biosynthesis, a precursor of mineralocorticoids (MCs) and GCs, including cortisol in humans or corticosterone in rodents (hereafter referred to as CORT) [29]. The circadian clocks and GC production regulate physiological processes throughout the day by modulating the gene expression of key targets [32]. In basal conditions, GC levels peak at the beginning of the active phase, which is morning for humans and nighttime for most rodents.

The HPA axis is repeatedly activated during chronic stress, inducing the secretion of GCs, which generate a GC burden and enhance adrenal sensitivity to ACTH [29]. The intensity of the chronic stress response depends on the degree and nature of the stressor and the ability of the organisms to cope [29]. CORT regulates the degree and duration of the HPA axis response by controlling negative feedback mechanisms through its actions on glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in multiple areas of the brain, including the PVN, hippocampus, and amygdala [33]. Chronic stress may impair the CORT negative feedback control of the HPA axis through downregulation of the GR in these regulatory areas of the brain [12].

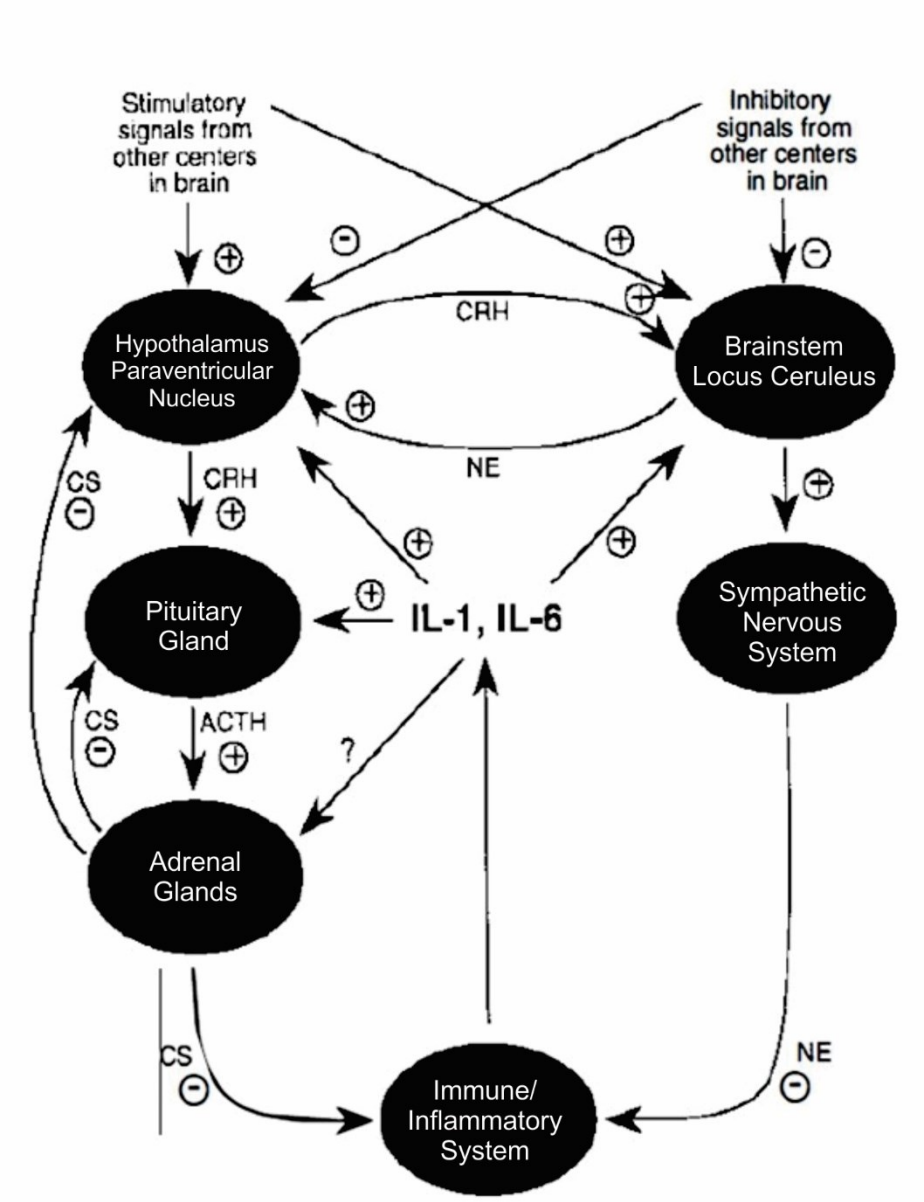

Figure 1.3: Schematic of the feedback regulation of the interrelated systems forming the neuroendocrine-immune network.

HPA axis regulation on the left: several signals activate neurons of the paraventricular nucleus of the hypothalamus to release CRH. In turn, CRH stimulates the anterior pituitary gland corticotroph cells to produce ACTH. ACTH binds to receptors on the adrenal cortex to stimulate the release of cortisol/corticosterone. (-): depicts negative feedback and (+) positive feedback. CS: corticosteroids, NE: norepinephrine, CRH: corticotrophin-releasing hormone, ACTH: adrenocorticotropic hormone, IL: interleukins. Used with permission from Annual Reviews, Inc., ref [34]; permission conveyed through Copyright Clearance Center, Inc., Copyright © 1995, as outlined in Appendix D.

1.2.3 Corticosteroids and their functions

Corticosteroids have important functions in normal and pathophysiological mechanisms [35,36], modulating skeletal growth, cardiovascular and immune functions, and reproduction. Steroid hormones are divided into GCs (e.g., CORT) and MCs (e.g., aldosterone), and are released from the cortex of the adrenal glands in response to stressful stimuli or physiological signals [36].

Endogenous GCs are lipophilic steroids released into the blood stream by the adrenal glands [36]. Approximately 80-90% of GCs are bound to proteins in the bloodstream (i.e., corticosteroid-binding globulin (CBG)), while ~10% are bound to albumin with a lower affinity [37]. The bioavailability of GCs is mainly determined by a balance between the active CORT and inactive cortisone/11-dehydrocorticosterone forms. The two enzymes 11β-hydroxysteroid dehydrogenase type 1 and 2 (11β-HSD1 and 11β-HSD2) coordinate this balance [36]. 11β-HSD1 is a bidirectional enzyme that primarily acts as a reductase to produce active CORT. The 11β-HSD2 exclusively functions as an oxidase enzyme and inactivates CORT in MC target tissues (i.e., placenta, kidney, colon, and salivary glands) [38]. It is noteworthy that synthetic GCs cannot bind to CBG and MR and are not metabolized by the 11β-HSD enzymes [36].

The primary MC steroid hormone is aldosterone, with a prominent role in regulating water and electrolyte homeostasis [69]. The MR has been shown to modulate immune system function, oxidative stress, and release pro-inflammatory cytokines [70]. The placenta is a MC target tissue where aldosterone participates in placental development and adaptation [69]. The 11β-HSD2 enzyme regulates the actions of steroid hormones during pregnancy by metabolizing CORT and controlling its binding to MR [72].

1.2.4 11β-Hydroxysteroid dehydrogenases (HSDs): the fetal-placental glucocorticoid regulator

GCs play beneficial roles in fetal organ development and maturation, regulation of energy demands, and immunomodulatory functions [39]. Tight control of CORT movement from mother to the fetus is essential to prevent its detrimental effects. Prenatal exposure to excess GCs has been associated

with fetal programming and may lead to severe consequences in postnatal physiology by altering fetal growth and maturation [40]. These changes may produce long-term effects on brain structure and development, behaviour, and HPA axis dysregulation [41]. Although administering exogenous synthetic GCs to non-humans and pregnant women at risk of preterm birth is effective in accelerating fetal lung maturation, it may decrease birth weights [42]. In turn, low birth weight is linked with cardiovascular, metabolic and neuroendocrine disorders [43]. Exogenous GCs administration is associated with postnatal immunity dysfunction, altered HPA axis responsiveness [39], cardiovascular dysfunction, metabolic impairments [40], and behavioural changes [44-46] in both humans and rodents.

11β-HSD1 was identified in gestational tissues such as chorion, decidua, and placental villous, while 11β-HSD2 has been mainly found in the syncytiotrophoblast layer and fetal tissues [47]. 11β-HSD2 controls the maternal-fetal GC transfer [44], preventing fetal exposure to excessive GC levels despite its higher concentrations in maternal serum [48].

Several studies show the essential role of the fetal-placental GC barrier in protecting the fetus against excessive amounts of cortisol [49,50]. In rats, maternal stress decreases placental 11β-HSD2 expression in late gestation [51]. Stress-released catecholamines (epinephrine and norepinephrine) inhibited 11β-HSD2 mRNA expression in early- and late-gestation human trophoblasts, contributing to the deleterious effects of GC programming [52]. Furthermore, pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) showed an inhibitory effect on placental 11β-HSD2 activity in term human explants [46]. In contrast, spontaneous human labour is associated with increased 11β-HSD1 expression and CORT levels [53]. Changes in the equilibrium of the 11β-HSD enzymes are a potential molecular mechanism involved in the detrimental offspring effects of maternal stress.

1.2.5 Mineralocorticoid (MR) and glucocorticoid receptors (GR)

GR is ubiquitously expressed in the body, regulating around 1000-2000 genes or up to 20% of all genes in the body [37]. Without its ligand, GR remains inactive in the cytoplasm, sequestered by chaperone proteins [36]. It is encoded by the human gene nuclear receptor subfamily 3 group c member

1 (NR3C1) and activated by GCs during circadian peaks and stress-induced GC secretion. The MR is encoded by the human gene nuclear receptor subfamily 3 group c member 2 (NR3C2). MR has a higher affinity for GCs than GR during basal conditions when MRs are mainly occupied by GCs [54]. In turn, GR occupancy for GCs increases during stress.

Active and free GCs diffuse into the cells and bind to intracellular GRs and MRs, members of the nuclear receptor family of transcription factors. The binding of GCs to their receptors activates signalling via both genomic and non-genomic pathways. In the genomic pathway, GR and MR interact with glucocorticoid response elements (GREs) in the DNA and induce changes in messenger RNA (mRNA) transcription and posttranslational modifications [55]. The nongenomic pathway consists of various rapid actions that do not affect gene expression and, therefore, involve interactions with the membrane-bound GRs (mGRs), cytosolic GRs, and nonspecific interactions with the cell membrane [56]. These interactions may affect the inflammatory response, ion cycling across the membranes, oxidative stress, and mitochondrial processes [56].

1.3 Inflammatory response: maintaining homeostasis

The body is constantly changing to maintain equilibrium. External and internal insults, including stressors, noxious and infectious agents, and mechanical damage, continuously disrupt homeostasis and trigger a stress response [57]. An inflammatory response is only initiated when the stress response is insufficient to return the physiological factors to baseline levels [57]. The inflammatory response is activated by tissue-resident cells (e.g., macrophages and mast cells) and sensory neurons in response to infection, injury, and tissue stress when the autonomous defences are inadequate [57]. The insult is sensed indirectly by recognizing functional features such as tissue damage and structural components of the noxious agent via pattern recognition receptors (PRRs) [57].

The PRRs are membrane-bound and cytoplasmic receptors on immune and non-immune cell types that sense PAMPs released from microbial structures and endogenous signals including damage-associated molecular patterns (DAMPs) [58]. The activation of these receptors (e.g., toll-like receptors (TLR)) initiates downstream intracellular cascades, including the activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) pathways. The production of inflammatory mediators such as cytokines and

chemokines is then upregulated. These mediators trigger vasodilation and increase vascular permeability, allowing specialized pro-inflammatory cells to be recruited from the circulation (i.e., neutrophils, monocytes, natural killer cells) into the tissue [59].

To facilitate cell recruitment, cytokines, prostaglandins (PGs), and nitric oxide (NO) act on vascular smooth muscle to increase blood flow through relaxation mechanisms [60]. Activating endothelial cells also increases surface adhesion molecules, leading to higher permeability to proteins and fluids [60]. This further facilitates leukocyte capture and extravasation. Inflammatory signals act upon multiple cell types to alter their core functionalities, allowing for a proper inflammatory response [59]. Therefore, these processes ultimately lead to the five cardinal signs of the inflammatory response: heat, swelling, redness, pain, and loss of tissue function [58].

1.3.1 Cytokines

Cytokines are small proteins released from immune and non-immune cells, and they have pivotal roles in various physiological and pathological processes, such as cell signalling, growth, survival, and differentiation [61]. They act as immunomodulating agents with autocrine, paracrine, and endocrine actions. Cytokines are divided into several categories according to their structural homology and function, including interferons, chemokines, interleukins, and TNF.

1.3.1.1 IL-6 family: pleiotropy and redundant functions

The IL-6 family has ten cytokine members, including IL-6, IL-11, IL-27, IL-35, IL-39, etc. [62]. The multifunctional and redundant biological functions of the IL-6 family cytokines highlight their potential for therapeutic target clinical applications. This is partially explained by the pleiotropic actions of the glycoprotein 130 (gp130) signal-transducing receptor [63]. The IL-6 cytokines are released during chronic inflammation and autoimmune diseases and participate in physiological processes during early development, hematopoiesis, bone metabolism, and neuronal and cardiovascular system development [62]. Their members are characterized by a four α-helical bundle structure, whereby the receptors have similar molecular structures and activate the same intracellular signalling pathways [64]. They are part of a large group that signal via the JAK-STAT pathway. This pathway always needs a cytokine, cytokine

receptor, JAK kinase, STAT signal transducer, and suppressor of cytokine signalling (SOCS) negative feedback regulators [65].

The IL-6 receptor (IL-6R) complex is characterized by the ligand-binding subunit IL-6Rα and the gp130 homodimer [65]. In the classic signalling pathway, when IL-6 binds to the membrane-bound IL-6R, a conformational change brings the JAK kinases into proximity, where they are autophosphorylated and activated (Figure 1.4) [66]. The activated JAKs subsequently phosphorylate tyrosine residues on gp130, to which the STAT transcription factors (STAT1, STAT3, and to a lesser extent, STAT5) bind and become phosphorylated by JAKs. Once phosphorylated, the STATs detach from gp130 and reorient themselves into homo- or heterodimers that translocate to the nucleus, where they regulate gene transcription [66].

For the IL-6 *trans*-signalling pathway, IL-6 binds to a soluble form of the IL-6Rα (sIL-6Rα) to create the IL-6/sIL-6Rα complex [65] (Figure 1.4). This complex interacts with the membrane-bound gp130 to initiate signal transduction in cells lacking IL-6Rα. Soluble gp130 act as a physiological antagonist of the *trans*-signalling pathway. It binds to the IL-6/sIL-6Rα complex and neutralizes its activity [65]. Evidence indicates that the *trans*-signalling pathway primarily manages IL-6's pro-inflammatory functions, whereas the IL-6's anti-inflammatory activities are mediated via the classic pathway [64].

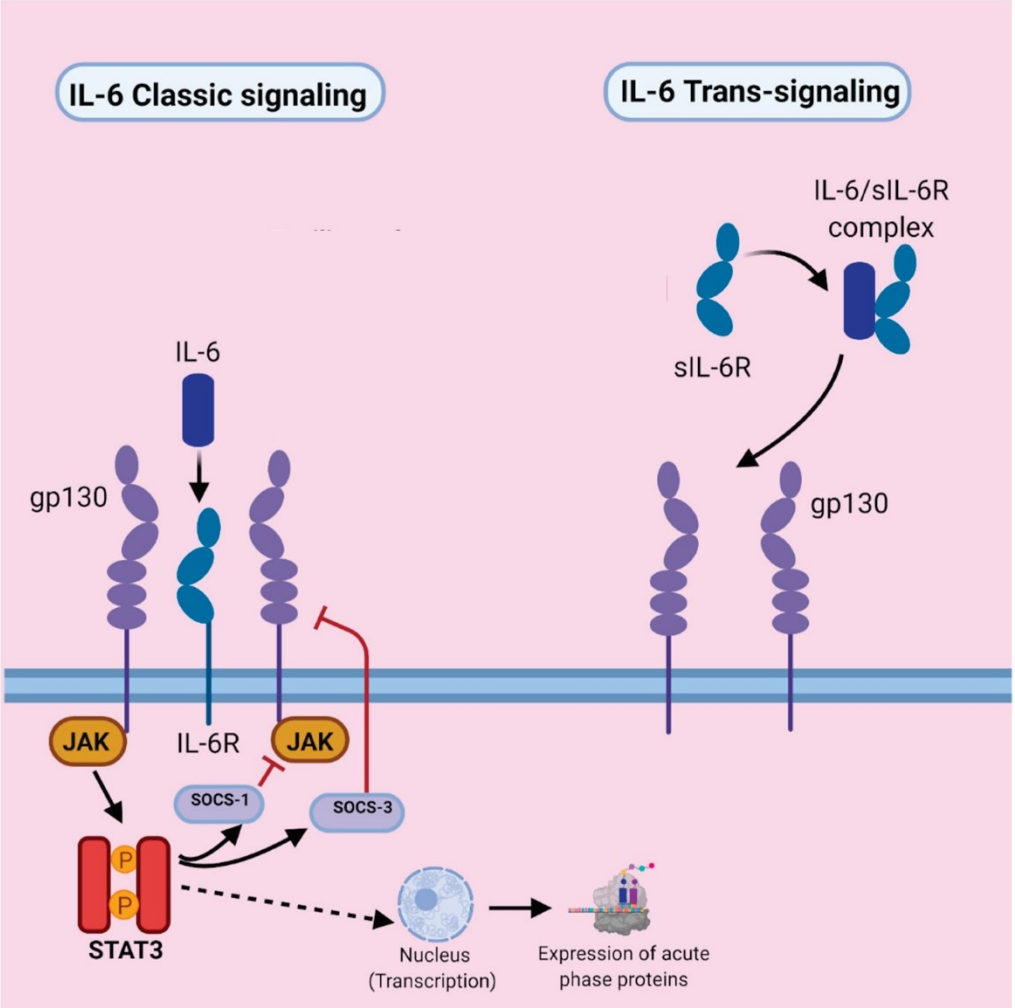

Figure 1.4: Schematic of IL-6 classical and trans-signalling pathways.

The classical signalling pathway involves the binding of IL-6 cytokine to the membrane-bound IL-6Rα. The IL-6-IL-6Rα complex associates with membrane-bound gp130, which dimerizes and activates the JAK kinases by phosphorylation. This triggers STAT3 and STAT1 binding, phosphorylation, and dimerization. This dimer then translocates to the nucleus to regulate gene transcription. SOCS1 and 3 functions as negative regulators by inhibiting JAKs. The soluble form of IL-6Rα is a trans-signalling agonist and binds IL-6 to create the IL-6/sIL-6Rα complex, which interacts with the membrane-bound gp130 to initiate signal transduction in cells lacking IL-6Rα. Adapted from ref [67]: this article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

1.3.1.2 IL-1 family: broad spectrum immunological responses

The IL-1 family participates in a broad spectrum of immunological and inflammatory responses, possessing the nonspecific functions of innate immunity and specialized adaptive immunity [68]. There are 11 cytokine members, including IL-1α, IL-1β, IL-1 receptor accessory protein (IL-1Ra); and 10 receptors, including IL-1 receptor 1 (IL-1R1), IL-1 receptor 2 (IL-1R2), and IL-1 receptor 3 (IL-1R3 or IL-1RAP) [69]. All members are potent modulators of inflammatory responses and function at picomolar levels to trigger downstream signalling effects [69].

Both IL-1α and IL-1β bind to the same receptor and are structurally similar, but they are encoded by different genes [69]. The IL-1α precursor is constitutively produced by most cells in its already active form. It has dual functions as it is considered an alarmin (an endogenous molecule that acts as a danger signal to activate the immune system [70] or DAMP) that binds to the extracellular IL-1R1 to initiate an inflammatory cascade. It also modulates gene expression as a transcription factor in the nucleus [68].

The IL-1β cytokine is produced by a limited number of cells (e.g., monocytes, macrophages, dendritic cells) and is primarily induced under pathological conditions [69]. Upon recognition of PAMPs and cytokines, TLR stimulates immune cells' transcription of IL-1β. The PPR recognition of DAMPs and PAMPs activates the NLR family pyrin domain containing 3 (NLRP3) inflammasome that mediates caspase-1 activation [71]. Caspase-1 is a cysteine protease that converts inactive pro-IL-1β and pro-IL-18 to active and mature IL-1β inflammatory cytokine. Mature IL-1β also triggers its own activation as an auto-inflammatory induction [69].

The IL-1R family consists of 10 structurally-similar receptors that contain an extracellular domain for ligand binding, a transmembrane domain, and an intracellular domain [72]. The toll-IL-1-receptor (TIR) domain is located in the intracellular portion of the receptor and initiates signal transduction (Figure 1.5). They are alarm receptors that rapidly activate the innate immune system upon sensing exogenous (PAMPs acting on TLR) or endogenous (IL-1 cytokines acting on IL-R1) ligands [72]. The cytokines IL-1α, IL-1β and IL-1Ra bind to two receptors: the ubiquitously expressed IL-1R1 and IL-1R2, which are expressed primarily on B lymphocytes, neutrophils, and monocytes [68,73].

The IL-1R1 receptor undergoes conformational changes upon binding to an IL-1 cytokine. This allows a shared co-receptor IL-1RAP (also known as IL-1R3) to bind to IL-1R1 and form a heterodimer

[68] (Figure 1.5). This trimeric complex (i.e., cytokine, primary receptor and accessory protein) allows the juxtaposed TIR domains to recruit intermediate molecules for downstream signalling via the activation of NF-κB, AP-1, and the mitogen-activated protein kinase (MAPK) pathways [74].

The activities of IL-1 cytokines are regulated by transcriptional control, cleavage of the proforms, rates of secretion from the cell, and control of receptor binding by antagonists and decoy receptors [69]. The IL-1R2 acts as a decoy receptor with an extracellular domain for ligand binding. It binds to IL-1β with high affinity and less efficiently to IL-1α and IL-1Ra [72]. However, it lacks the intracellular TIR domain required for downstream signalling [74]. Thereby, it neutralizes IL-1α and IL-1β cytokines and sequesters the accessory protein (IL-1RAP/IL-1R3). Both IL-1R1 and IL-1R2 exist in a soluble form and can easily capture IL-1 ligands and interact with the soluble IL-1RAP, further enhancing their inhibitory activity [72,74].

The IL-1Ra (also known as anakinra) is an endogenous antagonist of IL-1α and IL-1β. It binds IL-1R1 competitively but does not recruit the IL-1RAP needed for activation [74]. The association of IL-1Ra to IL-1R1 impedes the IL-1RAP from forming a trimeric complex for receptor activation [69]. It exists in a secreted glycosylated form (sIL-1Ra) and an intracellular form (icIL-1Ra). Together with the membrane-bound and secreted IL-R2, they function as endogenous inducible anti-inflammatory molecules to reduce the IL-1 pro-inflammatory effects [73].

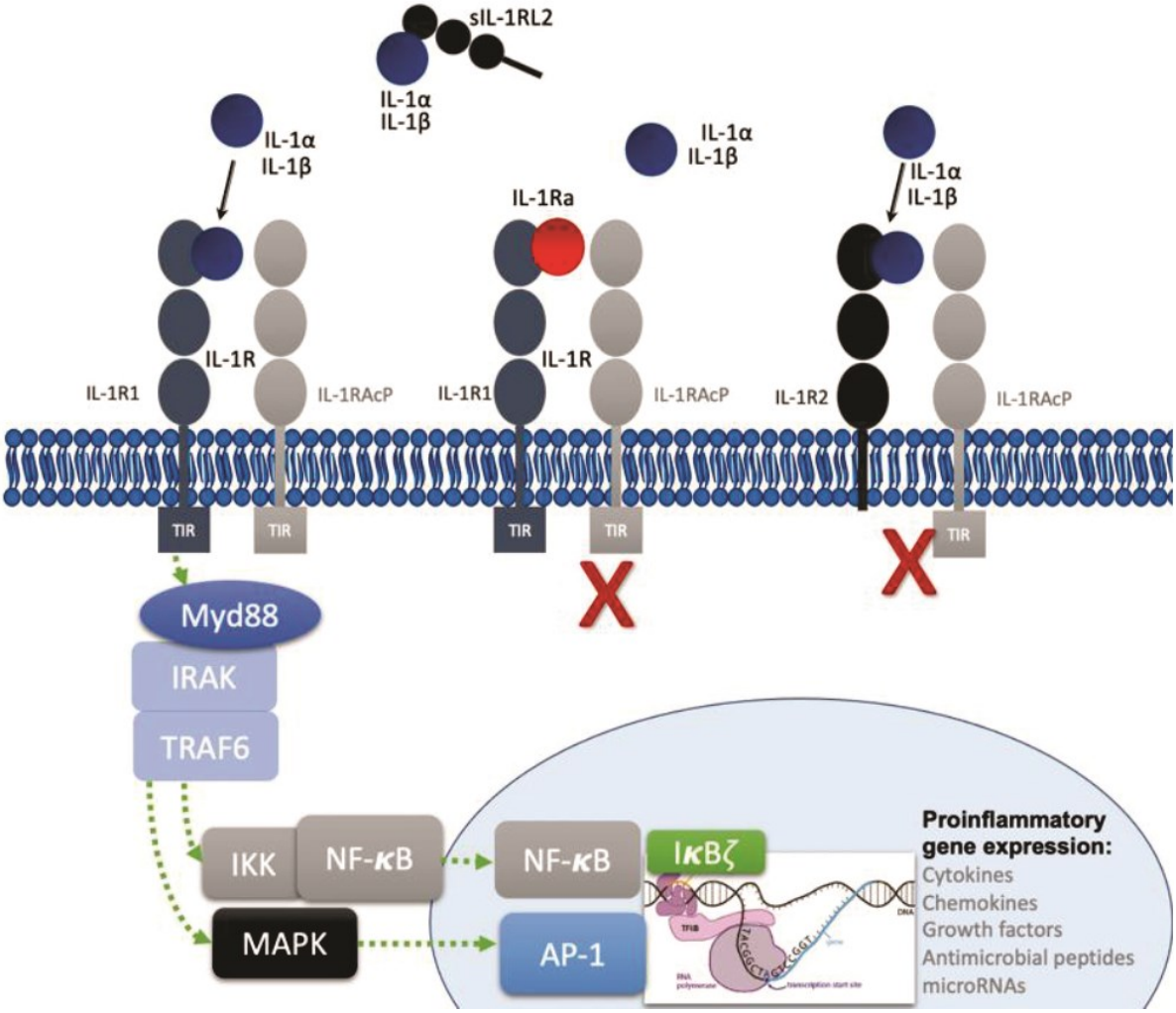

Figure 1.5: Schematic of IL-1 cytokine and receptor signalling pathways.

IL-1 (α or β) forms a functional complex with IL-1R1 and IL-1RAP. This ligand-receptor complex signals via two canonical pathways: the kinase pathway that activates AP-1 and the NF-kB transcription factors. The nuclear translocation of transcription factors induces the transcription of key pro-inflammatory and pro-labour genes, including cyclooxygenase-2 (COX-2), IL-6, and IL-8. This system is tightly regulated by endogenous IL-1 inhibitors such as IL-1Ra, sIL-1RAP, and IL-1R2 (soluble and membrane-bound). Adapted from [75]: this article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

1.3.1.3 IL-6 and IL-1 cytokines and their association with term and preterm labour

The physiological pathways leading to the onset of labour are, at least in part, orchestrated by pro-inflammatory cytokines that induce leukocyte migration to gestational tissues and upregulation of uterine activation proteins (UAPs) [73]. The IL-6 and IL-1 pro-inflammatory cytokines play a role in the coordination and regulation of these mechanisms.

Intra-amniotic inflammation is the most studied factor associated with preterm birth. This clinical condition is often associated with infection or sterile inflammation [71]. Preterm premature rupture of membranes (pPROM) and preterm birth are associated with higher IL-6 and IL-1 in gestational tissues and blood [76-79]. Indeed, the IL-1α alarmin, or the molecular danger signal released by leukocytes that triggers the immune system activation [70], has been found as a key cytokine upregulated in the amniotic fluid of women with sterile intra-amniotic inflammation [80]. Other important and recently discovered cytokines that function as alarmins are the IL-33, IL-1F7b, IL-16, and high-mobility group box-1 protein [81]. Administration of IL-1α alarmin to pregnant mice results in preterm birth, and treatment with IL-1R1 antagonist prevents IL-1-induced parturition [82]. Studies have also shown that IL-1α, IL-1β, and IL-6 are the primary cytokines elevated in sterile intra-amniotic inflammation [80,83,84], suggesting that they play a central role in the inflammatory milieu in PTB cases.

The mechanisms involved in the production of pro-inflammatory cytokines involved in sterile intra-amniotic inflammation, infectious conditions, and preterm birth include PAMPs- and DAMPs-induced release of IL-6 and activation of the NLRP3 inflammasome to release mature forms of IL-1β and IL-18 cytokines [84,85]. IL-6 has been shown to exert its biological roles in amniotic fluid through classical and *trans*-signalling pathways [86]. Both IL-6 and IL-1β upregulate UAPs contributing to the events of parturition, such as PGE2, PGF2α, and oxytocin receptor (OTR) [87]. In mice fetal membranes, the administration of IL-1α induced NLRP3 inflammasome activation, followed by increased caspase-1 and IL-1β proteins [71]. Injection of IL-1α and increased levels of IL-1β were associated with a higher risk of preterm birth and neonatal mortality [88]. These findings are supported by increased IL-1β and IL-6 levels in the cervix, myometrium, and fetal membranes with the onset of labour [88,89] and higher concentrations in the amniotic fluid of infection-induced preterm birth [76,77]. Furthermore, a larger IL-1β/IL-1Ra ratio and increased IL-1β levels were observed in decidual samples from women in labour [90].

The pro-labour actions of IL-1β were demonstrated by its induction of CRH expression in human placenta [91] and CRHR1 in human myometrium [92], while both IL-6 and IL-1β inhibit 11β-HSD2 in placental villi [46]. IL-1β modulates progesterone metabolism in human cervical fibroblasts through the regulation of 20α-hydroxysteroid dehydrogenases activities and expression [93]. Moreover, IL-1β was shown to downregulate the expression of tissue inhibitors of metalloproteinase-1 and -2 and increase matrix metalloproteinases (MMPs) in the human cervix, which contributes to cervical ripening [94]. In a global inflammatory transcriptional response of term decidual cells, treatment with IL-1β resulted in differential expression of various cytokines, chemokines, and other inflammatory mediators [95]. Micro RNAs (miRNAs) involved in MAPK inflammatory cascades and TLR signalling were also altered by IL-1β treatment, suggesting that decidual miRNAs may regulate inflammatory pathways *in utero* and perhaps be exploited as therapeutic target [95].

1.4 Crosstalk between stress and inflammation: neuroendocrine-immune interface

Immune and neuroendocrine system communication is required to maintain homeostasis upon internal and external insults. Both systems sense environmental cues and respond by relaying signals between immune cells and neurons [96]. The immune, nervous, and endocrine systems are interconnected, and communication is established by nerve pathways and regulatory molecules such as cytokines, neurotransmitters, steroids, and neuropeptides that bind to their receptors to activate specific signalling pathways [31,97] (Figure 1.3). For example, immune cells express receptors for neuropeptides and hormones, including GCs [98], while neuronal and endocrine cells express receptors for cytokines and chemokines [98]. Immune cells produce hormones (e.g., growth hormone, prolactin, ACTH, CRH), neurotransmitters (e.g., NE and E) and neuropeptides (e.g., endorphins) that operate in an autocrine and paracrine fashion to regulate immune functions [31] and peripheral immunity [99]. Conversely, the brain's innate immune and non-immune cells (e.g., astrocytes and glial cells) release inflammatory mediators that act locally to affect the nervous system, including the HPA axis [98,100]. These cytokines and chemokines cross the blood-brain barrier to regulate neuronal properties [99].

A series of orchestrated events involving behavioural and neuroendocrine-immune responses are elicited to produce and secrete GCs and cytokines/chemokines [101]. Negative feedback loops between the HPA axis and sympathetic-adreno-medullar axis with the immune cells maintain homeostasis within the neuroendocrine-immune regulatory network [98]. GCs are widely known for their immunosuppressive actions to prevent overreaction of inflammatory responses [98,102]. The GCs' anti-inflammatory actions are mediated by direct interactions between the GR and nuclear transcription factors (e.g., NF-κB) to repress pro-inflammatory genes or by interactions with mGR [31]. However, the dual action of GCs has been shown in the immune system as both anti- and pro-inflammatory mediators [103]. The pro-inflammatory actions of GCs in response to chronic stress may stimulate exacerbated peripheral immunity and elevate IL-1 concentrations in the central nervous system (CNS) [104,105]. Indeed, inflammation is arguably involved in the pathogenesis of stress-related diseases [106]. The effect of GCs, therefore, depend on the nature and duration of the stimulus (acute or chronic) and the physiological status of the immune system [31,103].

1.5 Physiological changes during pregnancy, labour, and parturition

Pregnancy is a time of significant physiological changes to nurture and accommodate a growing fetus. Inflammation and neuroendocrinological processes are essential for normal pregnancy, labour, and parturition. Several studies have attempted to unravel fundamental aspects of human conception, pregnancy, uterine transition for labour, and time of delivery. Still, the exact mechanisms remain unclear due to the complexity and integrated physiology within multiple tissues, cells, and systems. This section will review the most studied pathways involved in pregnancy establishment and the transition of the uterus to a contractile state.

1.5.1 Pregnancy is an inflammatory event

The immune system is active and functional throughout pregnancy. It is tightly controlled to allow implantation, decidualization, vascular remodelling, and placentation [107]. It is clear now that pregnancy is not an immune suppression state [108]. In fact, the deletion of NK cells diminishes trophoblast invasion and affects placental development [109]. Depletion of uterine dendritic cells in mice results in impaired

blastocyst implantation and decidual formation, causing embryo resorption [110]. Macrophages are the second largest population of leukocytes in the decidua. They control trophoblast invasion, tissue and vascular remodelling, apoptotic cell clearance, and innate and adaptive immunity [111,112].

The first and early second trimesters are characterized by a potent pro-inflammatory response with invading cells, apoptotic state, and repairing cells to guarantee a successful establishment of pregnancy [108]. The second trimester features an anti-inflammatory dominance, with a shift to T helper type 2 cell profile [108,113]. This stage is characterized by rapid fetal growth and an environment favouring uterine quiescence [113]. Recent data, however, revealed a novel mechanism by which the NLRP3 inflammasome is activated in placental trophoblast cells during midgestation, thus increasing IL1β secretion into the circulation [114]. This primes circulating maternal monocytes, poises them for inflammasome signalling in the periphery and protects the chorionic villi and the fetus from potential infections. Thus, the second trimester is also a dynamic immunological state whereby the placenta acts as a protective barrier and promotes maternal peripheral host defence [115].

Birth is an inflammatory process whereby the gestational tissues act in coordination and parallel to drive the amplification of the inflammatory cascade. This occurs through endocrine, paracrine, and autocrine interactions between pro-inflammatory and pro-contractile mediators to shift the uterus from a quiescent state to a contractile organ until it reaches an irreversible endpoint that triggers parturition [85,116].

1.5.2 Inflammation and adverse pregnancy and infant outcomes

Inflammation is a reported feature of PNMS, suggesting that stress during pregnancy yields similar outcomes to acute or chronic maternal immune activation (MIA). MIA can occur following exposure to pathogens and sterile inflammation during critical windows of fetal development [117]. It has been implicated in neurodevelopmental disorders with immunological bases, such as schizophrenia [118] and autism spectrum disorder [119]. The underlying mechanisms of the adverse effects of MIA may involve early-life programming of the fetal brain and immune system [120]. It is suggested that MIA induces a cascade of downstream cellular and molecular changes whereby cytokine imbalance [121,122], metabolic

and oxidative stress [123], neuroendocrine changes [124], and epigenetic mechanisms [125] can induce adverse effects on the fetus (Figure 1.6).

High levels of pregnancy-specific distress were associated with increased IL-6 and TNF-α in women who delivered preterm, and these factors were predictive of shortened gestational age [126]. High anxiety and depression were correlated to increased levels of pro-inflammatory cytokines, including IL-6, IL-2, IL-9 and IL-17A [127]. The regulation of the maternal GC-immune coordination, characteristics of the stress (i.e., type, duration, time of exposure), genetic and epigenetic factors, and lifestyle likely contribute to prenatal programming and disease risk in the offspring.

Another adverse health outcome of inflammatory imbalance is chronic endometritis, also called persistent impaired inflammatory state of the endometrium [128]. Chronic inflammation of the endometrium is also closely related to infertility, abnormal placentation-related diseases, miscarriage, and endometrial polyps [128-130]. Such reproductive disorders are also attributed to stress-induced immune-endocrine imbalance [129]. Leukocyte impairment is also linked with menstrual disorders and preeclampsia [84].

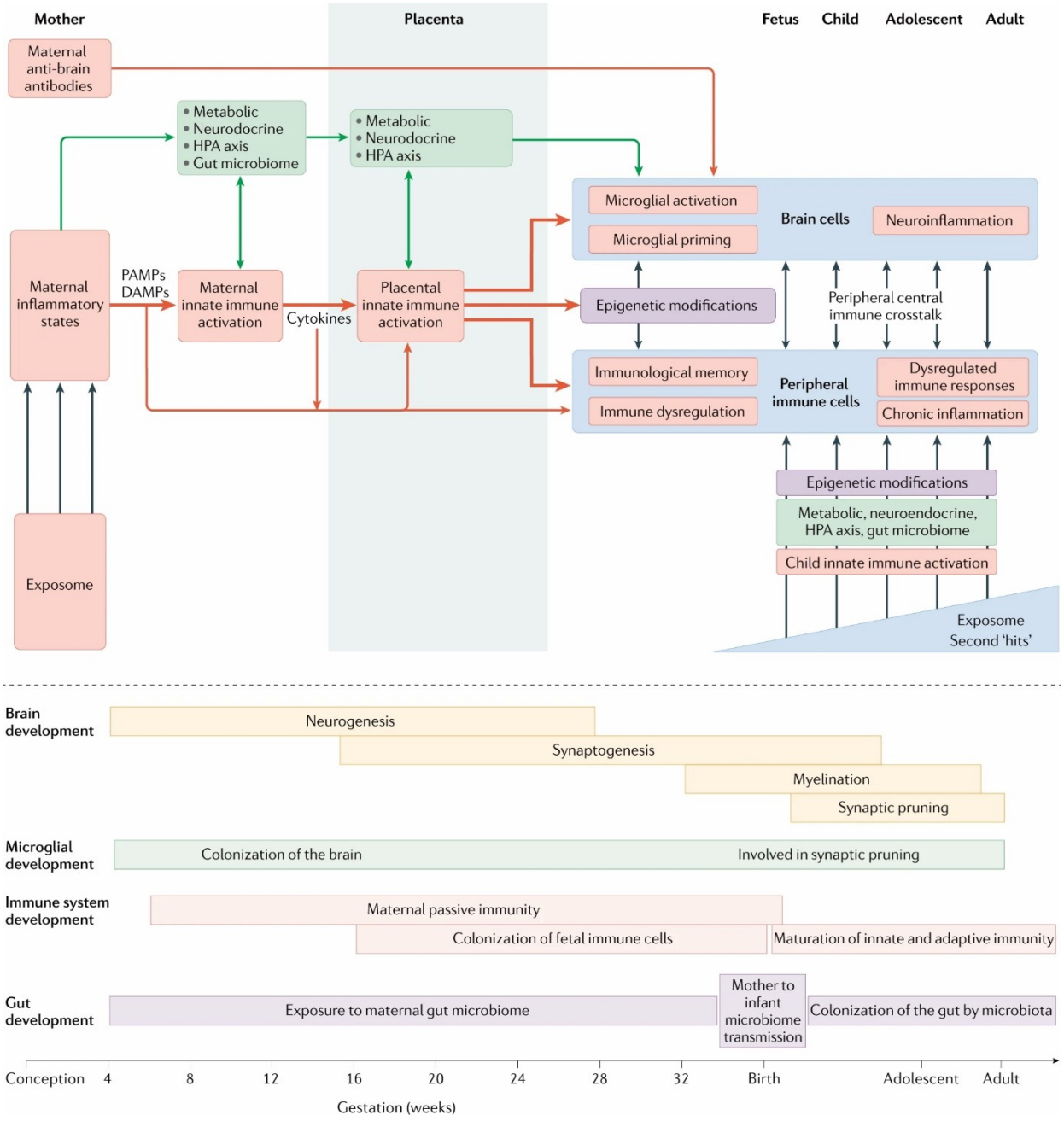
Mother
Placenta
Fetus
Child
Adolescent
Adult
Maternal anti-brain antibodies
• Metabolic
• Neurodocrine
• HPA axis
• Gut microbiome
• Metabolic
• Neurodocrine
• HPA axis
Microglial activation
Microglial priming
Brain cells
Neuroinflammation
Maternal inflammatory states
PAMPs
DAMPs
Maternal innate immune activation
Cytokines
Placental innate immune activation
Epigenetic modifications
Peripheral central immune crosstalk
Immunological memory
Immune dysregulation
Peripheral immune cells
Dysregulated immune responses
Chronic inflammation
Epigenetic modifications
Metabolic, neuroendocrine, HPA axis, gut microbiome
Child innate immune activation
Exposome
Exposome
Second 'hits'
Brain development
Neurogenesis
Synaptogenesis
Myelination
Synaptic pruning
Microglial development
Colonization of the brain
Involved in synaptic pruning
Immune system development
Maternal passive immunity
Colonization of fetal immune cells
Maturation of innate and adaptive immunity
Gut development
Exposure to maternal gut microbiome
Mother to infant microbiome transmission
Colonization of the gut by microbiota
Conception
4
8
12
16
20
24
28
32
Birth
Adolescent
Adult
Gestation (weeks)

Figure 1.6: The influence of environmental factors on MIA and adverse fetal development.

Maternal inflammatory activation by heterogenous agents (e.g., stress, lifestyle factors, infection, etc.) induces the release of PAMPs and DAMPs. They activate TLRs on gestational tissues and/or maternal peripheral innate immune cells, stimulating cytokine release. Cytokines in the gestational tissues and placenta may affect metabolic, neuroendocrine, and stress signalling pathways. These changes can cause long-lasting epigenetic modifications on fetal tissues and cells (e.g., fetal brain and immune cells) during critical times of development, including critical periods of CNS and immune system development and gut microbiota colonization. Postnatal peripheral-central immune crosstalk responds to environmental factors and may trigger inflammatory signals following interactions with second immune hits. This can result in chronic inflammation in the brain and periphery, which intensity depends on factors such as aberrant immune programming, genetic risk, sex, and type and intensity of second hits. MIA has also been shown to induce epigenetic modifications in the fetus, affecting its life-long health trajectories. Reprinted by permission from Springer Nature: ref *[122]*, Copyright © 2021 as outlined in Appendix D.

1.5.2.1 Preterm birth: global health burden

Preterm birth is defined as any live birth before 37 completed weeks of gestation [131]. It is the leading cause of death in children under five years of age [132] and a major risk for morbidity and mortality among infants worldwide [131]. Preterm birth rates range from about 5% to 18% globally, corresponding to an estimated 15 million births annually [133]. Its incidence in Canada fluctuated between 7.5% and 8.2% of live births from 2000 to 2010 [134], while the rate in the United States was approximately 8.4% in 2020 [135].

Preterm births resulted in approximately 1 million deaths in 2015 in the 194 WHO member states [132], where three-quarters were preventable with feasible and cost-effective interventions [131]. Despite advances in knowledge and technology, preterm birth numbers, and the economic and health care burden of prematurity, are rising [131]. The cost of preterm births for the Canadian health system exceeds $8 billion per year [136].

1.5.2.1.1 Classification and etiology of preterm birth: a heterogeneous syndrome

Preterm birth is a syndrome with many causes. Indicated or iatrogenic preterm birth occurs when medical providers initiate labour via induction or elective cesarean delivery [137]. It accounts for 30-35%

of all preterm births. Its etiology is heterogeneous and determined by maternal or fetal complications (i.e., preeclampsia, fetal distress, intrauterine growth restriction, etc.) [138]. Idiopathic spontaneous preterm birth occurs in 40-45% of cases. It is subdivided into preterm labour with intact membranes and pPROM, with the latter accounting for 25-30% of spontaneous preterm births [138,139].

Spontaneous preterm birth is a heterogeneous and multi-factorial syndrome involving various endogenous and exogenous risk factors rather than a single component [139] (Figure 1.7). The interplay between these factors stimulates mediators of parturition, including a shift to pro-inflammatory cytokine signalling and increased expression of UAPs. This induces the transformation of the myometrium from a quiescent to a contractile state before 37 completed weeks of gestation [131,140].

Endogenous factors associated with spontaneous preterm birth include sterile intra-amniotic inflammation, intra-amniotic infection, cervical insufficiency, hemorrhage, decidual senescence, and others [83,141,142]. Exogenous risk factors include physical and psychological stressors, history of preterm delivery, low socio-economic status, social isolation, smoking, low body mass index, etc. [139,143,144] (Figure 1.7). Psychological stressors have been linked with increased inflammatory mediators and shorter gestational length in humans [196,207] and animals [117,118]. Also, life-long accumulated socioeconomic inequalities and racial disparities may lead to an increased allostatic load and have been associated with a higher risk of preterm birth [195]. The rising rates of conception using assisted reproductive technologies are associated with uterine overdistension of multiple pregnancies [138], which is a risk factor for PTB around ten times higher than singleton births [145]. Identifying risk factors for preterm birth contributes to categorizing at-risk women and facilitates population-targeted interventions.

Advances in neonatology technology allow many vulnerable premature infants to survive and reach adulthood. However, the life-long disabilities of prematurity span from neurodevelopmental deficits, cerebral palsy, visual and hearing impairments, asthma, cardiovascular and metabolic diseases, and many more [146-148].

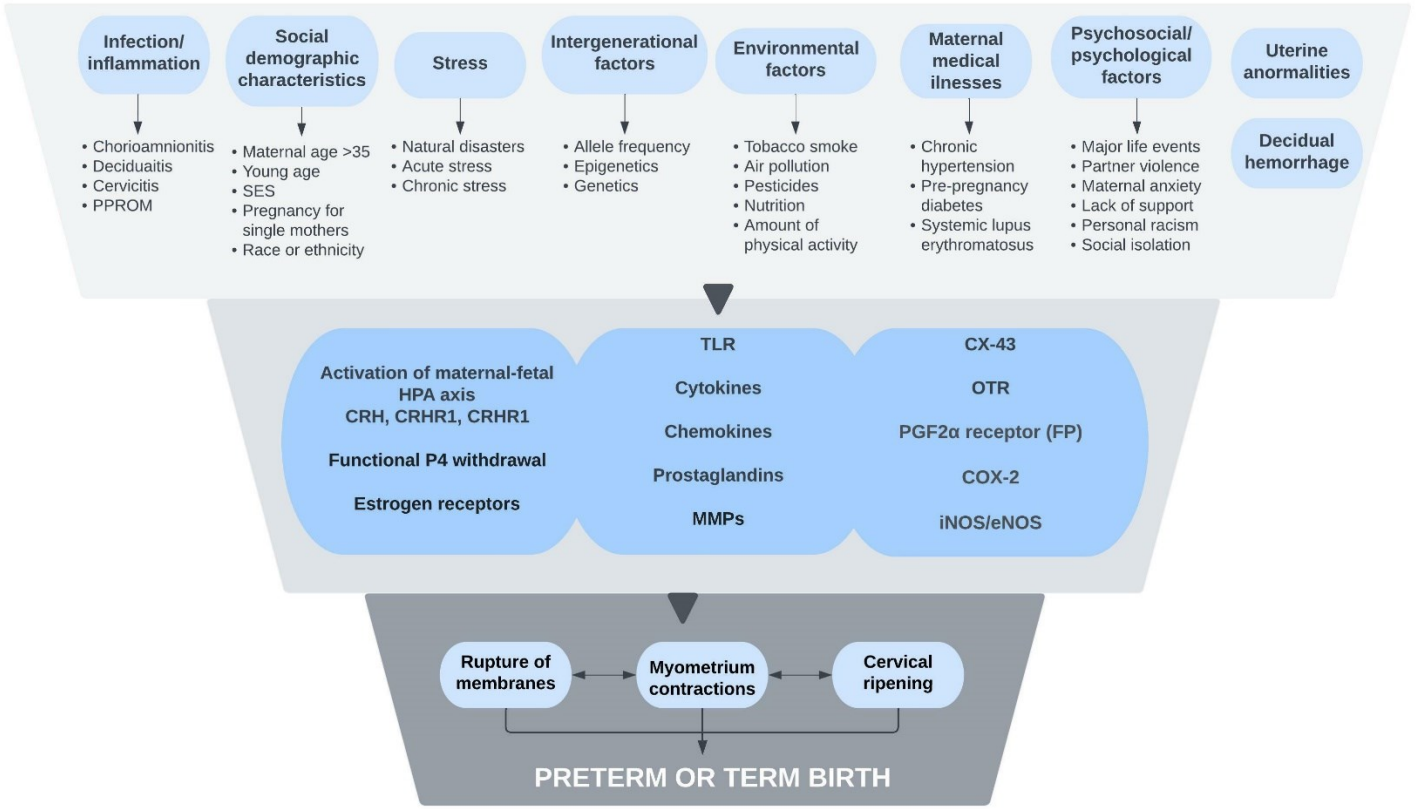

Figure 1.7: Heterogenous pathophysiology of preterm and term birth.

Preterm birth is a multifactorial syndrome with numerous initiating factors that stimulate mediators involved with the onset of labour. Ultimately, the activation of multiple pathways culminates in the common events leading to birth, including rupture of membranes, myometrium contractions and cervical ripening. The complexity of the syndrome is an obstacle to effectively treating preterm birth. However, targeting upstream mediators of the inflammatory cascade (i.e., TLR and cytokine receptor inhibitors) is a promising strategy to delay preterm birth while minimizing unintended adverse effects. Created with www.lucidchart.com.

1.5.2.1.2 Allostatic load and preterm birth conceptual framework

Studies have shown a clear relationship between preconceptional and prenatal adversity to the risk of preterm birth and low birth weight [149-152]. Although previous AL assessments during pregnancy by have not revealed clear associations between AL stress biomarkers and adverse pregnancy outcomes [153,154], a large prospective observational cohort study found that high AL was linked to preterm birth [155]. Populational differences such as cultural, structural, and social contexts might influence outcomes. These AL markers may be used to produce an index score of multisystemic physiological and epigenetic markers to develop a risk model for preterm birth and other adverse pregnancy outcomes [156].

The proposed AL and preterm birth conceptual framework integrates the contributions of environmental factors, epigenetic inheritance, and physiological mediators of AL (Figure 1.8) [156]. In this framework, generation programming directly influences individual lifetime cumulative stress. The individual perceives stress and produces a downstream stress response. The net effect of the adaptative responses and epigenetic signatures leads to high AL, representing the cumulative stress burden throughout the life course. The stress accumulation can eventually reach a point when an individual can no longer cope, leading to an elevated risk of preterm birth, other adverse pregnancy outcomes, and adverse developmental outcomes in the offspring.

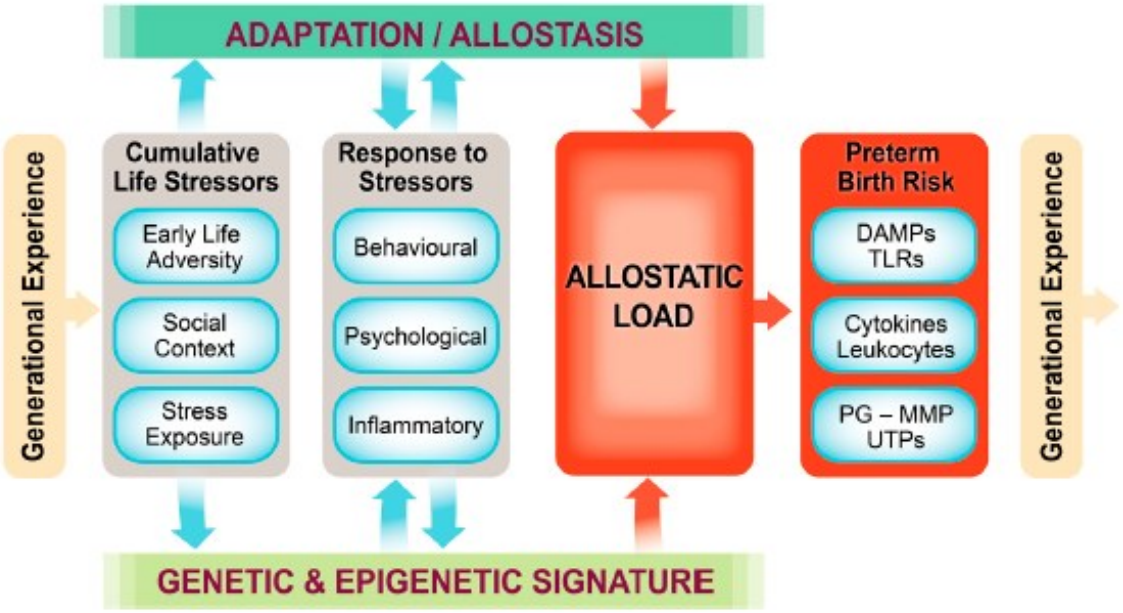

Figure 1.8: The preterm birth conceptual framework.

The preterm birth conceptual framework postulates that transgenerational (TG) programming occurs through cumulative hits of life stressors, which produce downstream responses, including genetic and epigenetic signatures. When the net effect of stress responses outweighs the capacity to adapt, high AL results in a series of inflammatory and molecular responses that induce uterine activation for labour and preterm birth. These cumulative changes are transmitted to the germ cells, programming physiological and inflammatory responses in the progeny. From ref *[156]*, Creative Commons Attribution licence (CC BY).

1.5.3 Mechanisms involved in the uterine activation for labour

1.5.3.1 A localized intrauterine inflammatory response

The decidua, myometrium, fetal membranes, and cervix must work together to secrete pro-inflammatory cytokines and chemokines to activate maternal peripheral leukocytes. These inflammatory mediators act on the uterine vascular endothelium to enable the influx of leukocytes into the uterine compartments [85,157] (Figure 1.9). The invading leukocytes release more cytokines and chemokines, driving a feedforward upregulation of inflammatory mediators. They further activate adjacent tissues and trigger the amplification of the inflammatory cascade by stimulating UAPs, changing receptor bioavailability and neuroendocrine hormones, and inducing positive feedback interactions between cells,

ligands, and tissues [85,158,159]. Some of the UAPs include cyclooxygenases, uterotonins, receptors, and connexins that function in a complex and cooperative fashion. Many contain binding sites in their promoter regions for inflammatory transcription factors such as AP-1 and NF-κB, which may be activated by pro-inflammatory cytokines, hypoxic insults, and lipopolysaccharide (LPS) [160]. These transcription factors have been shown to regulate downstream genes such as COX-2, OTR, cytokines (e.g., TNF-α, IL-1β, IL-6, IL-8), and MMP-9 [161,162].

This process is known as uterine transition and encompasses physiological and cellular changes that occur in a short period of time and ultimately lead to the physiological events of delivery, including myometrial contraction, rupture of membranes, and cervical ripening.

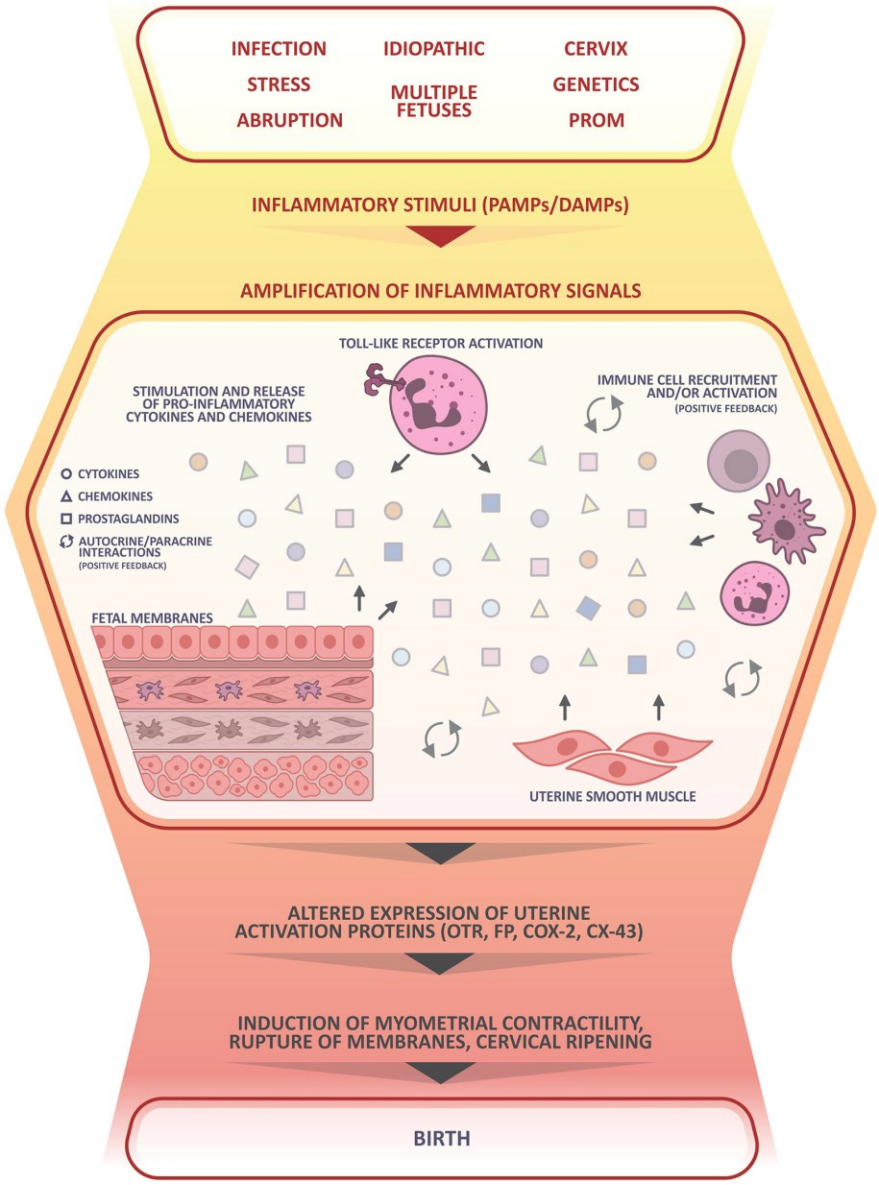

Figure 1.9: Activation of the inflammatory pathway to term or preterm birth.

Several signals, including stress, induce the release of PAMPs/DAMPs that activate TLR to produce pro-inflammatory cytokines and chemokines. These pro-inflammatory mediators stimulate leukocyte activation and migration to the uterus and further release of cytokines and chemokines, amplifying the inflammatory cascade in a feed-forward loop. The inflammatory mediators have paracrine actions and stimulate the adjacent tissues to release more inflammatory signals. The amplification of inflammatory mediators stimulates the expression of UAPs such as OTR, COX-2, prostaglandin F receptor, connexin-43, etc. The increase in UAP mediators and the amplification of the inflammatory cascade ultimately leads to the transition of the uterus from quiescent to active and contractile uterus of delivery. The upregulation of inflammatory mediators also induces the physiological events of delivery, including myometrial

contraction, rupture of membranes, and cervical ripening. From ref *[85]*, Creative Commons Attribution licence (CC BY).

1.5.3.2 Oxidative stress

Reactive oxygen species (ROS) are metabolic by-products generated from physiological enzymatic processes such as arachidonic acid metabolism (via COX), inflammation, and cellular respiration [163]. The free radicals include superoxide radicals, hydrogen peroxide, NO, hydroxyl radicals, and singlet oxygen. These radicals play physiological roles in immune cell activation, cellular signalling pathways, neuronal activity, and blood flow regulation [164]. However, excess ROS are harmful by damaging nucleic acids, proteins, and lipids. They are implicated in many reproductive complications (e.g., preterm birth) [165], cancer, and neurological and cardiovascular diseases [164].

The redox status is characterized as the balance between oxidants and antioxidants through the regulation of ROS. Endogenous enzymes (e.g., superoxide dismutase (SOD), catalase, glutathione peroxidase) and exogenous molecules obtained from nutrition (e.g., vitamins A, C, E, carotenoids) maintain this balance [165]. Oxidative stress occurs when there is an imbalance between free radicals and antioxidants. There is growing evidence linking oxidative stress with preterm birth and pPROM, including higher F2-isoprostanes (a marker of oxidative stress) in the amniotic fluid and plasma of women at risk of preterm birth, lower levels of antioxidants in maternal and cord blood of preterm birth cases, as well as shorter telomere lengths and higher oxidative stress in preterm birth and pPROM [164,166]. The onset of labour is also associated with an increase in oxidative stress at the fetal membranes overlying the cervix at term, as demonstrated by lower SOD antioxidant activity and higher oxidative stress markers [167]. This contributes to increased membrane permeability, loss of integrity, and susceptibility to rupture.

Environmental factors such as maternal psychosocial stressors [168], pro-inflammatory cytokines [169,170], and infection [171] may disrupt the redox balance and cause premature aging of fetal tissues, and increase parturition mediators to induce early labour or pPROM. The excess ROS production cause tissue damage and cell death and generate a vicious cycle of ever-increasing inflammation and ROS production [164]. Increased ROS production also leads to the consumption of antioxidant defences,

telomere length reduction, inducing fetal membrane senescence and apoptosis, and activation of the NF-kB inflammatory pathway [164].

1.5.3.3 DAMPs or PAMPs

DAMPs are released by progressive physiological cellular stress, aging of gestational tissues (i.e., placenta and fetal membranes), mechanical stretch, and fetal growth. DAMPs-induced downstream transcriptional response elicits expression patterns of sterile inflammation. They trigger the expression and release of pro-inflammatory cytokines, chemokines, PGs and their receptors, leukocyte activation, upregulation of UAPs, and inflammasome assembly [85,172,173]. The mechanistic pathways of PAMPs-induced inflammation are similar to DAMPs but originate from pathogens via bacterial or viral infections [173]. DAMPs and PAMPs-driven inflammation causes fetal membrane weakening, tissue remodelling, and increases in inflammatory cytokines and chemokines in amniotic fluid [174], placenta, and maternal circulation [175], leading to preterm birth.

1.5.3.4 Neural-immune interactions: crosstalk between signalling pathways

The neuropeptide CRH has several roles in pregnancy maintenance, parturition, fetal maturation, and vascular, inflammatory, and stress responses [176]. CRH is locally produced in the decidua, placenta, and fetal membranes, and concentrations increase while CBG decreases throughout gestation [158]. In the mother, CRH-induced elevated CORT levels stimulate CRH local production in gestational tissues, where it produces several mediators involved with the onset of labour and parturition, including oxytocin (a potent uterotonic agonist) and PGs [177]. Placental and endometrial CRH stimulate the release of ACTH and cortisol in the fetus, which promotes fetal organ maturation [176]. Fetal-derived CORT further drives CRH secretion by the decidua, trophoblast and fetal membranes, creating a feed-forward loop.

Myometrial contractions are also directly regulated by CRH through its actions on CRHR1 and CRHR2 receptors [178], which modulate signalling cascades and downstream expression of PGs in the placenta and fetal membranes [179,180] and pro-inflammatory cytokines and chemokines via the NF-kB pathway [181]. These inflammation-sensitive mediators further induce other contraction-related mediators

and UAPs [85]. CRH's direct and indirect actions in gestational tissues and its interrelated interaction with inflammatory mediators demonstrate its critical role as a key hormone required for the events leading to uterine activation and labour.

1.6 Developmental origins of health and disease (DOHaD)

The fetal origins of adult disease hypothesis [182,183], frequently called Barker's hypothesis, postulates that exposure to environmental insults during critical windows of growth and development may have significant consequences on short and long-term health. The fetus is thought to respond to a hostile uterine environment to ensure survival by matching and adapting to the early environment, thereby preparing for the conditions it expects to encounter later in life [184]. Fetal adaptation induces developmental trajectory changes and permanent changes in the tissue's structure and physiological mechanisms. This predictive adaptive response, known as a form of developmental plasticity, is advantageous if the early acquired phenotype matches the physiology of its post-natal life [185]. However, if the pre-and postnatal environments are mismatched, the phenotype would manifest as a disease risk in adulthood.

With growing evidence that the life-long effects of early-life adversity were apparent not only during fetal development but also through childhood, the term fetal origins of adult disease became inappropriate [186]. Therefore, the name was updated to developmental origins of health and disease or DOHaD, encompassing the broader windows of susceptibility from embryo to child development.

1.6.1 Developmental windows of susceptibility: vulnerable time for fetal programming of epigenetic signatures and organ structure

The early stage of embryo development is a dynamic and vulnerable period in which epigenetic modifications in response to environmental cues play crucial roles in normal development, physiological processes, and embryo survival [187]. Epigenetics is defined as heritable alterations in gene expression that are not caused by changes in the DNA sequence, that is, changes in phenotype without changes in the genotype [188]. The epigenome is a record of biochemical modifications made to an organism's DNA and histone proteins [189]. Epigenetic modifications involve DNA methylation (DNAm), histone

modification and chromatin remodelling, and non-coding RNAs (ncRNAs) that control development, function, plasticity, and pathological processes. The epigenome controls the genome structure and function and dictates when genes are turned on and off, and regulates gene expression over a lifetime and across several generations [190]. Many epigenetic modifications are heritability maintained but are temporally regulated and reversible, potentially becoming irreversible [188].

Reprogramming waves of DNAm occur during early life to erase the parental methylation profiles to establish totipotency before embryo implantation. The susceptibility windows extend throughout pregnancy, where dynamic changes occur, including rapid growth, cell and tissue differentiation, and neuroendocrine and metabolic circadian rhythms are established. During these times, even small environmental factors can cause lasting effects on growth and development through induced epigenetic reprogramming, which includes undernutrition, endocrine disruptors, and stressful experiences [187,191].

Environmental exposures during critical times of development can lead to fetal programming. Fetal programming, also known as prenatal programming, suggests that environmental cues occurring during critical times of fetal development may cause changes that persist throughout the entire lifespan [192]. The term “programming” describes the effects of environmental imprinting and life-long malfunctions and disease [193,194]. The concept was later expanded to recognize the contributions of the fetal developmental period and early infancy in the long-term predisposition of diseases [195]. Thus, disease development is a consequence of an individual’s genetic and environmental interactions, whereby these inherited traits may be subsequently passed on to the offspring modifying their health outcomes [196].

1.6.2 Early-life stress: a critical period for disease programming

Adverse experiences in early life have long-lasting consequences on children’s health across various domains, including immunological, neuronal, behavioural, and metabolic development [197]. Early-life stress refers to any stress experienced during prenatal and early postnatal life and extends to childhood and adolescence [198]. Compelling evidence demonstrates that maternal nutrition status, mental health (e.g., perceived social isolation, depression, anxiety, etc.), maternal and child abuse, violence, poverty, and day-to-day hassles have lasting adverse effects on the offspring's health [198].

Animal models of early-life stress have significantly advanced our knowledge about the mechanistic pathways affecting long-term development, but the mechanisms of early-life reprogramming remain not well understood despite such advances. The complexity of such regulations might be attributed to the multidimensional aspects of stress and individual resilience.

1.6.3 Prenatal maternal stress (PNMS): definition and challenges in conducting its investigation

Prenatal maternal stress (PNMS) is any type of stress that pregnant women and animals experience [199]. Depending on its magnitude, it disrupts the balance that supports normal pregnancy and may affect maternal health and fetal development. The stressors range from mild, moderate and severe and vary by nature and time of exposure during gestation [200].

It was reported that 78% of pregnant women experience low to moderate stress while 6% experience high-stress levels [201]. The elevated levels of prenatal psychosocial stress are primarily associated with depression, panic disorder, domestic violence, drug use, and multiple medical comorbidities [201]. Higher prenatal stress levels are also linked to downregulation of placental transcripts related to cytokine signalling, T-cell regulation, and innate immune responses [202].

Assessing stress during pregnancy is accompanied by significant challenges, including the complexity of individual stress response, the relationship between individual perception of stress and environmental demands, and pregnancy symptoms acting as confounders for psychological and environmental factors [203]. Susceptibility to adversity is also subject to individual resilience, time of stress exposure, and the intensity of the challenge. The number of direct and indirect variables, their degree of intensity, and the subjectivity of stress perception make it hard to standardize and measure the effects of stress on health outcomes.

1.6.3.1 Consequences of PNMS on maternal and infant health outcomes

PNMS influences maternal and child's long-term health and development through direct and indirect pathways. Its indirect impacts on children's health are governed by adverse pregnancy and birth outcomes [200] (Figure 1.10). Maternal stress has been associated with an increased risk of infection

[204], gestational diabetes [205], preterm birth [206,207], preeclampsia [208], and unplanned caesarean section [209]. Altered maternal health, behaviour, and poor postnatal care may also indirectly affect infants' health [200,210]. Perinatal mood disturbances are linked with poor postnatal care, whereby lack of maternal-infant bonding and breastfeeding may lead to negligible behavioural development in infants [200,211]. Women subjected to high psychosocial stress tend to follow unhealthy behaviours such as poor nutrition and smoking, which are known risk factors for preterm birth and suboptimal infant health [149].

The direct effects of PNMS comprise alterations in neurobiological development, including changes in organ systems' function and structure. Prenatal exposure to maternal stress hormones, including CORT during stressful experiences or exogenous administration, has been shown to affect infants' long-term behaviour and physiology [40]. Gestational psychobiological stress was associated with delayed infant development and impaired cognitive performance [212]. Furthermore, clear evidence shows the lasting consequences of prenatal GC exposure to infant development and its association with low birth weight, developmental delays, organ dysfunction, and altered microbiota composition [40,213,214] (Figure 1.10).

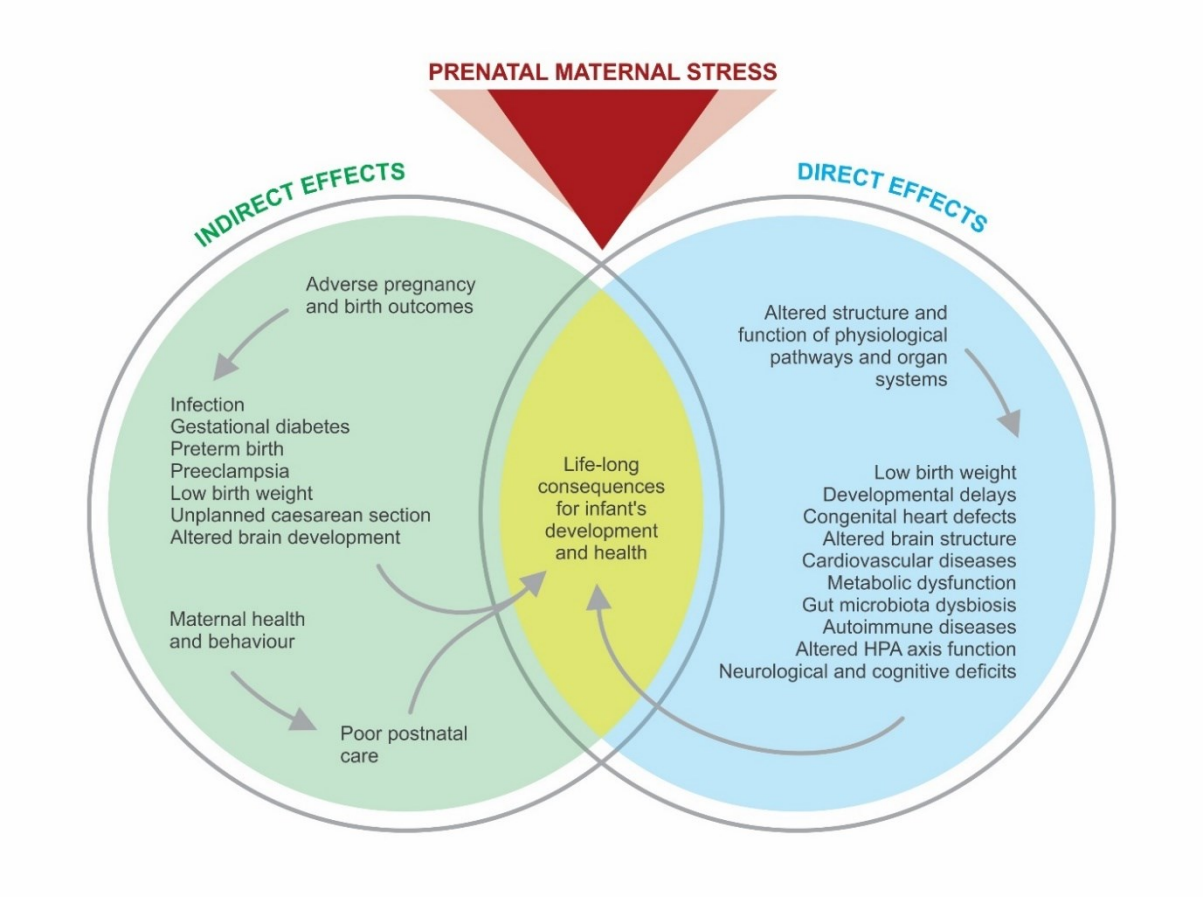

Figure 1.10: Consequences of PNMS on maternal and infant health outcomes.

The effects of PNMS are experienced by direct changes in the fetal development of organ systems or indirectly through increased risk of adverse pregnancy and birth outcomes and altered maternal health. Adapted from ref [200], use with permission from SAGE journal as outlined in Appendix D.

1.6.4 How about the fathers?

Most studies on the prenatal effects of stress focus on maternal events occurring during the preconceptional and prenatal periods. Despite that, solid research shows that the preconceptional health of fathers can also influence pregnancy and infant health [215-217]. It is suggested that parental insults acquired during the lifetime alter the health outcomes of the offspring through complex maternal-fetal interactions (e.g., maternal behaviour, germ cell reprogramming, intrauterine environment, and lactation). Fathers influence offspring health and development through direct paternal transmission via germ cell

reprogramming, seminal plasma, and indirect mechanisms via paternal behaviour in biparental species [216,218].

The DOHaD concept was expanded to include the Paternal Origins of Health and Disease, where paternal factors other than genetics dictate offspring's lifetime health [219]. Using animal models to study paternal inheritance can exclude the effects of paternal behaviour, social factors, and microbiota transfer [220]. A paternal high-fat diet in rodents has been associated with metabolic dysfunction early in life [221], changes in gut microbiota [222], and increased mammary cancer risk in their daughters [223]. Furthermore, the paternal obesity phenotype was transgenerationally transmitted to the first filial (F)1 generation males, increasing their risk of metabolic diseases following an additional short challenge with a Western-style diet [224]. It was shown that a truly inherited paternal transmission of disease risk when the F1 sons' programmed phenotype was observed in the F2 grandsons not exposed to the dietary challenge. Furthermore, chronic variable stress (CVS) administration to male mice during the preconceptional period revealed HPA axis dysfunction and epigenetic reprogramming in regulatory brain regions in the male offspring [225].

Although animal models have shown a clear link between paternal preconceptional exposure to environmental stressors and offspring disease risk, limited data are available on humans. Paternal stress contributions to offspring disease were noticed in epidemiological studies, including the 'Överkalix' study, where the ancestral effects of low food supply were evaluated. The Överkalix population suffered from a severe fluctuation in food supply in the 19 century [226]. The analysis of three generations revealed that good food supply in the paternal grandfathers during their slow growth period, which is the period before prepubertal peak in growth, was linked with higher mortality risk of diabetes mellitus in their grandchildren. Opposite findings were found with poor food supply, whereby grandsons whose paternal grandfathers had a poor food supply in the slow growth period exhibited reduced mortality risk of cardiovascular diseases [227]. Strikingly, the grandfather and father's low food supply was protective against cardiovascular death in future offspring [227]. The effects of paternal early-life stress were also assessed in the prospective FinnBrain Birth Cohort, whereby higher early-life stress in fathers was linked with altered white-matter development in the offspring's brain [228]. These early-life changes can have far-reaching implications during adulthood and may be passed on to later generations.

1.6.4.1 Underlying mechanisms of paternal transmission of environmental cues

The offspring's TG programming via the male-lineage appears to be regulated by epigenetic mechanisms. Accumulating studies have shown the influence of sperm and seminal fluid in the transmission of epigenetic marks and phenotypes across generations [222,229,230], programming future progeny to risk of disease or adaptation to the anticipated environment. Environmental-driven alterations of the sperm epigenome are possible due to the dynamic changes occurring during spermatogeneses, such as DNAm and histone modifications [230]. The epigenetic modifications control germ cell function and modulate embryonic development post-fertilization. The paternal inheritance through sperm has been proved with *in vitro* fertilization to eliminate confounding factors such as environmental effects, animal behaviour, and seminal plasma.

Spermatogenesis is the continuous process of producing spermatozoa from stem cells (prospermatogonia) in males, comprising four differentiation stages: spermatogonia, spermatocytes, spermatids, and spermatozoa (mature sperm) [217]. At puberty, sperm production is initiated with multiple mitosis and cell differentiation rounds to produce spermatocytes[231]. Spermatocytes go through meiosis to give rise to spermatids, a process known as spermiogenesis [231]. The spermatid paternal genome undergoes an extensive transition from nucleosomal core histone-to-protamine chromatin to protect the DNA and facilitate condensation of the sperm head [232].

In humans, 15% of nucleosomes are retained in mature sperm during the histone-to-protamine transition and post-translational modifications [233]. This indicates that epigenetic transmission of marks to future offspring may occur through retained histones that escaped global remodelling [217]. Thus, the histone-to-protamine transition and the continuous cycles of mitosis and meiosis are vulnerable periods for accumulating epigenetic modifications following environmental insults and could be transmitted to future offspring [234].

Spermatozoa RNA populations may also be vulnerable to environmental insults and altered by intercellular communication via epididymosomes. In fact, somatic-germline interactions mediated by extracellular vesicles (EVs) establish epigenetic memory. These EVs can acquire environmental cues that will be transferred to the sperm [235]. Similarly, seminal fluid contains microbiota that releases molecules akin to EVs that may also transfer RNA cargos to the sperm [235,236].

Alterations in sperm methylation patterns before fertilization have the potential to modulate the fetal programming of the offspring. Compared to somatic cells, DNAm patterns are unique in the spermatozoa and are formed during the two waves of methylation/demethylation reprogramming events after fertilization and in the primordial germ cells (PGC) of the developing embryo [237,238]. De novo methylation and demethylation also happen far beyond the fetal developmental stage, suggesting that DNAm patterns can be acquired during spermatogenesis and at various times during the postnatal life [239]. Thus, exposure to stress during fetal development, early life, and across a lifetime may predispose the offspring to epigenetic changes and postnatal diseases.

1.6.4.2 Effects of stress in early life and adulthood on male reproductive health

Basal GC levels during pubertal development have supportive roles in cell development and steroidogenesis [240]. However, it is increasingly clear that environmental stressors affect male health and fertility. Stress impairs testicular function [241], spermatozoa parameters [242], suppresses testosterone secretion [243], and may lead to infertility [244]. Lifestyle choices (e.g., alcohol and cigarette consumption), infections, and systemic inflammation are linked with testicular dysfunction and infertility [245] (Figure 1.11). However, the underlying mechanisms of stress-induced testicular dysfunction are not yet fully understood.

Several studies indicate that early-life origins of testicular dysfunction arise from environmental exposure to insults that permanently modify testicular germ cells [246-249]. Germline epimutations acquired during fetal gonadal sex determination may become mitotically stable and can be transmitted to subsequent generations via sperm and egg [250]. These modifications are passed on to the embryo's stem cells and ultimately to all somatic cells, leading to epigenetic TG inheritance of disease risk.

Stress-induced activation of the HPA axis and release of gonadotropin-inhibitory hormone suppresses the hypothalamic-pituitary-gonadal (HPG) axis and the Leydig cells in the testes [244]. Leydig cells are the primary source of testosterone production in the testes under the control of luteinizing hormone (LH) [244]. The inhibitory effects of stress contribute to the fall in testosterone levels, which in turn impairs testicular structure, cell function, and spermatogenesis [248,249]. In fact, work and chronic

life stressors in men were shown to reduce testosterone levels, sperm motility, count and lead to sperm morphological defects [251,252].

Little is known about the roles of GCs, the MR, and GR in testicular function and development [253,254]. It is known that GR polymorphism modulates sperm parameters [255]. Given that the human testis is a target for GCs and testicular cells express GR, it is plausible that stress may alter polymorphism of the GR and cause testicular and sperm dysfunction. In sheep, acute exposure to synthetic GC (i.e., betamethasone) at mid-gestation impaired fetal testicular development by changing testis morphology and increasing GR expression in Leydig cells of male fetuses [256], suggesting that environmental factors early in life modulate long-term male reproductive health. Stress-induced CORT in rodents inhibited testosterone-biosynthetic enzyme activity, partly through its actions on GR present in Leydig cells [257], and caused apoptosis of Leydig cells and low testosterone levels [258].

Inflammatory mediators participate in physiological roles in the testis, including intercellular communication in the seminiferous epithelium, Sertoli cell development, spermatogenic cell division and differentiation, testicular steroidogenesis, etc. [259,260]. However, excessive production of inflammatory molecules and ROS may impair normal physiological processes in the testis. This occurs through downstream signalling pathways that ultimately affect hormone production, organ structure and function, and fertility [259] (Figure 1.11). The testis's Sertoli, Leydig and immune cells express PRRs such as TLRs and produce pro-inflammatory cytokines [259]. The IL-1 cytokines have inhibitory effects on LH-induced androgen formation by Leydig cells [261] and in steroidogenic enzymes in the testis [262], which may lead to testicular dysfunction.

A rat model of systemic inflammation with LPS injections induced oxidative stress and reduced antioxidant enzymes, including SODs [263]. The systemic inflammation caused testicular increases in IL-1β and COX-2. These inflammatory mediators may impair the function of steroidogenic Leydig cells and further intensify ROS production. Similarly, a single LPS injection in mice decreased serum testosterone, steroidogenic proteins, and increased oxidative stress damage in Leydig cell membranes through lipid peroxidation [264]. The LPS- induced ROS disrupted Leydig cell mitochondria, suggesting that testicular inflammation adversely impacts steroidogenesis.

Stress exposure may also affect the redox balance set by the NADP+ to NADPH ratio within testicular cells and alter the oxidase and reductase activities of the 11β-HSDs enzymes that control local GC and, indirectly, affect testosterone concentrations [265]. Both isoforms have been shown to protect testicular tissues from the adverse effects of excessive CORT on testosterone production [240]. However, the effects of stress on testicular expression and activity of 11β-HSDs are poorly investigated.

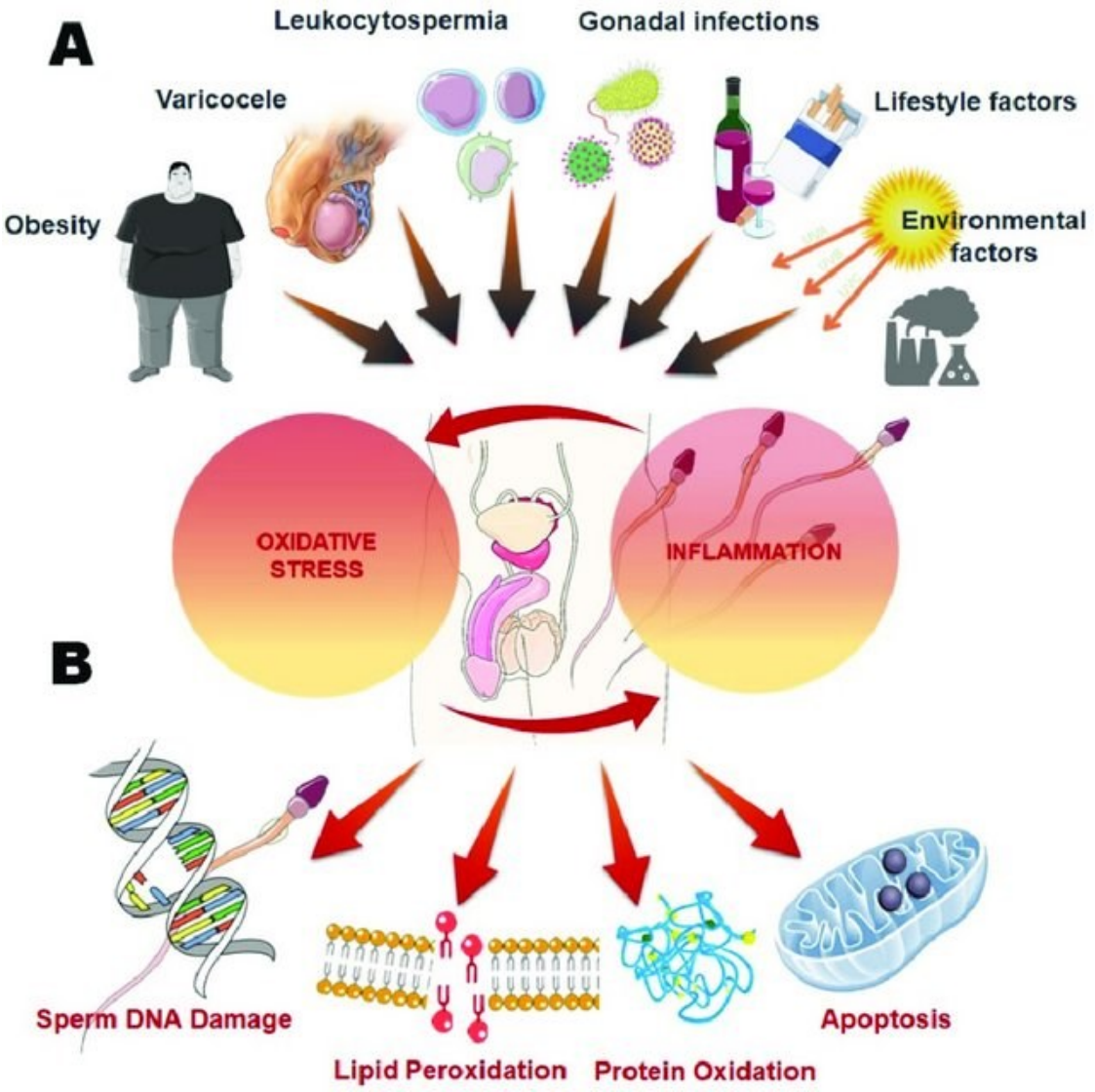

Figure 1.11: Influence of several stressors on testicular structure and function.

(A) External and internal factors may disrupt normal testicular functioning through the induction of inflammation and oxidative stress. (B) Inflammatory and oxidative stress mediators generate a vicious continuous cycle of feed-forward activation. This creates cellular damage and induces testicular dysfunction via downstream activation of inflammatory and apoptotic cascades and cellular and molecular damage. From ref *[259]*, Creative Commons Attribution licence (CC BY).

1.6.5 Mechanisms of early-life programming

There are several mechanisms hypothesized for how PNMS affects life-long fetal programming. While the association between prenatal stress and offspring adverse outcomes is now established, the underlying mechanisms are poorly understood. Understanding these mechanisms is vital to comprehend the effects of gene-environment interplay on human development. This knowledge would open possibilities for prevention and treatment, resulting in favourable outcomes on human health trajectories. This thesis outlines some potential mechanisms underlying the psychosocial and psychological effects on maternal and fetal health.

1.6.5.1 Permanent changes in organ structure, volume, and/or tissue composition

Early-life insults during critical periods of organ development and tissue differentiation can cause permanent changes in organ structure. Indeed, multigenerational (MG) exposure to recurrent restraint and forced swimming stressors induced neuromorphological changes in the parietal cortex of rats [266]. Moreover, maternal undernutrition during pregnancy had long-lasting impacts on male adult offspring testicular morphology, altering seminiferous tubules and epithelium parameters [267].

1.6.5.2 Inflammatory mediators

Cytokines, chemokines, and ROS have interrelated relationships, where each one contributes to the other's regulation [268]. Inflammatory imbalance or MIA during pregnancy may activate fetal immune responses and permanently change neuroimmune function and fetal brain development [269,270]. However, how maternal cytokines enter the fetal circulation and affect fetal brain development remains unclear.

Studies have shown that cytokines can cross the placenta [271]. Low amounts of IL-1α and IL-1β and high amounts of IL-6 were transferred through intact fetal membranes [272]. Furthermore, a study showed that IL-6 had bi-directional movement across the human placenta, while the transfer of IL-1α and TNF-α was low [273]. In animal studies, MIA-induced downstream signalling of IL-6 through the placenta resulted in immunological programming of the fetal brain and behavioural impairments [274].

Despite the increase evidence, the capacity of cytokines and chemokines to cross the placenta remains controversial. As such, stress-induced cytokine activation in the placenta and fetus is another potential mechanism to explain the presence of inflammatory mediators in fetal circulation [269].

1.6.5.3 Excessive GC exposure and HPA axis dysregulation

Modifications to GC metabolism by altered 11β-HSDs enzyme activities may influence early-life fetal programming. Inactivating 11β-HSD2 is thought to permanently change fetal cardiovascular and endocrine systems and metabolism [275]. Prenatal stress from gestational days (GD)14-20 resulted in reduced 11β-HSD2 expression and DNAm changes in the HSD11B2 gene promoter in the placenta of rats [276]. These changes could lead to higher fetal exposure to GCs and increase the risk of preterm birth and delayed intrauterine growth [277].

Other hypotheses involve maternal and fetal disruption of the HPA axis and altered negative feedback responses and expression of GR in the fetal brain [278,279]. Prenatal corticosteroid therapy during premature labour also exposes the fetus to excess GCs and contributes to HPA axis alterations and cardiovascular and metabolic disorders in adulthood [275].

1.6.5.4 Epigenetics

Epigenetic processes control cell differentiation, gene expression, genomic imprinting, and metabolism [188] and are key to fetal programming. This is supported by TG studies demonstrating the epigenetic transmission of marks from one generation onwards and the consequent adverse health outcomes in the progeny. Stressing pregnant rats with forced swimming and restraint altered the expression of uterine and placental microRNAs (miRNAs) involved in pregnancy maintenance and labour.

These changes were transmitted across three generations, which might explain the shorter gestations observed in the progeny [152].

Epigenetic inheritance is also transmitted through the sperm. For example, subjecting males to a low-protein diet caused global hypomethylation of sperm DNA and impacted their offspring's health [222]. The underlying mechanisms in the paternal programming of the offspring may involve sperm carrying epigenetic marks that escape reprogramming or seminal plasma-mediated reprogramming, possibly through its microbiota and PGs (i.e., regulating the maternal reproductive tract and immunological responses required for pregnancy success) [222,280]. Transcription factors are also subjected to epigenetic regulation, affecting a wide range of downstream target genes through gene expression control [281].

1.6.5.5 Intergenerational (IG), multigenerational (MG), and transgenerational (TG) inheritance

Intergenerational, multigenerational, and transgenerational (herein referred to as IG, MG, and TG, respectively) inheritances are used to distinguish different environmental exposures in their frequency and longitudinal duration (Figure 1.12). IG inheritance is when the gametes are exposed to the environmental factor, and the transmission of information occurs directly from the parents to the child [282]. MG influence refers to the direct exposure of multiple generations to the environmental factor [283], revealing the cumulative effects of stress. One would expect that the adverse effects of MG exposure would be enhanced in the grandchild or great-grandchild. Prenatal stress in the parental F0 generation has been shown to reduce the pregnancy duration of children and grandchildren, where the shortest pregnancy length occurred in the MG stress lineage [152].

TG inheritance represents the transmission of information to generations where the germline has not been directly exposed to the environmental factor [283]. In the female germline, when a gestating female (F0) is directly exposed to environmental insults, her developing fetuses (F1 generation) somatic and germ cells (the PGC of the embryo producing the F2 generation) experience *in-utero* exposure to the insult (Figure 1.12). A true TG inheritance involves persistent phenotypic changes in the non-exposed F3 generation through epigenetic mechanisms [283]. When the environmental event occurs in the male

germline, the somatic cells of the F0 father are directly exposed to the insult along with its sperm (F1 generation). Thus, the heritable phenotype present in the F2 generation onwards is derived from epigenetic mechanisms and represents a true TG inheritance [188].

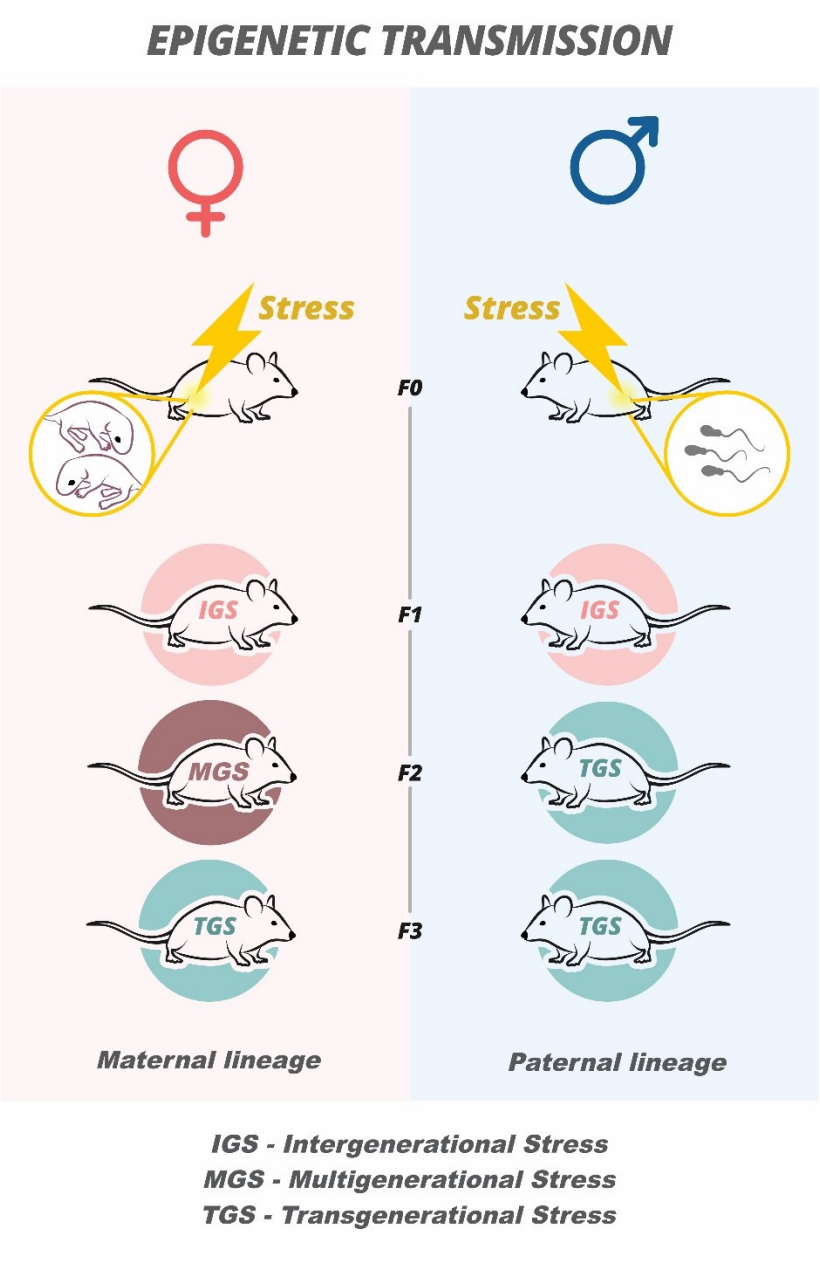

Figure 1.12: Schematic of IG, MG and TG inheritance of stress.

Exposing F0 gestating females to stress results in direct exposure of the F1 generation embryo (IG exposure) and F2 generation germline (MG exposure). The F3 generation is the first that has not been directly exposed to the insult, demonstrating a genuinely TG phenomenon. Exposing F0 males to stress

affects their somatic and germ cells (i.e., sperm), which will produce the F1 generation. The F2 generation is the first that has not been exposed to stress; thus, their phenotypes are transmitted by a true TG inheritance.

1.7 Coping with stress: contribution of early-life experiences

Coping is a critical process involving individual strategies to manage internal and external challenges during the stress response [284]. Part of the process of coping involves the individual subjective cognitive appraisal through evaluation and interpretation of a particular encounter, which has the potential to cause harm or benefit. A situation perceived as a threat triggers cognitive, behavioural, and physiological efforts to manage the demands of the perturbation by dealing with the problem and regulating emotion to minimize internal disturbances. If the challenge or cumulative stress during a lifetime exceeds the ability of one to cope, adverse health outcomes may arise [156].

Various factors support the development of an individual's coping strategies, including personal, social, and cultural factors, either experienced early in life, throughout life or acquired from others [285]. Indeed, studies have shown that early-life developmental programming influences coping skills during a lifetime and that stress-coping phenotypes are transgenerationally transmitted to the offspring to facilitate adaptation to a later predicted environment [284,286].

1.8 Strategies to mitigate the effects of prenatal maternal stress: enriched environment therapy

As extensively reviewed in the previous sections, maternal stress significantly impairs normal physiological adaptations of pregnancy and disrupts the neuroendocrine and immune systems and fetal development [287]. Coping strategies to reduce stress include regular exercise, meditation, therapy, avoiding unhealthy habits, increasing social interaction, etc. Stress management approaches have improved anxiety and depressive disorders in pregnant women [288]. Expressive writing is a low-cost and simple intervention involving 15-20 min of writing that provides emotional disclosure, anxiety and worry relief, resilience, and improves physical and immune parameters [289,290]. Interventions targeting infants have also shown beneficial outcomes. Early developmental interventions for preterm

birth infants significantly improved cognitive and motor outcomes [291]. Yet, little is known about the most effective intervention type and implementation time and duration.

Enriched environment is used to reduce or reverse the adverse effects of programming by stress in animals [292]. Enriched housing is characterized by social, cognitive, motor, and sensory stimulation. In these cages, animals can interact with their counterparts in larger spaces and access different toys, shelters, and novel types of food [292,293]. Enriched environment has been shown to attenuate prenatal effects of stress on cognitive, behavioural, and motor function [294,295] as well as neuronal morphology and immune function [295,296]. Enriched environment also improved epigenetic modifications due to stress [292,297] and reduced inflammatory mediators in the uterus and cervix of pregnant mice subjected to systemic inflammation induced with LPS, contributing to pregnancy maintenance and reducing preterm birth rates by 40%. [298]. While enriched environment therapy has primarily produced beneficial effects on stressed animals, its outcomes are inconsistent and depend on the type of enrichment, species, individual variability, and variable under analysis (e.g., behavioural, specific stress biomarkers) [299].

1.9 Animal models of stress used in this dissertation

Animal models have long been used in biomedical research to investigate a large range of mechanisms and to test novel therapies and approaches to medical care. The advantage of using animals in translational research is the ability to strictly control the environment, make feasible genetic and physiological manipulations, and replicate human biological conditions in animals [300].

Animal models of stress are utilized to reproduce the conditions that humans are exposed to daily. Given that the stress response is complex with multifaceted manifestations, researchers should consider the translational interpretations of the duration of the challenge, the time of assessment of indicators/mediators of stress, the phenomenon of habituation, and the physiological and anatomical differences between humans and animals [301].

1.9.1 Psychosocial and psychological stressors

Psychosocial and psychological stressors have been shown to impair normal physiological and biochemical functions. They are considered risk factors for the development of diseases such as depression, anxiety, and cardiovascular and immunological diseases [302]. The stressors discussed below were used in this dissertation's animal models of stress.

1.9.1.1 Social isolation stress (SIS)

Humans are social beings that need to engage in social interactions and require a sense of belonging to maintain a healthy mental state and feel emotionally fulfilled [303]. Social isolation in humans is a quantitative lack of social contact. Loneliness, however, is a subjective or emotional perception of social isolation with a lack of social contact and an absence of belongingness [304]. The former is associated with poor physical health, whereas the latter influences mental health. Both are interrelated and, if left unattended, can lead to cognitive, emotional, behavioural, and physical health complications [304]. Around 15-30% of the general population suffers from chronic loneliness [305], with fewer social interactions occurring with increasing age [304].

Social isolation is considered a psychosocial stress linked to higher morbidity and mortality rates [303], and is commonly associated with depression, anxiety, and low resilience [306,307]. Perceived social isolation is particularly interesting given the global pandemic imposed by the novel SARS-CoV-2 virus in early 2020. To contain the virus, governments worldwide initiated mass physical distancing (i.e., lockdowns) for everyone and reinforced isolation/quarantine protocols for infected and exposed people. These protocols caused worldwide social, economic, and life-long health consequences, including global impacts on mental health [308]. Therefore, studying the effects of social isolation is timely and essential to understanding its impact on adverse health outcomes.

To induce social isolation stress (SIS) in animals, they are placed alone in a cage to limit social interactions while conserving normal access to olfactory, auditory, and visual contact with their counterparts [309]. SIS has been reported as mild psychosocial stress in rodents [292,295,310], resulting in functional, morphological, and neurochemical alterations in distinct brain areas, HPA axis dysfunction, and altered behaviour in adult offspring [295,311-313]. However, SIS's effects are inconsistent across

animal studies, given the variations in species and laboratory strain, sex differences, and individual resilience [314]. Other factors that might influence these contrasting findings include the handling procedures, duration, and time of the isolation protocol.

The most commonly used SIS procedures include a low intensity of SIS in the first days of life [315-318], social separation during GD1 to 21 [319], post-weaning SIS [320-323], juvenile and gestational SIS [292,324], and isolation during adulthood with additional stress exposures [313,325]. As far as we know, the evaluation of the effects of preconception and gestational SIS on pregnancy outcomes has not been studied before.

1.9.1.2 Restraint stress

Restraint stress is the most used stressor in rodents and is commonly used as a prenatal stressor [287]. The rodent is placed in a plastic tube or wire-mesh container to restrict their movements and block them from moving and turning [326]. Even though it is considered a form of physical stress, restraint is arguably mostly a psychological stressor because the animal's cognitive perception of its inability to move/turn precedes the physical discomfort of being restricted in a tube [327]. The effects of restraint are well documented with impairments in cognitive, affective [328,329], morphological [330], neuroendocrinological and immunological functions [331], and epigenetic responses [332]. Restraint protocols are adaptable to each stress study and vary in frequency, duration, severity, the instrument used, and time of exposure (light or dark phase).

1.9.2 Animal models of human pregnancy

Pregnancy typically lasts 40 weeks of gestation in humans, which presents a challenge in studying mechanistic pathways related to parturition [333]. Along with ethical reasons, other significant obstacles include the availability of reproductive and fetal tissues and economic barriers [334]. Hence, animal models of human pregnancy represent a powerful strategy to overcome these difficulties, besides the remarkable anatomical and physiological similarities to humans. It is important to consider each animal model's limitations and to evaluate the literature for the most relevant model depending on experimental designs (Table 1.1).

Nonhuman primates are similar to humans at physiological, immunological, behavioural, and genetic levels [335]. However, their shortcomings include small litter, costs, long gestation times, ethics/legislation, and lack of genetic control. Rodents (rats and mice) are often used in biomedical research because of their similar anatomy and physiology to humans [336]. They have economic advantages, share 95% of genes between all three species, have shorter reproduction lengths, weaning, and lifespan, produce larger litter sizes, and allow for substantial genetic manipulation. Rodent models also have differences from humans, including, but not limited to, uterine and placental structural differences, gene expression and proteins abundance, HPA axis regulation, length of gestation, and heavily reliability on progesterone withdrawal at the end of pregnancy [337].

Table 1.1: Comparisons of reproductive characteristics of different animal models with humans.

From ref *[333]*, used with approved permission of the American Physiological Society, Copyright © 2009, as outlined in Appendix D.

Characteristic	Rabbit	Sheep	Rat	Mouse	Guinea pig	Human
Gestation, days	32±3	147±4	22±1	20±1	67±3	266±14
Usual litter size, no.	8±4	1–2	10±6	10±5	3±2	1
Placental morphology	Hemodichorial, labyrinthine	Epithelial-chorial, cotyledonary	Hemotrichorial, labyrinthine	Hemotrichorial, labyrinthine	Hemomonochorial, labyrinthine, discoid	Hemomonochorial, villous, discoid
Source of progesterone	Corpus luteum	Corpus luteum, then placenta	Corpus luteum	Corpus luteum	Corpus luteum, then placenta	Corpus luteum, then placenta
Progesterone withdrawal?	Yes	Yes	Yes	Yes	Partial at *days 40–50*; not at parturition	No
Induction of preterm birth	Antiprogestin, ovariectomy	Fetal ACTH, glucocorticoid, antiprogestin	Antiprogestin, ovariectomy	Antiprogestin, ovariectomy, LPS	Antiprogestin plus oxytocin	Cervical ripening (PGE_2 or antiprogestin) plus oxytocin

Values are means ± SD.

Chapter 2

Hypotheses and aims

It is clear from the extensive background discussion that parental experiences acquired through the lifetime can influence offspring health outcomes. These phenotypic variations are transgenerationally passed on to the offspring through the maternal and paternal lines. However, there is a gap in the literature regarding the effects of environmental insults on reproductive tissues' inflammatory and stress-related markers and its associated risk of pregnancy complications such as preterm birth. To fill this gap, the main aims of this dissertation were:

1) to examine the transgenerational (TG) effects of different prenatal maternal stressors (PNMS) on uterine and testicular inflammatory and stress biomarkers and their health consequences,

2) to assess the contribution of environmental insults to preterm birth in the stressed parental generation and the offspring exposed to ancestral stress,

3) to examine the TG effects of long-term enriched environment exposure to one generation of ancestrally stressed rats (F1 generation of stressed females), and

4) to identify sex differences in reproductive tissue programming across generations of rats exposed to ancestral stress through the maternal and paternal lineages.

To answer these questions, we first subjected rats to long-term SIS from preconception and throughout pregnancy (Chapter 4). To the best of my knowledge, no studies evaluate the SIS's effects on pregnancy outcomes and the inflammatory profile of reproductive tissues. Additionally, SIS's TG and multigenerational (MG) effects were not previously investigated in reproductive tissues. The impact of SIS was assessed across four generations of rats in a TG and MG prenatal stress fashion. Ancestral and cumulative prenatal stressors have been shown to alter behaviour and produce physiological dysfunctions in various organ systems in the offspring. Based on this knowledge, I hypothesized that:

• Pre-pregnancy and gestational SIS increases the risk of preterm birth in mothers and offspring and low birth weight in their pups.

• Long-term SIS induces altered uterine markers of stress and inflammation across four generations of rats in both a TG- and MG-dependent manner.

The cumulative effects of different stress hits have been shown to enhance maternal stress load and cause immediate and long-lasting implications on maternal and offspring health. Based on that information, we investigated the effects of two psychological and psychosocial CVSs using restraint and

SIS in pregnant rats over four generations. This cohort aimed to assess the TG effects of CVS. Thus, only the parental F0 generation was stressed, while the F1-F3 offspring were undisturbed. From the F1 offspring onwards, the cohort was subdivided into two separate studies: maternal prenatal transgenerational stress (MTPS) and paternal prenatal transgenerational stress (PTPS) to assess the transmission of information through both maternal and paternal lineages.

In the MTPS study of chapter 5, I hypothesized that:

• PNMS induces uterine molecular changes of key inflammatory and stress markers across generations.

• CVS-induced uterine imbalance of inflammatory and endocrine markers increases the risk of preterm birth in stressed mothers and offspring.

• The TG effects of adversity are mitigated by one-generation exposure to enriched environment housing in the F1 offspring.

In the PTPS study of chapter 6, I hypothesized that:

• Gestational CVS in the F0 parental generation induces fetal programming of inflammatory and stress markers in the F1 offspring's testes.

• Paternal F1 altered phenotypes are transgenerationally passed on to the second and third generations' reproductive tissues via the paternal lineage, affecting the progeny's uteri and testicular inflammatory and stress profiles.

• The CVS-induced altered uterine inflammatory and endocrine markers increase the risk of preterm birth in the female offspring of F1-stressed fathers.

A schematic of this dissertation aims is described in Figure 2.1.

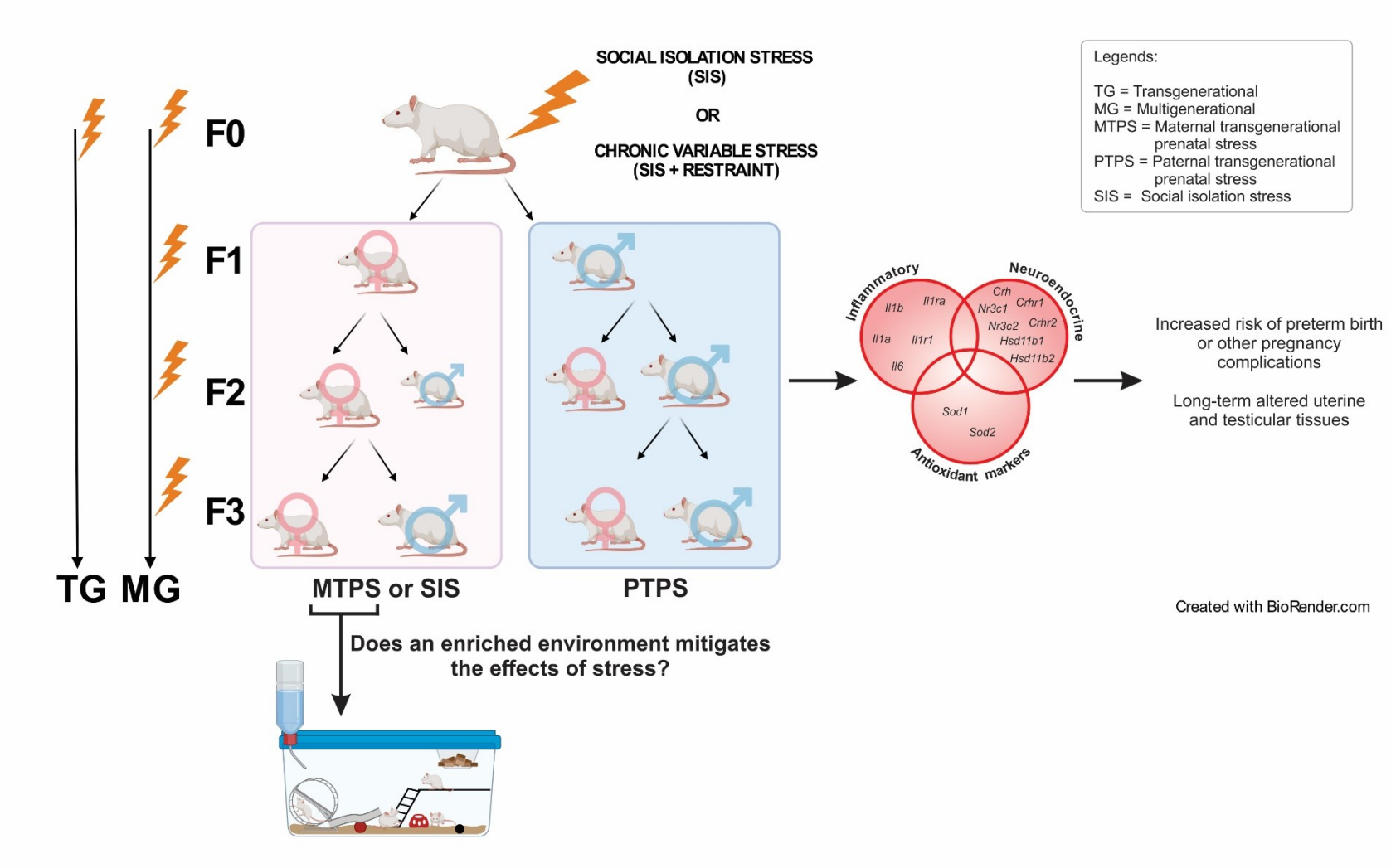

Figure 2.1: Overview of the main hypotheses and aims described in this dissertation.

A summary of the studies' objectives and hypotheses for the social isolation stress, maternal transgenerational prenatal stress (MTPS), and paternal transgenerational prenatal stress (PTPS) projects.

CHAPTER 3

General methods

3.1 Tissue Collection

Social isolation stress (SIS) study (Chapter 4)

Uterine tissue

In F0, F1 and F2 generations, dams were euthanized at weaning (lactational day (LD)21) via intracardiac injection of euthanyl (sodium pentobarbital) 300 mg/kg (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada). In the F3 generation, uteri were collected from virgin females at P90. Following decapitation, uterine horns were extracted immediately and transferred into tubes placed on dry ice, then stored at −80 °C. Uterine horns were used for gene expression (RT-qPCR) and protein (Multiplex assay) analyses. The number of animals varied between generations and experiments: Controls N = 20-31; F0 N = 8-10; F1 N = 5-8, F2 + F3 N = 4-12.

Blood

Blood draws (0.5 mL) occurred at baseline prior to social isolation between days 90-105 and on gestational day (GD)18. Blood sampling was performed as previously described by Faraji et al. 2020 [338]. Briefly, blood samples were collected between 9:00 and 11:00 am from the tail vein of animals anesthetized with 4% isoflurane. We isolated plasma by centrifuging the blood samples at 5000 rpm for 5 min. Plasma samples were stored at −80 °C until corticosterone (CORT) level analysis. Non-fasting blood glucose was measured using an Ascensia Breeze Blood Glucose Meter with test strips (Bayer, Mississauga, ON, Canada).

Maternal transgenerational prenatal stress (MTPS) study

Uterine tissue

Dams were euthanized with euthanyl (sodium pentobarbital) 300 mg/kg (Cambridge, ON, Canada) anesthesia on LD21 or postnatal day (P)115. Uterine horns were dissected and snap-frozen for mRNA and protein analyses (N = 6-12 and N = 3-4, respectively). Snap-frozen tissues were placed at –20 °C prior to tissue embedding. Pieces of uterine horns were embedded in optimal cutting temperature medium compound (Tissue-Tek® OCT, Sakura Finetek, CA, USA) and snap-frozen for subsequent analysis. Tissues embedded in OCT were cut at 5 µm, mounted at –20 °C, and stored at –80 °C until use.

Blood

Animals under 4% isoflurane (Fressenious Kabi Canada Ltd. Toronto, ON, Canada) had blood samples (0.5 mL) collected from the lateral tail vein between 9:00 and 10:00 am on GD18 for dams and P110 for tested offspring. Plasma was isolated by centrifuging the blood at 5,000 rpm for ten minutes and then stored at −80 °C until further analyses.

Paternal transgenerational prenatal stress (PTPS) study

Uterine and testicular tissue

Males were euthanized with euthanyl (sodium pentobarbital) 300 mg/kg (Cambridge, ON, Canada) anesthesia when successful pregnancy was confirmed in their partners, while females were euthanized on LD21. Tested adults were euthanized on P115 when their uteri or testes were collected. Uteri and testes were dissected and snap-frozen for mRNA and protein analyses (N = 7-22 and N = 5, respectively). Snap-frozen tissue was stored at –80 °C until use.

Blood

Animals under 4% isoflurane (Fressenious Kabi Canada Ltd. Toronto, ON, Canada) had blood samples (0.5 mL) collected from the lateral tail vein between 9:00 and 10:00 am on GD18 for dams and P110 for tested offspring. Plasma was isolated by centrifuging the blood at 5,000 rpm for ten minutes and then stored at −80 °C until further analyses.

3.2 CORT Assay

SIS study

CORT levels were measured using enzyme-linked immunosorbent assay (ELISA) commercial kits (Cayman Chemical, Ann Arbor, MI, USA) as per the manufacturer's protocol.

MTPS and PTPS studies

CORT levels were measured by commercial ELISA kits (Abcam, ab108821d; Cambridge, UK) according to the manufacturer's instructions.

3.3 Molecular analysis

3.3.1 Gene Expression

RNA Extraction

Total ribonucleic acid (RNA) from F0-F3 uterine horns was extracted using Trizol (Thermo Fisher Scientific, Wilmington, DE, USA) and Qiagen RNeasy Mini Kit on QIAcube (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the total RNA. A 260/280 nm absorbance ratio of ~2.0 was considered pure

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was used to quantify genes involved in parturition, inflammation, and stress-related pathways in the uterine horns. The genes selected for each project were:

SIS study: inflammatory markers: *Il1a, Il1b, Il1r1, and Il6*. Stress markers: *Crh, Crhr1, Crhr2, Hsd11b1* and *Hsd11b2*.

MTPS study: inflammatory markers: *Il1a, Il1b, Il1ra, and Il6*. Stress markers: *Crh, Crhr1, Crhr2, Hsd11b1, Hsd11b2, Nr3c1, Nr3c2, Sod1, and Sod2.*

PTPS study: inflammatory markers: *Il1a, Il1b, Il1ra, Il1r1, Il1rap, and Il6*. Stress markers: *Crh, Crhr1, Crhr2, Hsd11b1, Hsd11b2, Nr3c1, Nr3c2, Sod1, and Sod2.*

The reverse transcriptase reaction was performed with total RNA (500 ng) to produce complementary deoxyribonucleic acid (cDNA) using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's protocol. The primers used in these studies were previously designed by our group, assuring that the 3′ and 5′ primers spanned over an exon-exon boundary to avoid primers binding to genomic DNA. Primer sequences, annealing temperatures, and accession numbers are described in Table 3.1. The PCR was completed in duplicates by adding 0.5 µL forward and 0.5 µL reverse primer (10 µM), 10 µL iQ SYBR Green Supermix (Bio-Rad Laboratories, Mississauga, ON, Canada), and 9 µL of cDNA (25 ng/µL) for a total reaction of 20 µL/well. Two-step quantitative RT-PCR (amplification and melt curve analysis of nonspecific products) with

denaturation at 95°C for 10 min, annealing and elongation for 15 s at 95°C, and 1 min at the primer-specific annealing temperature (Table 3.1) were run in iCycler iQ thermal cyclers (Bio-Rad Laboratories, Mississauga, ON, Canada). A pooled sample was prepared with three different cDNA samples combined to assess batch-to-batch repeatability between the same gene experiments. The pooled sample was included in all PCR plate analyses with proper threshold cycle (Ct) adjustments prior to data analysis.

Data analyses were conducted as previously described by Leimert et al. [172]. In brief, cDNA samples were serially diluted to produce a standard curve for each PCR reaction (target genes and the housekeeping gene *Ppia*) and analyzed with iCycler IQ software (Bio-Rad Laboratories, Mississauga, ON, Canada). The equation $E = 10^{-1/slope}$ was used to determine the reaction amplification efficiency using the slope of the standard curve. The average Ct value for each sample was corrected by the efficiency of the reaction. This was repeated for all genes selected in this study. The final threshold cycles were expressed relative to the pooled sample. Target genes data were analyzed according to the Pfaffl method [339] relative to Cyclophilin A (Peptidilprolyl Isomerase A or *Ppia*) gene expression using the formula:

$$expression\ ratio\ = \frac{E_{Target}{}^{\Delta Ct(Control-Sample)}}{E_{Ref}{}^{\Delta Ct(Control-Sample)}}$$

Table 3.1 Primer forward and reverse sequences and annealing temperatures for RT-qPCR.

Gene function	Target gene	Forward primer (5' → 3')	Reverse primer (5’ → 3’)	Annealing temperature (°C)	Amplicon size (bp)	NCBI reference sequences*
Housekeeping gene	***Ppia (Cyclophilin A)***	CAC CGT GTT CTT CGA CAT CAC	CCA GTG CTC AGA GCT CGA AAG	60	114	NM_017101.1
Cytokines implicated in the inflammatory process of labour	***Il1a***	AAGACAAGCCTGTGTTGCTGAAGG	TCCCAGAAGAAAATGAGGTCGGTC	55	85	NM_017019.1
	Il1b	CTCAATGGACAGAACATAAGCC	GGTGTGCCGTCTTTCATCA	51	143	NM_031512.2
	Il6	TCCTACCCCAACTTCCAATGCTC	TTGGATGGTTTGGTCCTTAGCC	65	78	NM_012589.2
	Il1ra	AAGACCTTCTACCTGAGGAACAACC	GCCCAAGAACACATTCCGAAAGTC	55	117	NM_022194.2
	Il1r1	CCTGTGATTATGAGCCCACG	CGTGTGCAGTCTCCAGAATATG	58	298	NM_013123.3
	Il1rap	GGGCAACATCAACGTCATTTTAG	CAGCTCTTTCACCTTCAAGTCCTT	68	64	NM_012968.1
Enzymes that regulate CORT action in gestational tissues and testis	***Hsd11b1***	GAAGAAGCATGGAGGTCAAC	GCAATCAGAGGTTGGGTCAT	60	113	NM_017080.2
	Hsd11b2	CGTCACTCAAGGGGACGTAT	AGGGGTATGGCATGTCTCC	55	61	NM_017081.2
Crh peptide and receptors involved in HPA axis regulation and control of myometrium activity	***Crh***	ATCTCACCTTCCACCTTCTG	GTGTGCTAAATGCAGAATCG	60	147	NM_031019.1
	Crhr1	GGTGACAGCCGCCTACAATT	AAGGTACACCCCAGCCAA	60	149	NM_030999.4
	Crhr2	TGGTGCATACCCTGCCCTAT	GTGGAGGCTCGCAGTTTTGT	60	200	NM_022714.1
GC receptor involved in metabolic processes, implantation, fetal development, onset of labour, testicular maturation, Leydig cell steroidogenesis, etc.	***Nr3c1***	TGTATCCCACAGACCAAAGCA	AATCCTCATTCGTGTTCCCTTC	52	183	NM_012576.2
MC receptor modulates the	***Nr3c2***	GGCAAACAGATGATCCAGG	CAACTCAAAGCGAACGATGA	60	128	NM_013131.1

immune system function and cytokine production in gestational tissues						
Antioxidant enzymes that regulate the balance of ROS production	***Sod1***	GCAGAAGGCAAGCGGTGA	GGTACAGCCTTGTGTATTGTC CC	60	112	NM_017050.1
	Sod2	GTCTGTGGGAGTCCAAGGTT	GTTCCTTGCAGTGGGTCCTGATTA	60	105	NM_017051.2

*NCBI—National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

3.3.2 Luminex Cytokine Assays

Cytokine levels for IL-1α, IL-1β, and IL-6 from F0 and F1 uteri were quantified simultaneously in Chapter 4 (SIS study). Analyses were performed using Bio-Plex 200 suspension array system and Bio-Plex 200 software, version 6.0 (Bio-Rad Laboratories, Mississauga, ON, Canada). We utilized Rat Luminex Discovery Assay, a pre-customized magnetic bead-based multiplex assay (R&D Systems, Minneapolis, MN, USA), and followed the manufacturer's protocol. In brief, uterine horns (3 mm) were weighted and diluted with 1x phosphate-buffered saline (PBS) to a concentration of 0.1 mg/mL. Tissues were homogenized using Tissue Lyzer II (Qiagen, Toronto, ON, Canada) with 7 mm stainless steel beads four times for 2 min, 25 Hz cycles. Tissue homogenate protein concentrations were quantified using a BCA Protein Assay kit (Thermo Fisher Scientific, Wilmington, DE, USA) and then immediately stored at −80 °C until use. Multiplex assay was calibrated and validated before sample analyses. Reagents' preparation and assay were conducted following the manufacturer's protocol.

3.3.3 Western Blot

Pieces of snap-frozen uterine horns were homogenized using a Qiagen TissueLyser II (2 min at 25 Hz, 3 times; Qiagen, Toronto, ON, Canada) in radio immunoprecipitation assay (RIPA) buffer (1 M Tris pH 8, 5 M sodium chloride, 500 mM Ethylenediaminetetraacetic acid (EDTA) pH 8, Triton X-100, 200 mM phenyl methane sulfonyl fluoride (PMSF)) containing freshly added HALT protease inhibitor cocktail (100x; Thermo Fisher Scientific, Wilmington, DE, USA). Protein lysate was stored at –80°C until western blot analysis. The protein concentration of samples was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) with Precision red advanced protein assay reagent (Cytoskeleton Inc, Denver, CO, USA). The total protein for each sample (20 µg) was combined with 1x loading buffer (250 mM Tris-hydrochloric acid (HCl) containing 4% sodium dodecyl sulphate (SDS), 10% glycerol, 2% β-mercaptoethanol, and 0.002% bromophenol blue) and denatured at 95°C for 5 min. The protein lysates were separated by SDS (12% w/v)-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with intercept TBS blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature and were subsequently incubated with the primary antibodies anti-IL-1RAP at 1:1000 (Abcam, ab8110;

Cambridge, UK) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at 1:5000 (Thermo Fisher Scientific, PA1-987; Wilmington, DE, USA) overnight at 4°C. Membranes were then washed three times with PBS containing 0.1% Tween 20 (Sigma-Aldrich, San Luis, MO, USA) and incubated with secondary IRDye 800CW antibody (LI-COR Biosciences, Lincoln, NE, USA) at 1:5000 at room temperature. The intensities of the light-emitting bands were quantified using the Odyssey LI-COR Biosciences Infrared Imaging System and application software V3.0 (LI-COR Biosciences, Lincoln, NE, USA). The relative levels of IL-1RAP were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band intensities, and a ratio of the relative values to an internal blot control was obtained. Data were expressed relative to F0 controls (F0N).

3.3.4 Superoxide detection assay

Intracellular superoxide levels were measured by staining with 25 μM of dihydroethidium (DHE 25mg; Biotium Inc., Fremont, CA, USA) to detect oxidative stress in uterine horns. Uterine tissues were washed with Hank's Balanced Salt Solution (Gibco™ HBSS, calcium, magnesium; Thermofisher, ON, Canada) and incubated for 10 min at 37 °C in a humid chamber. Subsequently, 25 μM of DHE was added and incubated for 30 min at 37 °C. Excess DHE was washed away after the incubation time, and the sample was quickly cover-slipped and imaged. Images were captured using a fluorescence microscope (IX81; Olympus, Tokyo, Japan) with a CoolSNAP HQ2CCDcamera (Photometrics, Huntington Beach, CA, USA) using cellSens Dimensions, version 1.9 (Olympus, Japan) with TRITC at 532 nm wavelength. Images were analyzed with Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA) to assess mean fluorescence intensity (MFI). Duplicate images were taken for each sample from four regions (i.e., top, bottom, left, and right) at 20× magnification. All images were corrected to background fluorescence, and their respective MFIs were averaged and normalized to the average of the F0 control animals.

3.4 Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics in Chapter 4 (version 26; IBM Corp, Armonk, NY, USA) and GraphPad Prism in Chapters 4, 5, and 6 (version 5.0 and 9.0; GraphPad Prism, La Jolla, CA, USA).

SIS study

Biological Data

Bodyweights, litter size, blood glucose, and CORT were analyzed using the Kruskal-Wallis test (a non-parametric one-way analysis of variance (ANOVA)) with subsequent pairwise comparisons adjusting *p*-values (Bonferroni's correction). An independent *t*-test was used to assess differences in gestational length between controls and F0 animals, while the Kruskal-Wallis test was used to evaluate differences in pregnancy duration between stressed offspring and controls. Pearson correlation coefficient (r) was used to determine the relationships between biological parameters. Fisher's exact test was used to verify the association between treatment (SIS and controls) and adverse health outcomes (breeding and pregnancy success, health-related outcomes, and pregnancy-related outcomes). The strength of association was assessed by the Phi (φ) coefficient. Gestational length, litter size, and bodyweight data are presented as mean ± standard error of the mean (SEM).

Molecular Data

Independent-samples median test was performed on the F0-F3 control groups. For each gene, controls that displayed the same median across generations (F0-F3) were pooled (N = 20-31). Changes in gene expression across generations were estimated using the Kruskal-Wallis test with subsequent pairwise comparisons adjusting p-values (Bonferroni's correction). TG and MG lineages were analyzed separately. For uterine cytokine abundance using the multiplex immunoassay, data were tested for normal distribution and, when applicable, data were log-transformed to achieve normality. An independent sample t-test was used to analyze differences between control and SIS in the F0 generation, while 1-way ANOVA was applied to analyze F1 animals' data. Significant ANOVA results were further analyzed using Tukey post-hoc testing for multiple comparisons. Levene's median test was conducted when unequal variances were detected. Data were presented as concentration (pg/mL). Significance was assumed whenever $p < 0.05$. Data coloured in grey represent animals from the TG group, whereas data in yellow display animals from the MG group.

MTPS study

Results were analyzed with a one-way ANOVA test and log10-transformed when necessary, followed by Tukey post hoc test when significance was achieved ($p < 0.05$). The non-parametric Kruskal-Wallis test was used when the data did not follow the assumptions of parametric one-way ANOVA and is indicated in the figure legends. The interaction between treatment and housing for each generation was measured using Two-way ANOVA. Significant results were explored using Tukey's or Šidák's post-hoc tests for multiple comparisons. Significance is denoted in each figure where asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Results are expressed as mean ± SEM. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

PTPS study

Gestational lengths, CORT levels, maternal and offspring weights, and gene and protein expression of inflammatory and stress markers were analyzed with a one-way ANOVA test or the non-parametric Kruskal-Wallis tests. Data were log10-transformed when necessary and analyzed with a one-way ANOVA test, followed by Tukey's post hoc test for multiple comparisons when significance was achieved ($p < 0.05$). When data did not follow the normality assumptions of parametric tests, the non-parametric Kruskal-Wallis test was used instead. The adjusted coefficient of variation for the gestational lengths of dams was calculated by subtracting the mean from each pregnancy duration value and then dividing by the mean of the respective group. Significance is denoted in each figure where asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Results are expressed as mean ± SEM. All statistical analyses were performed using GraphPad Prism (version 9.0; GraphPad Prism, La Jolla, CA).

CHAPTER 4

SOCIAL ISOLATION STRESS (SIS) STUDY

Social Isolation Stress Modulates Pregnancy Outcomes and the Inflammatory Profile of Rat Uterus

A version of this article was published in: Lopes, N.A[1,2].; Falkenberg, E.A. [3]; Wiley, C.[1]; Patel, V. [1]; Serrano-Lomelin, J. [1]; Fang, X. [1]; Weiler, A.M. [3]; McCreary, J.K. [3]; Metz, G.A.S. [1,3*]; Olson, D.M. [1,2,3,4*]. Social Isolation Stress Modulates Pregnancy Outcomes and the Inflammatory Profile of Rat Uterus. Int. J. Mol. Sci. 2022, 23, 6169. https://doi.org/10.3390/ijms23116169.

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The animal cohort was conceptualized and designed by Gerlinde A.S. Metz and David M. Olson. Nayara A. Lopes and Xin Fang designed and planned the experiments. Nayara A. Lopes, Vaishvi Patel, and Camille Wiley performed the experiments. Data collection and analysis were done by Nayara A. Lopes, Erin A. Falkenberg, Jesus Serrano-Lomelin, and Xin Fang. Nayara A. Lopes wrote the first draft. Nayara A. Lopes, Jesus Serrano-Lomelin, Gerlinde A.S. Metz, and David M. Olson performed all revisions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors thank Lucas Costa Vieira for designing Figure 4.1. We thank Anna Noga for her assistance in preparing this manuscript in accordance with Good Publication Practices.

Research funded by the Canadian Institutes of Health Research (CIHR)

4.1 Introduction

A successful pregnancy requires complex cooperation and interdependence between physiological systems, including neuroendocrine, cardiovascular, and immune systems [340,341]. Environmental stressors may disrupt the homeostatic mechanisms within these systems, leading to pregnancy complications and affecting fetal health and development [200]. Stress is known to activate the hypothalamic-pituitary-adrenal (HPA) axis, increase maternal levels of glucocorticoids (GCs) [279], and upregulate pro-inflammatory cytokines [342] in a complex and non-linear fashion. When acute and/or chronic stress sustains endocrine and immune system activation earlier than 37 weeks of gestation, the risk of preterm birth increases [141,207].

The environment an individual is exposed to in early life may be a determinant for long-term disease risk and phenotypic changes [343]. This process is called fetal programming where the health trajectory of the offspring is impacted by adversity *in utero* during critical periods of rapid growth and organ development [344,345]. Stress experienced by the mother during pregnancy influences fetal programming, and it contributes to her offspring's allostatic load. The allostatic load conceptual framework describes the cumulative burden of chronic stress over the course of life resulting in wear and tear on the body [7,346]. Such cumulative effects in the form of allostatic load may dysregulate the inflammatory cytokine milieu predisposing pregnant individuals to complications such as preterm birth. Thus, the profound consequences of allostatic load might be transmitted across generations promoting adverse pregnancy outcomes and poor health in the offspring [156].

Our previous studies in rat stress models showed that different types of prenatal maternal stress (PNMS) result in various degrees of adverse pregnancy and offspring outcomes [152,313,331]. In our primary series of studies, we introduced a new paradigm whereby a family history of stress programmed physiological and epigenetic pathways that regulate parturition, brain plasticity, and behaviour culminating in shorter gestation of the daughters and grand-daughters and delayed growth of the offspring [152,347,348]. This was accomplished using single-hit stress in pregnant rats by forced swimming and restraint in a single generation or across four generations. Given that humans rarely face only a single stress during pregnancy, we further explored the effects of PNMS on pregnancy outcomes using a two-hit stress model combining psychological stressors and intraperitoneal injections of IL-1β [331]. This stress

paradigm resulted in increased variation in F0 gestational lengths, affected the growth trajectories of the offspring, increased the occurrence of adverse pregnancy outcomes, and altered uterine markers of stress and inflammation.

The present study presents the next iteration of our PNMS series in which we examined whether social isolation leads to adverse pregnancy and newborn outcomes. Social isolation stress (SIS) is characterized by prolonged lack of social support and interactions which can cause psychological distress, and its prevalence is increasing worldwide [349,350]. Perceived SIS is associated with an increased risk of coronary heart disease and stroke [351], cancer [352], psychiatric disorders [353-355], and early mortality [304,356]. When rats are deprived of physical social interactions during SIS rearing, while maintaining regular olfactory, auditory, and visual contact with their counterparts [309,357], they experience mild psychosocial stress [292,295,310]. Consequences of SIS include functional, morphological, and neurochemical alterations in distinct brain areas, HPA axis changes, disrupted inflammatory responses, and altered behaviour in adult offspring [295,311-313,358-360]. While the effects of SIS have been reported in the literature, little is known about its effects on pregnancy outcomes. In humans, several studies found an association between SIS and depression [25,37,38]. Heightened inflammatory response [127,361,362] and prenatal cortisol levels (HPA dysfunction) [363,364] are known outcomes in depressed pregnant women. Indeed, antenatal depression has been linked to high rates of preterm birth [365,366] and preeclampsia [367].

In the present study, we subjected rats to SIS from preconception and during pregnancy to evaluate its effects on pregnancy outcomes and uterine tissue inflammatory profile. Most rodents are social mammals that live in groups and participate in constant social interaction with their conspecifics [309,355]. The impact of SIS was assessed across four generations of rats in a transgenerational (TG) and multigenerational (MG) prenatal stress fashion (Figure 4.1). Stress only occurred in the F0 dams in the TG lineage, while the F0-F3 generations were exposed to cumulative stress in the MG lineage. Both ancestral and cumulative prenatal stress paradigms have been shown to alter behaviour and produce physiological dysfunctions in the offspring [152,295,313]. We hypothesized that pre-pregnancy and gestational SIS would increase the risk of preterm birth and low birth weight and that uterine markers of

stress and inflammation would be upregulated across four generations of rats in both a TG- and MG-dependent manner.

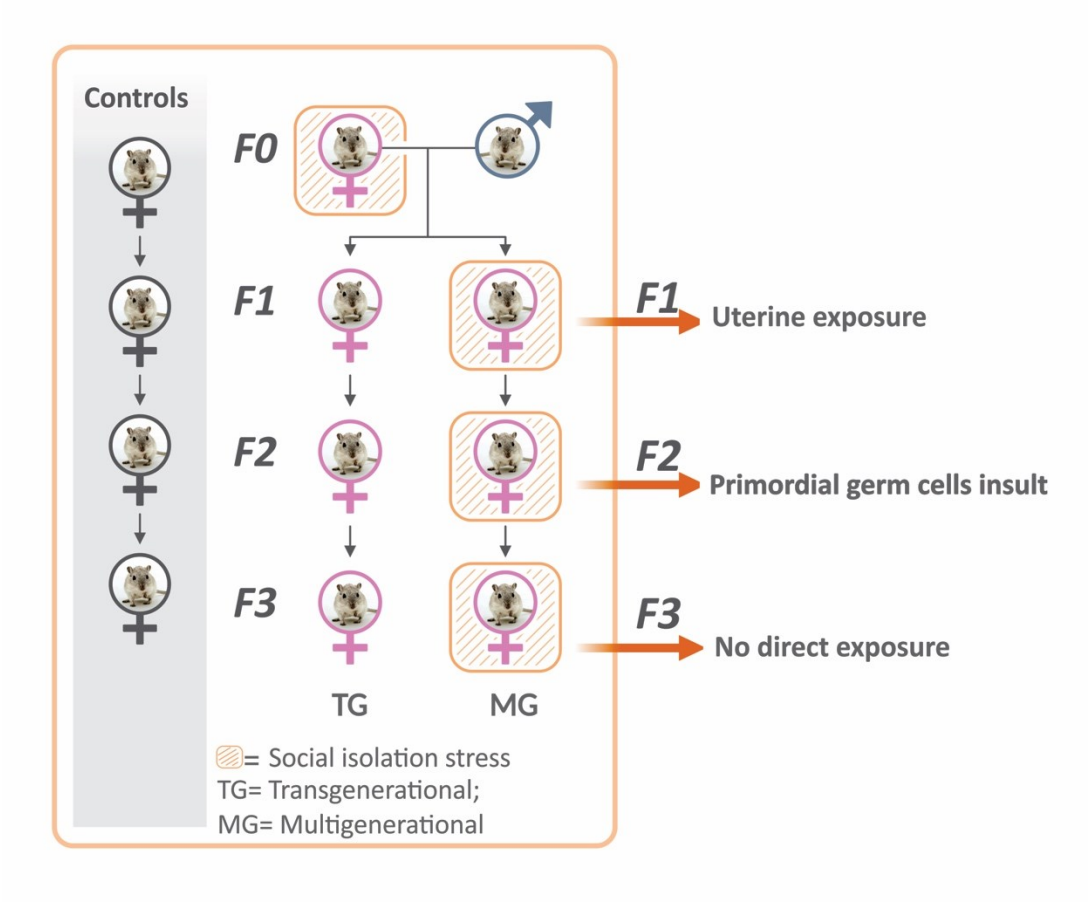

Figure 4.1: Social isolation stress schematic.

Flow chart illustrating the SIS experimental design. F0 rats were subjected to SIS, and their filial generation F1 was split into TG and MG groups. In the TG group, stress was only implemented in the parental F0 generation, while each parental and offspring generation was exposed in the MG group. F0-F3 generations of non-stressed rats served as controls. In the TG lineage, exposing the gestating female F0 generation to SIS implies that only the F1 fetuses experience direct uterine exposure to stress, and the F2 generation's exposure to stress is through their mother's primordial germ cells (F1 generation's germ cells). Therefore, the F3 generation of the TG lineage is the first unexposed generation, considered a true transgenerational inheritance. In the MG lineage, all generations of offspring experience both direct and cumulative uterine exposure to stress through the maternal primordial germ cells. TG = transgenerational; MG = multigenerational; F = filial generation.

4.2 Specific methods

4.2.1 Animals and experimental design

Timed-pregnant female Long-Evans hooded rats (N = 111) were used to produce a cohort of four generations (F0-F3) of stressed animals. Female rats were individually paired with stress-free males for one hour per day for mating. Breeding was initiated at 110 days of age and continued until pregnancy was detected or females reached 180 days of age (Figure 4.2). Pregnancy was confirmed by progressive maternal weight gain. Weight measurements were performed on GD18 for dams and P1 for pups. The gestational length was video-recorded from GD20 until birth continuously using an infrared cage site camera (Panasonic WV-BP330, Panasonic, Minato-ku, Tokyo, Japan), and was determined by the total number of hours between final mounting and delivery of the first pup. All pregnant dams were housed individually from GD19 until delivery. The pups were kept with their mothers until weaning (lactational day (LD)21) and then housed with same-sex siblings. Unhealthy dams were excluded from the study and adverse health outcomes were recorded for further analyses.

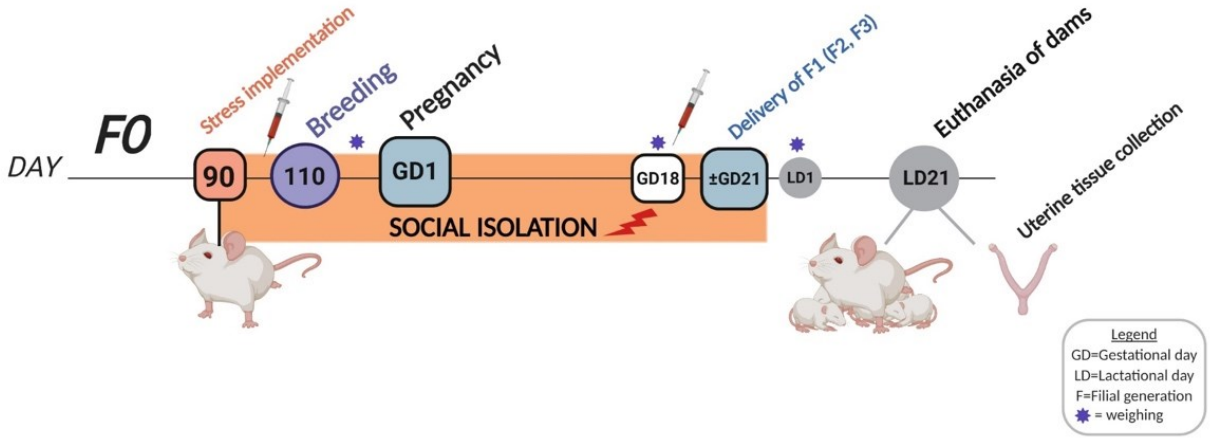

Figure 4.2: Social isolation stress timeline illustrating the experimental design and tissue collection.

Female rats were exposed to SIS during preconception (days 90-110) and pregnancy (GD1-~21). Syringes depict blood draws once at baseline (between days 90-105) and on GD18, while asterisks illustrate weight measurements. Dams were sacrificed at the weaning of their offspring (LD21) when uterine tissues were collected. Created with BioRender.com (accessed on 12 May, 2022).

Females from the parental generation (F0, N = 37) were randomly divided into control or SIS groups and bred with stress-free males to produce the filial (F) generation (F1), and their subsequent F2 (granddaughters) and F3 (great-granddaughters) offspring. Timed-Pregnant female rats from the F1-F3 generations were randomly split into TG or MG stress lineages (Figure 4.1).

Animals were bred and raised locally at the vivarium of the Canadian Centre for Behavioural Neuroscience, University of Lethbridge, AB, Canada. All experiments were conducted in agreement with the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee (Protocol #1715). All animals were housed in standard cages (45.5 × 25.5 × 20 cm) with the room temperature set at 20 °C and 30% relative humidity. They were subjected to a 12-h light/dark cycle (lights were on at 7:30 and off at 19:30) with *ad libitum* access to food and water. Litter size was not normalized. Animals were handled the same amount daily throughout the experiments and housed in the same room to control for environmental factors where they could hear and smell their counterparts.

4.2.2 Pre-conceptional and gestational stress

Psychological SIS consisted of housing a dam alone from P90 for a minimum of two weeks before being paired with a naïve, pair-housed male for breeding (1 h/day). After mating, SIS dams were housed alone until delivery (Figure 4.2), and they stayed with their pups until LD21. A control group of rats was bred alongside the treatment group, in which animals were housed in pairs throughout the experiment (preconceptionally and during pregnancy) until GD21.

4.3 Results

SIS shortened gestational lengths in F0 dams

Gestational lengths were significantly decreased in the F0 stress groups compared to group-housed controls (519.23 ± 24.73 h vs. 530.06 ± 9.26 h, p = 0.045; Figure 4.3A). Pregnancy duration was not reduced in the F1-F3 offspring of the TG group (F1 529.35 ± 7.00 h; F2 528.08 ± 5.78 h; and F3 529.27 ± 3.72 h; p = 0.751; Figure 4.3A) compared to controls. Similarly, gestational length did not change in the F1-F3 offspring of the MG group (F1 527.81 ± 6.53 h; F2 527.59 ± 4.96 h; F3 531.19 ± 5.64 h; p = 0.412; Figure 4.3A). Although the overall gestational length was not affected in SIS-exposed F3

offspring, increased blood glucose levels on GD18 were associated with shorter gestation length (r(16) = −0.493, p = 0.038; Figure 4.3B).

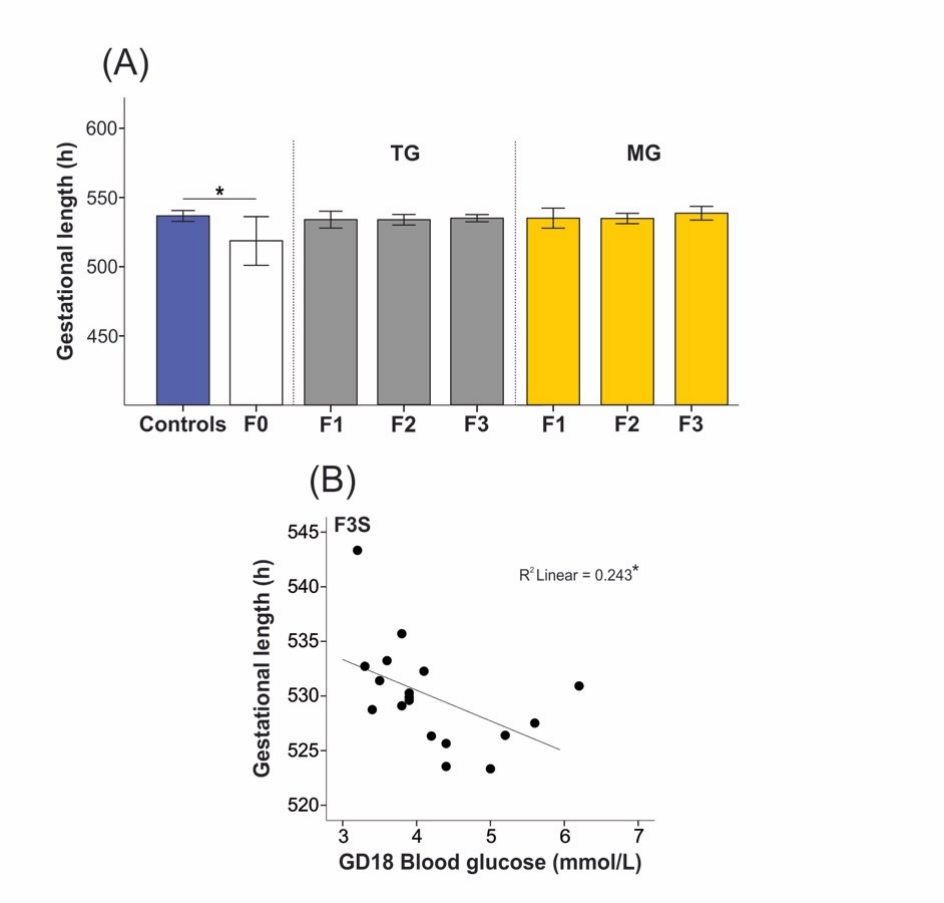

Figure 4.3: SIS significantly reduced the gestational lengths of the F0 dams but did not impact the offspring's pregnancy duration.

(A) Gestational length recordings demonstrated shorter gestation in the parental generation F0, while no changes were seen in the TG or MG offspring. (B) For the F3 generation animals of both TG and MG groups, higher blood glucose levels were associated with shorter gestation in F3-stressed animals on GD18. Asterisks indicate significance: * $p < 0.05$. Controls N = 20-28; F0 N = 10-11; F1 N = 6-8, F2 N = 10-12; F3 N = 8-11. Mean ± SEM. An Independent t-test was used to assess gestational length between controls and F0 animals, while Kruskal-Wallis was used to evaluate differences in pregnancy duration between the offspring and controls.

SIS moderately impacted the breeding of dams

We performed pathological analyses in noticeably unhealthy dams across all groups. Observed adverse health outcomes included disinterest in breeding, inability to become pregnant, pregnancy-related complications, and other health-related adverse events (Table 4.1). The phi coefficient indicated a moderate relationship between stress and breeding success ($\varphi = 0.326$), but the association was not significant (13.5%, $p = 0.067$). The occurrence of health complications such as kidney dysfunction and idiopathic disease was higher in controls (8.1%) compared to stressed (5.4%) animals, although the association was low and not significant ($p = 0.337$, $\varphi = 0.157$). Little if any association was observed between treatments (SIS or control) for the variables: inability to become pregnant ($p = 1.000$, $\varphi = 0.023$) or pregnancy-related complications ($p = 1.000$, $\varphi = 0.046$).

Table 4.1: Results of the Fisher's exact test for association between treatment (SIS and controls) and adverse health outcomes.

Adverse health outcomes were assessed in noticeably unhealthy dams from both control and stress groups.

Outcomes % (N)	Controls (N = 15)	Stressed (N = 22)	p	Strength of Association (φ)
Inability to become pregnant	8.1 (3)	10.8 (4)	1.000	0.023
Health complication	8.1 (3)	5.4 (2)	0.377	0.157
Pregnancy-related complication	2.7 (1)	2.7 (1)	1.000	0.046
Disinterest in breeding	(0)	13.5 (5)	0.067	0.326

Blood glucose levels were reduced in the offspring exposed to TG or MG SIS

Basal glucose levels were unaltered in the TG ($p = 0.494$) and MG ($p = 0.826$) lineages during preconception. However, we observed changes in the SIS animals' late-pregnancy blood glucose concentrations. Blood glucose levels were significantly impacted in the TG ($p = 0.001$) and MG ($p < 0.0001$) stress protocols (Figure 4.4A,B). In the stressed TG group, glucose levels dropped significantly in the F1 generation (F0-F1, $p < 0.01$) on GD18 (Figure 4.4A). However, glucose levels returned to baseline in F2 animals ($p = 0.025$). Gestational blood glucose levels were lowest in the F3 generation of the re-current stress MG group when compared to controls ($p = 0.047$), and the F0 ($p < 0.0001$) and F2 generations ($p = 0.011$) (Figure 4.4B).

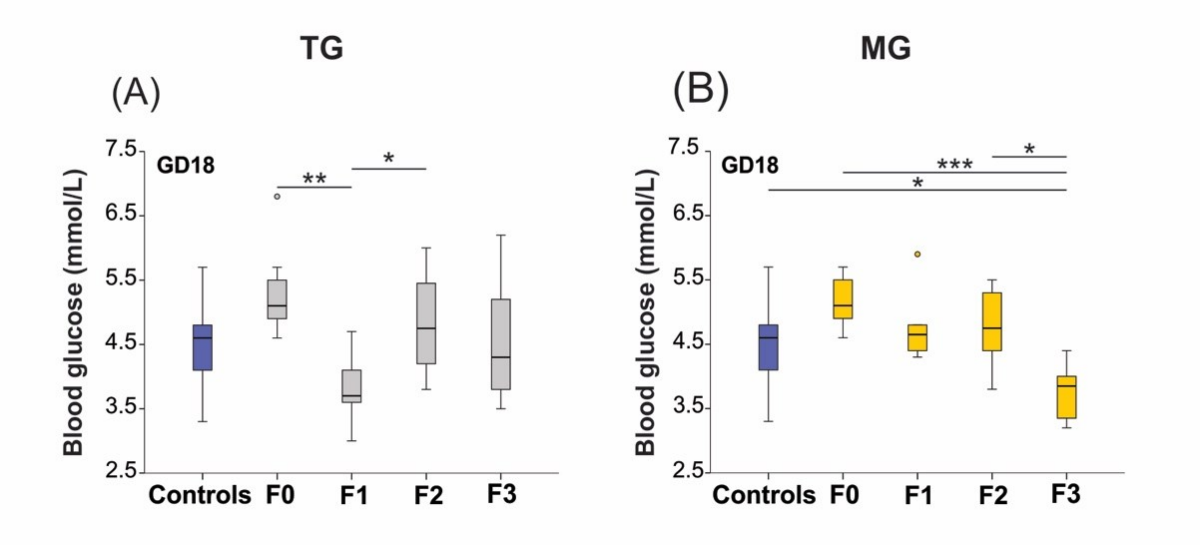

Figure 4.4: Blood glucose levels decreased on GD18 in the offspring of animals exposed to SIS.

(A) Blood glucose levels significantly decreased in F1 animals of the TG group and (B) the F3 generation of the MG group on GD18. Asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Controls N = 20-28; F0 N = 10-11; F1 N = 6-8, F2 N = 10-12; F3 N = 8-11. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

Maternal weight and litter size were unchanged by TG and MG SIS

Maternal (TG $p = 0.212$; MG $p = 0.372$; Table 4.2) and gestational weights (TG $p = 0.631$; MG $p = 0.565$; Table 4.2) were unchanged in all groups and generations. Maternal weight gain calculations revealed no changes in either group (TG $p = 0.189$; MG $p = 0.167$; Table 4.2). Furthermore, no differences in litter size were observed between generations in the TG ($p = 0.355$) and MG ($p = 0.351$) stress lineages (Figure 4.5).

Table 4.2: Preconceptional and gestational SIS did not affect maternal weight at baseline or during pregnancy.

		Controls (N = 20–28)		F0 stress (N = 10–11)		F1 stress (N = 6–8)		F2 stress (N = 10–12)		F3 stress (N = 8–11)		
	Bodyweight (g)	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p value**
Transgenerational (TG) group	Baseline (preconception)	306.87	29.02	322.68	23.50	299.83	22.18	302.37	19.21	312.95	29.41	0.212
	GD18	406.35	34.36	419.86	38.71	396.29	20.20	413.62	25.85	410.21	30.54	0.631
Multigenerational (MG) group	Baseline (preconception)	306.87	29.02	322.68	23.50	313.48	24.62	310.27	28.74	306.51	29.96	0.372
	GD18	406.35	34.36	419.86	38.71	401.88	40.70	419.01	33.96	396.85	37.09	0.565

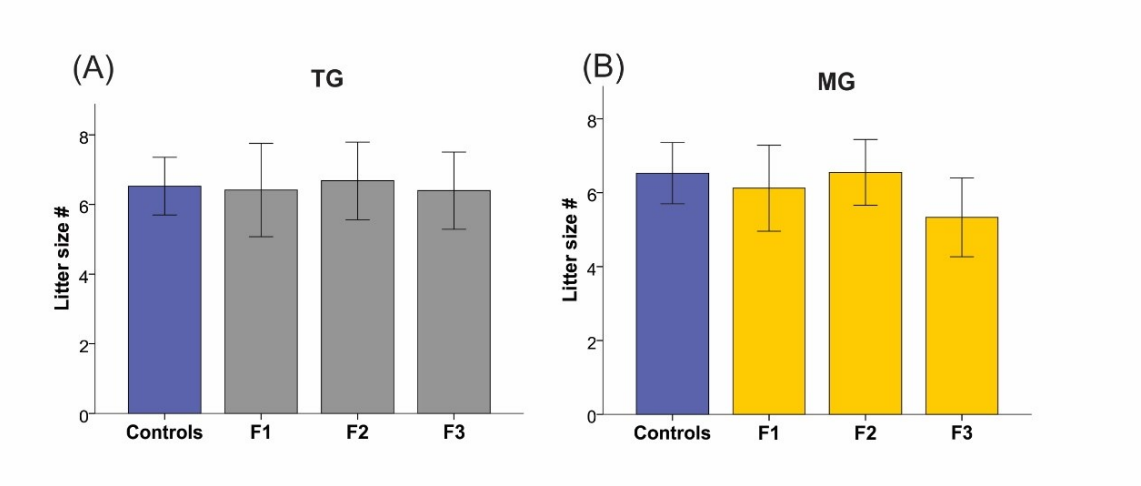

Figure 4.5: Litter sizes remained unchanged.

Litter sizes of dams exposed to SIS did not change in the (A) TG and (B) MG groups. Controls N = 18; F1 N = 6-8, F2 N = 11; F3 N = 9-10. Mean ± SEM.

Neonatal growth was affected in animals exposed to SIS, while plasma levels of corticosterone (CORT) remained unchanged in the parental generation

Plasma CORT levels are measured to confirm the presence of stress. Unexpectedly, plasma collected on GD18 displayed basal CORT levels in F0 stressed animals (543 ± 261 ng/mL; Figure 4.6A) compared to controls (582 ± 332 ng/mL). Levels of CORT were significantly increased in the F1 generation (1399 ±722 ng/mL, p = 0.001) of the TG lineage than those of F2 animals (350 ± 179 ng/mL; Figure 4.6A). The MG lineage demonstrated an overall increase in CORT levels (p = 0.037) (Figure 4.6B), and higher CORT levels were associated with increased blood glucose levels on GD18 in F2 TG-stressed animals (Figure 4.6C,D).

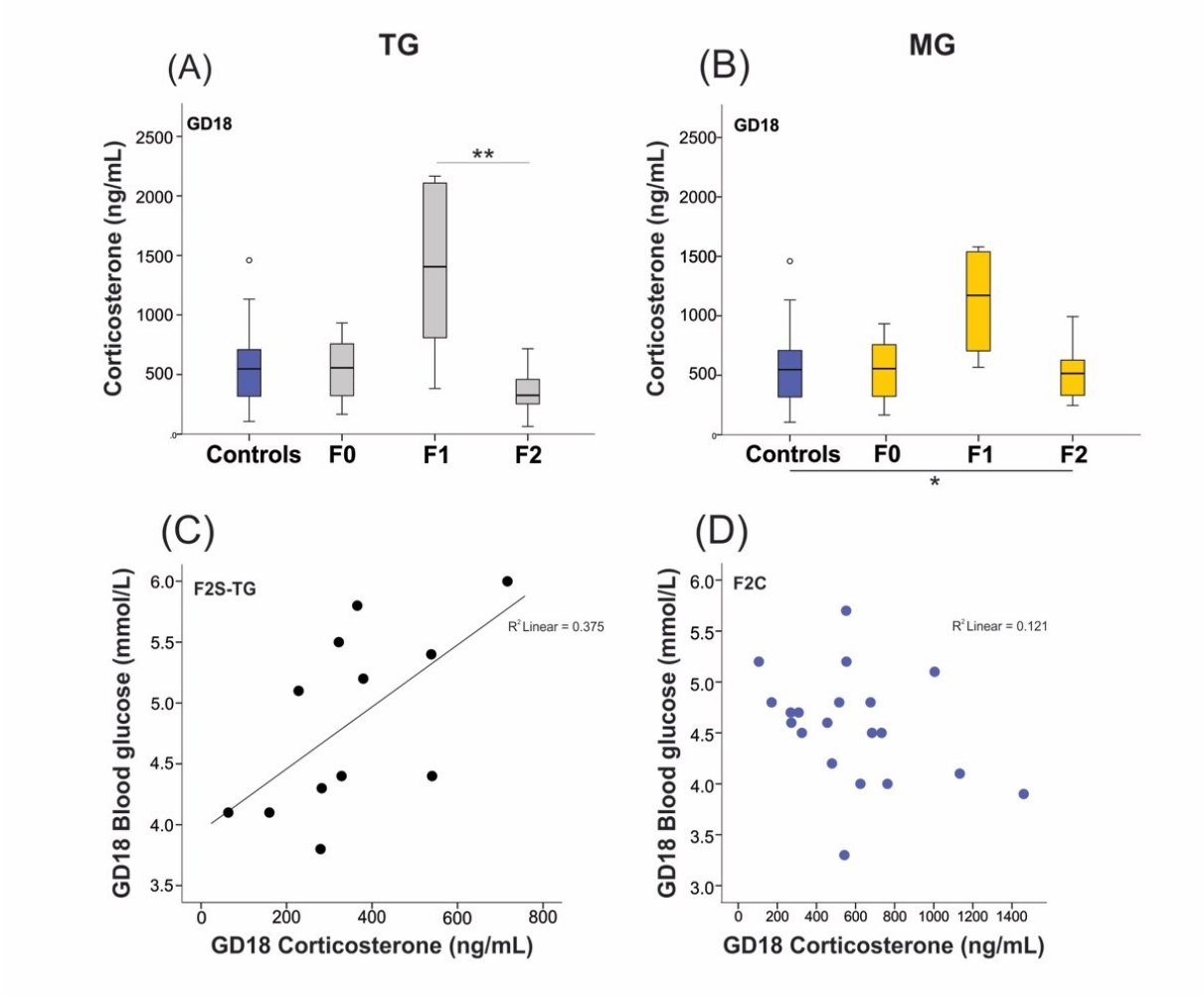

Figure 4.6: Elevated plasma corticosterone (CORT) levels in F1 animals of TG and MG stress groups.

CORT levels were significantly elevated in the F1 dams of the (A) TG (B) and MG groups on GD18 but returned to baseline levels in the F2 generation. (C) Higher CORT levels on GD18 were associated with increased blood glucose levels in the F2 generation of the TG group. (D) No correlation was found between CORT and glucose levels in F2 controls. Asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$. Controls N = 20-28; F0 N = 10-11; F1 N = 6-8, F2 N = 10-12. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

Even though CORT amounts in the parental generation were unaltered, the growth of F1 and F2 offspring significantly changed in a sex-specific manner (Figure 4.7). Daughters (6.34 ± 0.63 g; $p = 0.019$), sons (6.68 ± 0.59 g; $p < 0.001$), and grandsons (6.88 ± 0.57 g; $p = 0.024$) exposed to TG stress were significantly lighter on postnatal day (P)1 compared to controls (females 6.61 ± 0.57 g; males 7.14 ±

0.06 g; Figure 4.7A,B). However, by the F3 generation, TG-male neonate weights normalized to baseline (controls 7.14 ± 0.06 g), and they were significantly heavier than F1 male neonates (7.06 ± 0.46 g vs. 6.68 ± 0.59 g, p = 0.019; Figure 4.7B).

In the cumulative SIS-exposed MG lineage, F1 and F2 females (F1 6.24 ± 0.48 g, p = 0.001; F2 6.34 ± 0.50 g, p = 0.010) and males (F1 6.70 ± 0.53 g, p = 0.001; F2 6.74 ± 0.57 g, p < 0.001) displayed significantly lower weight on P1 than controls (Figure 4.7C,D). Conversely, F3 MG-stressed females were significantly heavier at birth than F1 neonates (6.59 ± 0.54 g vs. 6.24 ± 0.48 g, p = 0.009; Figure 4.7C).

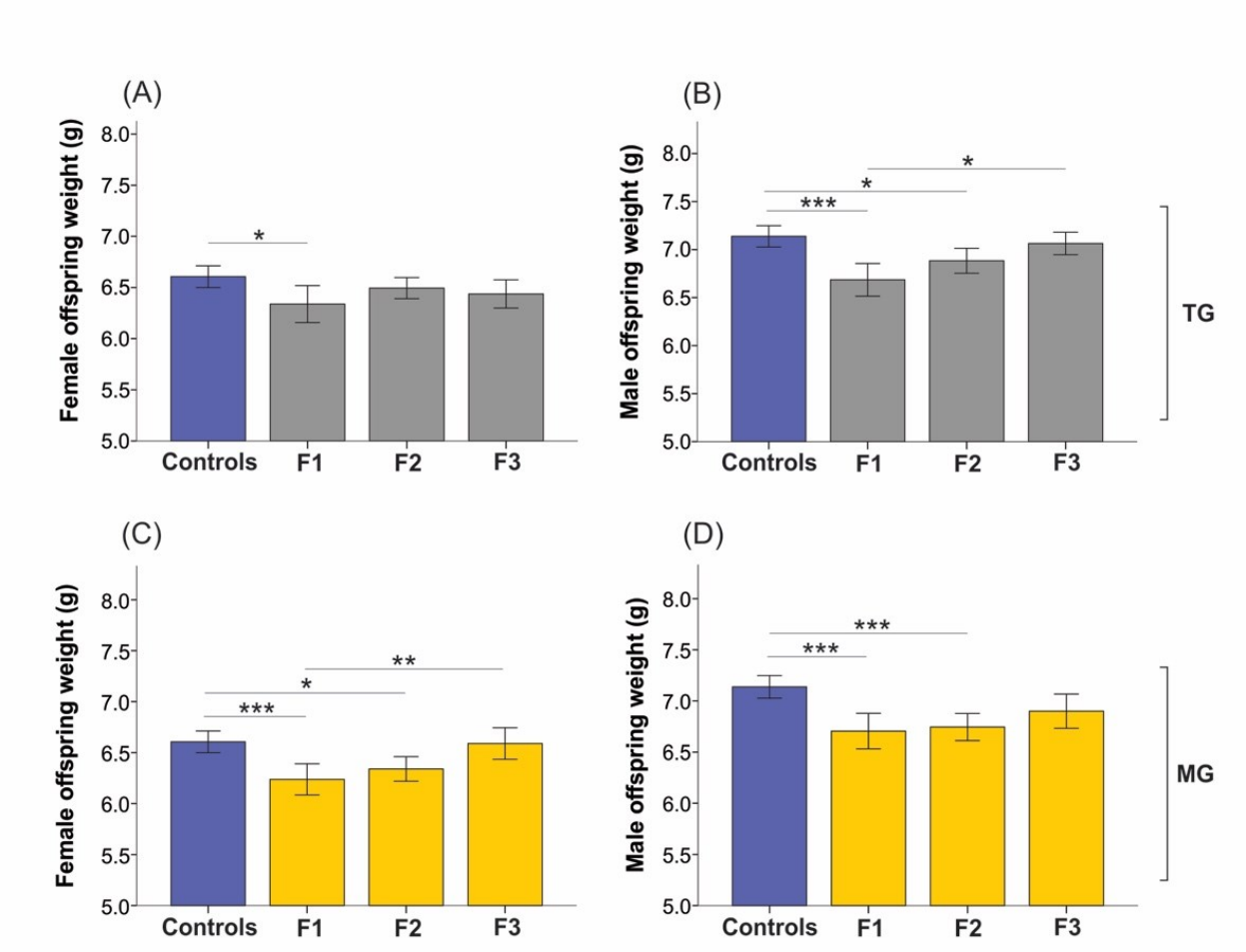

Figure 4.7: The birth weights of F1 females and males from both SIS lineages were significantly decreased.

Birth weights of (A) F1 female and (B) F1 and F2 male neonates significantly decreased in the TG stress group. (B) Yet, F3 TG-stressed males were heavier than F1 male pups. (C) Females from the MG group

displayed significantly reduced weight on P1 in the F1 and F2 generations, while F3 females were heavier than F1-stressed animals. (D) F1 and F2 MG-stressed males were significantly lighter than controls on P1. Asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean ± SEM. Controls N = 115-121; F1 N = 37-50, F2 N = 68-79; F3 N = 47-65 neonates.

Uterine mRNA expression of inflammatory and stress response genes was altered in both SIS TG and MG in a generation-dependent manner.

Pro-inflammatory cytokines and their receptors

Uterine expression of the pro-inflammatory cytokine interleukin-1α (*Il1a*) was not significantly different in either TG ($p = 0.498$) or MG ($p = 0.744$) stress groups (Figure 4.8A,D). On the contrary, *Il1b* mRNA expression doubled in the stressed F0 generation compared to controls ($p = 0.026$), but then it dropped in the TG F1-F3 offspring as compared to its parental generation (overall $p < 0.0001$; Figure 4.8B). Similarly, *Il1b* expression in the MG lineage ($p < 0.0001$) displayed a pattern akin to TG stress, except that the differences in F3 animals did not reach significance (F0-F3 $p = 0.112$) (Figure 4.8E). In the MG group, *Il1b* abundance decreased significantly in the F1 ($p < 0.0001$) and F2 ($p = 0.003$) progeny compared to F0 uteri (Figure 4.8E). The expression of interleukin-1 receptor 1 (*Il1r1*) significantly increased in the F3 generation of the MG group (F0-F3 $p = 0.005$; Figure 4.8F), but its expression was unchanged in the TG lineage ($p = 0.205$; Figure 4.8C). Results for the interleukin-6 (IL-6) pro-inflammatory cytokine were not included in the analysis, as the mRNA expression in control animals displayed variability within generations.

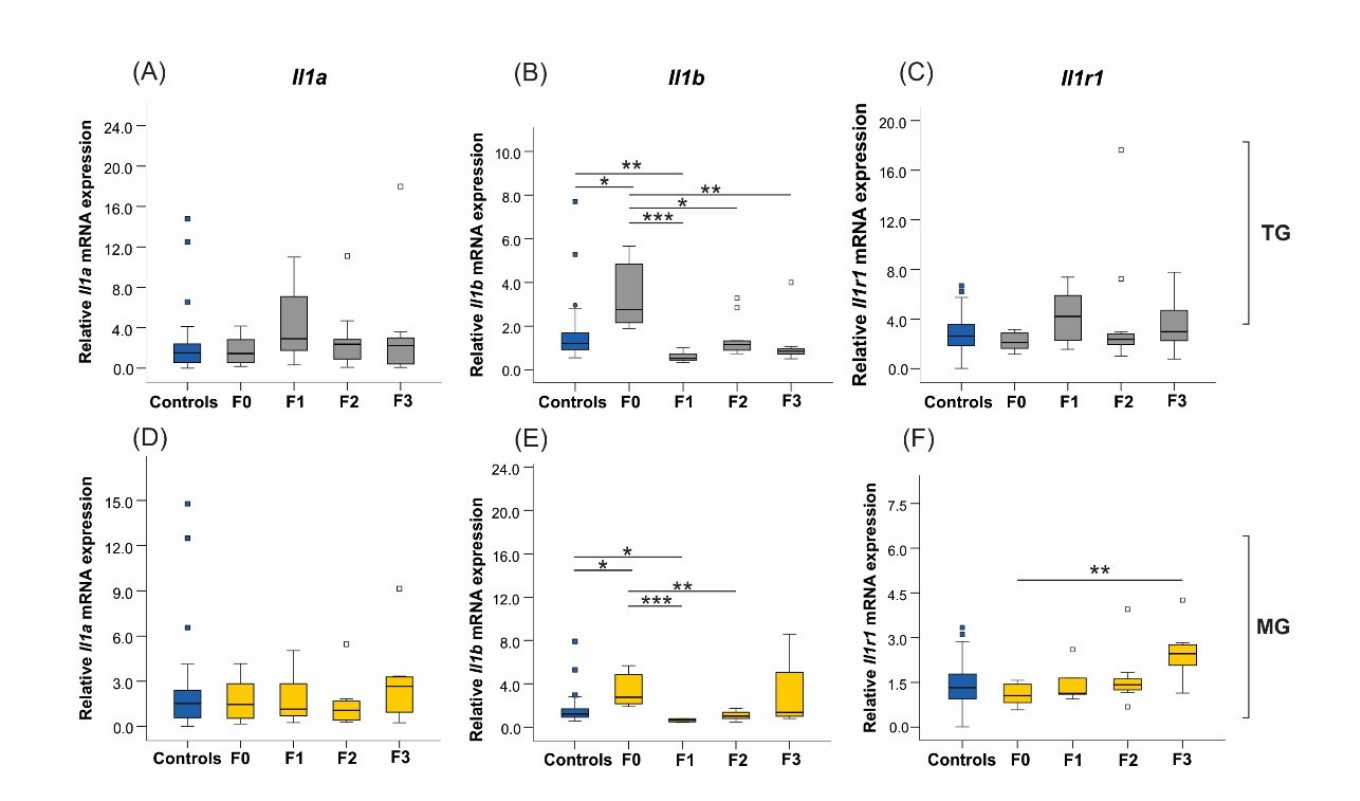

Figure 4.8: The uterine expression of the *Il1b* pro-inflammatory cytokine and the *Il1r1* receptor was significantly altered across generations in TG and MG-stressed animals.

Results from TG and MG groups will be presented alongside each other to compare the effects of stress between the two cohorts. (A,D) Expression levels of *Il1a* were unaffected in both TG and MG groups. (B) The abundance of *Il1b* doubled in the F0 generation and decreased significantly in F1-F3 offspring. Similarly, (E) MG-stressed rats presented similar *Il1b* uterine mRNA expression patterns as the TG dams, with significantly increased expression in F0 and a drop in the F1 and F2 generations. The *Il1r1* receptor was significantly upregulated in the F3 uteri of the MG group (F), while its expression was unaffected in all generations of the TG group (C). Asterisks indicate significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Controls N = 20-31; F0 N = 8-10; F1 N = 5-8, F2 + F3 N = 4-12. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

Corticotrophin-Releasing Hormone (*Crh*) and its Receptors

There was a significant downregulation of *Crh* expression in the uteri of F3 animals from the TG lineage compared to controls ($p = 0.007$; Figure 4.9A), but it was unaffected in the MG stress group ($p = 0.080$; Figure 4.9D). Its receptor, *Crhr1*, was significantly downregulated in the parental generation uteri of the TG (Controls-F0 $p = 0.007$; Figure 4.9B) and MG (Controls-F0 $p = 0.007$; Figure 4.9E) stress

groups. Although stress exposure differed in the progeny, *Crhr1* expression patterns were similar in both TG and MG lineages. We observed a doubling of *Crhr1* expression in the F1 uteri (F0-F1 p = 0.024) exposed to MG stress, whereas its abundance tripled in the F2 uteri of the TG group (F0-F2 p = 0.007) when compared to F0 generation. No changes were seen for the corticotrophin-releasing hormone receptor 2 (*Crhr2*) mRNA levels when only F0 animals were socially isolated (p = 0.438; Figure 4.9C), but it was significantly upregulated in the F3 generation of the cumulative stress group (F0-F3 p = 0.035; Figure 4.9F).

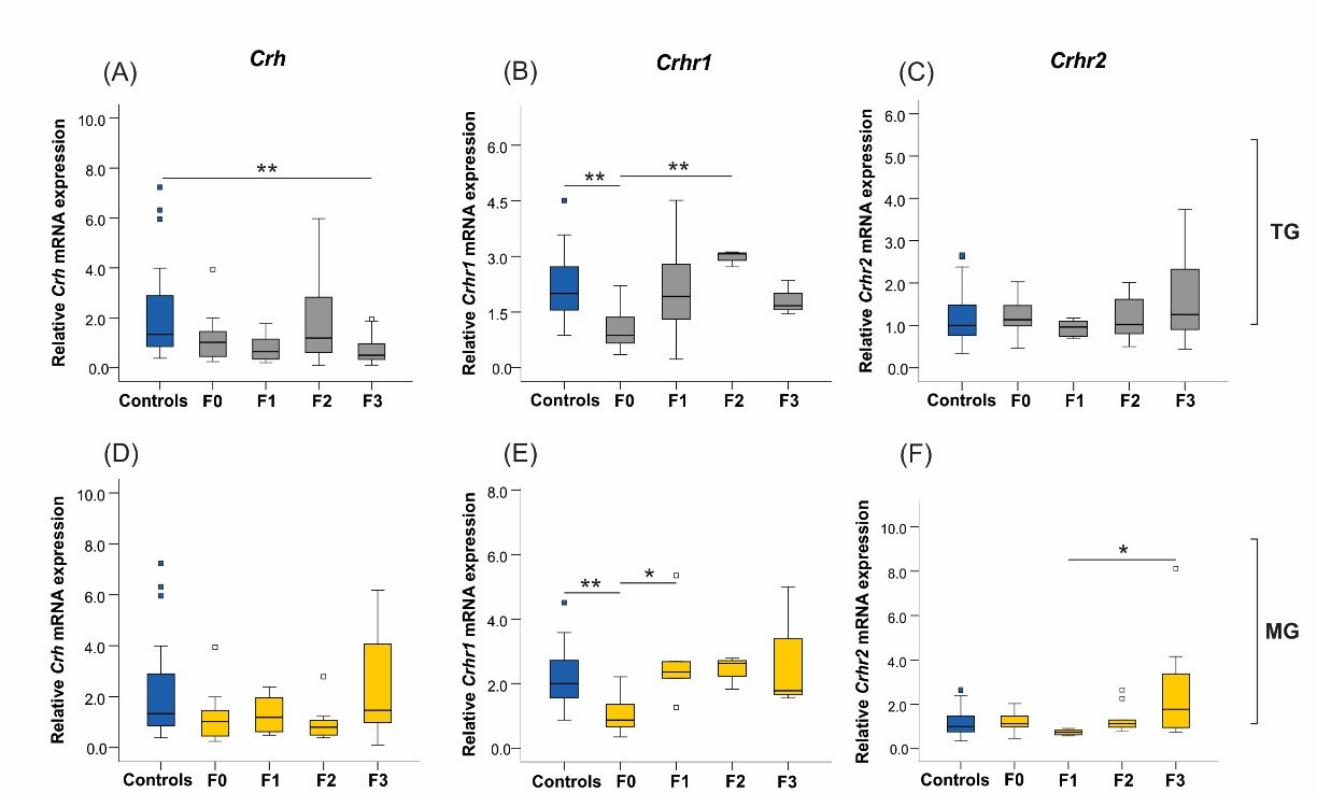

Figure 4.9: The uterine expression of *Crh* and *Crhr2* reacted differently in TG and MG lineages, while *Crhr1* expression patterns were similar.

(A) The mRNA expression of *Crh* was significantly reduced in the F3 generation of the TG group, (D) yet, no changes were observed in the MG lineage. (B) The *Crhr1* expression significantly decreased in F0 animals while its abundance tripled in the TG F2 generation. (E) The same pattern was observed in the MG lineage, where uterine expression of *Crhr1* was significantly downregulated in the F0 generation (C) but doubled in the F1 offspring. The expression of *Crhr2* was unchanged in the TG lineage, while (F) it significantly increased in the F3 animals exposed to cumulative MG stress. Asterisks indicate significance. * p < 0.05; ** p < 0.01. Controls N = 20-31; F0 N = 8-10; F1 N = 5-8, F2 + F3 N = 4-12. Box

plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

11β-hydroxysteroid dehydrogenase type 2

The 11β-HSD enzyme isoforms 1 and 2 modulate local GC metabolism in the uterus and placenta and regulate the timing of labour [368]. The 11β-Hsd2 isoform transforms CORT into its inactive dehydrocorticosterone form. In the TG lineage, *Hsd11b2* mRNA levels tripled in F1 daughters (Controls-F1 $p = 0.023$, F0-F1 $p < 0.0001$; Figure 4.10A) and then normalized in the F2 generation (F1-F2 $p = 0.036$). In contrast, the uterine expression of the *Hsd11b2* gene was unaltered in the MG group ($p = 0.092$; Figure 4.10B). The *Hsd11b1* results were excluded due to significant variability observed in controls.

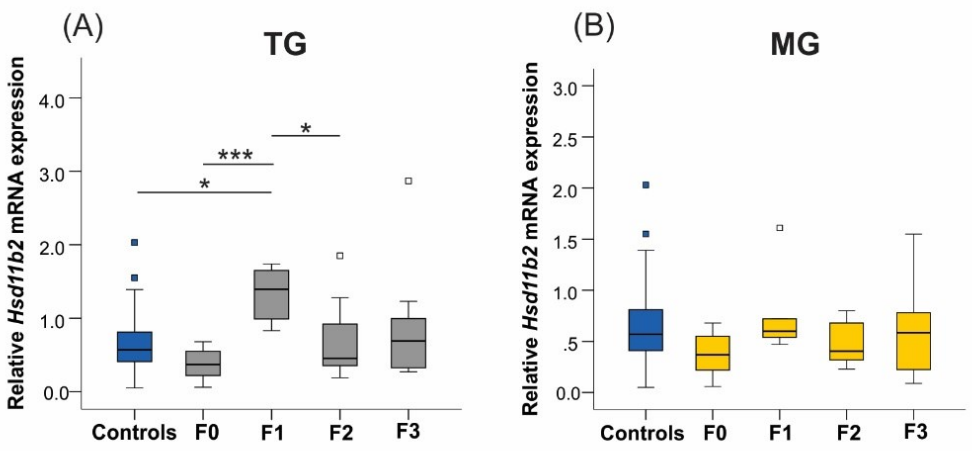

Figure 4.10: The uterine expression of *Hsd11b2* significantly increased in the F1 generation of the TG lineage.

(A) The abundance of *Hsd11b2* tripled in the daughters' uteri. (B) Expression levels of *Hsd11b2* were unaltered when exposed to MG SIS. Asterisks indicate significance: * $p < 0.05$; *** $p < 0.001$. Controls N = 20-31; F0 N = 8-10; F1 N = 5-8, F2 + F3 N = 4-12. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges.

SIS reduces protein concentration of IL-1β in F1 offspring uteri of exposed mothers

We performed multiplex protein analysis on three pro-inflammatory cytokines (IL-1α, IL-1β, and IL-6) related to the onset of labour [88]. Overall, protein concentrations of IL-1α, IL-1β, and IL-6 were unaltered in all F0 treatment groups (F0C-F0S $p = 0.24$; $p = 0.65$; $p = 0.62$, respectively; Figure 4.11A,C,E). In the F1 generation, however, IL-1β protein levels were significantly reduced in the TG lineage ($F(2, 9) = 5.21$, $p = 0.031$; Figure 4.11D) compared to controls ($p < 0.05$). A similar drop occurred in the F1 MG animals, but it was not significant. Conversely, IL-1α and IL-6 protein concentrations did not show significant differences in the F1 generation ($F(2, 11) = 0.65$, $p = 0.54$ and $F(2, 14) = 0.97$, $p = 0.40$; Figures 4.11B,F, respectively). The levels of IL-1α in the MG lineage demonstrated a decrease, but it was not significant (Figure 4.11B).

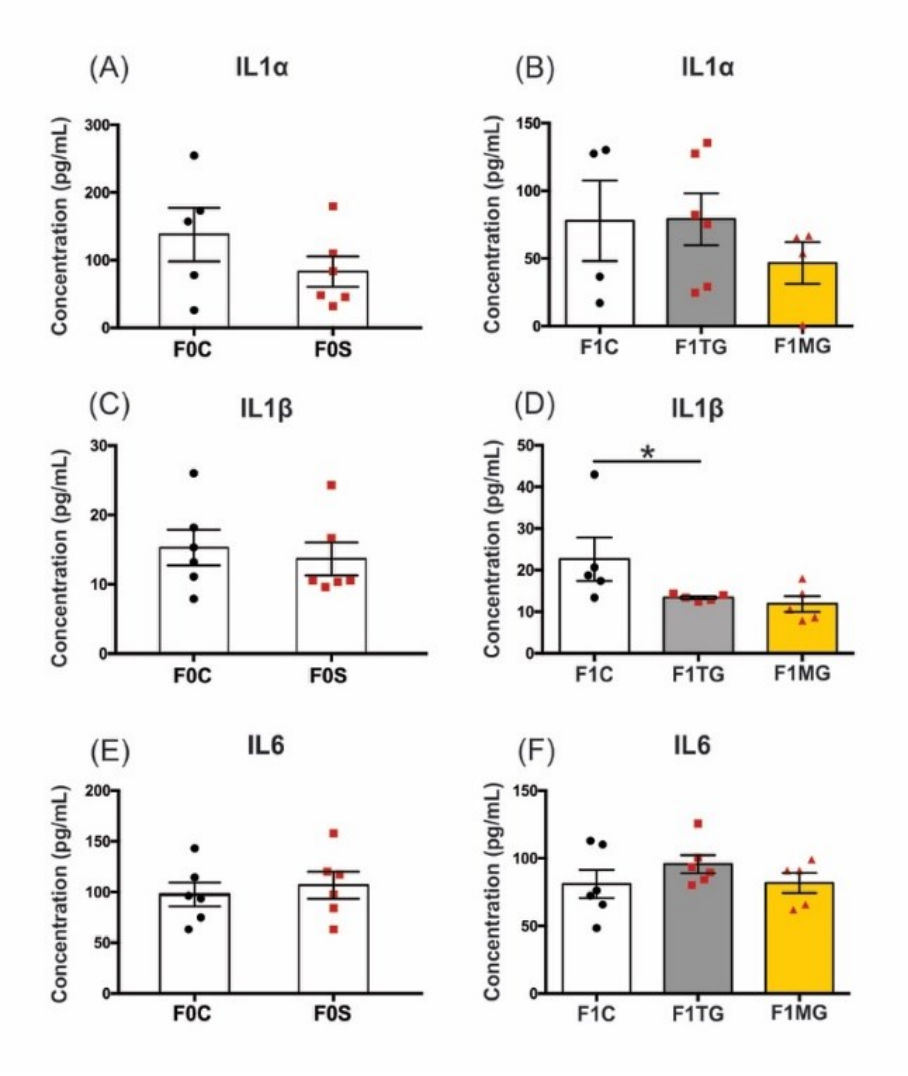

Figure 4.11: Decreased uterine IL-1β protein concentrations in F1 generation of SIS-exposed dams.

(A,B) The levels of IL1α protein were unaltered in F0 and F1 uteri, although they showed a tendency to decrease in the F1 dams of the MG lineage. (C) The IL-1β protein levels in F0 uteri were unchanged (D) but they significantly decreased in the TG lineage compared to F1 controls. In the MG lineage, IL-1β protein also showed a tendency to decrease its levels but did not reach statistical significance. (E,F) Concentrations of IL-6 were unchanged in both generations and treatment groups. Data are presented as concentration (pg/mL), mean ± SEM. The F0 tissue homogenates were analyzed by independent t-test, whereas F1s were analyzed by ordinary one-way ANOVA with Tukey post-hoc test. Statistical significance: * $p < 0.05$, N = 4-6.

4.4 Discussion

In this study, we demonstrated over four generations of rats that PNMS from SIS: (1) induces early birth of the parental generation pups, (2) changes gestational blood glucose levels in the offspring, (3) does not alter maternal gestational weight, (4) leads to increased CORT in the F1 generation, (5) affects neonatal birth weights, and (6) induces fetal programming changes in uterine gene expression of stress and inflammatory markers.

Preterm labour is a syndrome that remains the leading cause of death during infancy with potentially life-long neurodevelopmental and chronic consequences [132,369]. Current preterm birth therapeutics cannot provide a long-lasting delay of preterm birth and are ineffective in reducing neonatal morbidities [370]. Our primary finding was that SIS reduces pregnancy duration of the parental generation but not enough to cause preterm birth. This finding is consistent with other stress studies where rodents were exposed to restraint [371], a combination of four stressors [372], and immunological challenges [373]. Pregnant mothers that experienced the Quebec ice storm in 1998 during the first and second trimesters had shorter gestational lengths (38.9 and 38.7 weeks, respectively) compared to those exposed during preconception or the third trimester (39.4 and 39.7 weeks, respectively) [374]. However, in the current study, gestation durations normalized in subsequent generations.

The present data revealed a moderate association between SIS and a disinterest in breeding by the dams. There was, however, no significant change in the occurrence of adverse health outcomes in SIS-exposed rats. In contrast, other rodent stress models by our group demonstrated adverse health outcomes such as resorption, preterm, and post-term delivery [152,331] (see Appendix Tables A1,A2). A

possible explanation for the differences observed in health outcomes in our studies is the nature of the stress exposure and the number of hits used to stress the animals.

Preconceptional and gestational SIS did not modify the baseline or gestational weights of dams between the groups, and litter sizes remained normal. Previous SIS studies also did not observe differences in body weight [323,375], suggesting some SIS protocols may not be salient to elicit body weight changes in rodents, or differences in strains and species may contribute to these discrepancies.

The SIS study is the third in a series of PNMS studies. We previously subjected pregnant rats to psychological stressors to investigate the programming of physiological and epigenetic pathways by using forced swimming and restraint in a single generation and across four generations—named here single-hit stress [152]. Furthermore, we tested the effects of two-hit stress on pregnant rats' health outcomes by using a combination of psychological stressors and intraperitoneal injections of IL-1β [331]. A full summary of results and comparisons can be found in Appendix Tables A1 and A2.

In all of our PNMS studies, we found that the offspring of stressed rats showed low birth weights regardless of the stress type [152,331]. Females and males of both SIS paradigms displayed reduced birth weights in the F1 generation. Sex-specific variations were evident in the F2 generation, where the male birth weights were still low in both paradigms, and female birth weights remained low only in the MG group. These data are consistent with the two-hit stress model [331] and chronic variable mild stress in rats [376]. Birth weights, however, returned to control levels in the F3 generation, indicating a resilience or adaptation to SIS. These findings support the premise that PNMS programs developmental trajectories across generations of offspring in a sex-specific manner.

Exposure to PNMS in a single or over multiple generations of rats has immediate and long-lasting effects on metabolic parameters in the offspring. We observed low blood glucose levels on GD18 in the F1-TG and F3-MG offspring. These findings indicate other layers of regulatory mechanisms on gestational glucose levels in animals exposed to stress. For example, the normalization of blood glucose levels in SIS TG animals after the F1 generation may indicate an adaptation to SIS. Another possible explanation is that stress affects the oscillation of blood glucose levels in rodents [377], which can permanently disrupt glucose metabolism.

We also found an association between higher blood glucose levels and shorter gestation in F3 SIS-exposed animals. This finding agrees with previous observations made in pregnant women [378] and PNMS-exposed rats [152], where elevated glucose levels were associated with shorter pregnancy lengths and altered fetal growth patterns [379]. Increasing CORT levels are associated with elevated blood glucose concentrations in F2-TG animals, suggesting a relationship between higher stress with shorter gestational lengths and higher gestational glucose concentrations. This finding is in accordance with our single-hit stress that only used psychological stressors to expose the rats [152]

Social isolation is considered a mild psychosocial stressor for most rodents and resembles perceived isolation observed in depressive disorders [292,380]. GCs (e.g., CORT) and HPA axis mediators are commonly used parameters to evaluate the effects of maternal stress. We did not see changes in plasma CORT in the SIS-treated parental generation, similar to our two-hit stress study [331]. However, unlike the two-hit study, we found elevated CORT levels only in F1 animals in both stress paradigms. A MG psychological stress study induced HPA axis dysregulation and blunted CORT levels, especially in F4 generation males [381]. Furthermore, absent [382,383] or reduced [384,385] basal CORT levels were reported in rodents due to SIS.

Social stressors in rodents were shown to affect neurobiological mechanisms implicated in depression, including the activation of pro-inflammatory cytokines, increased levels of GCs, and upregulation of CRH and its receptors [386]. The pro-inflammatory IL-1β and IL-6 cytokines are key mediators involved in the inflammatory events of parturition [85] and are commonly upregulated in sterile inflammation and intrauterine infection associated with preterm birth [83,141]. Moreover, the peptide hormone CRH plays a central role in regulating the maternal and fetal HPA axis [387] and exerts its actions by activating two types of receptors, CRHR1 and CRHR2. [178]. Intrauterine CRH and its receptors have crucial roles in parturition [178,181], and their interplay and synergistic effects with pro-inflammatory cytokines, prostaglandins, and uterine activation proteins regulate the uterus' transition to a pro-contractile state [85].

The allostatic load conceptual framework describes how the consequences of recurrent or chronic stress accumulate causing progressive wear and tear on the body. Thus, the concept of allostatic load also applies to the stress passed on to the offspring through the maternal lineage [156].

Observations in all of our PNMS studies (Appendix Tables A1 and A2) support a relationship between preterm birth and transgenerational transmission of stress and inflammatory markers [152,331]. Shorter gestational lengths in the SIS-exposed F0 animals correlated with high uterine *Il1b* mRNA expression levels. This observation is likely explained by the role IL-1β plays in orchestrating downstream signalling of pro-labour mediators [85]. The *Il1b* mRNA expression and protein abundance were reduced in offspring of MG and TG lineages in line with a return to normal gestation lengths.

The cumulative effects of SIS in the MG group resulted in elevated *Il1r1* receptor abundance in the F3 uteri, which may represent a maladaptation. In fact, a study has found increased *Il1r1* mRNA expression and higher protein abundance before parturition in rats [388]. However, there were no changes in the *Il1r1* and *Il1a* cytokine abundance in uteri of stressed rats from the TG and both stress paradigms, respectively. This intriguing effect may result from an underlying coping mechanism developed by the F1-F3 offspring of dams exposed to chronic SIS, which were revealed as baseline *Il1a* and *Il1r1* receptor expression and decreased *Il1b* expression in the uteri.

The actions of IL-1β have been shown to modulate the uterine expression of CRH receptors and their variants, and they control the onset of labour [92,389]. The isoform CRHR1 is frequently associated with relaxation of the myometrium, while the isoform CRHR2 is likely involved as a pro-contractile stimulus [178]. Here, we showed a pro-labour profile in the F0 parental uteri, with upregulation of *Il1b* expression and downregulation of *Crhr1*. In the F1-F2 SIS-exposed offspring, the *Crhr1* abundance increased, while the *Crh* mRNA expression decreased in the F3-TG lineage. These data suggest that the offspring across several generations may be programming uterine gene expression adaptations in response to SIS as a protective and evolutionary mechanism to mitigate future adverse pregnancy outcomes such as preterm birth.

Cumulative SIS, however, raised *Crhr2* mRNA expression in F3 animals. This change in generational programming would shift the uterus to a pro-labour status, thus increasing the risk of preterm birth in future pregnancies. The offspring preterm birth protective programming patterns of *Crh* and *Crhr1* versus the pro-preterm birth patterns of *Crhr2* mRNA expression demonstrate the complex and multifaceted functions of CRH and its receptors in regulating various cellular responses and myometrial

muscle tone throughout pregnancy. Additional research is warranted to address the cumulative and ancestral effects of stress on the HPA axis and CRH physiological mechanisms in the uterus.

The 11β-Hydroxysteroid dehydrogenase type 2 (Hsd11β2) enzyme isoform inactivates CORT into its dehydrocorticosterone form, regulating fetal exposure to GC and the onset of labour [368]. In the current study, intergenerational transmission of traits was observed in the F1 generation of the TG lineage, with increased *Hsd11b2* expression, while its levels returned to baseline in the F2 generation. This result suggests that higher *Hsd11b2* abundance in the F1 dams is correlated with reduced CORT levels locally in the uterus. This observation further indicates that the offspring are adapting to counteract the effects of SIS. We only assessed the *Hsd11b2* expression in the uterus given that the dams eat their placentas.

Due to the generational nature of this study, we assessed uterine tissues collected on LD21 when the pups were weaned. Keeping dams and pups together for 21 days could represent a form of enriched environment and potentially act as a confounding factor for the metabolic and molecular changes observed in the study. However, it was essential to house them together for the proper nutrition and development of the offspring. We would need to perform a separate study to examine the uterine gene expression patterns during parturition.

Early life is a stage of developmental plasticity where phenotypic changes are influenced by the environment [390]. Our past and present data fit within the match/mismatch and fetal programming hypothesis, where early programming of adaptive responses to adversity in anticipation of the postnatal environment occurs in response to stress [195,390,391]. The results of these PNMS studies depict the distinct immediate and generational effects caused by the different stress types [152,331] (Appendix Tables A1 and A2).

A common finding among our PNMS studies was low birth weights in pups regardless of the stress protocols. This indicates that PNMS directly affects early-life outcomes of the progeny and that these effects are transmitted to future offspring. In humans, low birth weight is associated with risks for short and long-term complications and disabilities [392,393].

We demonstrated that the expression of key mediators of parturition change in the uteri of stressed animals in all three PNMS studies. These modifications are stress-specific and vary with the

duration of the stress protocol [152,331] (Appendix Tables A1 and A2). Ultimately, these changes lead to the transmission of inflammatory and stress markers and adverse outcomes over generations of rats thereby increasing future adverse pregnancy outcomes. Prenatal stress was also linked to preterm birth in humans [206,207] and it was shown to be passed on to the offspring, where it altered the health outcomes of the progeny [394,395].

Our study is timely considering the unprecedented global SIS imposed by the SARS-CoV-2 virus starting in 2020 [25,27,85]. Pregnant women experienced various degrees of isolation during their pregnancies, and a rise in depression and anxiety was observed [396]. Worry, financial pressure, and rates of domestic violence increased while prenatal care visits decreased [397-399]. A combination of perceived stress and physiological changes related to infection by the SARS-CoV-2 virus may explain the strong association with increased rates of preterm birth, preeclampsia, low birth weight, and gestational diabetes [400]. Indeed, loneliness and social isolation were associated with chronic inflammation during the COVID-19 pandemic [401]. The long-term impacts of the COVID-19 pandemic are expected to be experienced years later, where mothers may leave epigenetic imprints of their stressful experiences on their children, leading to life-long consequences [402].

We found a link between preconceptional and gestational SIS to shorter gestational lengths in the first generation of rats, possibly due to inflammatory imbalance in the uteri. These effects were not observed in the offspring, suggesting the activation of adaptive mechanisms through the generations. Despite the encouraging findings of this study, our society should give cautious attention to this issue, given its possible impacts on pregnancy outcomes of future generations. An integrated multidisciplinary approach and immediate strategies should be implemented worldwide to increase the social support and wellness of pregnant women and reduce the burden of stress and anxiety and build resilience.

CHAPTER 5

MATERNAL TRANSGENERATIONAL PRENATAL STRESS (MTPS) STUDY

Environmental enrichment promotes transgenerational programming of uterine inflammatory and stress markers comparable to gestational chronic variable stress

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Currently in preparation for submission
October 2022

Acknowledgments: I would like to thank Ms. Anita L. Quon (laboratory technician) and Roberto Villalobos Labra (postdoctoral fellow) at the Sandra T. Davidge laboratory, Edmonton, AB, for training and assistance in the fluorescence experiments. We also thank Dr. Anna Noga for her assistance in preparing this manuscript in accordance with Good Publication Practices.

Research funded by the Canadian Institutes of Health Research (CIHR)

5.1 Introduction

Evidence from many animal and human studies suggests that prenatal maternal stress (PNMS) is associated with shortened gestation times and adverse life-long health outcomes in the offspring [152,372,403-405]. Adversity during critical periods of embryonic and fetal development may lead to modifications of the transcriptome that alter gene expression and phenotypes in adulthood [195,283]. This concept is known as the "Barker hypothesis" [406,407]. However, the PNMS mechanisms underlying adverse pregnancy outcomes and fetal programming are not well understood.

When a pregnant female experiences adversity, the impacts of that stress affect the somatic tissues (F0 generation), the fetuses (F1 generation), and the fetuses' germline (F2 generation) [408]. A true transgenerational inheritance arises when the phenotypic changes observed in the stressed individuals persist in the unexposed F3 offspring. This occurs through germline epimutations that are transmitted to the descendants [250,408].

Maternal neuroendocrine responses to stress and immune function must adapt significantly during pregnancy to support fetal growth and development [409], characterizing a vulnerable time to stress. The hypothalamic-pituitary-adrenal (HPA) axis and the immune system are two of the most relevant systems affected by gestational stress [410]. Several studies show a link between prenatal stressors and altered endocrine and inflammatory mediators in the gestational tissues [152,279,405,411,412]. Excess maternal cortisol/corticosterone (CORT) is released during stress and may cross the placenta, directly affecting the fetal brain and HPA axis [413]. This elevated CORT concentration surpasses the protective actions of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts active glucocorticoids (GCs) into inactive ketone products [414]. Therefore, stress disrupts pregnancy homeostasis and may lead to adverse maternal and child outcomes.

During the inflammatory process of labour, cytokine and chemokines are amplified and modulate coordinated physiological processes that promote uterine transitioning into an active state [85,415]. When exposed to acute and chronic stressors, pregnant women exhibited higher plasma concentrations of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumour necrosis factor-alpha [416-418], and lower levels of the anti-inflammatory cytokine IL-10 [416]. Further studies demonstrated that changes in the immune system and cytokine milieu during PNMS are associated with poor pregnancy outcomes,

including preterm birth [126,206,372,419]. Moreover, prenatal exposure to social isolation and restraint stress in rats induced changes in the uterine expression of inflammatory markers [331,412].

Maternal stress is also associated with cellular redox dysregulation [420-423], which contributes to poor outcomes such as neuropsychiatric disorders [421] and preterm birth [166]. Both pro-inflammatory cytokines [46,424] and hypoxia [425,426] downregulate placental 11β-HSD2, potentially leading to excessive fetal exposure to maternal CORT. Therefore, the stress-induced impaired coordination of the HPA-immune system and reactive oxygen species (ROS) production in mothers may, at least in part, explain the early programming of diseases [427].

Strategies to reduce the effects of stress include healthy environments and lifestyle choices that promote physical and mental benefits to life-long human health. In animals, the enriched environment intervention is a stress-reduction technique that improves the physical and social environment [292,428]. It promotes social interaction by housing more animals in larger cages. Animals are also provided with various toys with diverse textures, colours, and shapes to encourage cognitive, sensory, and motor stimulation [428].

Preconceptional and gestational enriched environment housing contributed to pregnancy maintenance and reduced preterm birth rates by 40% in an inflammatory mouse model by dampening the inflammatory response and pro-labour mediators [298]. The enriched environment intervention also improved behavioural, morphological, and molecular parameters resulting from the adverse programming of ancestral stress [287,295]. Yet, little is known about the efficacy of enriched housing on improving transgenerational uterine programmed effects of PNMS and stress-induced preterm birth risk.

The present study investigated the transgenerational effects of prenatal psychological and psychosocial stress over four generations (1) on offspring birth weights, (2) uterine expression of inflammatory and stress markers, and (3) preterm birth risk. Only the parental F0 generation was subjected to social isolation stress (SIS) and restraint, while the F1-F3 generations were left unstressed. We also assessed (4) whether housing F1 daughters exposed to ancestral stress under enriched housing would improve stress-induced adverse outcomes in their uteri and future generations. We hypothesized that PNMS induces uterine molecular changes of key inflammatory and stress markers and leads to

preterm birth in a transgenerational fashion. We also postulated that the transgenerational effects of adversity would be mitigated by enrichment in the progeny.

5.2 Specific methods

5.2.1 Animals

A total of 1,495 Long-Evans hooded rats (Rattus norvegicus) were used to produce a female lineage of rats. In pairs, nulliparous females were bred and raised at the University of Lethbridge - Canadian Centre for Behavioural Neuroscience vivarium. On postnatal day (P)95, females were individually paired with a stress-free male for one hour a day until successful mating (Figure 5.1). Pregnancy was confirmed by steady weight gain. Pregnant dams were individually housed from gestational day (GD)20 until delivery, and their gestational hours were video-monitored by continuous infrared light cameras (Panasonic WV-BP330, Panasonic, Minato-ku, Tokyo, Japan). Pregnancy duration was measured as the time between the final mounting and delivery of the first pup. Maternal data are referred to as GD or lactational days (LD), while offspring-related data are described in postnatal days (P). Gestational lengths were monitored, and offspring weights were measured on P1. The pups stayed with their mothers until weaning on P21, followed by housing with same-sex siblings. All experiments were conducted according to the Canadian Council for Animal Care and were approved by the University of Lethbridge Animal Welfare Committee, protocols 1705 - Rat Breeding Colony and 1715 -Adverse Pregnancy Outcome.

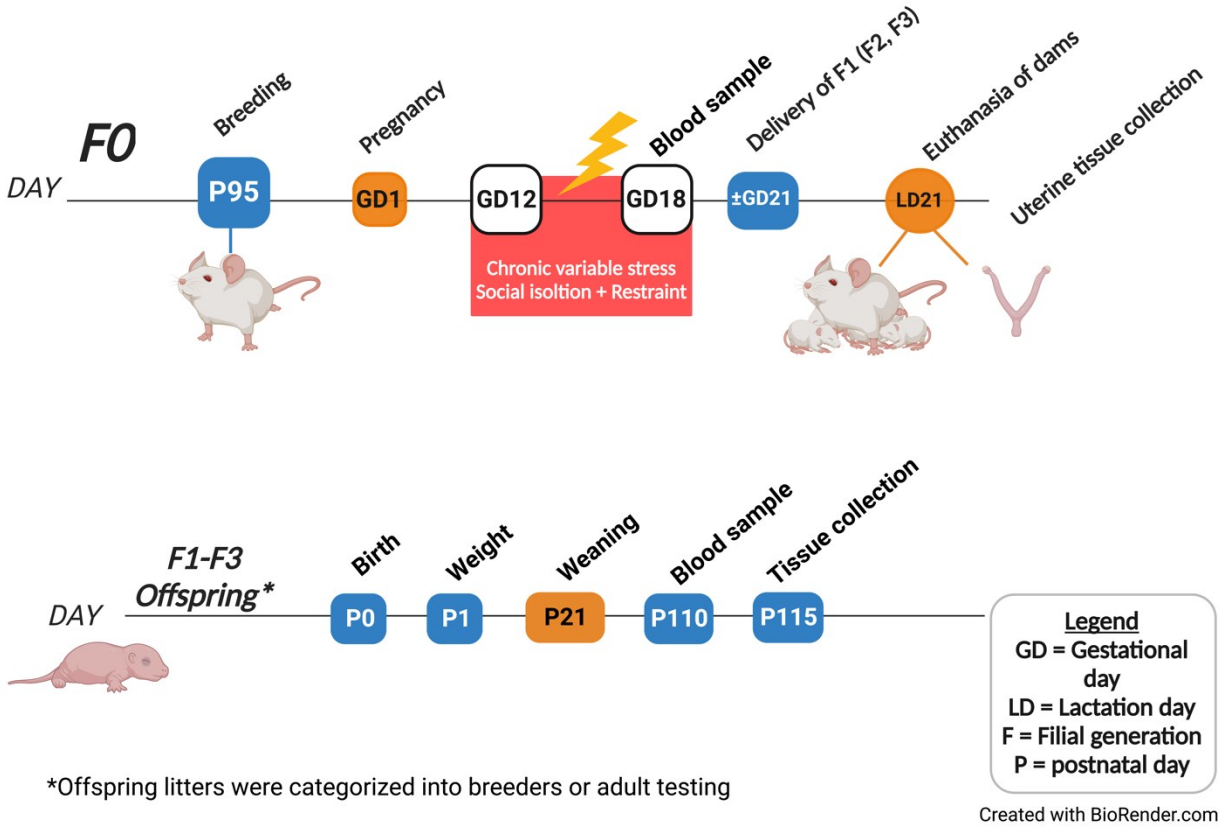

Figure 5.1: Timeline illustrating the stress protocol, tissue collection, and offspring analyses.

Gestational stress was implemented from GD12 to GD18 using restraint and social isolation stressors, creating our psychological and psychosocial chronic variable stress (CVS) model. Blood collection occurred on GD18 in the mothers and P110 in the offspring. Dams were sacrificed at the weaning of their offspring (LD21) when uterine tissues were collected. Tested offspring were euthanized and had their tissues collected on P115. Created with BioRender.com (accessed on 19 July 2022).

5.2.2 Experimental design

Three generations of female rats were bred with unstressed control males under standard or enriched conditions, whereby dams and offspring were split according to treatment in each generation (Figure 5.2). Timed-pregnant females (N = 31-40) from the parental F0 generation were stressed during mid-late gestation (F0S; GD12-18). Their F1 female offspring (F1S; N = 32-48) were bred to produce the subsequent F2S generation. The F2S female offspring (N = 80-104) were again bred to yield the F3S generation (N = 48-56). This cohort generated a transgenerational prenatal stress model of female rats,

where only the F0 pregnant dams were subjected to stress. A non-stressed control group of rats was bread alongside the chronic variable stress (CVS) treatment group. Each generation's treatment is depicted by the letters after the filial generation, including stressed rats F0S, F1S, F2S, F3S, and control rats F0C, F1C, F2C, and F3C, where C depicts non-stressed controls, and S stressed (Figure 5.2). A transgenerational enriched housing lineage was produced by splitting F1 pups into standard housing or enriched environment housing conditions at weaning (LD21), yielding two lineages of standard housing or enriched environment through the F1-F3 generations. The animals were kept in their assigned housing condition until GD20, when they were moved to a cage equipped with a camera system to monitor gestational length and maternal behaviour.

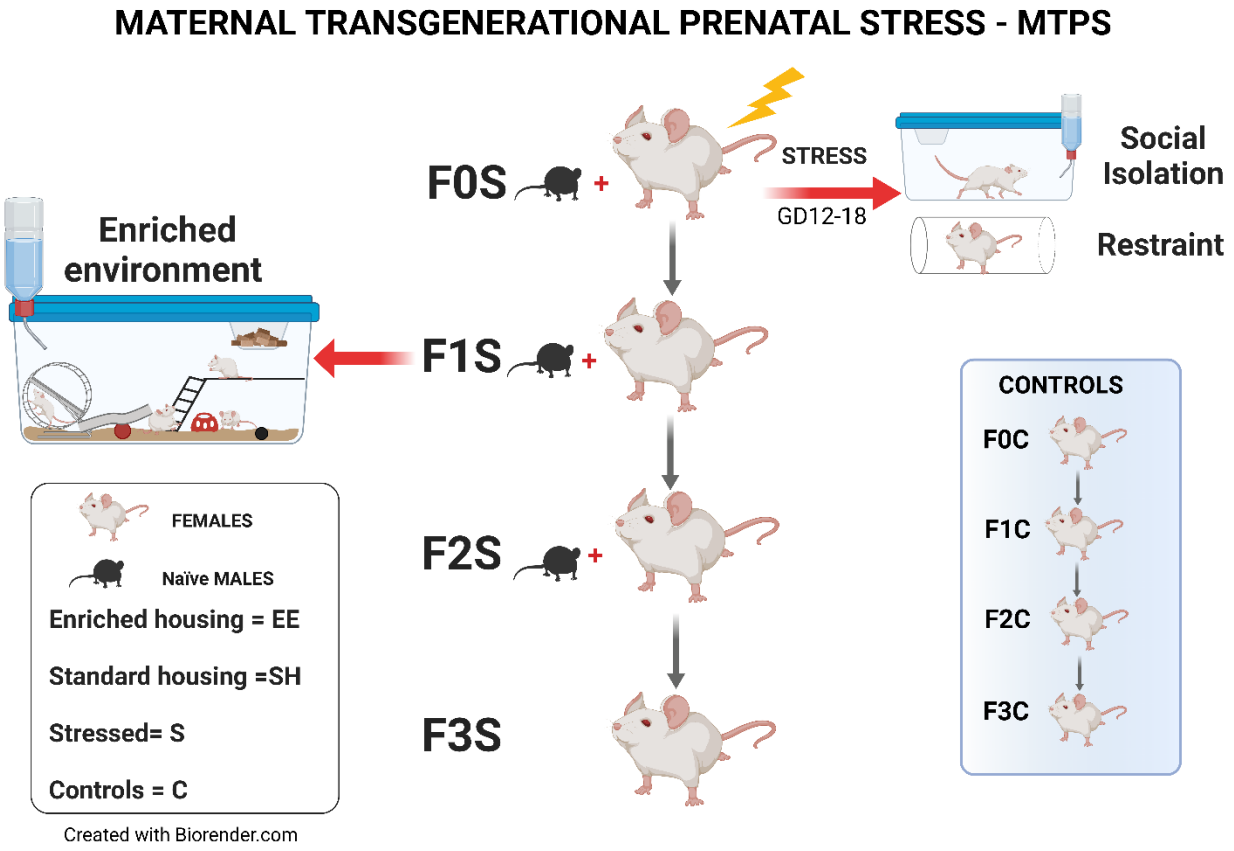

Figure 5.2: Maternal transgenerational prenatal stress (MTPS) experimental design.

Flow chart illustrating that F0 pregnant dams were subjected to social isolation and restraint stress from GD12-18. Enriched environment housing included a combination of physical and sensorimotor

enrichment and was implemented only in the F1 generation from weaning to GD20. The F2 and F3 generations were not directly exposed to enriched housing or stress but experienced their transgenerational effects. F = filial generation. Created with Biorender.com (accessed on 15 July 2022).

5.2.3 Prenatal stress procedures

Timed-pregnant F0 rats underwent CVS encompassing periods of restraint and social isolation stress (SIS) from GD12 to 18. Stress procedures were implemented at different times and days to avoid habituation to the stressor (see Table 5.1). For the restraint protocol, animals were placed in a customized transparent plexiglass container for 15-60 minutes in the morning or evening. The container was placed vertically and adjusted to the animals' size to prevent them from turning but without compressing their body. The animals were also subjected to 17 hours of overnight SIS on GD14 and GD17, where they were housed alone from 16:00-09:00 of the following morning but could still hear and smell their counterparts. The F1-F3 offspring were left unstressed throughout the experiment.

Table 5.1: Description of stress procedures implemented through GD12-18 using restraint and social isolation.

Gestational day	**G-12**	**G-13**	**G-14**	**G-15**	**G-16**	**G-17**	**G-18**
Treatment (am)	Restraint **60 minutes**	N/A	Restraint **45 minutes**	N/A	Restraint **15 minutes**	N/A	Restraint **45 minutes**
Treatment (pm)	N/A	Restraint **30 minutes**	Isolation **Overnight**	Restraint **60 minutes**	N/A	Restraint **30 minutes** Isolation **Overnight**	N/A

5.2.4 Rearing environments

The rats were housed under a circadian cycle (12:12h light/dark cycle) with lights on at 7:30 am, room temperature set at 20 °C and relative humidity at 30%. The animals had *ad libitum* access to water and food throughout the experiments. The F1 generation animals were assigned to standard housing or enriched environment housing conditions from P21 to GD20. For the standard housing conditions, rats were housed with non-sibling pairs in a standard shoebox-sized plexiglass cage of 8 in. height x 8 in. width x 16 in. depth. They were also offered a standard rodent diet and water *ad libitum.* Rats assigned to enriched environment were housed in communal condos measuring 24 in. height x 33 in. width x 22 in. depth. They were housed with 4-5 counterparts and were given a standard rodent diet and novel types of food, toys/wheel, and shelters that were changed weekly.

5.3 Results

Gestational lengths were unchanged in the stress groups across two generations

The gestational lengths for the F0-F2 stress groups remained unchanged compared to controls (F0C 526.3 ± 5.12 h, F0S 526 ± 3.05 h, F1S 528 ± 2.67 h, F2S 526.4 ± 2.85 h; $F(3,39) = 0.5783$, $p = 0.633$; Figure 5.3A). We did not find any statistically significant interactions between treatment and housing for the F1 and F2 generations ($F(1, 28) = 0.0002$, $p = 0.988$; $F(1, 28) = 1.481$, $p = 0.234$, respectively) where the implementation of the enriched environment intervention did not modify gestational lengths in the F1 and F2 generations compared to standard housing conditions ($p > 0.05$; Figure 5.3B,C).

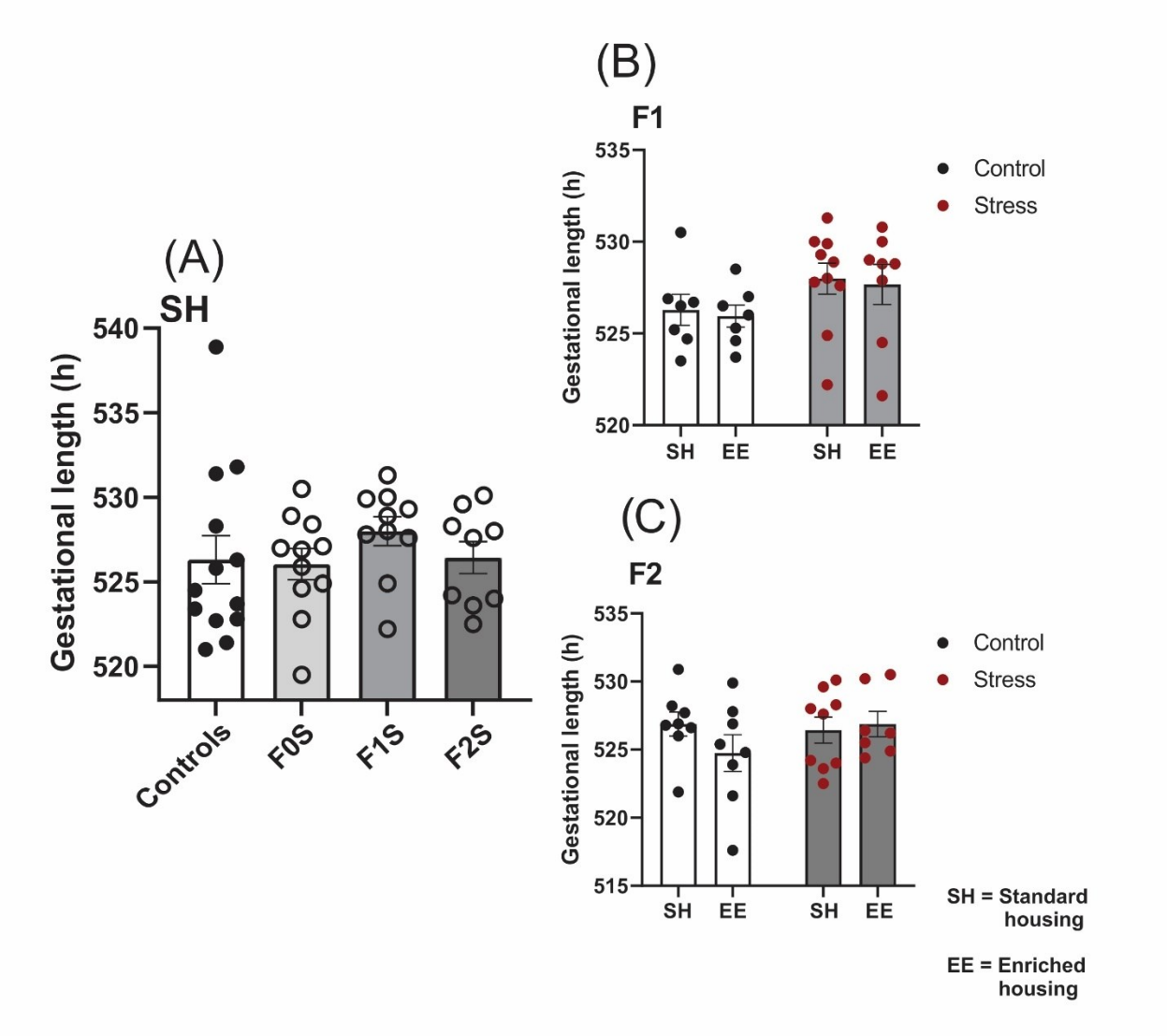

Figure 5.3: Gestational lengths were unchanged in animals subjected to PNMS.

(A) Gestational lengths among treatment groups. Gestational lengths in control and stress groups exposed to either standard housing or enriched environment housing conditions in the (B) F1 and (C) F2 generations. Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C) analyses were used. N = 9-13 (A) or 7-10 (B,C).

Offspring's birth weights were unchanged in the stress group but were increased with environmental enrichment

Stress has known adverse effects on neonatal birth weights [152,331,412]. We measured female and male weights on P1 to determine if a combination of psychological and psychosocial stressors

impacted offspring weight. We also tested the effects of enriched housing on neonatal weight. Birth weights were unchanged in either the female (F1C 6.45 g ± 0.52, F1S 6.43 ± 0.51, F2S 6.54 ± 0.48, F3S 6.51 ± 0.52; p = 0.673; Figure 5.4A) and male (F1C 6.89 g ± 0.5, F1S 6.84 ± 0.55, F2S 7.01 ± 0.48, F3S 6.82 ± 0.82; p = 0.575; Figure 5.4B) offspring between control and stress groups. However, we observed a significant effect of housing on P1 weights of female neonates (F2 generation; $F(1, 188) = 5.239$, $p = 0.023$; Figure 5.4C), while no interaction or treatment effects were observed ($F(1,188) = 1.053$, $p = 0.306$ and $F(1, 188) = 0.741$, $p = 0.390$, respectively). The birth weight analysis of male neonates exposed to ancestral effects of enriched environment housing revealed no interaction in the F2 generation ($F(1, 193) = 3.655$, $p = 0.057$; Figure 5.4D), despite significant main effects of treatment and housing in F2 male pups ($F(1, 193) = 7.029$, $p = 0.009$ and $F(1, 193) = 9.135$, $p = 0.003$, respectively). Two-way ANOVA revealed no differences in offspring weight between treatment and housing types for both F3 females and males (Appendix Figures B1A,B).

Litter sizes and fetal sex ratios of the treatment groups remained unchanged ($p = 0.983$ and $F = (3, 51) = 1.001$ and $p = 0.398$, respectively; Figures 5.5A and B).

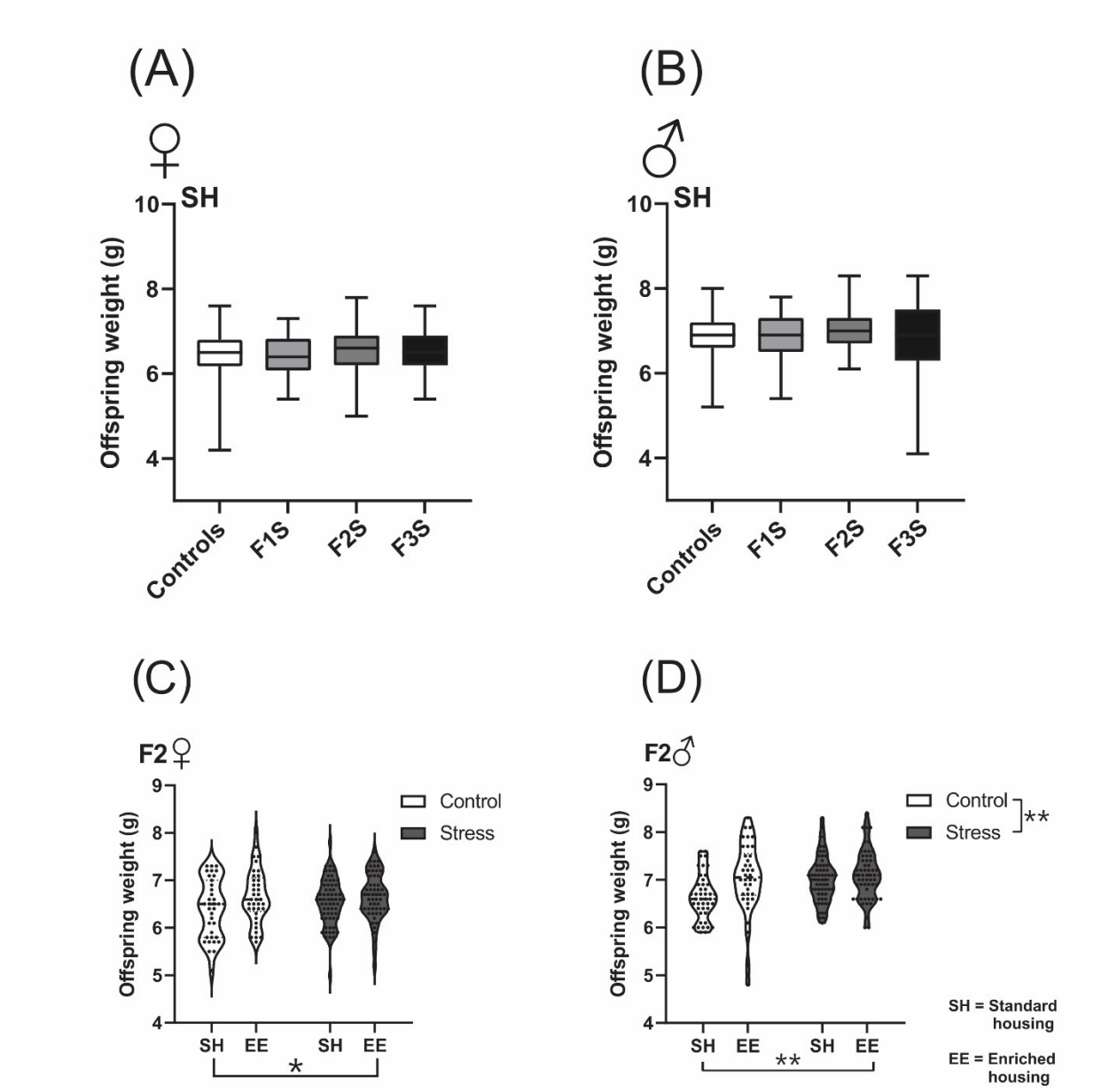

Figure 5.4: Offspring weights remained unchanged between treatment groups, while enriched housing significantly increased neonatal birth weights of females and males in the F2 generation.

Offspring weights between treatment groups in (A) females and (B) males. Pup weights of control and stressed (C) females and (D) males subjected to standard or enriched environment housing. Data are compared to F1C (A,B) and F2C (C,D), mean ± SEM. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges. Kruskal-Wallis test (A,B) or two-way ANOVA (C,D) analyses were used. N = 35-65 (females, A,C); 38-66 (males, B,D). Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***).

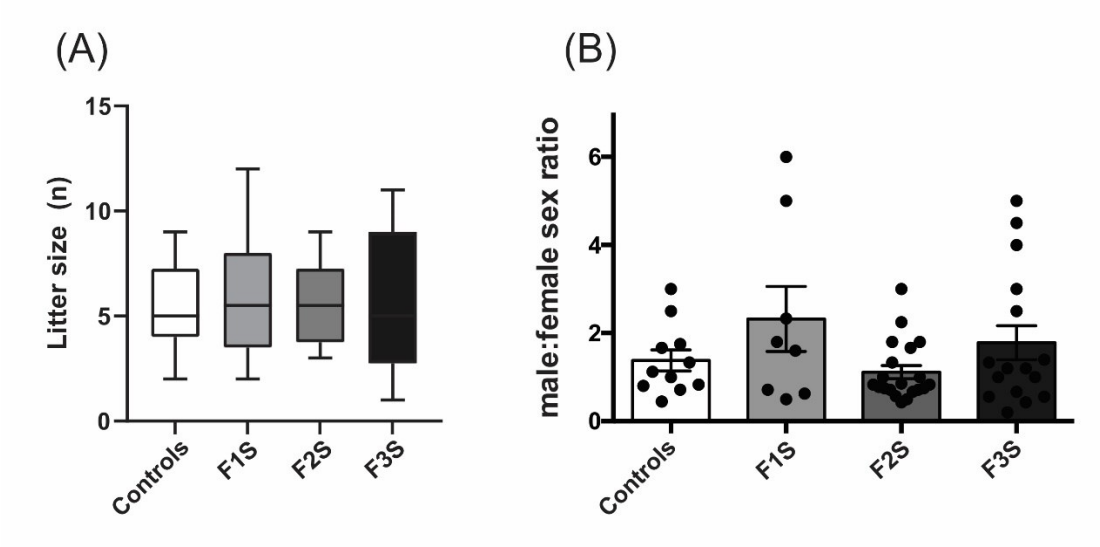

Figure 5.5: Litter sizes and fetal sex ratios were unchanged between treatments.

(A) Litter sizes and (B) fetal sex ratio between treatment groups. Data are compared to F1C and were analyzed using the (A) Kruskal-Wallis test and (B) one-way ANOVA, mean ± SEM. Box plots mid-lines indicate medians, whiskers indicate min-max values, and boxes indicate interquartile ranges. (A) N = 14-22 and (B) 8-18 per group.

Concentrations of plasma CORT levels were increased in F1 and F2 stressed animals and were not mitigated by enriched environment intervention

We measured CORT levels in plasma collected on GD18 (stressed dams) and P110 (adult offspring) to assess HPA axis activation and the release of GCs. CORT levels increased in the F1S and F2S offspring ($F(4,19) = 6.534$, $p = 0.002$) when compared to F0S animals ($p = 0.021$, $p = 0.001$, respectively; Figure 5.6A). Two-way ANOVA revealed no significant interaction between the effects of stress and housing type on CORT levels in the F1-F3 offspring ($F(1,16) = 3.105$, $p = 0.09$; $F(1,16) = 2.728$, $p = 0.118$; $F(1,15) = 0.2423$, $p = 0.630$, respectively; Figures 5.6B-D). However, simple main effect analysis in the F1 generation showed a statistically significant effect of treatment and housing on CORT concentrations ($F(1,16) = 18.40$, $p < 0.001$; $F(1,16) = 13.10$, $p = 0.002$, respectively; Figure 5.6B).

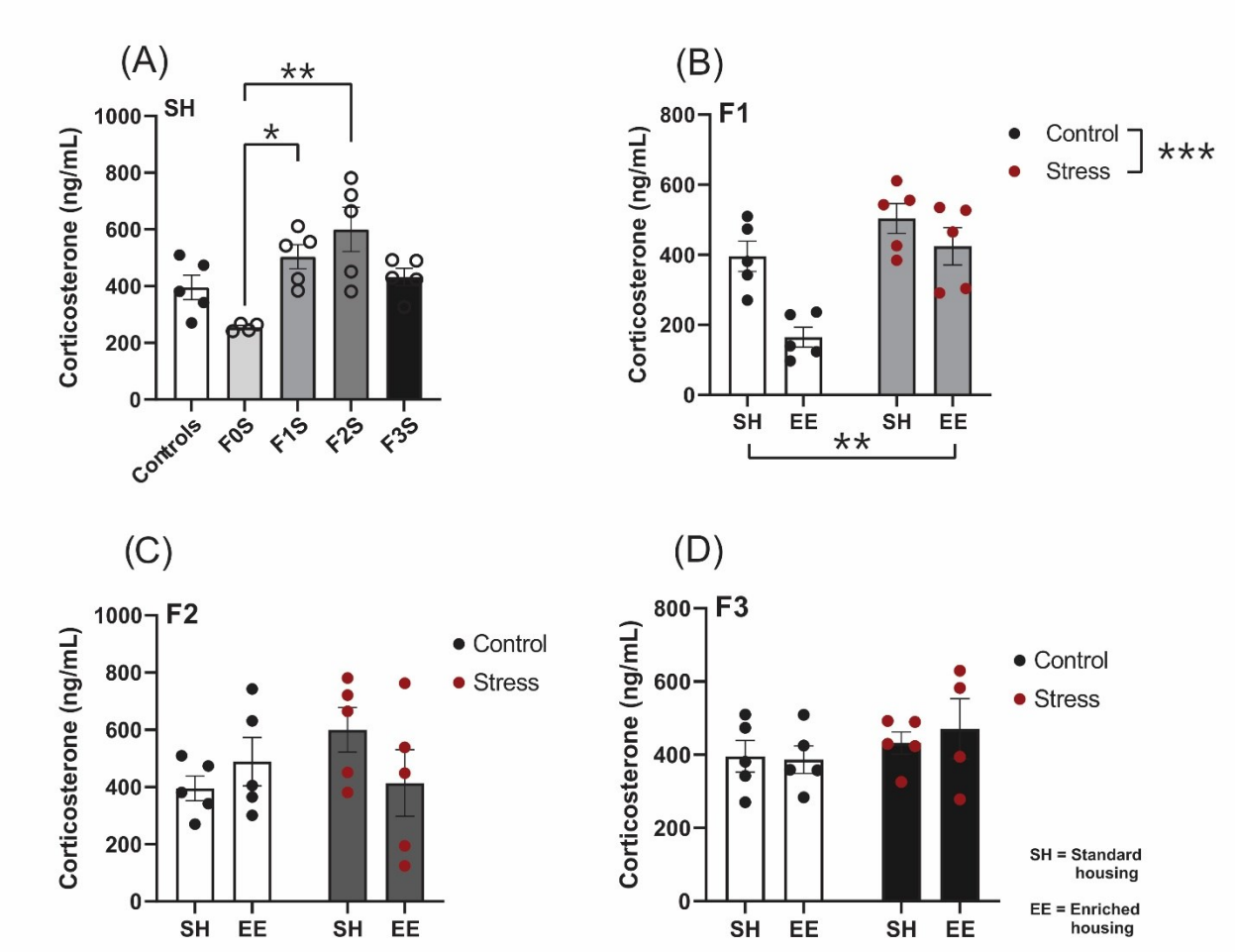

Figure 5.6: Elevated CORT concentrations in the F1 and F2 stressed offspring despite enrichment therapy.

(A) Plasma CORT levels in the F0-F3 stressed and control animals. Effects of housing and treatment on the (B) F1, (C) F2, and (D) F3 generations. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C,D) analyses were used. N = 4-5 per group.

Uterine ROS levels are unchanged in animals exposed to gestational and ancestral stress

Dihydroethidium (DHE) staining is widely used to assess the intracellular formation of ROS (originally designed to detect superoxide). DHE is a cell-permeable fluorescent dye that reacts with intracellular and extracellular superoxide to produce ethidium bromide, which binds to nuclear DNA and

generates red fluorescence (excitation/emission wavelengths of 518/605 nm) [429]. We tested the effects of gestational (F0 generation) and ancestral (F1-F3 generations) stress on the formation of ROS in the uteri of exposed dams and evaluated whether enriched environment housing would mitigate these effects.

Evaluation of ROS levels revealed unchanged levels between control and stress groups over the F0-F3 generations ($F(4,20) = 1.201$, $p = 0.341$; Figure 5.7B). We did not observe a significant interaction between the effects of treatment and housing in both F2 ($F(1,16) = 1.830$, $p = 0.195$; Figure 5.7C) and F3 generations ($F(1,16) = 0.095$, $p = 0.762$; Figure 5.7D). Two-way ANOVA showed insignificant effects of treatment and housing in the F2 and F3 offspring ROS levels ($p > 0.05$; Figure 5.7C,D).

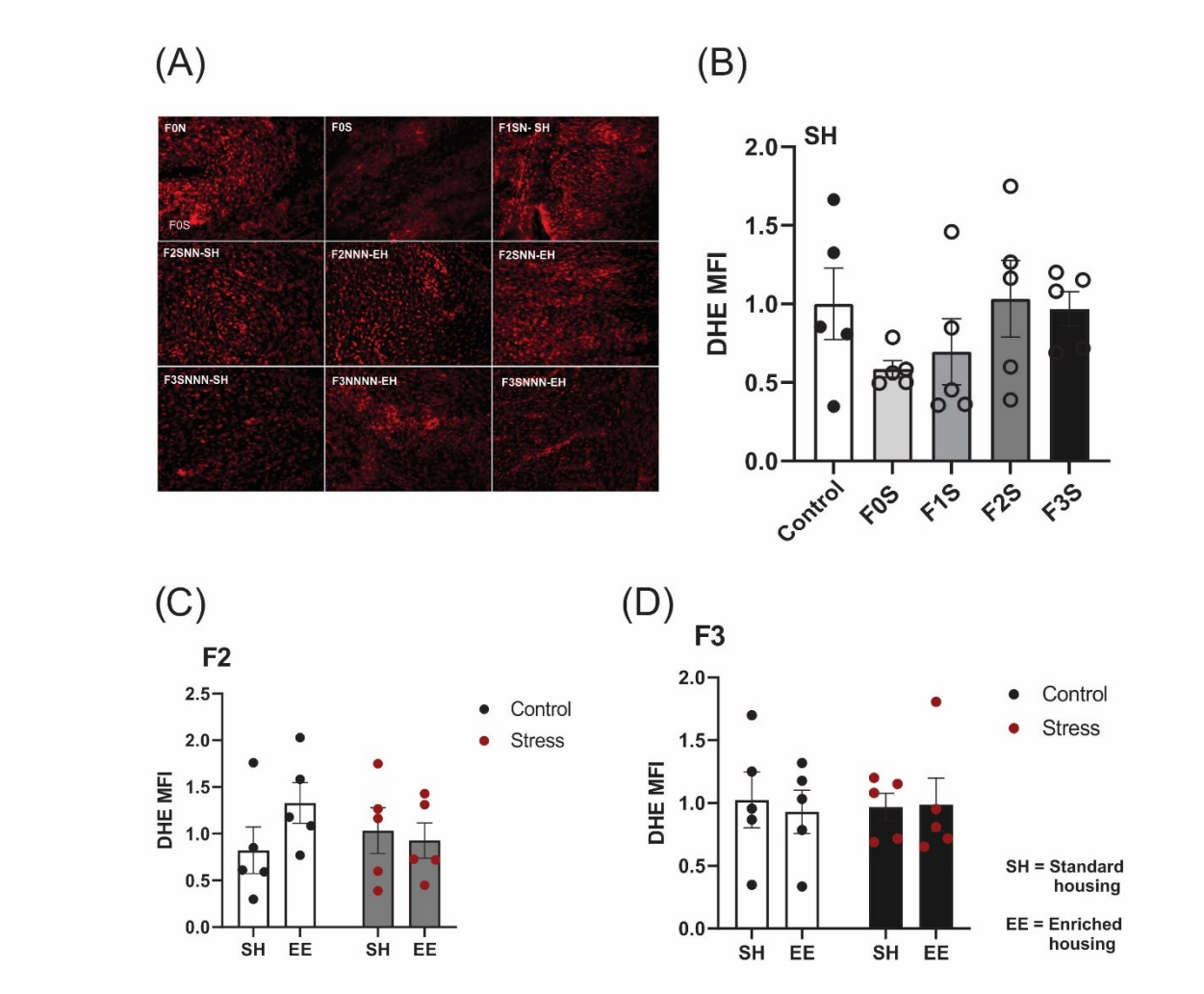

Figure 5.7: Uterine ROS levels were unchanged between treatments and housing conditions across the F0-F3 generations.

Four random regions of each uterus were used to measure mean fluorescence intensity (MFI) with DHE staining. (A) Representative images for each treatment and housing group. (B) Analysis of ROS levels (MFI) in uterine samples from stressed dams compared to controls. Assessments of the effects of enriched environment housing on uterine ROS levels in the (C) F2 and (D) F3 generations of animals subjected to ancestral stress. Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (B) and two-way ANOVA (C,D) analyses were used. N = 5.

Uterine expression of candidate genes and proteins involved in local inflammatory responses are impacted in rats exposed to ancestral stress

Pro-inflammatory cytokines: interleukin-1α (*Il1a)* and interleukin-1β (*Il1b)*

Stress has been shown to alter pro-inflammatory cytokine expression in the uterus and levels in the blood [331,342,412,416]. Since pro-inflammatory cytokines are essential mediators of the inflammatory events leading to the activation of the birth cascade [85], we measured *Il1a* and *Il1b* gene expression in the uteri of rats that experienced gestational and ancestral stress. Uterine mRNA expression of *Il1a* decreased in the stressed F1 and F3 generations ($F(4,38) = 8.774$, $p < 0.001$; Figure 5.8A) compared to controls (F0C vs. F1S $p < 0.001$; F0C vs. F3S $p < 0.001$) and F0S (F0S vs. F1S $p < 0.001$).

We observed a main effect of treatment on *Il1a* expression in the F1 generation ($F(1,30) = 18.180$, $p < 0.001$; Figure 5.8B), but there was no significant interaction or main effect of housing ($F(1,30) = 3.477$, $p = 0.072$; $F(1,30) = 0.237$, $p = 0.630$, respectively). Also, there was no significant interaction between treatment and housing on *Il1a* mRNA expression in the F2 generation ($F(1,28) = 1.259$, $p = 0.271$; Figure 5.8C). Two-way ANOVA revealed no significant interaction or main effects of treatment and housing on F3 uterine *Il1a* gene expression (Appendix Figure B2A).

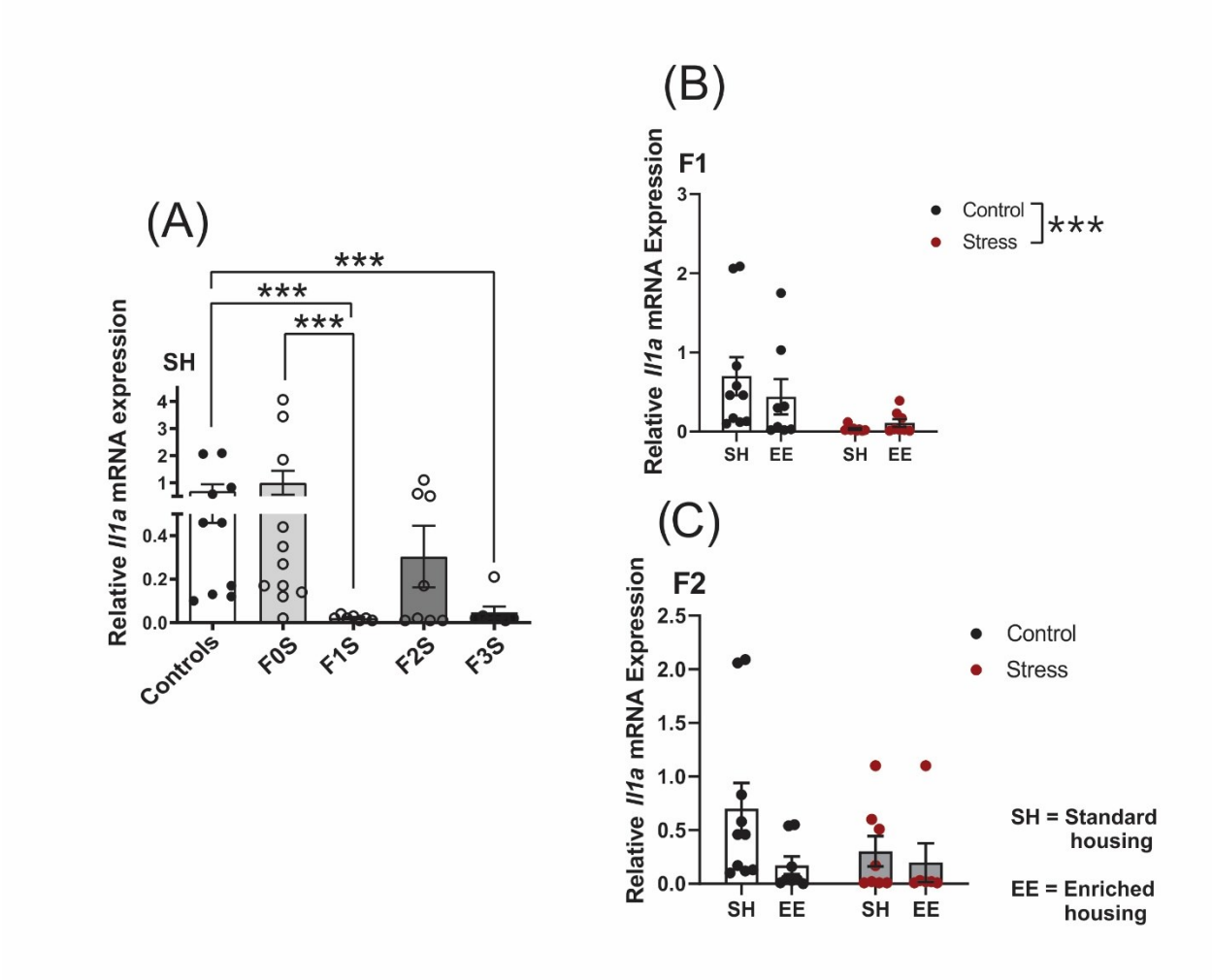

Figure 5.8: Uterine expression of *Il1a* was significantly downregulated in the F3 generation of stressed dams raised in standard housing and F1-stressed animals exposed to both housing conditions.

(A) Gene expression of *Il1a* in uteri of control and stressed dams across the F0-F3 generations. Uterine expression of *Il1a* in (B) F1 and (C) F2 animals exposed to different treatments and housing conditions. Asterisks indicate significance: < 0.001 (***). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C) analyses were used. N = 7-11 (A); 8-10 (B); or 6-10 (C).

The expression of *Il1b* increased significantly in the F2 generation ($F(4,42) = 5.491$, $p = 0.001$; Figure 5.9A) as compared to F0C and F0S ($p < 0.002$ for both). A statistically significant interaction between treatment and housing ($F(1, 31) = 7.915$, $p = 0.008$; Figure 5.9B) and a significant main effect of housing ($F(1, 31) = 4.239$, $p = 0.048$) was revealed in the *Il1b* expression analysis of the F1 generation. Pairwise comparisons showed a significant increase in *Il1b* expression in the F1C-enriched group

compared to F0C-standard housing controls (p = 0.002; Figure 5.9B). There was a significant interaction between treatment and housing for *Il1b* expression in the F2 generation (F (1, 29) = 8.244, p = 0.008; Figure 5.9C), whereby *Il1b* significantly increased in the F2C-enriched and F2S-standard housing groups compared to F0C-standard housing controls (p = 0.007 and p = 0.019, respectively). Two-way ANOVA revealed no significant interaction or main effects of treatment and housing on *Il1b* mRNA expression in the F3 uteri (Appendix Figure B2B).

Uterine mRNA expression of *Il6* and *Il1ra* demonstrated no significant differences between control and stressed animals across generations (Appendix Figure B3A,B).

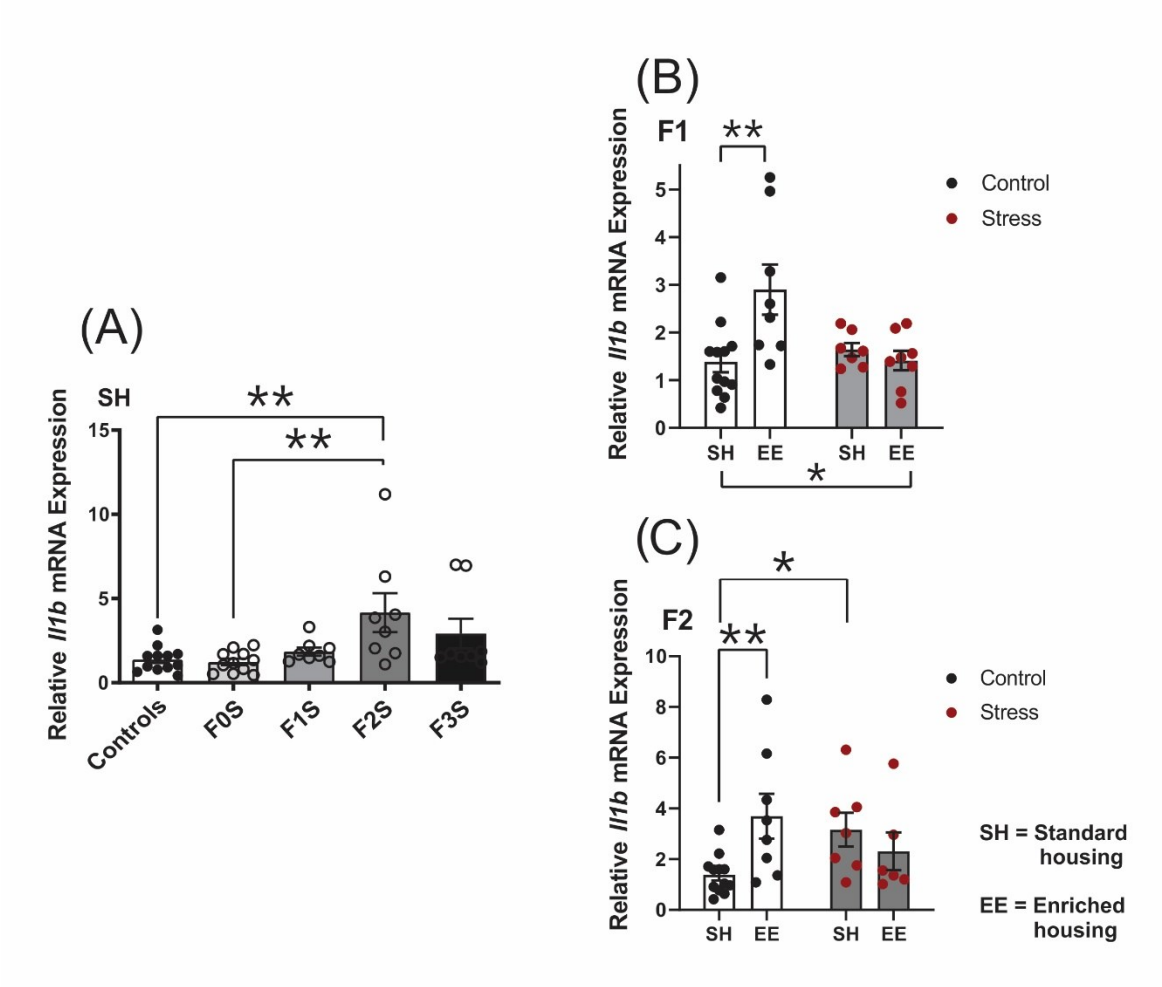

Figure 5.9: The mRNA expression of *Il1b* increased significantly in the F2 generation exposed to transgenerational stress and in the F1 and F2 controls exposed to an enriched environment.

(A) *Il1b* expression in F0-F3 dams subjected to CVS under standard housing. Uterine gene expression of *Il1b* in the (B) F1 and (C) F2 generations of controls and stressed animals exposed to standard or

enriched environment housing. Asterisks indicate significance: < 0.05 (*); < 0.002 (**). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C) analyses were used. N = 8-12 (A); 7-12 (B); or 6-12 (C).

Psychological and psychosocial stress in tandem did not induce changes in IL-1RAP protein levels in rat uteri.

The IL-1RAP protein forms a complex with the IL-1R1 and, upon binding of IL-1 agonists, initiates downstream signalling events to active IL-1 responsive genes [430]. Protein abundance of IL-1RAP remained unchanged across the stress-exposed F0-F3 generations, as shown by western blot analysis ($p = 0.970$; Figure 5.10B). Furthermore, we did not observe a significant interaction or main effects of treatment and housing types on IL-1RAP abundance in the uterus of F1-F3 adult offspring ($p > 0.05$; Figure 5.10C-E, respectively).

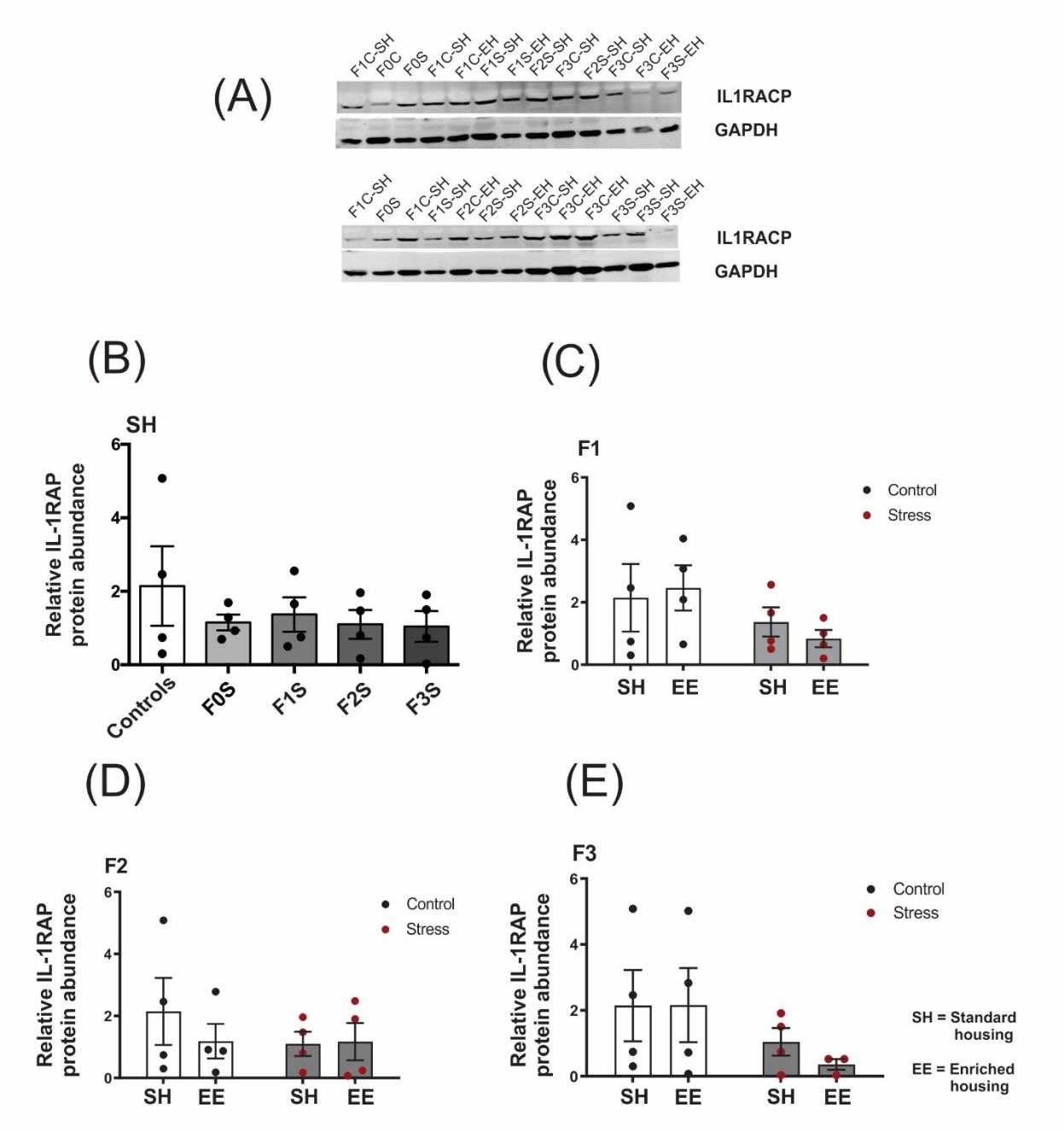

Figure 5.10: The protein abundance of IL-1RAP remained unchanged over the stressed F0-F3 generations regardless of housing type.

(A) IL-1RAP protein abundance quantified using densitometry (representative blots included). (B) Uterine protein abundance of IL-1RAP in F0-F3 stressed dams compared to controls. (C-E) IL-1RAP abundance according to treatment and housing across the F1-F3 offspring. Blots were quantitated using Odyssey software. All groups were compared using the Kruskal Wallis test (B), and the effects of treatment and

housing were assessed using two-way ANOVA (C-E). Data are normalized to GAPDH and compared to the F0C, mean ± SEM. N = 4 (B) or 3-4 (C-E) per group.

Uterine expression of candidate genes involved in local stress responses is impacted in rats exposed to ancestral stress

11β-Hydroxysteroid Dehydrogenase Type 2 (*Hsd11b2*)

Uterine gene expression of stress markers involved with GC metabolism, inflammation, and parturition was analyzed. Expression levels of *Hsd11b2* were decreased in the F1-F3 offspring ($F(4, 42) = 9.376$, $p < 0.001$; Figure 5.11A). Further pairwise comparisons showed significantly decreased expression of *Hsd11b2* in the F1S ($p < 0.002$), F2S ($p < 0.002$) and F3S ($p < 0.001$) generations compared to F0C. A significant interaction was observed between treatment and housing on *Hsd11b2* mRNA expression in the F1-F3 offspring ($F (1, 32) = 6.808$, $p = 0.014$; $F (1, 30) = 15.050$, $p < 0.001$; $F (1, 30) = 9.624$, $p = 0.004$, respectively; Figure 5.11B-D). The expression of *Hsd11b2* decreased in all the F1 generation groups regardless of treatment and housing conditions (F0C vs. F1C-enriched $p < 0.001$; F0C vs. F1S-standard housing $p = 0.001$; Figure 5.11B). Similarly, we demonstrated a significant drop in *Hsd11b2* expression in the F2C-enriched and F2S-standard housing groups compared to controls ($p < 0.001$ for both; Figure 5.11C). We also observed reductions in the expression of *Hsd11b2* in the F3C-enriched and F3S-standard groups compared to controls ($p < 0.001$ for both; Figure 5.11D).

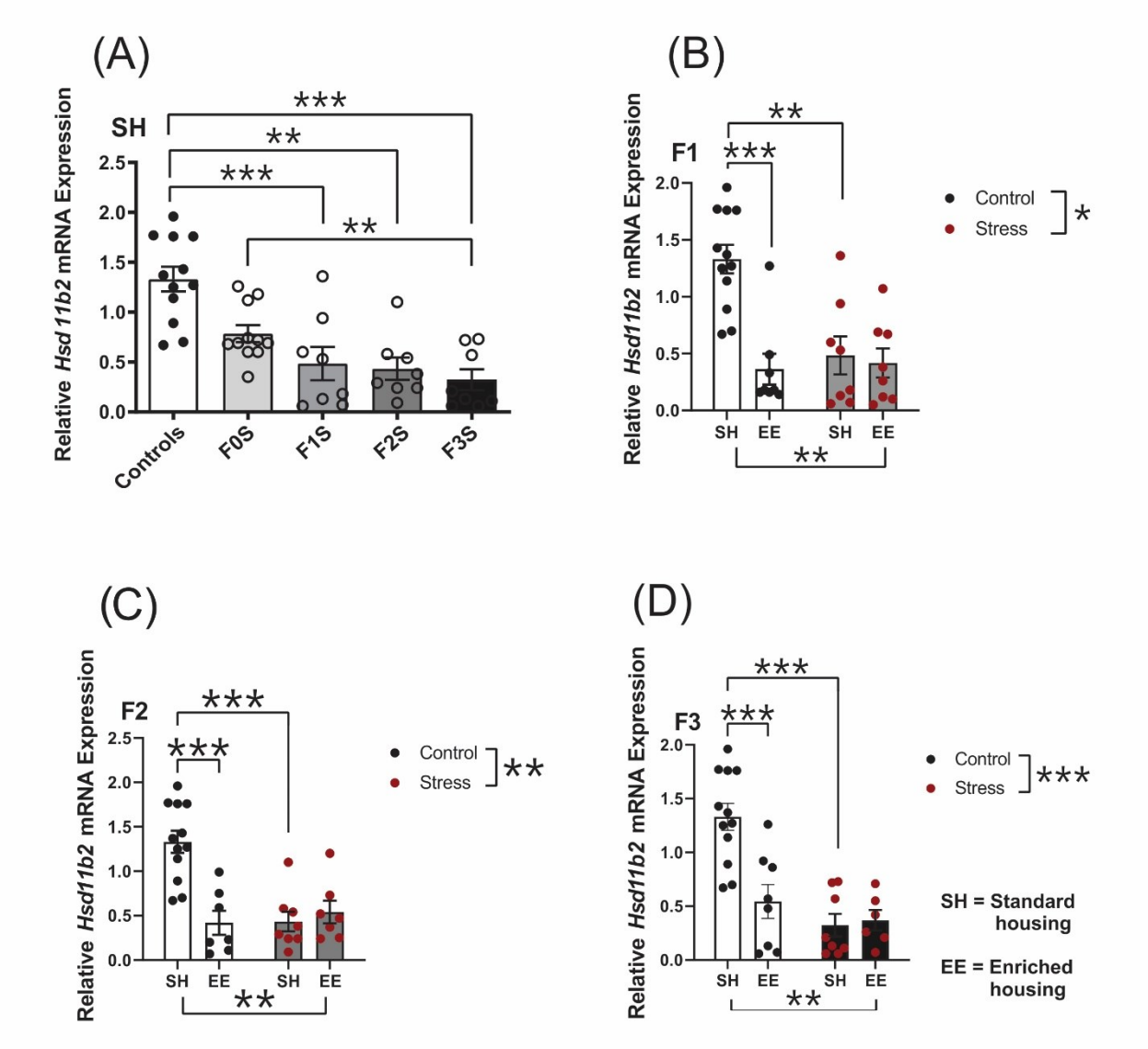

Figure 5.11: Expression of *Hsd11b2* is significantly decreased in the uteri of F1-F3 stressed dams and in the animals exposed to enriched environment housing.

(A) Gene expression of *Hsd11b2* in uteri of control and stressed dams across the F0-F3 generations. Uterine expression of *Hsd11b2* in (B) F1, (C) F2, and (D) F3 animals exposed to different treatments and housing conditions. Asterisks indicate significance: 0.002 (**); < 0.001 (***). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C,D) analyses were used. N = 8-12 (A,B); 7-12 (C); or 6-12 (D) per group.

Mineralocorticoid receptor (*Nr3c2*)

Uterine expression of *Nr3c2* was upregulated in stressed offspring ($F(4,42) = 4.584$ $p = 0.004$; Figure 5.12A), with increased levels in the F2S and F3S generations compared to F0C ($p < 0.002$ and $p < 0.05$, respectively) and F0S (F0S vs. F2S $p < 0.05$). The analysis of the effects of housing and treatment revealed a significant interaction between factors on *Nr3c2* expression in the F1 and F2 generations ($F(1, 32) = 6.040$, $p = 0.02$ and $F(1, 31) = 6.915$, $p = 0.013$, respectively; Figure 5.12B,C). Pairwise comparisons showed no significant changes in *Nr3c2* expression between housing conditions in F1S animals ($p = 0.741$), although its expression increased significantly in the F1C group compared to controls ($p = 0.014$). We did not observe a main effect of housing on *Nr3c2* expression in the F2 generation ($F(1, 31) = 0.289$, $p = 0.595$), but we did see a significant increase in the F2S-standard housing group compared to controls ($p < 0.001$; Figure 5.12C). Two-way ANOVA revealed no significant interaction or main effects of treatment and housing on *Nr3c2* mRNA expression in the F3 uteri (Appendix Figure B2C).

Gene expression of *Crh, Crhr1, Crhr2, Hsd11b1,* and *Nr3c1* were unchanged in the uteri of stressed animals when compared to controls (Appendix Figures B3C-G).

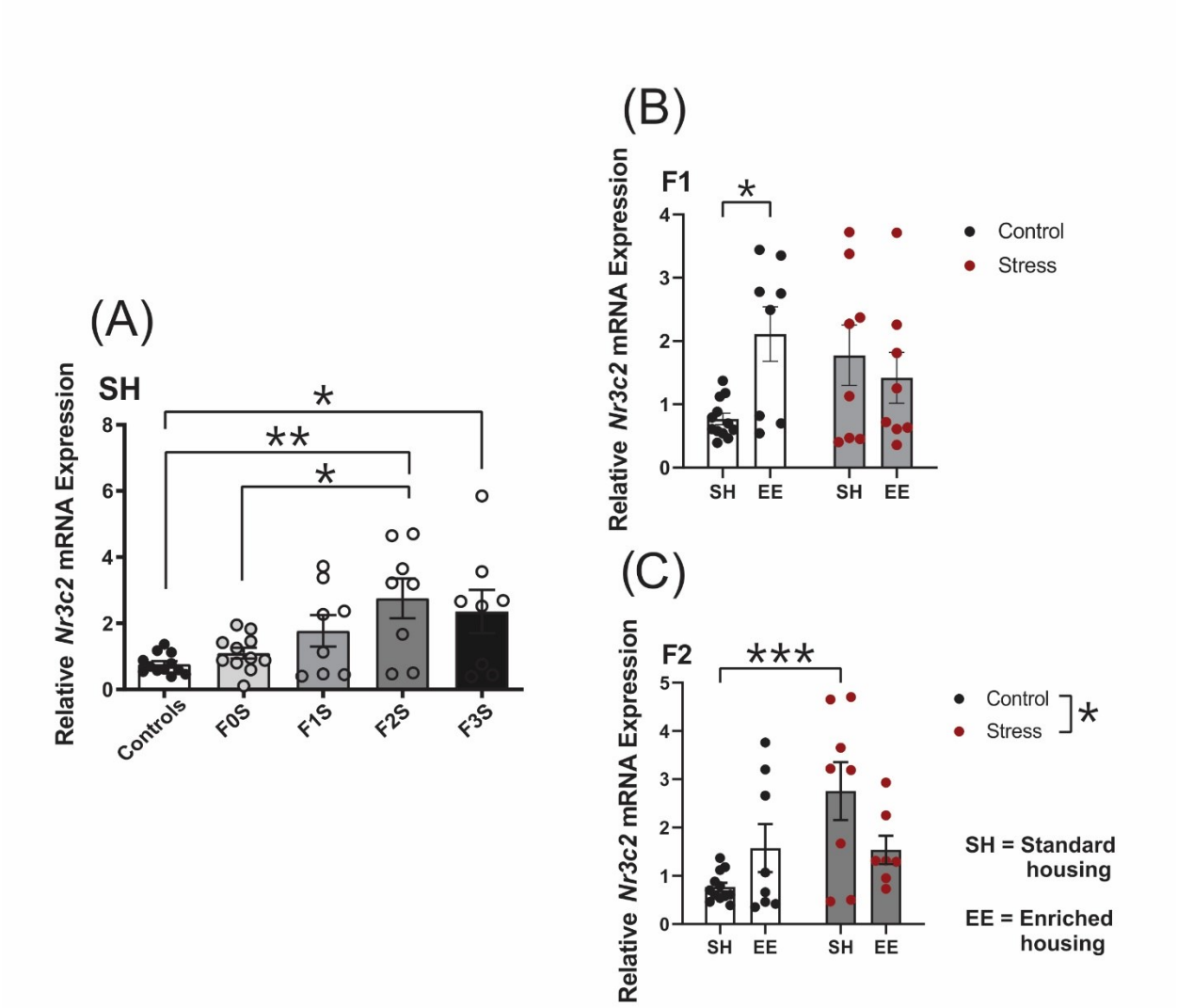

Figure 5.12: Gene expression analysis of *Nr3c2* showed significant increases in the uterus of stressed F2 and F3 dams under standard housing, with similar effects shown when raised under enrichment.

(A) *Nr3c2* mRNA levels across the F0-F3 generations of stressed dams compared to controls. Analysis of *Nr3c2* expression in the (B) F1 and (C) F2 generations of dams exposed to different treatments and housing types. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B,C) analyses were used. N = 8-12 (A,B) or 7-12 (C) per group.

Antioxidant enzyme gene expression: superoxide dismutase type 1 (*Sod1*)

The analysis of antioxidant enzymes in the uteri of rats revealed a significant upregulation of *Sod1* mRNA expression in the F1S group compared to controls ($F(4, 41) = 3.503$, $p = 0.015$; Figure 5.13A). We also observed a significant interaction between treatment and housing conditions on *Sod1*

expression in the F1 generation (F (1, 32) = 5.289, p = 0.028; Figure 5.13B), but no significant main effects (treatment F (1, 32) = 0.459 p = 0.503; housing F (1, 32) = 1.521, p = 0.227). Two-way ANOVA revealed no significant interaction or main effects of treatment and housing on *Sod1* mRNA expression in the F2 and F3 uteri (Appendix Figure B2D,E). Gene expression of *Sod2* was unchanged in the uteri of stressed animals under standard housing when compared to controls (Appendix Figure B3H).

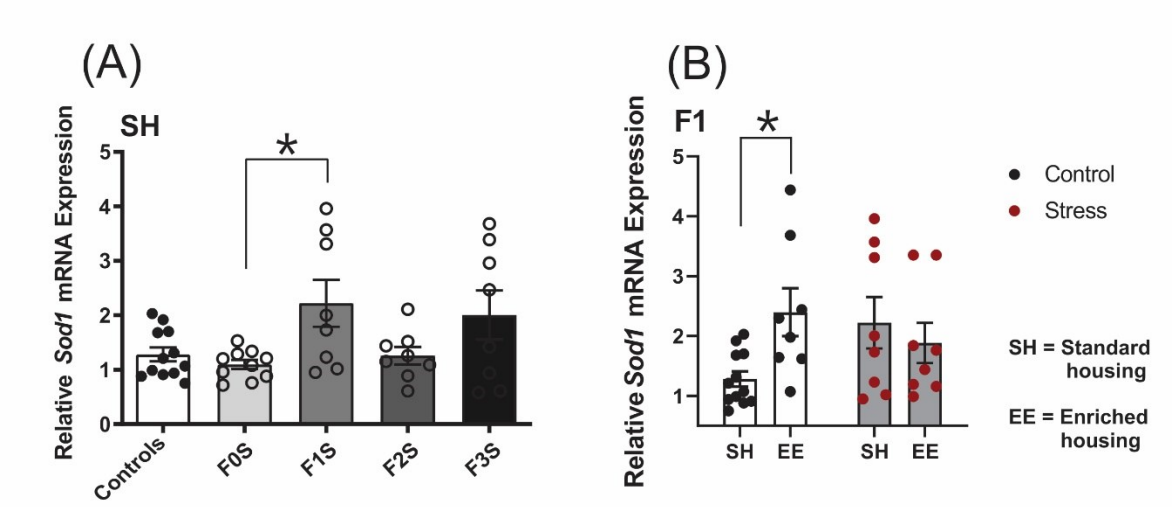

Figure 5.13: Uterine expression levels of the antioxidant enzyme *Sod1* were significantly increased in F1-stressed animals and in F1 controls subjected to enriched environment conditions.

(A) Expression of *Sod1* in uteri of stressed F0-F3 dams compared to controls. (B) Uterine expression of *Sod1* in F1 females exposed to stress and enriched environment housing as compared to controls and standard housing. Asterisks indicate significance: < 0.05 (*). Data are compared to F0C, mean ± SEM. Ordinary one-way ANOVA (A) and two-way ANOVA (B) analyses were used. N = 8-12 per group.

5.4 Discussion

We provide evidence that psychological and psychosocial CVS alter the inflammatory status and endocrine markers in the uterus of adult dams through transgenerational programming of the female germline over four generations. Our CVS model was insufficient to induce preterm birth or influence neonatal birth weights. Instead, we observed a boost in resilience to stress in this cohort. To our knowledge, this is the first study to confirm that enrichment therapy in the offspring of rats exposed to

ancestral stress has no beneficial effects on uterine expression of inflammatory and endocrine markers, but it rather generates patterns similar to the stress group.

Both preterm and term labour are inflammatory events characterized by the infiltration of leukocytes into gestational tissues and increased levels of pro-inflammatory cytokines and chemokines [85,431]. Maternal stress may disrupt this sensitive immunological balance and lead to birth complications (i.e., preterm birth [149,207,372], preeclampsia [208,432]) and inflammation in the placenta and fetal brain [405,433,434]. Restraint and social isolation stressors have been associated with uterine inflammatory, endocrine, and epigenetic modifications in gestational tissues [152,412]. Other consequences of such stressors include metabolic, behavioural, and adverse pregnancy outcomes in rats [287,313,338]. We previously showed that gestational exposure to SIS shortens gestation times in the stressed dams [412], while restraint and forced swimming hastened parturition in the exposed offspring [152]. We proposed that a combination of two variable stressors would lead to preterm birth in the dams exposed to gestational stress and in their offspring subjected to ancestral stress. Unexpectedly, no changes in pregnancy duration were observed in this study across the F0-F2 generations of stressed rats. These data align with previous maternal stress studies where gestational stress did not influence pregnancy lengths [331,435].

The F1-F3 offspring's birth weights remained unaltered in this CVS protocol as opposed to our previous studies [152,331,412]. Perhaps, the dams exposed to CVS became more resilient to offset the risk of stress on pregnancy and offspring outcomes. We also speculate that individual susceptibility to stress may play a role in the differences observed between different stressors [436], possibly through epigenetic mechanisms. Despite these findings, significant differences between control and stress animals were observed when comparing housing conditions in the F2 generation.

Our CVS protocol reduced the mRNA expression of *Il1a* in the uteri of the F1 and F3 generations exposed to ancestral stress, while it remained unchanged in the F2 generation. Other reports of transgenerational effects of stress mysteriously skipping generations and reappearing in future progeny in a sex-specific fashion exist [412,437]. Our results are similar to another study where a combination of psychological and immunological stressors also showed downregulation in uterine *Il1a* expression in prenatally-stressed F1 neonates [331]. It was also reported that multigenerational PNMS in mice elicited

immunosuppressive effects in the F2 generation, which were resolved in the F3 animals, suggesting a compensatory mechanism against cumulative stress [372]. We believe the reduced *Il1a* expression observed here may be a compensatory downregulation initiated by fetal programming of the F1 and F3 offspring in response to adversity.

In contrast, there was an upregulation of uterine *Il1b* expression in the F2-stressed animals. It is possible that transgenerational CVS increased the AL and exceeded the ability of the F2 generation to cope, resulting in the activation of inflammatory pathways, although not enough to cause preterm birth. These findings are expected given that preterm birth is a complex syndrome that involves early activation of multiple pathways that ultimately trigger labour.

We sought to investigate IL-1RAP abundance in the uteri of stressed animals. The IL-1RAP is a component of the IL-1R complex required for IL-1 signal transduction, and it presumably regulates inflammatory responses and uterine sensitivity to IL-1 during labour [438]. We hypothesized that IL-1RAP levels would increase in the uteri of stressed dams, given that stress is a potential regulator of IL-1 receptors and accessory proteins [388] and that IL-1RAP levels are increased in preeclamptic placentas [438]. However, there were no differences in IL-1RAP protein abundance between the stressed and control groups across the F0-F3 generations or between housing situations. This finding was observed in a rat study where IL-1RAP protein abundance was unchanged during labour, although *Il1rap* expression increased in the upper uterus [388].

Neuroendocrine mechanisms regulate the physiological responses to stress by controlling the maternal HPA axis [439]. PNMS often intensifies CORT secretion contributing to adverse pregnancy outcomes and detrimental offspring neurodevelopment [440]. Although the causal link between PNMS and HPA axis disruption to adverse pregnancy outcomes has been well-studied, contradictory findings suggest that HPA axis regulation during gestation is complex and related to the stress type. We showed increased CORT concentrations in F1 and F2 adult rats exposed to ancestral stress, suggesting changes in GC programming. These findings align with our prior work where multigenerational restraint and forced swimming stressors elevated CORT levels in the F2 offspring [152].

Fetal exposure to maternal GCs is partially controlled by the 11β-HSD2 enzyme types 1 and 2 [40] expressed in the placenta and uterus of rodents [441,442] and humans [368]. The 11β-HSD1 isoform

generates active CORT from their inactive 11-dehydro metabolites. However, 11β-HSD2 performs the opposite reaction and converts active CORT to its inert form, thereby acting as a physiological "barrier" to the adverse developmental effects of GCs [442].

We found reduced gene expression of *Hsd11b2* in F1-F3 offspring of rats exposed to CVS. This finding suggests that lower *Hsd11b2* expression in the offspring's uteri is associated with higher local tissue CORT levels. Interestingly, in the F2-stressed uteri, we also observed increased *Il1b* expression. This matches previous studies where pro-inflammatory cytokines inhibited human placental 11β-HSD2 activity [46], but it opposes the findings on uterine *Hsd11b2* expression in response to SIS [412]. The attenuation of 11β-HSD2 activity by prenatal stressors is linked to preterm birth, low birth weights, and neurodevelopmental delays in the progeny [40,443]. Our CVS did not alter offspring birth weights or pregnancy lengths, but future analysis of behaviour and brain development would clarify the effects of ancestral stress on the offspring.

Mineralocorticoids (MCs) are steroid hormones (e.g., aldosterone) with prominent roles in regulating water and electrolyte homeostasis [444]. Although their roles in pregnancy are unclear, aldosterone and the mineralocorticoid receptor (MR) modulate the immune system function, and regulate oxidative stress and the release of pro-inflammatory cytokines [445]. The placenta is a MC target tissue, and aldosterone modulates placental development and adaptation [444]. The intracellular homologous glucocorticoid receptor (GR) and MR mediate the actions of CORT and aldosterone, and both receptors act as transcription factors [446]. During pregnancy, 11β-HSD2 activity controls CORT binding to MR; therefore, it strictly regulates the actions of steroid hormones [447]. However, stress may disrupt this delicate control.

CVS increased uterine expression of *Nr3c2,* a gene encoding MR, in the F2 and F3 generations. This finding correlates with the reduced expression levels of *Hsd11b2* in those animals. Excessive GCs due to stress would occupy more MRs resulting in an increased risk for adverse pregnancy outcomes such as gestational hypertension and tissue inflammation. We postulate that MR expression in the uterus modulates inflammatory responses during pregnancy, and stress alters this balance and leads to inflammatory disruption. This is passed on transgenerationally, as indicated by increased *Nr3c2* and *Il1b* expression and reduced *Hsd11b2* in the uteri of stressed F2 offspring.

Oxidative stress results from an imbalance between ROS and antioxidant activity in cells and tissues [165]. ROS can damage cell components and contribute to the pathophysiology of reproductive diseases, including preterm birth, preeclampsia, and miscarriage when in excess [165]. The antioxidant defences that prevent ROS formation, repair damage, or promote ROS scavenging protect against their damaging effects [448].

SODs are antioxidant enzymes that catalyze the conversion of two superoxide anions to hydrogen peroxide and molecular oxygen [448], and they regulate the balance of ROS production during pregnancy [449]. Measurements of ROS have been widely determined by staining with DHE fluorescent probe [450]. In this study, ROS levels were unchanged across four generations of transgenerationally stressed rats, although we found increased *Sod1* uterine expression in F1-stressed animals. This indicates that a compensatory mechanism was triggered in the F1 generation to counterbalance the effects of ancestral stress experienced *in utero*.

Most of the literature describes enrichment intervention as an optimal housing condition that produces beneficial albeit variable effects on neuroanatomical and behavioural assessments [292,293,295,451-454]. Enriched environment is also used to alleviate or reverse the transgenerational effects of stress [294]. To our knowledge, this is the first study to show the transgenerational effects of enrichment interventions on the uterine inflammatory and endocrine status of stressed rats. Enriched environment arguably has transgenerational effects supported by the data of this study. Although the enriched environment intervention was only implemented in the F1 generation, the expression patterns were unique and transmitted across generations that only experienced the ancestral effects of enriched environment (F2 and F3 generations), affecting uterine gene expression in the progeny in a complex fashion.

Enriched environment was insufficient to mitigate the altered inflammatory and stress markers in the uteri of stressed rats. The gene expression patterns of animals raised in enriched environment housing were similar to those of stressed offspring raised in standard housing. Similar expression patterns for *Il1a* and *Il1b* pro-inflammatory cytokines were seen between stressed-standard and -enriched environment animals for the F1 and F2 generations. The transgenerational effects of enriched environment were also observed with increases in *Il1b* expression in F2 controls. Comparable findings

were also noted for the *Hsd11b2*, *Nr3c2*, and *Sod1* expression of offspring raised in enriched environment, where the expression patterns of controls were similar to the stressed offspring. These findings are supported by previous studies where enrichment interventions promoted stress-like effects [294,455-457], anxiety, and reduced social interactions in a sex-specific manner [294].

The sudden change in environment may have been perceived as a threat to the rats. They were involuntarily and unexpectedly introduced to a novel cage to which they were not acclimated. This hypothesis is supported by previous findings where birds initially exposed to a short-term enriched environment perceived the introduction of new objects as a stressor [457]. Also, male rats subjected to 40 days of enriched environment showed signs of chronic stress depicted as altered neuroendocrine regulation by enhanced adrenocortical function and larger adrenals [458]. Although we found no changes in CORT levels in the enriched offspring, enriched environment housing could have been perceived as over-enrichment even in a long-term intervention protocol as used in this study. The animals were introduced to various novel objects, food, and wheels, allowing for more extensive physical exercise. The recurrent change in the environment may have prevented the animals from habituating. However, the stress response triggered by enriched environment indicates a type of positive stress, where the modifications in uterine markers of stress are similar to the negative PNMS. Still, it might not produce the adverse health consequences of chronic stress [458].

How the enrichment paradigms produce diverged results among studies is not fully understood. The inconsistent enriched environment results are attributed to each variable in analysis and are context-dependent, which may produce resilience to stress or stress-like effects [294]. Furthermore, different genotypes, species, and enrichment paradigms (mild or intense) have produced a non-genetic individuality in vulnerability or resilience to PNMS [292,299]. The inter-individual traits observed may also originate from complex and hard-to-measure microenvironmental effects [299,459]. Furthermore, physical exercise has been associated with both heightened stress response and a vital component of the beneficial effects of an enriched environment [287].

The evolutionary rationale for the transgenerational inheritance of environmental effects is to prepare the offspring for anticipated adversity later in life. The transmission of environmental cues possibly occurs through epigenetic inheritance of stress via the gametes, where the parental phenotypic

traits are passed down to the progeny [188]. When the adaptations are maladaptive, the inherited traits may lead to pathologies and detrimental modifications that persist through generations yet may be reversible by a phenotypic switch [188,460].

A shortcoming of the present study is that uterine tissues were collected at LD21 instead of right after labour due to the transgenerational design of this stress model. Therefore, the uterine expression of inflammatory and stress markers may be regulated differently during labour. The findings of this study, thus, may reflect the uterine transcriptome programmed by maternal and ancestral CVS, which may affect future health and pregnancy outcomes. Also, analysis of pro-inflammatory cytokine concentrations and 11β-HSD activities in the uterus and placenta would further elucidate the interactions between these mediators and their contributions to intrauterine inflammation and labour initiation in pregnancies subjected to chronic stressors.

Our results indicate that stressing F0 pregnant rats with CVS conveys transgenerational effects to the offspring, including long-lasting modifications in the inflammatory and endocrine status of their adult uteri. These alterations may produce adaptive or disruptive outcomes in the offspring. This study also demonstrates that enrichment had adverse rather than beneficial effects on uterine biomarkers of stress over generations of rats. Enrichment can be perceived as stressful depending on the context and variable in the analysis. However, further research should clarify the efficacy of enriched environment housing in ameliorating the effects of CVS on behaviour, pro-inflammatory, and neuronal factors in the brain. Finding an effective therapy to reduce the effects of prenatal stress and identifying predictive biomarkers of stress that can be translated to humans may improve maternal and child health over multiple generations.

CHAPTER 6

PATERNAL TRANSGENERATIONAL PRENATAL STRESS (PTPS) STUDY

The effects of prenatal stress are passed down from mothers to sons and then to grandchildren through the paternal lineage affecting inflammatory stress markers in the uteri and testes of rats

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Currently in preparation for submission
October 2022

Acknowledgments: We also thank Dr. Anna Noga for her assistance in preparing this manuscript in accordance with Good Publication Practices.

Research funded by the Canadian Institutes of Health Research (CIHR)

6.1 Introduction

Stress occurs when a perceived external or internal threat disrupts the body's homeostasis and elicits a stress response [6]. The stress response involves an interplay between the immune, neuro, and endocrine systems [461]. When the central stress systems identify a stressor, the sympathetic-adreno-medullar and hypothalamic-pituitary-adrenal (HPA) axes release catecholamines and glucocorticoids (GCs), respectively, to establish the stress response [17]. Depending on its magnitude, stress exposure during pregnancy may impact the neuroendocrine and immune systems that maintain a healthy pregnancy and affect the maternal HPA axis, maternal-fetal GC levels, and the inflammatory activity that regulates pregnancy and birth [200].

Prenatal maternal stress (PNMS) is known to affect both the mother and the developing fetus by altering the homeostatic milieu of pregnancy [200]. Higher incidences of adverse pregnancy outcomes, including preterm birth [462,463], low birth weight [464], and preeclampsia [208] have been observed with PNMS. Among the most common complications of maternal stress, preterm birth is a multi-factorial syndrome characterized by the early activation of the uterus from a quiescent to a contractile state before 37 weeks of gestation [207,369].The rationale for linking PNMS to preterm birth is that maternal stress is associated with increased levels of pro-inflammatory mediators and alterations in the maternal-fetal neuroendocrine axis [149].

Several studies demonstrate that stress exposure during critical periods of fetal development has life-long consequences for the health trajectory of infants and contributes to the onset of early pathologies [195,406]. As described by the Developmental Origins of Health and Disease (DOHaD) hypothesis, early life (fetal and early childhood) environmental experiences may induce *in-utero* prenatal programming of the offspring, permanently changing fetal structure and function during a lifetime [195,465]. The consequences of PNMS span across generations, a phenomenon known as intergenerational (IG) and transgenerational (TG) transmission of traits from parents to offspring [466]. The transmission of inflammatory and stress markers across generations may cause enduring changes in the reproductive tissues of the offspring [331,412] and increase their susceptibility to pregnancy complications (e.g., miscarriage, infertility, preterm birth [467]) and alter reproductive health.

Most studies that investigate the impact of an adverse early environment have focused on maternal factors, but fathers also play critical roles in fetal development. Accumulating evidence demonstrates the damaging effects of prenatal stress on the male offspring's metabolic patterns [468,469], behaviour [470,471], and testicular structure and function [267]. PNMS exposure in the male offspring alters testicular morphology, causes redox dysregulation, affects testicular gene expression of inflammatory mediators, and disrupts the hypothalamic-pituitary-gonadal (HPG) axis, spermatogenesis, and sperm parameters [472,473].

Studies have also examined how these altered phenotypes of *in-utero* stressed males are transmitted along the germline, altering neurodevelopment [474]. The underlying mechanisms of paternal transmission of traits to the offspring may involve the epigenetic transmission of information through the sperm, indirect seminal fluid influence on the female reproductive tract, direct seminal fluid effects on sperm after ejaculation, and paternal postnatal behaviour in biparental species [216,280].

The effects of maternal stress on reproductive tissues are complex. They involve an interrelated relationship between pro-inflammatory, neuroendocrine mediators, reactive oxygen species (ROS) and antioxidants, and local GC metabolism regulators such as the 11β-Hydroxysteroid dehydrogenases (11β-HSDs) [46,158,424]. Excess or low production of such mediators and poor coordination of the GC-immune system contributes to early-life fetal programming of diseases by interfering with tissue structure, function, and physiological mechanisms in the offspring [427].

We aimed to investigate if *in-utero* stressed males transmit inflammatory and stress markers of prenatal programming to future offspring. Preterm birth was assessed in the female offspring of the first generation (F1) prenatally stressed to investigate whether preterm birth risk is passed through the paternal lineage. This is a transgenerational study where F0 pregnant dams were stressed by chronic variable stress (CVS) during gestation, whereas the F1-F3 offspring were undisturbed. We hypothesized that gestational CVS in the parental generation induces fetal programming of inflammatory and stress markers in the F1 offspring's testes. The paternal F1 altered phenotypes are transgenerationally passed on to the second and third generations' reproductive tissues via the paternal lineage, affecting the progeny's uteri and testicular inflammatory and stress profiles. These stress-induced alterations in uterine

inflammatory and endocrine markers increase the risk of preterm birth in the female offspring of F1-stressed fathers.

6.2 Specific methods

6.2.1 Animals

Animals were bred and raised at the University of Lethbridge - Canadian Centre for Behavioural Neuroscience vivarium individually (males, from postnatal day (P)90 onwards) or in pairs (males, from weaning to P90; females, weaning to gestational day (GD)20). A total of 1,128 Long-Evans Hooded rats were used to produce a paternal lineage. F0 females were bred with control males and underwent CVS from GD12-18 (Figure 6.1). Female F1 controls (n = 16-24) were then exposed to F1-stressed or control males (n = 16-20) on P95 for 20 minutes per day until mating was observed, where they remained together for 2 hours. This protocol produced the F2 generation, which was again bred with female controls to yield the F3 generation (Figure 6.1). Pregnancy was confirmed by daily female weight gain.

Upon confirmed successful pregnancy, breeder males were euthanized (Figure 6.2). Pregnancy duration and delivery times were assessed by continuous video-recording with infrared light cameras (Panasonic WV-BP330, Panasonic, Minato-ku, Tokyo, Japan), and gestational duration was measured as the period between copulation and the delivery of the first pup. Maternal data are referred to as gestational days (GD) or lactational days (LD), while paternal- and offspring-related data are described as postnatal days (P). Maternal weight was measured at baseline (a couple of weeks before breeding) and GD18. Offspring weights were measured one day after birth (P1), and litter size and sex count were recorded (Figure 6.2). Offspring stayed with their mothers until weaning or LD21. All experiments were conducted according to the Canadian Council for Animal Care and were approved by the University of Lethbridge Animal Welfare Committee, Protocols 1705 - Rat Breeding Colony and 1715 - Adverse Pregnancy Outcome.

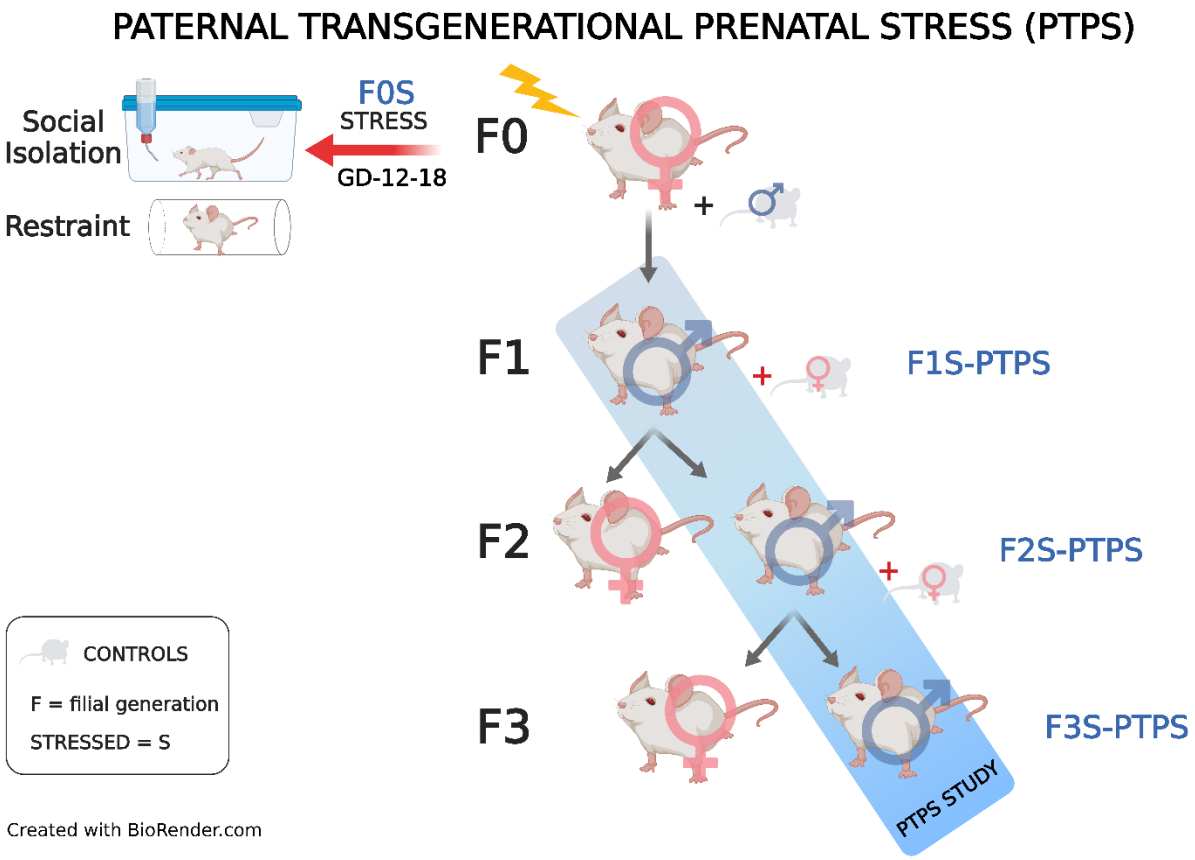


Figure 6.1: Schematic demonstrating the paternal transgenerational prenatal stress (PTPS) model.

F0 pregnant dams were prenatally stressed by restraint and social isolation from GD-12-18 to create both MTPS and PTPS studies. Their F1 sons were used to produce the paternal lineage of rats exposed to ancestral stress, also named the PTPS study. The F2-stressed male rats were bred to yield the F3 generation to investigate the effects of stress on the male line. F = filial generation, S = stress, C = controls, PTPS = animals derived from the PTPS project. Created with Biorender.com (accessed on 22 August 2022).

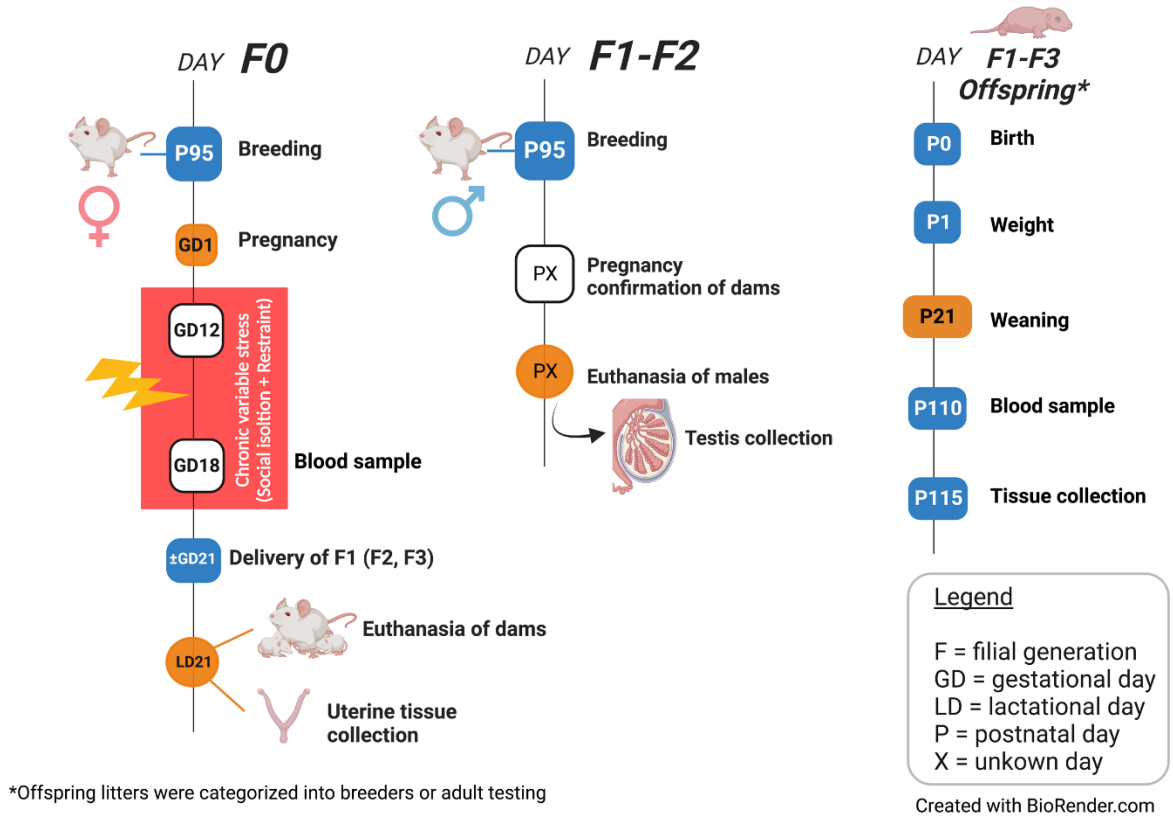


Figure 6.2: Parental F0 generation, F1 and F2 males, and F1-F3 offspring timelines and tissue collection.

Our PTPS stress model using chronic variable stress (CVS) was produced by exposing F0 pregnant dams to restraint and social isolation stressors from GD12 to GD18. Dams were sacrificed at the weaning of their offspring (P21), and uterine tissues were collected. The F1 and F2 male offspring were then mated to control females. Male euthanasia and testis collection occurred when pregnancy was confirmed in the partner (usually 3-4 days after breeding). Offspring birth weights were recorded on P1. Blood samples were collected from the mothers on GD18 and from the offspring on P110. Created with BioRender.com (accessed on 08 September 2022).

6.2.2 Rearing environments

Animals were exposed to a circadian cycle (12:12h light/dark cycle) with lights on at 7:30 am, the room temperature was set at 20°C and relative humidity at 30%. Rats were raised and bred in standard housing consisting of regular shoe box cages of 8 in. height x 8 in. width x 16 in. depth. *Ad libitum* access to water and regular food was provided throughout the experiments (pre-mating, breeding, pregnancy, and postnatal).

6.2.3 Experimental design and prenatal stress procedures

Female F0 rats were subjected to prenatal chronic variable stress (CVS) as described in the methods section in chapter 5. In summary, prenatal CVS consisted of restraint stress and 17-hour overnight social isolation stress (SIS) implemented at various durations and times of the day from GD12 to 18 (Table 6.1). The restraint protocol in a transparent plexiglass container varied from 15-60 minute procedures at different times of the day, while 17 hours of overnight SIS occurred on GD14 and GD17. The effects of transgenerational prenatal stress were examined in the F1 male generation born to F0-stressed mothers (Figure 6.1).

To produce a transgenerational paternal lineage, F1 males that experienced stress *in utero* (F1S-PTPS) were bred with control females to create the F2 generation. The F2 males exposed to ancestral CVS (F2S-PTPS) were again bred with control females to yield the F3 generation (F3S-PTPS). Each generation's treatment is depicted by the letters after the filial generation, including stressed rats F0S, F1S, F2S, and F3S and control rats F0C, F1C, where C depicts non-stressed controls and S stressed animals. The F1, F2, and F3 stressed offspring derived from the PTPS study are named F1-PTPS, F2-PTPS and F3-PTPS. Red circles depict gene expression of interest genes in uterine tissues, whereas blue circles describe gene expression in the testes.

Table 6.1: Description of stress procedures implemented through GD12-18 using restraint and social isolation.

Gestational day	**G-12**	**G-13**	**G-14**	**G-15**	**G-16**	**G-17**	**G-18**
Treatment (am)	Restraint **60 minutes**	N/A	Restraint **45 minutes**	N/A	Restraint **15 minutes**	N/A	Restraint **45 minutes**
Treatment (pm)	N/A	Restraint **30 minutes**	Isolation **Overnight**	Restraint **60 minutes**	N/A	Restraint **30 minutes** Isolation **Overnight**	N/A

6.3 Results

Gestational lengths remained unchanged in the daughters of stressed F0 mothers and ancestrally stressed F1 males

The schematic illustration of females selected for gestational length analysis, including the F0 stressed dams, F1 dams prenatally stressed *in utero*, and F2 females from the paternal line born to stressed F1 males is presented in Figure 6.3A. The gestational lengths of F1 females (514.4 ± 25.80 h) stressed *in utero* and F2 females (518.5 ± 24.28 h) born to males exposed to ancestral stress did not change compared to controls (527.1 ± 5.163 h, $p = 0.786$; Figure 6.3B). The adjusted coefficient of variation was used to assess the dispersion of the gestational lengths dataset relative to the group mean. Despite there being no changes in pregnancy duration among stressed females, the coefficient of variation for gestational lengths significantly varied in the female offspring of stressed F0 dams or F1 sires. A higher coefficient of variation was observed in the F1S generation and F2S-PTPS females ($p = 0.0001$) compared to F0 controls (F0C vs. F1S $p < 0.002$) and F0 stressed rats (F0S vs. F1S $p < 0.002$, and F0S vs. F2S-PTPS $p < 0.002$; Figure 6.3C).

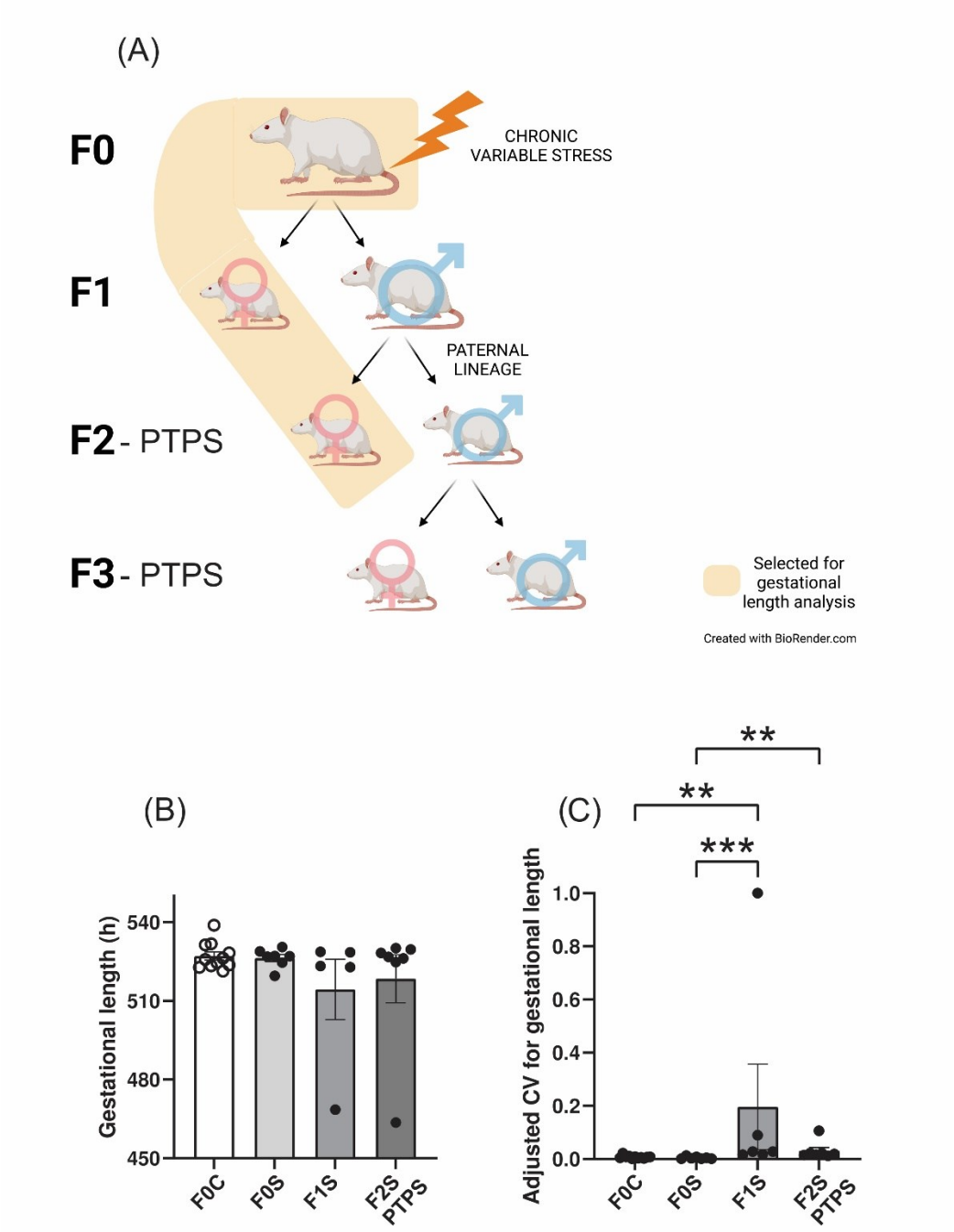


Figure 6.3: Gestational lengths of females exposed to prenatal and ancestral PNMS were unchanged.

(A) A schematic figure of females selected for pregnancy duration evaluation is presented. (B) Gestational lengths of stressed and control females. (C) The adjusted coefficient of variation of gestational lengths among control and stressed females. Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 5-11.

Plasma levels of corticosterone (CORT) were unchanged between females and males across generations

Analysis revealed no changes in CORT plasma levels in female offspring on P110 between control and stress groups (p = 0.186; Figure 6.4A). Similar findings were observed in males, where plasma CORT levels were unchanged between control and stressed animals on P110 (p = 0.793; Figure 6.4B).

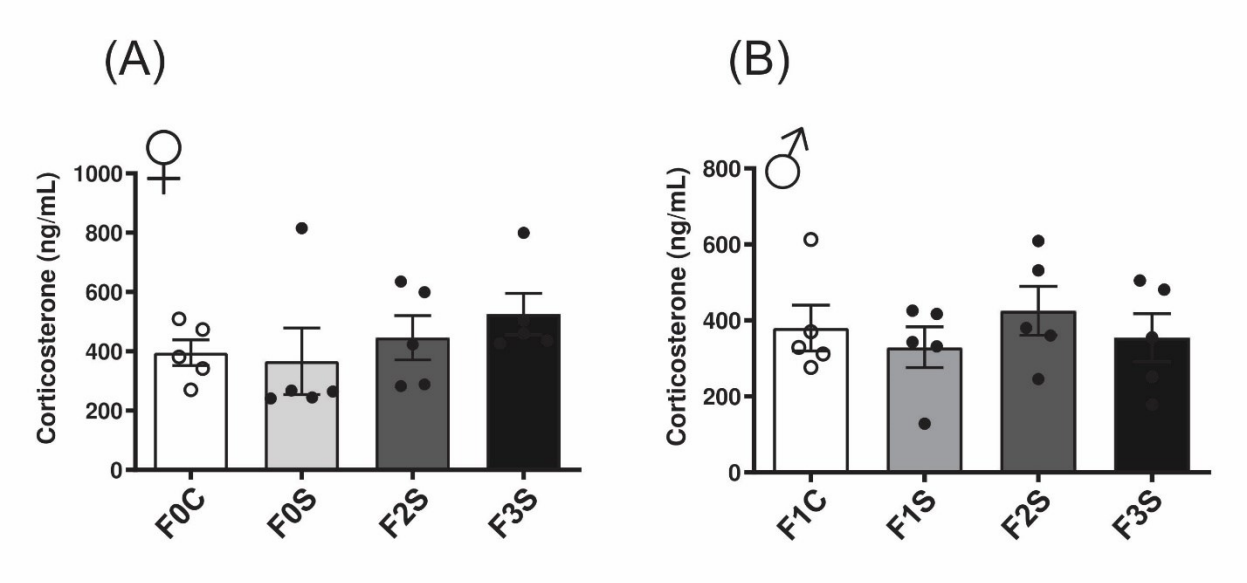


Figure 6.4: Plasma levels of CORT did not change between controls and stressed female and male offspring on P110.

Comparison of CORT levels between controls and stressed (A) female and (B) male animals across generations. Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 5.

Maternal weight increased in the F1 and F2 generations exposed to ancestral PNMS

Maternal weight analysis revealed a significantly higher baseline weight in F1S (293.1 ± 13.4 g) and F2S-PTPS (301.9 ± 24.2 g) animals exposed to ancestral stress (p < 0.001) compared to controls (255.2 ± 19.6 g; F0C vs. F1S p = 0.009, F0C vs. F2S-PTPS p < 0.001) and F0 stressed (267.1 ± 20.7 g; F0S vs. F2S-PTPS p = 0.01; Figure 6.5A). Maternal body weights remained elevated during gestation (GD18) for the F2S-PTPS stressed females (386.5 ± 33.1 g) compared to controls (331.5. ± 25.9 g; F0C vs. F2S-PTPS p < 0.001) and the F0 stressed animals (338.7 ± 23.6 g; F0S vs F2S-PTPS p = 0.009; Figure 6.5B).

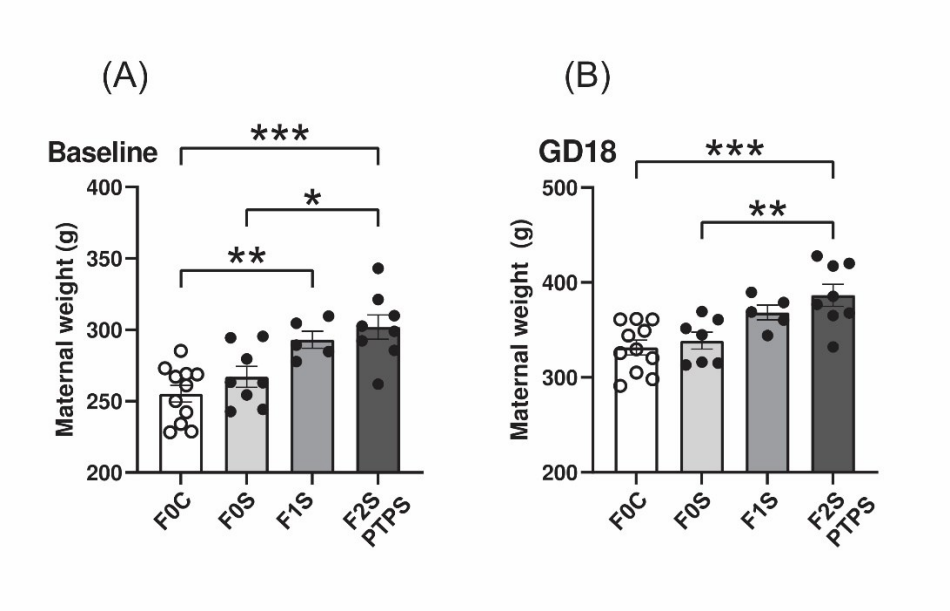


Figure 6.5: Maternal weights significantly increased in offspring subjected to ancestral PNMS.

(A) Baseline maternal weight measurements of control and stressed females. (B) Comparisons of GD18 gestational weights between controls and stressed groups. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data were analyzed with the one-way ANOVA. Mean ± SEM, N = 5-11.

The birth weights of F2-stressed males were significantly increased

Female neonatal birth weights did not change in the F1-F3 stressed offspring (F1S 6.43 ± 0.5 g; F2S 6.76 ± 0.58 g; F3S 6.39 ± 0.66 g) exposed to ancestral stress compared to controls (6.5 ± 0.43 g, p = 0.118; Figure 6.6A). Birth weights of F2S stressed offspring significantly increased in male neonates (7.33 ± 0.55 g; p = 0.002; Figure 6.6B) compared to controls (6.89 ± 0.5 g; F1C vs. F2S p < 0.002) and F1S animals (6.84 ± 0.55 g; F1S vs. F2S p < 0.002), while offspring weights returned to baseline values in the F3S group. Litter sizes and fetal sex ratios remained unchanged across the F1-F3 generations (F (3, 24) = 0.164 p = 0.919 and p = 0.874, respectively; Figures 6.6C and D).

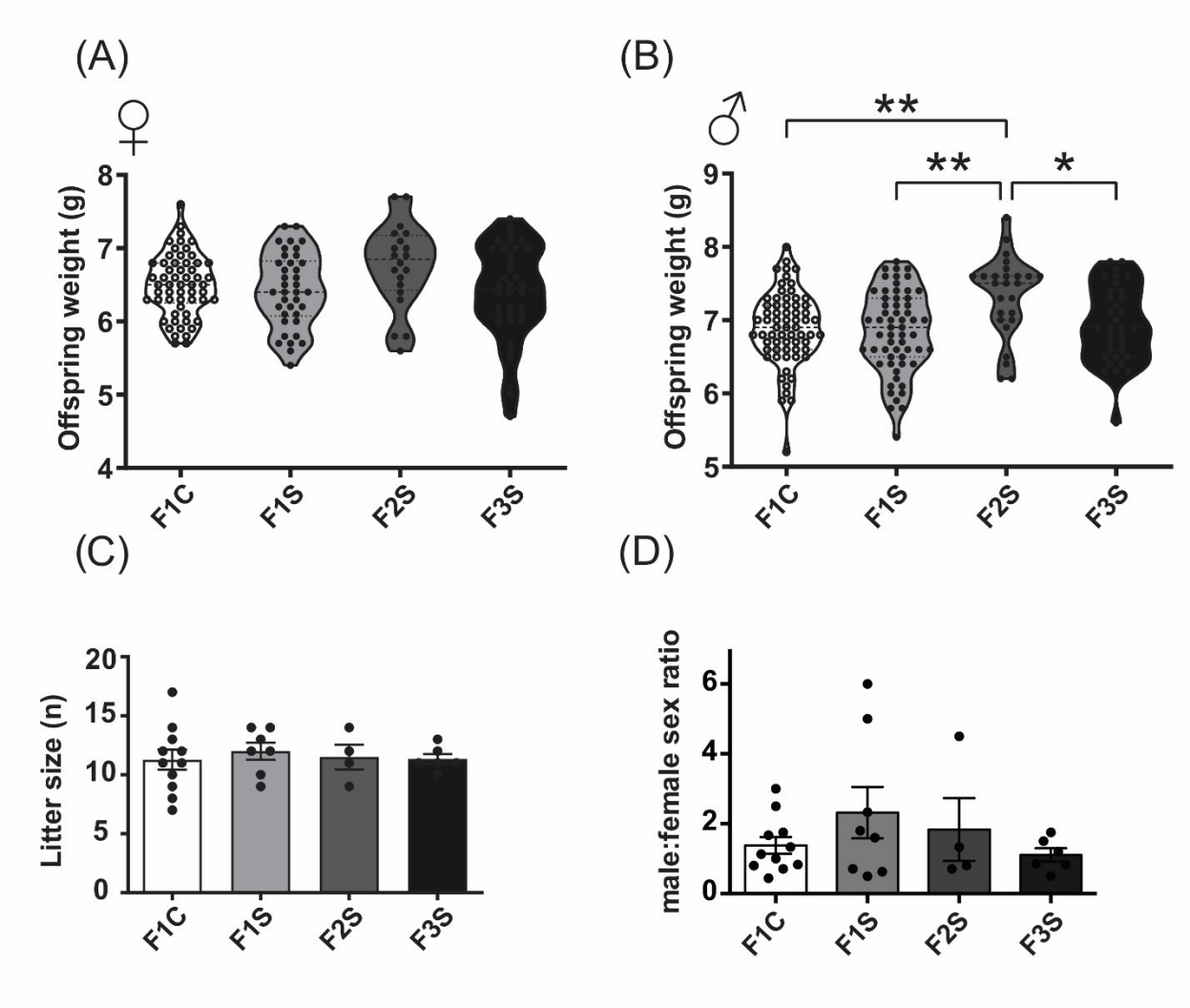


Figure 6.6: Neonatal male birth weights increased in the F2 generation exposed to ancestral PNMS, while weights were not changed in control and stressed females. Litter sizes and fetal sex ratios were unchanged across generations.

Birth weights of (A) female and (B) male neonates, (C) litter sizes, and (D) fetal sex ratios across generations. Asterisks indicate significance: < 0.05 (*); < 0.002 (**). Data were analyzed with the Kruskal-Wallis test (A,B, D) or one-way ANOVA (C). Mean ± SEM, N = 20-66 (A,B), 4-11 (C), and 4-12 (D).

Female uterine gene expression of inflammatory and stress mediators

Pro-inflammatory mediators were significantly altered in the F2S and F3S female offspring from the paternal lineage

Pro-inflammatory cytokines: *Il1a, Il1b, Il6*

The mRNA expression of the interleukin *Il1a* in the uterus was significantly decreased (p = 0.003) in the F2 and F3 stressed animals compared to controls (F2S p = 0.003 and F3S p = 0.010; Figure 6.7A). In contrast, the uterine expression of *Il1b* was significantly increased in the F3 generation (p = 0.007) when compared to controls (p = 0.034; Figure 6.7B). Uterine expression of *Il6* showed a similar trend, with a significant increase (p < 0.001) in the F2 and F3 stressed animals compared to F0S (p = 0.001 and p < 0.001, respectively; Figure 6.7C).

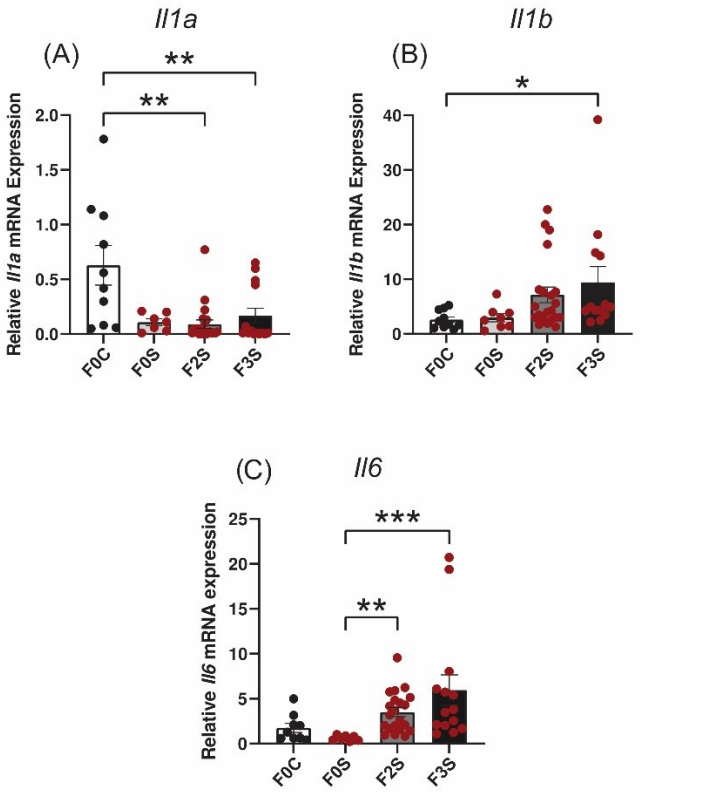


Figure 6.7: The uterine expression of *Il1b* and *Il6* cytokines was upregulated, while *Il1a* was decreased in the offspring of males exposed to ancestral PNMS.

Uterine expression levels of (A) *Il1a* (B) *Il1b* (C) *Il6* in stressed and control animals over generations. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-21. Red circles depict gene expression of interest genes in the uterine tissues.

Members of the interleukin (IL)-1 cytokine family

Uterine expression of *Il1ra*, the endogenous IL-1 receptor (IL-1R) antagonist, was significantly elevated ($p < 0.001$) in the F2 and F3 stressed offspring compared to controls and F0S animals ($p = 0.026$ and $p < 0.001$, respectively; Figure 6.8A). Similarly, the expression of *Il1r1* also increased ($p < 0.001$) in the F2S and F3S uteri when compared to controls (F2S $p < 0.001$ and F3S $p = 0.010$; Figure 6.8B). The analysis for the *Il1rap* molecule required for IL-1/IL-1R1 signalling revealed no changes in *Il1rap* mRNA levels ($p = 0.154$) or protein abundance ($F(3, 16) = 0.7834$, $p = 0.5205$) in control or stress groups across generations (Figure 6.8C,D).

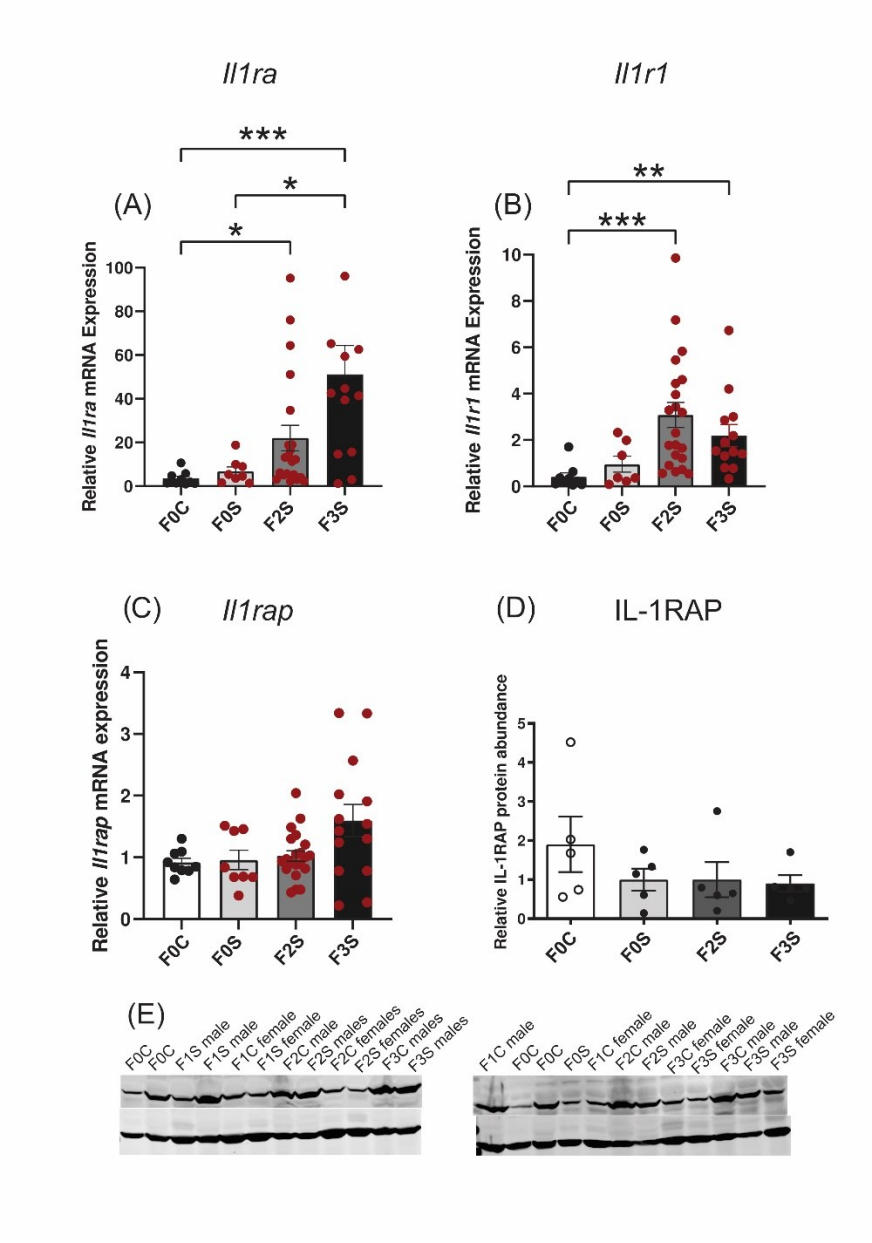


Figure 6.8: The uterine expression of *Il1r1* and its antagonist *Il1ra* were increased in the F2S and F3S animals, while the expression and protein abundance of *Il1rap* remained unchanged.

Expression levels of (A) *Il1ra,* (B) *Il1r1,* (C) *Il1rap* in the uterus of rats exposed to ancestral CVS. (D) IL-1RAP protein abundance in the uterus of stressed dams and their offspring, quantified using densitometry. (E) Representative western blots for IL-1RAP protein abundance. Blots were quantitated using Odyssey software. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data were analyzed with the Kruskal-Wallis test (A-C) or one-way ANOVA (D). Mean ± SEM, N = 7-21 (A-C) or N = 5 (D).

The uterine expression of stress mediators was predominantly upregulated in the F2 and F3 generations of stressed animals

Crh and its receptors (*Crhr1* and *Crhr2*)

The expression of both corticotropin-releasing hormone *Crh* ($p = 0.001$) and *Crhr1* ($p = 0.023$) was upregulated in uterine tissues of the F2 and F3 generations when compared to F0 stressed dams (*Crh*: F2S $p = 0.002$ and F3S $p = 0.003$; *Crhr1*: F2S $p = 0.023$ and F3S $p = 0.033$; Figures 6.9A,B). Similar observations were made for the uterine expression of *Crhr2* ($p < 0.001$), where *Crhr2* increased in F2 and F3 stressed offspring compared to F0C (F2S $p = 0.002$ and F3S $p = 0.017$; Figure 6.9C).

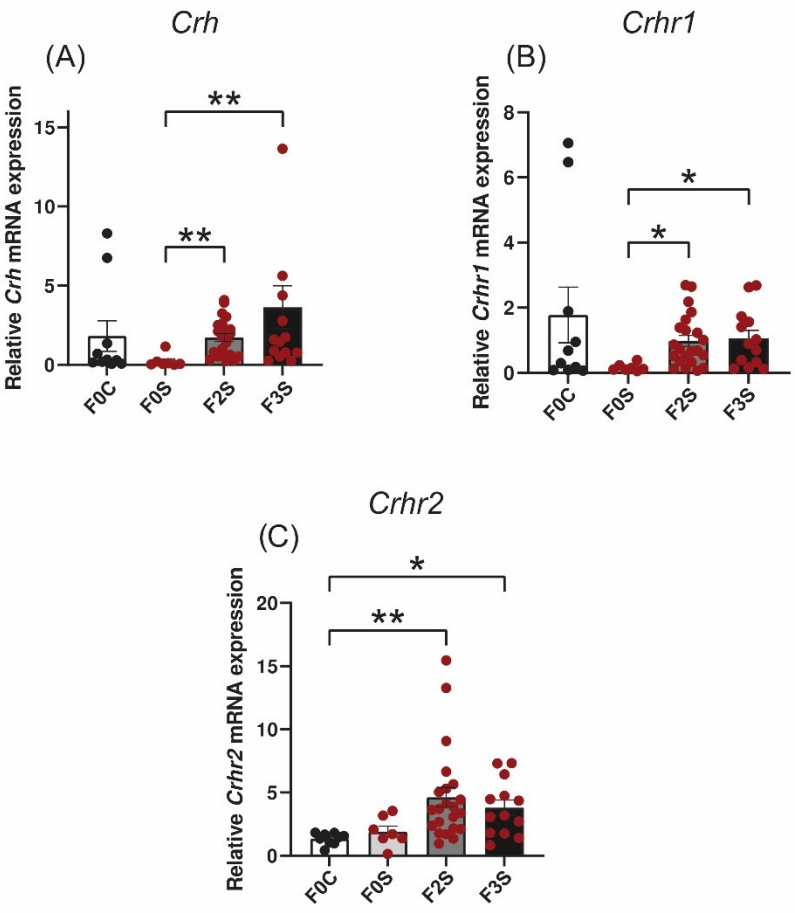


Figure 6.9: The mRNA expression of *Crh* and the receptors *Crhr1* and *Crhr2* was upregulated in the ancestrally stressed F2 and F3 offspring.

Expression levels of (A) *Crh,* (B) *Crhr1,* and (C) *Crhr2* between stress and controls. Asterisks indicate significance: < 0.05 (*); < 0.002 (**). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-22.

Glucocorticoid (*Nr3c1,* GR) and mineralocorticoid (*Nr3c2,* MR) receptors

We observed significantly upregulated expression of the *Nr3c1* (GR) in the uteri of F2 stressed dams ($p < 0.001$; Figure 6.10A) and of *Nr3c2* (MR) in the F3 generation of ancestrally stressed animals compared to F0 controls ($p = 0.039$; Figure 6.10B).

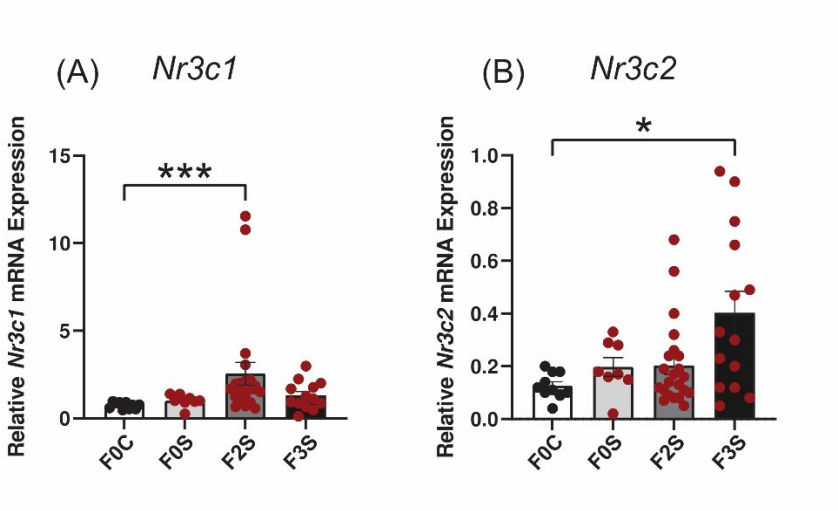


Figure 6.10: The expression of GR (*Nr3c1*) and MR (*Nr3c2*) increased in the F2S and F3S uteri, respectively.

Uterine expression levels of (A) *Nr3c1* and (B) *Nr3c2* receptors in control and stressed dams across generations. Asterisks indicate significance: < 0.05 (*); < 0.001 (***). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 8-22.

11β-Hydroxysteroid Dehydrogenase Types 1 and 2 (*Hsd11b1* and *Hsd11b2*)

Uterine gene expression analysis of *Hsd11b1* revealed significantly increased expression ($p = 0.040$) in the F2 stressed offspring compared to F0 controls ($p = 0.025$; Figure 6.11A), while no changes were observed for *Hsd11b2* expression ($p = 0.104$; Figure 6.11B).

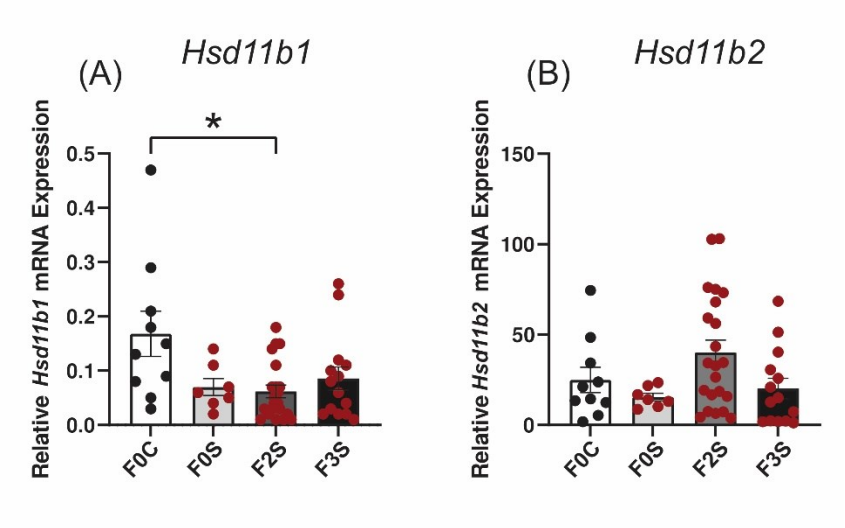


Figure 6.11: Uterine expression of the *Hsd11b1* gene increased in the F2-stressed animals, but *Hsd11b2* expression remained unchanged.

(A) *Hsd11b1* and (B) *Hsd11b2* gene expression in the uteri of control and stressed rats. Asterisks indicate significance: < 0.05 (*). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-22.

Antioxidant enzymes: superoxide dismutase types 1 and 2 (*Sod1* and *Sod2*)

The expression of the antioxidant enzyme *Sod1* was significantly increased ($p < 0.001$) in the F2 and F3 generations of stressed animals compared to F0 controls (F2S $p < 0.001$ and F3S $p < 0.001$) and F0 stressed animals (F2S $p = 0.002$ and F3S $p < 0.001$; Figure 6.12A). Similar findings were observed for *Sod2* expression, where its expression was significantly elevated ($p = 0.057$) in the F3 stressed offspring compared to F0S rats ($p = 0.046$; Figure 6.12B).

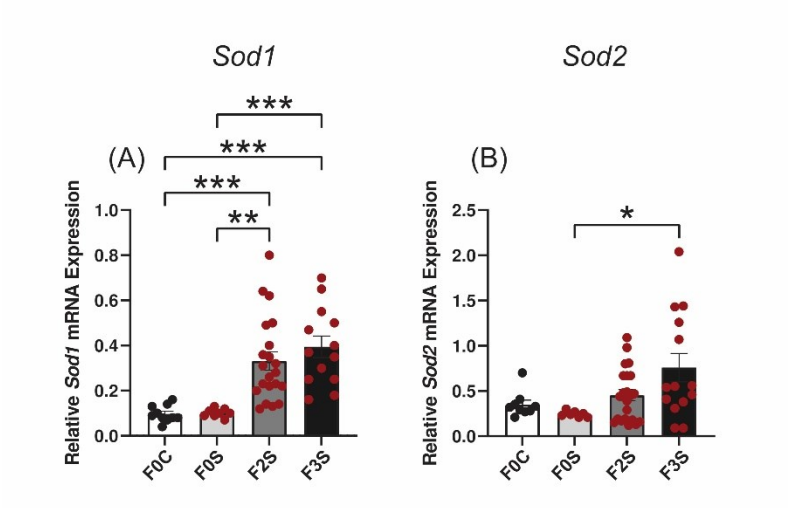


Figure 6.12: The uterine expression of the *Sod1* and *Sod2* was significantly increased in the F2/F3 and F3-PTPS generations of rats, respectively.

Expression of (A) *Sod1* and (B) *Sod2* in the controls and stressed uteri over the F0-F3 generations. Asterisks indicate significance: < 0.05 (*); < 0.002 (**); < 0.001 (***). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-22.

Male testicular gene expression of inflammatory and stress mediators

The testicular expression of *Il1a, Il1ra, Il1r1, Il6* inflammatory markers *and Crh, Nr3c1, Nr3c2, Sod1*, and *Sod2* in stressed animals was similar to controls (Appendix Figure C1A-I).

Testicular inflammatory mediators

The expression of *Il1b* was significantly decreased (p = 0.032) in the testes of the F3-stressed offspring compared to F1-stressed rats (p = 0.02; Figure 6.13A). However, the testicular expression of *Il1rap* increased significantly in the F2 and F3 generations of stressed animals (p = 0.031; Figure 6.13B), although the post-hoc test did not reveal significant differences between the groups. We did not observe any significant changes in testicular IL-1RAP protein abundance compared to F0 controls (F (3, 16) = 0.7834, p = 0.521; Figure 6.13D).

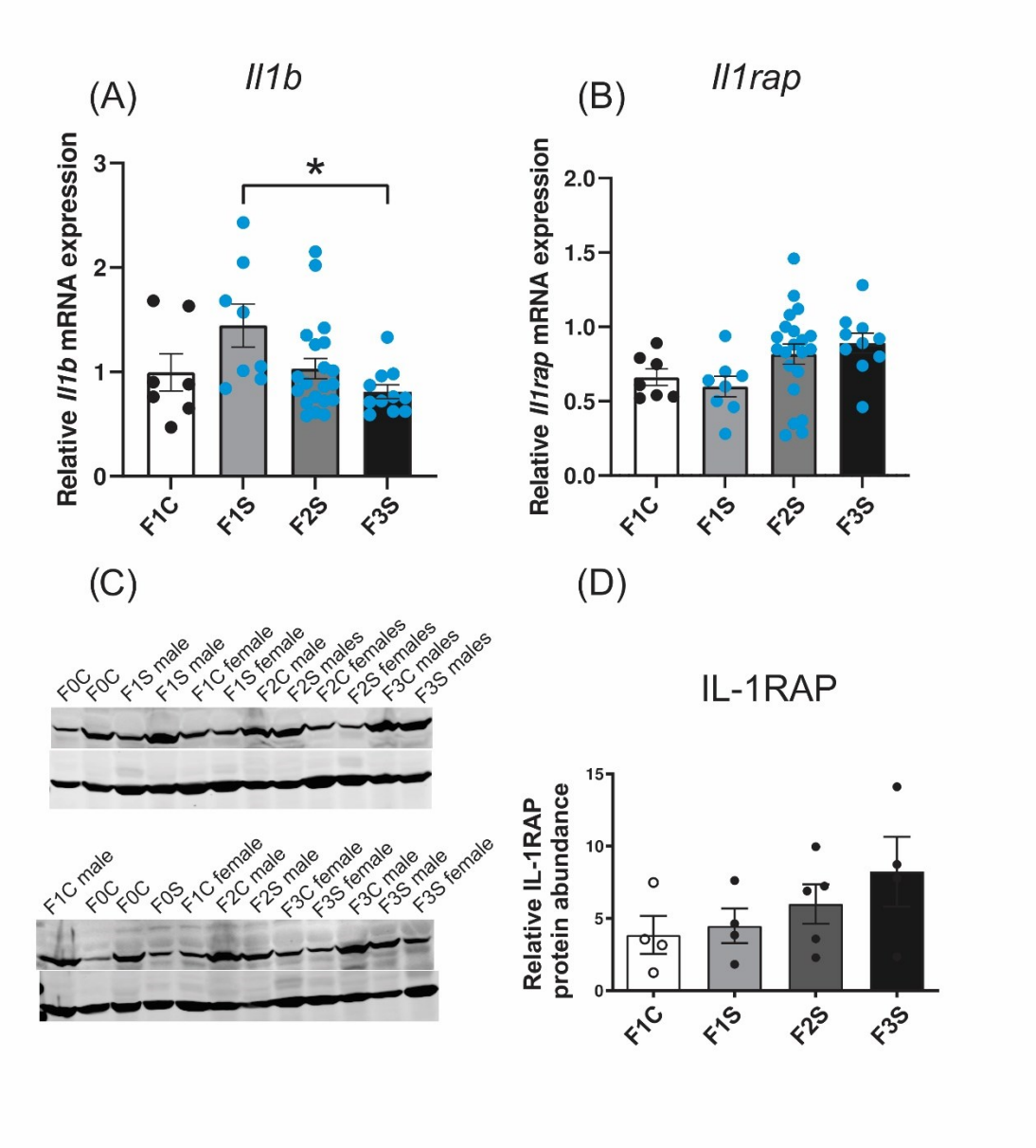


Figure 6.13: The expression of *Il1b* decreased in the testes of the F3S generation, while *Il1rap* increased in the F2 and F3 stressed offspring.

Testicular expression of (A) *Il1b* and (B) *Il1rap* in control and stressed males. (C) Representative western blots of IL-1RAP protein abundance. Blots were quantitated using Odyssey software. (D) Protein abundance of IL-1RAP in the testes of stressed animals and their offspring, quantified using densitometry. Asterisks indicate significance: < 0.05 (*). Data were analyzed with the Kruskal-Wallis test (A,B) or one-way ANOVA (C). Mean ± SEM, N = 7-21 (A,B) or N = 5 (C). Blue circles depict gene expression of interest genes in the testes.

Corticotropin-releasing hormone receptors (*Crhr1* and *Crhr2*)

The testicular expression of *Crhr1* decreased significantly (p = 0.007) in the F3S offspring compared to F2S animals (p = 0.009; Figure 6.14A). Conversely, the *Crhr2* expression was significantly downregulated (p = 0.019) in the testes of the F1S offspring compared to F1 controls (p = 0.038; Figure 6.14B).

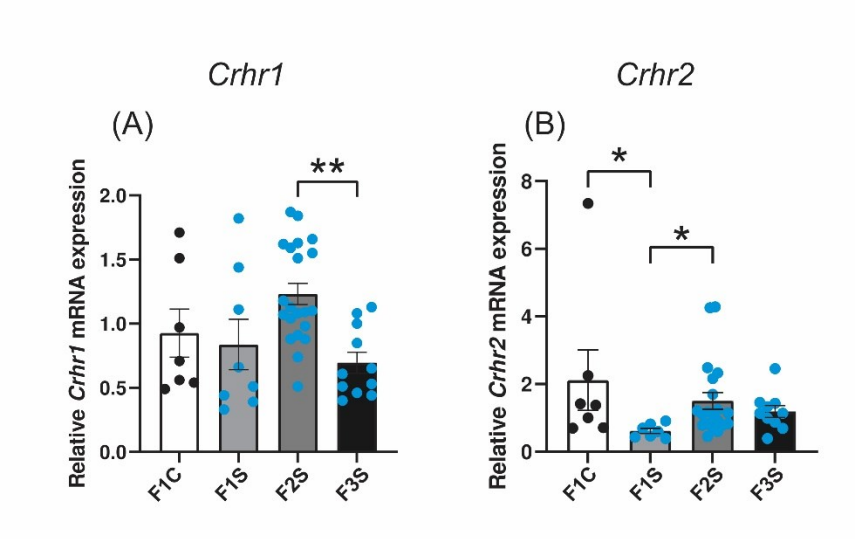


Figure 6.14: Testicular expression of *Crhr1* and *Crhr2* was decreased in the F3S and F1S offspring exposed to ancestral stress, respectively.

Gene expression of (A) *Crhr1* and (B) *Crhr2* in the testes of controls and stressed males over the F1-F3 generations. Asterisks indicate significance: < 0.05 (*); < 0.002 (**). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-21.

11β-Hydroxysteroid Dehydrogenase Types 1 and 2 (*Hsd11b1* and *Hsd11b2*)

The expression of *Hsd11b1* was reduced in the testes of F1S animals (p = 0.033; Figure 6.15A), despite no significant differences between groups revealed in the post-hoc test. The analysis of *Hsd11b2* expression in the testes across different groups revealed a significant downregulation (p < 0.001) in the F3S males compared to the F1 and F2 generations of stressed animals (F1S vs. F3S p = 0. 002 and F2S vs. F3S p < 0.001; Figure 6.15B).

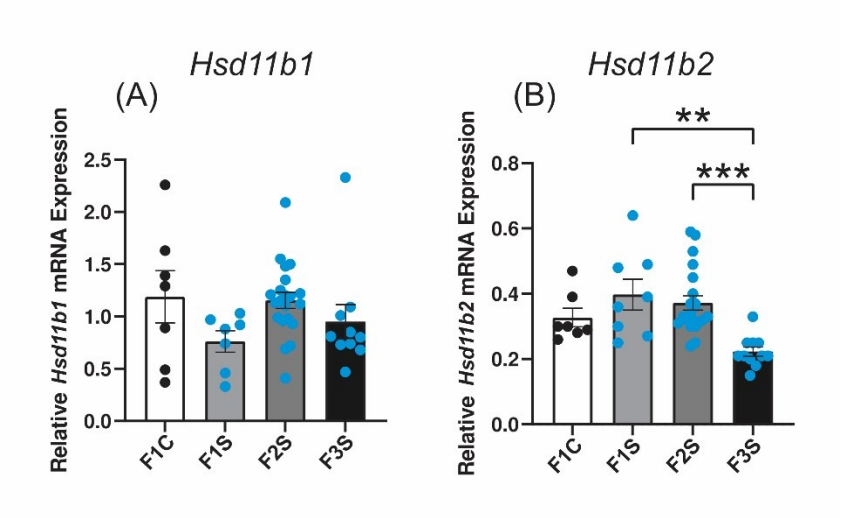


Figure 6.15: The testicular expression of *Hsd11b1* and *Hsd11b2* mRNA was downregulated in F1- and F3-stressed animals, respectively.

(A) *Hsd11b1* and (B) *Hsd11b2* gene expression in the testes of control and stressed animals over the F1-F3 generations of rats. Asterisks indicate significance: < 0.002 (**); < 0.001 (***). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-21.

6.4 Discussion

Our results provide evidence that paternal ancestral exposure to CVS (social isolation and restraint) can be transmitted to future generations. In this study, we saw an increase in the expression of many inflammatory and stress markers in the uteri of female offspring, and a decrease of these same markers in the testes of males. Stress vulnerability was transmitted to the offspring where male birth weights were significantly increased in the F2 generation. Stress also programmed F1 and F2 female offspring that had elevated body weights both at baseline and during gestation. Although the inflammatory and stress mediators involved with the onset of labour were altered, gestational lengths remained unchanged across generations of females exposed to stress either *in utero* or via the paternal lineage. Finally, our stress protocol did not modify plasma CORT levels of male and female offspring later in life at P110.

Our variable stress in the F0 dams was insufficient to induce shorter gestations or preterm birth in the prenatally stressed daughters (F1 generation) or the F2 offspring born to males exposed to ancestral stress (F2-PTPS). Similar results were observed in the F0-F2 daughters of the maternal lineage derived

from the variable stress protocol used in the present study (unpublished data - Chapter 5). Previous reports also showed that two-hit stress combining psychological and immunological stressors [331] and chronic mild stressors [435] did not reduce gestation duration in rats.

It is known that preterm birth is associated with the upregulation of pro-inflammatory cytokines and chemokines in the gestational tissues, along with the infiltration of leukocytes [85,475]. While inflammatory and stress mediators were altered in the uteri of rats exposed to ancestral stress, pregnancy duration in the dams and offspring was unaffected. The lack of change in gestational lengths may be explained by the multifactorial nature of preterm birth syndrome and its many causes, and an individual's susceptibility to stress. It is possible that the dams and future offspring maintained normal pregnancy lengths using compensatory mechanisms to increase their resiliency to stress.

CORT levels increase during episodes of perceived threats, making it the hallmark of stress responses [29]. Early-life stress exposure during plastic developmental windows is associated with long-term metabolic and HPA axis dysfunction [40]. Unexpectedly, cumulative stress in the parental generation did not change plasma CORT levels, nor did it alter the offspring's CORT levels in adulthood. This finding may indicate that the offspring are adapting to the ancestral stress, or that changes to the regulation of CORT were not transmitted through the paternal line.

Although most PNMS studies find a link between stress and low birth weight, obesity susceptibility and altered metabolic function are also epigenetically transmitted across generations [476,477]. Ancestral exposure to CVS programmed the F1 daughters and F2 females from the paternal lineage to higher baseline and gestational body weight. We also showed that males (F2S offspring) born to fathers directly exposed to stress *in utero* were heavier than controls. Similar results were observed in mice subjected to a different CVS protocol late in pregnancy, where offspring birth weights were increased [478]. Our results concur with other rat studies where prenatal exposure to pro-inflammatory cytokines [479] and variable stressors [480] also resulted in higher birth weights. In some studies, however, PNMS induced fetal intrauterine growth restriction and low birth weights at term [481,482]. These discrepancies may be attributed to the type, duration, and time of stress exposure, and several other risk factors, given that metabolic dysfunction is a multifactorial outcome [476]. Perhaps stress-induced upregulation of pro-inflammatory cytokines and stress hormones in the fetal compartment during

critical periods of development affect peripheral targets that control metabolic function and body composition [476].

Evidence suggests that endocrine, metabolic, stress, and inflammatory phenotypes can be transgenerationally transmitted to the offspring after exposure to perturbations during pregnancy [437,474]. Studying the paternal lineage transgenerational phenotypic inheritance in rodents has advantages over maternal studies, in which postnatal rearing behaviour and maternal intrauterine environmental confounding factors can be excluded [483]. The underlying mechanism for transmission involves reprogramming the male germ cells by epigenetic modifications, including changes to sperm microRNA (miRNA) content [229]. The male-line transmission of traits, thus, occurs via germ cell epigenetic inheritance.

Prenatal stress has been associated with intrauterine and fetal inflammation, as well as changes in HPA axis function [40,405,419]. Therefore, we sought to investigate whether males exposed to *in-utero* stress transmit altered inflammatory and stress profiles in the reproductive tissues of their progeny. Specifically, we hypothesized that prenatally stressed males transfer stress phenotypes to their daughters, increasing their risk of preterm birth. While no changes in pregnancy duration were observed across generations of rats, we found that inflammatory markers were primarily upregulated in the uterine tissues of the daughters and granddaughters of ancestrally stressed males (F1S offspring).

The gene expression of *Il1b*, *Il6,* IL-1 receptor 1 (*Il1r1*), and *Il1ra* (the receptor antagonist) was increased in the F2 and F3 uteri. These findings suggest that the uteri of female offspring from the paternal lineage were primed into a pro-inflammatory state. If these F2 and F3 dams experienced further challenges, pro-inflammatory pathways could be further activated in these already primed uteri, potentially leading to preterm birth. These results are in line with other PNMS studies. For example, increased *Il1b* uterine expression was also observed in the stressed F2 dams of the maternal lineage of CVS derived from the stress cohort of the present study (unpublished data – Chapter 5). The placental expression of *Il6* and *Il1b* was increased in male fetuses of mice subjected to CVS [434]. In humans, chronic stress [484,485] and stressful life events [486] during pregnancy were associated with increased cord blood cell production and circulating levels of pro-inflammatory mediators, including IL-6, IL-1, and TNF-α, and poor coordination of the GC-immune response [484].

In contrast, *Il1a* expression was significantly decreased in the F2 and F3 generations' uteri. We have previously demonstrated that CVS in the maternal lineage induced downregulation of *Il1a* mRNA expression in the uteri of F1- and F3-stressed animals (unpublished data – Chapter 5). The mechanisms causing these changes in gene expression in the reproductive tissues of stressed animals are not known. It is possible that there is an adaptive response to cumulative stress displayed as a drop in *Il1a* in the uterine tissues of rats exposed to different stressors. This adaptive response was observed in rats exposed to two-hit stress, where a combination of psychological and immunological stressors during pregnancy also decreased *Il1a* uterine expression in the F1S offspring [331].

Prenatal stress and maternal immune activation (MIA) [487] were shown to induce oxidative stress and reduce SOD and total antioxidant capacity activities in the maternal uterus [488] and the offspring's brain [489]. The cytokines and chemokines produced during stressful stimuli recruit phagocytic cells, which are significant producers of ROS [449]. In turn, oxidative stress triggers local tissue inflammation and promotes cytokine release creating a feed-forward cycle [490]. Here, *Sod1* expression was upregulated in the uterine tissues of the F2- and F3-stressed generations, while *Sod2* expression only increased in the F3S animals. These results suggest that the female offspring of stressed F1S males may have developed compensatory mechanisms by upregulating antioxidant enzymes to limit the effects of stress or increase the offspring's performance in a stressful environment they expect to encounter later in life.

PNMS impacts normal testicular function and sperm production in the offspring [472]. We explored the effects of maternal CVS during pregnancy on the inflammatory and stress profiles of the testes of male offspring. There was a significant reduction in *Il1b* expression in the F3S offspring's testes versus their F1S grandfathers who were directly exposed to CVS *in utero*. This reduced expression of *Il1b* may represent an adaptive mechanism of the F3S offspring to lessen the negative impacts of inflammation on Leydig cell functioning, androgen production, and spermatogenesis. Indeed, altered Leydig cell distribution and testosterone production in the testes of mice exposed to LPS-induced inflammation during gestation demonstrated testicular injury and impaired spermatogenesis [491]. In contrast, we observed an increase in *Il1rap*, a component required for IL-1R signal transduction, in the F2- and F3-stressed offspring.-These findings confirm that PNMS effects can be transmitted to future

generations in an intricate and generation-specific manner, altering inflammatory mediators in the testes of rats through the paternal lineage.

Reproductive tissues and immune cells produce CRH, a neuropeptide that helps to control pregnancy and parturition [181,492]. In this study, uterine expression of *Crh, Crhr1*, and *Crhr2* in the F2- and F3-stressed generations of females were increased. CRH levels rise with increasing gestational age, and its concentration significantly increases in the plasma of women with preterm birth [493]. This suggests the F2S and F3S offspring are at increased risk of early delivery. The underlying causes of such increases in *Crhr1* and *Crhr2* expression in F2- and F3-stressed animals' uteri are unknown, considering that the former is associated with myometrial relaxation, and the latter is linked with a pro-contractile stimulus [178]. Near labour, however, hypothalamic and placental CRH activities are controlled by several mediators, including cytokines, prostaglandins (PGs), cortisol, steroid hormones, etc. [492]. Previous studies demonstrate conflicting results regarding the uterine expression of stress markers. The expression levels of *Crh* and its receptors depend on the type and duration of the stress [331,412], among many other factors.

In males, CRH inhibits the secretion of hypothalamic gonadotropin-releasing hormone and human chorionic gonadotropin (which acts like a luteinizing hormone (LH) stimulus in Leydig cells) through CRHR1 activation. These decreases promote a fall in plasma concentrations of LH and testosterone levels in the adult rat Leydig cells, hormones that regulate spermatogenesis [494,495]. We found reduced testicular expression of *Crhr1* in F3-stressed animals and *Crhr2* in the F1S generation exposed to CVS *in utero*. These results suggest that stressed animals may have developed resilience to the adverse effects of stress on spermatogenesis by reducing CRH receptor expression across generations. However, CRH peptides and receptors are likely involved in several other physiological roles in the rat testis that are yet unknown and require further investigation.

GCs have significant roles in implantation, fetal development, and the onset of labour in female gestational tissues [447]. In males, GCs contribute to testicular maturation and Leydig cell steroidogenesis; however, they can also promote apoptosis of Leydig cells and suppress testosterone production when in excess [496]. The 11β-HSDs enzymes regulate GC levels in the reproductive tissues

by catalyzing the interconversion of active GCs and inert 11-keto forms and, thereby, controlling the equilibrium of local CORT levels.

In the maternal-fetal interface, 11β-HSDs protect the developing fetus from the deleterious effects of excess GC by controlling the passage of active GCs across the placenta [497]. The 11β-HSD1 isoform catalyzes bidirectional oxidoreductase activities and is guided by the developmental stage or physiological status of each cell type [51]. In the decidua and placenta, 11β-HSD1 predominantly functions as a reductase to elevate active GCs locally [51]. Both bidirectional 11β-HSD1 activities were reported in the testis, where their role is less clear [496]. The 11β-HSD2 isoform acts as an exclusive oxidase that catalyzes the conversion of active GCs to their inert forms in reproductive tissues.

The decreased uterine expression of *Hsd11b1* in the F2-stressed generation may indicate lower levels of active GCs in the uterus. This change was accompanied by an upregulation of *Nr3c1* (GR) expression. We also observed increased uterine expression of *Nr3c2*, a gene encoding the MR, in the F3-stressed generation. Expression levels of *Hsd11b1* and *Hsd11b2* in the testes of the F1S and F3S males, respectively, were also decreased. These results suggest that fetal programming of GC function and metabolism is established in the uteri and testes of future offspring whose F1 fathers were exposed to ancestral stress *in utero*.

A limitation of this study is that uterine tissues were collected at the weaning of the offspring (LD21), which prevents a more thorough investigation of the physiological changes that occurred during labour. Given the nature of the testicular tissue collection, sperm miRNA and DNA methylation (DNAm) analysis were unfortunately unattainable.

To our knowledge, our study on the transgenerational transmission of inflammatory and stress traits through the male lineage exposed to ancestral stress has not been studied before. We showed that transmission of such markers occurs through the paternal lineage and affects the offspring's reproductive tissues in a sex- and generation-dependent manner. The offspring of exposed fathers exhibited either adaptive responses to stress or maladaptive outcomes that could predispose the offspring to a variety of disease states. Such transmission is not well understood, and further studies are required to investigate the modes of transmission in order to develop preventative and corrective approaches to mitigate the effects of stress in future progeny.

CHAPTER 7

General discussion and future directions

7.1 Summary of key findings

Prenatal stress is an established risk factor for adverse pregnancy outcomes and impaired offspring health trajectories [200,498,499]. Stress exposure during pregnancy particularly impacts normal adaptations in the maternal systems that support pregnancy success, including the immune and neuroendocrine systems [200]. To our knowledge, the PNMS studies covered in this dissertation are unique and the first to assess the effects of different stressors on reproductive tissues' inflammatory and endocrine dysregulation and to investigate the causal link between prenatal stress and TG risk of preterm birth. I investigated the efficacy of enriched environment intervention in mitigating the effects of PNMS over the F1-F3 female progeny. Furthermore, the influence of *in-utero* prenatally stressed fathers on their progeny's pregnancy and health outcomes over two generations was examined.

Our previous study reported that a family history of cumulative or ancestral stress by restraint and forced swimming during gestation reduced gestational lengths progressively in the exposed F1 and F2 offspring [152]. Altered somatic tissue microRNA profiles were also observed in the ancestrally exposed offspring. They observed altered expression of miR-200 family members and mi-181a in the uterus and placenta, respectively, of F2 mothers. These miRNAs are involved with the parturition pathway and preterm birth in humans, suggesting that cumulative and ancestral stress transgenerationally programmed the offspring.

This study was the starting point for our investigation of the effects of prenatal stressors on adverse health outcomes in rats. We explored pregnancy and offspring outcomes of preconceptional and gestational exposure to psychosocial SIS in rats. I hypothesized that SIS in rats leads to a uterine imbalance of inflammatory- and stress-related mediators in the parental generation and offspring. This would increase the risk of preterm birth in the mothers and offspring. Understanding the consequences of social isolation is particularly important due to the current worldwide stress imposed by the COVID-19 pandemic and heightened rates of SIS [350,500]. I found that preconceptional and gestational SIS impacted the parental generation's pregnancy duration and altered their offspring's uterine stress and inflammation markers. These effects were observed in both TG and MG stress paradigms used in this study, indicating that stress is transmitted across generations in an intricate and generation-specific way. Despite no changes in the offspring's pregnancy length, their birth weights were reduced in the females

and males. SIS also programmed the offspring's long-term metabolic outcomes. Furthermore, chronic SIS induced disinterest in breeding among the rats, suggesting that stress affects their breeding behaviour and reproduction.

Next, I examined the immediate, long-term, and TG effects of stress by subjecting F0 pregnant rats to chronic variable stressors during mid- and late-gestation. CVS was implemented by exposing pregnant rats to restraint stress and overnight SIS. The rationale for combining two chronic stressors is to assess the effects of an additional hit during gestation, which would better represent real-life stressors. Additionally, stress exposure during mid- and late- pregnancy has been associated with a higher risk of pPROM, altered body composition, and metabolic and respiratory dysfunction in the offspring [501-504]. The F1 animals prenatally stressed *in utero* by CVS were subdivided into two stress cohorts: MTPS and PTPS (chapters 5 and 6).

The MTPS study explores the maternal transmission of stress to future offspring via the maternal lineage. We found that uterine markers of inflammation and stress were upregulated in the female progeny exposed to ancestral CVS. Nonetheless, we also observed signs of adaptation to stress in these animals, suggesting that stress-induced programming of the offspring is target-specific and depends on the generation under analysis. Opposed to the proposed hypothesis, CVS exposure in the parental generation did not cause gestational length changes in any generation. These results are expected given that preterm birth is a complex syndrome with intricate and interrelated pathways that, when activated, stimulate the transition of the uterus to a pro-contractile state, leading to the onset of labour [85]. Due to the observed signs of adaptation in the uteri of the offspring exposed to ancestral stress, I suggest that resilience to stress is also transmitted through generations, preventing the occurrence of preterm birth in animals. Also, vulnerability of resilience to stress depends on the stress type, time of exposure, and duration of the stress protocol. CVS exposure might not have been long enough or as severe to cause shorter gestation or preterm birth in the rats. The exact underlying causes of altered uterine inflammatory and stress markers in uteri of ancestrally stressed dams with absent changes in pregnancy duration are unknown and require further analysis. The translation of these findings to humans is likely to cause different stress programming effects in the uterus, given that perception of stress and resilience are differently regulated in humans [505].

The F1 MTPS animals were exposed to enriched environment housing from weaning to GD20. We hypothesized that enriched environment intervention would improve the future offspring's health outcomes. However, enriched housing induced uterine gene expression changes similar to those of stressed animals. We suggest that enriched environment in this cohort produced a type of positive stress generated by the involuntary modification of the environment by moving the F1 offspring to new cages and an unexpected environment for which they were not prenatally programmed. This supports previous literature findings where the introduction of novel objects and transfer to different cages is perceived as a stressor during short-term enrichment periods [457] and long-term enrichment through weaning to mating [294]. Although long-term enriched environment intervention was used, allowing the animals to become habituated to the new environment, genuine TG transmission of environmental cues occurred in the enriched lineage. Animals exposed directly or indirectly to enrichment exhibited stress-like effects, including the F3 generation who were not directly exposed to any stress or enrichment therapy. The inconsistent enriched environment findings are presumably context-dependent and attributed to several factors, through which non-genetic individuality, species, genotypes, microenvironmental effects, target specificity, and type of enrichment play significant roles on the final outcomes. Still, the results must be interpreted with caution in regards to conceivable variable outcomes, where enrichment may promote resilience to stress or may act as a stressor to animals.

Growing evidence shows that the early paternal life and preconceptional environment contribute mainly to offspring development and health through the transmission of epigenetic information to the zygote via sperm and seminal fluid [506]. Therefore, we explored whether *in-utero* CVS exposure to F1 males would alter preterm birth risk and inflammatory and stress markers, which would be transferred to the offspring, altering their pregnancy outcomes. This gave rise to the PTPS study described in Chapter 6. Preterm birth risk was not transmitted to the offspring through the paternal lineage, contrasting the proposed hypothesis. Furthermore, CVS in the F0 generation did not affect the adult offspring's CORT levels in plasma. The results confirmed that a family history of CVS conveys stress susceptibility to the offspring through the paternal lineage. The stress predominantly upregulated inflammatory and stress markers in the uterus of daughters and granddaughters of ancestrally stressed F1 males. Interestingly, maternal gestational and F2 male birth weights significantly increased, suggesting that CVS in F0

mothers may affect metabolic regulation in the offspring. However, further analysis of the long-term stress-induced metabolic consequences and growth trajectories in the offspring is recommended, adding measures of adiposity, glucose levels and tolerance, and longitudinal weight gain of the offspring.

Testicular inflammation induced by PNMS is associated with altered Leydig cell function, which impacts testosterone production and spermatogenesis [491]. To account for sex differences, we assessed the testes of F2 and F3 offspring exposed to ancestral stress. We found opposite inflammatory and stress patterns than the female offspring, whereby the selected markers were mainly downregulated in these animals. These findings indicate that prenatally stressed fathers may transmit stress resilience through the paternal lineage to mitigate the effects of stress on the male offspring.

7.2 Significance of results

Our PNMS studies make several contributions to the existing literature (Figure 7.1). First, we reported that SIS during pregnancy shortens the gestational lengths of stressed mothers, affects neonatal birth weights, and induces uterine reprogramming of future offspring, thereby altering inflammatory and stress profiles in the uterus of the progeny. Second, through our CVS protocol, whereby F0 rats were stressed during late pregnancy, we showed TG programming of females across four generations through the maternal lineage, affecting their adult uteri's inflammatory and stress profiles without inducing preterm birth. Third, long-term enriched environment intervention in the F1 offspring born to CVS-exposed F0 dams induced stress-like effects instead of beneficial outcomes and produced TG transmission of housing effects. Fourth, we demonstrated that CVS is transmitted through the paternal lineage and altered inflammatory and stress markers in the exposed offspring's uteri and testes.

Social isolation is associated with detrimental health outcomes such as anxiety and depression [306], dementia [507], cardiovascular diseases [351], and increased mortality [356]. The prevalence of social isolation among middle-aged and older Canadians was 5.1% in 2016 [508]. The prevalence rates of social isolation exceedingly increased with the unprecedented isolation imposed by the COVID-19 global pandemic [355,509,510]. Governments around the globe implemented closures and restrictions of in-person social interactions to limit the spread of the virus. Moreover, the increasing number of infections and lockdowns contributed to more social isolation [350]. A cross-sectional survey including 101 countries

found that 13% of participants reported a substantial increase in social isolation during the COVID-19 pandemic [509]. The rates of perceived and objective social isolation were 59.1% and 28.9%, respectively, in a survey conducted on the German population [510].

A significant literature gap remains on the effects of social isolation during pregnancy and its association with adverse pregnancy outcomes. Most of the literature investigates the lack of social support and mental health issues of being isolated. However, to our knowledge, this is a novel study that measures markers of stress and inflammation related to the onset of parturition in the uteri of animals exposed to preconceptional and gestational SIS. We showed that the impacts of SIS span from mental health and cardiovascular issues but also affect pregnancy duration and lead to TG programming of inflammatory and stress-related phenotypes in the uteri of future offspring, which may increase their risk of pregnancy complications (Figure 7.1).

In the context of the global COVID-19 pandemic, the rates of preterm birth varied among geographic locations and different studies, where preterm birth incidence decreased [511], increased [512], or remained unchanged [513]. It is difficult to assess the individual contribution of SIS to pregnancy complications such as preterm birth, given that several confounding factors may influence the observed risk. Some of these factors include the infection itself and the reduced number and quality of prenatal visits [514]. However, the effects of increased SIS prevalence during the pandemic are expected to be noted years after the initial exposure. Future studies aiming to investigate the effects of SIS on pregnancy outcomes are required to elucidate the underlying mechanisms and develop novel therapies to improve or prevent complications.

Maternal prenatal stress occurring during important windows of development promoted sex-specific responses, affecting stress sensitivity in the offspring differently in either sex. This suggests that maternal versus paternal transmission is distinct and yields different health outcomes (Figure 7.1). The developing offspring somatic and germ cells *in utero* receive environmental inputs and respond to these cues by altering epigenetic information transgenerationally inherited in the progeny. While preterm birth did not occur in the stressed parental generation and ancestrally exposed offspring of neither MTPS and PTPS, aberrant responses to stressors accumulated during a lifetime increase the allostatic load of these animals, which may result in a higher risk of disease onset. This is particularly possible considering the

existing upregulation of inflammatory and stress mediators in the offspring's uteri of both cohorts. Additional hits may exacerbate existing symptoms and cause further inflammation, preterm birth, and other pregnancy-related complications (Figure 7.1). We observed that stress is also transmitted to male testes through the paternal lineage, displayed as adaptability in inflammatory and stress markers in the testicular tissues of the offspring (Figure 7.1). In our model, downregulation of inflammatory and stress profiles may better prepare the offspring for a stressful environment. However, the testicular consequences of these compensatory effects are unknown. Although not covered in this dissertation, we expect that these alterations impact sperm parameters, steroid hormones, and fertility. These findings confirm the importance of the translation of animal model research to humans to clarify the effects of *in-utero* and preconceptional stress in fathers, which may contribute to the rising rates of infertility among men [515].

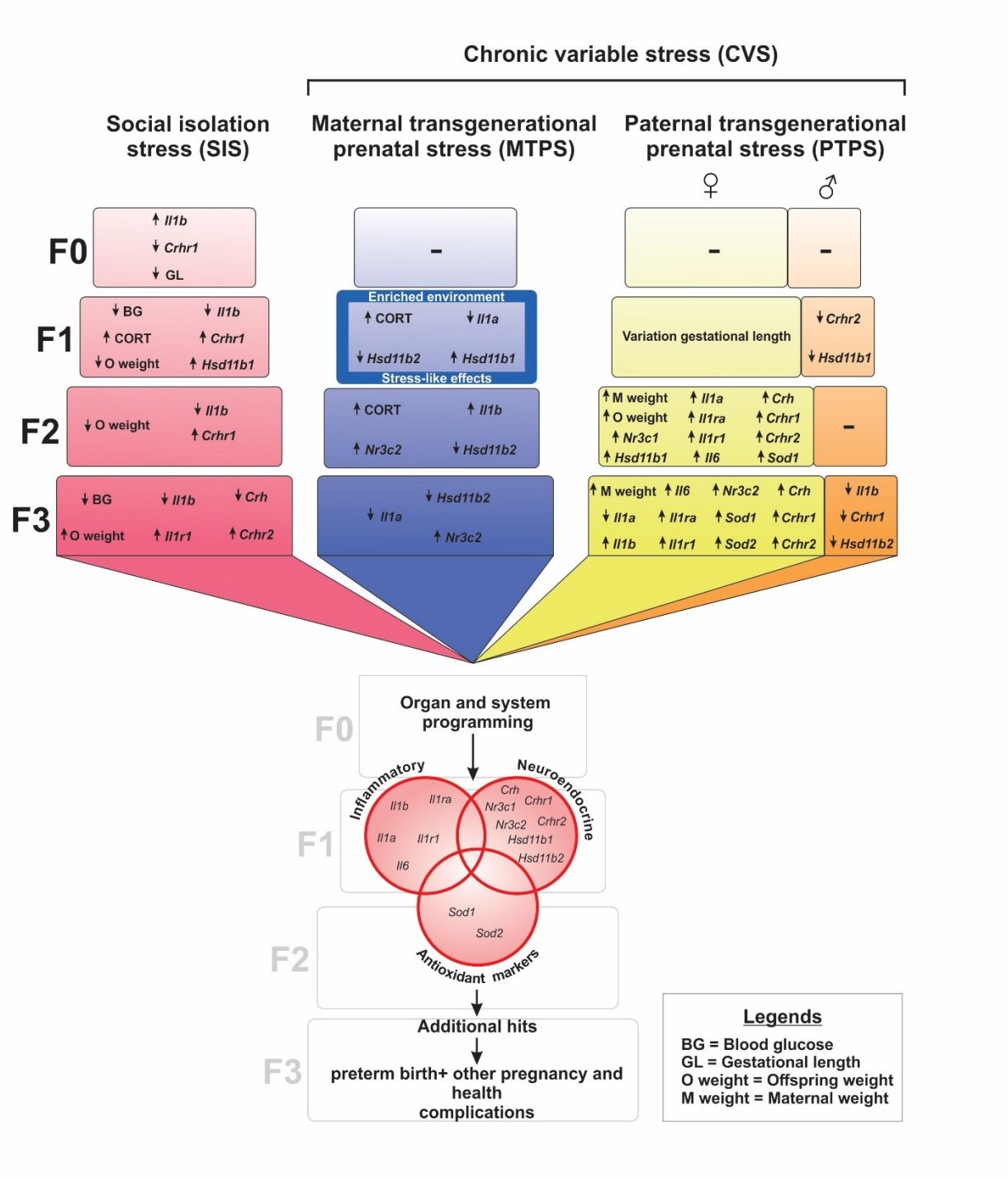
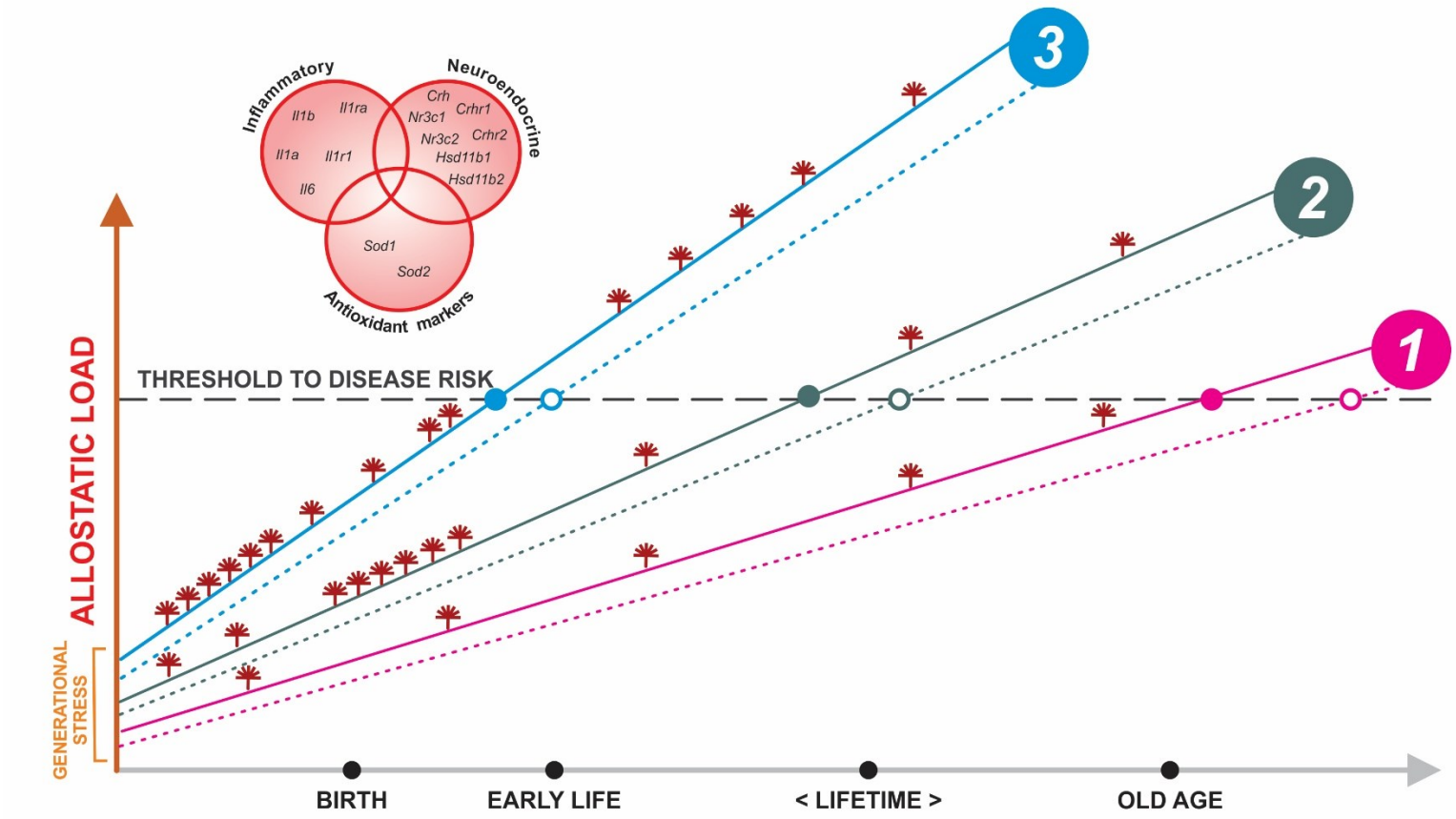


Figure 7.1: Illustration describing the main findings of the prenatal maternal stress models included in this dissertation.

Transgenerational transmission of stress may increase the allostatic load of future offspring and, consequently, predispose them to a higher risk of disease in adulthood upon exposure to additional stressors.

Early-life exposure to challenges has been shown to induce fetal programming of coping phenotypes that are transferred over generations [284,286]. When faced with a threat, an individual assesses the perturbation and produces an individual stress response to minimize internal disturbances. An individual is predisposed to adverse health outcomes when coping with the challenge or the cumulative stress across the lifespan does not occur [156] (Figure 7.2). Among several other factors, each individual's unique response to stress contributes to the extensive multifactorial etiology of preterm birth.



1 - NORMAL-LIFE STRESS
2 - EARLY-LIFE CHRONIC EXPOSURE TO STRESS
3 - EARLY-LIFE STRESS + ADDITIONAL HITS
* - STRESS HIT
- - - - COPING WITH STRESS

Figure 7.2: Cumulative lifetime stress conceptual framework describing the effects of multiple stress “hits” during life.

The cumulate effects of stress affect downstream regulatory responses of various organ systems and increase the allostatic load until it exceeds a certain threshold that, once crossed, can trigger adverse health conditions. If the stress effects induce an early activation of pro-inflammatory and neuroendocrine mediators to an irreversible point where the drive towards the amplification of the parturition cascade cannot be halted, preterm birth and other pregnancy complications can occur. These stressed-programmed markers are passed on to the next generations, affecting their health trajectories.

Individuality in the stress response is a potential reason for the diversity and contradictory results observed in the literature. Physiological mechanisms are regulated according to the organism’s ability to respond to the changing environment by either producing a favourable response to improve the offspring’s fitness or a maladaptive outcome whereby the individual is prone to diseases. The three studies covered in this dissertation displayed various levels of adaptation in the animals exposed to stress or its ancestral effects. The PNMS cohorts conveyed transgenerational information on risk and resilience to the offspring, suggesting that adaptation to stress depends on the context, the target under analysis, and stress-related factors. Additionally, stress was transmitted in a complex way, where biological and molecular modifications skip generations to reappear in future progeny, indicating that generation and sex of the offspring also play important roles in the programming of stress vulnerability and coping.

Environmental enrichment in humans has been shown to produce beneficial outcomes in the adult brain by increasing cognition, learning, and memory [516-518]. Yoga and music improve the functioning of several organ systems and reduce pain, anxiety/depression, and stress levels [519,520]. We initially hypothesized that exposing F1 ancestrally stressed rats to enriched housing would produce beneficial effects or reverse the adverse uterine outcomes of CVS in the F1-F3 female offspring. Conversely, enriched housing induced changes comparable to the stress group, even in future generation controls, indicating that housing effects can also be transmitted over generations. Despite that, implementing an enriched strategy is important to assess the plasticity of such TG modifications, which may be improved with simple interventions [521].

7.3 Limitations

Preconceptional and gestational SIS model

Intrinsic limitations are observed in TG models of stress, where a large cohort of four generations of rats is produced to investigate the transmission of stressed-programmed phenotypes across generations. The main limitation of the SIS study is the collection of uterine tissues at weaning of the offspring (LD21). The uterine tissue collection time is important in this study, considering that one of our main goals involved assessing the risk of preterm birth in the parental generation and the subsequent offspring. Thus, the inflammatory and endocrine changes in the uterus right after delivery might better represent the changes occurring during labour. The uterine inflammatory and stress profiles are expected to be somewhat differently regulated on LD21; thus, data assumptions of uterine expression of markers during labour should be cautiously made. However, we studied the effects of life-long and inherited stress and its contribution to AL, which is not affected by the time of collection. In future studies, this limitation can be tackled by subjecting pups to foster mothers, allowing the uteri collection to be done right after delivery.

We did not assess the estrous cycle stages of animals upon uterine tissue collection (at weaning of the offspring). A recent study confirmed that biological sex, but not fluctuations in circulating ovarian hormones across the estrous cycle stages, affect peripheral blood immune cell prevalence and variability in mice [522]. Also, the stimulation of the female rat's nipples during lactation has been shown to suppress the estrous cycle [523] through prolactin suppression of gonadotropin release [524], suggesting that the stage of estrus cycle determination might not be required in this project. Although uterine inflammatory mediators may be altered during the estrous cycle stages, communal living has been shown to induce synchronization of the estrous cycle of female rats [525]. This finding has also been observed in isolated groups of rats, where females synchronized their estrous cycle through airborne communication [525]. In addition, estrous cycle stage-specific differences might be equally spread among the dams in each treatment condition, potentially minimizing these variations.

This cohort not only investigated the stress-induced risk of pregnancy complications but also collected post-partum maternal behaviour and several other tissues for future analysis, thus limiting the availability of gestational tissues during labour. Another limitation related to the generational nature of the

study is that placental tissues were not available in any of the PNMS cohorts of this dissertation because the dams would eat their placentas. Although 11β-HSDs are expressed in the uterus, their expression and activity in the maternal-fetal interface of the placenta are of greater interest and could not be determined in these studies.

Some of the confounding factors concerning our SIS model comprise the post-natal social aspect of housing mothers and pups together until weaning, which could represent a form of enriched environment and could have impacted the accuracy of this study's findings. However, it is important to keep the mothers and pups together until weaning for proper neurobiological, behavioural [526], and gut microbiome development [527].

In this study, stressed animals were also housed in the same room and could hear and smell their counterparts, possibly reducing the level of isolation experienced by these animals. We have recently shown that the experimenter's biological sex contributes to the psychophysiological responses of rats, whereby exposure to a male experimenter altered the behavioural responses of female rats with increased CORT and decreased oxytocin levels [528]. Thus, the experimenters assigned to this stress cohort are another potential confounding aspect.

Although the rat perception of stress is likely different from humans, the SIS protocol might be somewhat similar in pregnant women, considering that rats are social mammals that partake in social interactions with their conspecifics [309]. Translating the SIS investigation to pregnant women would better clarify the pregnancy risk of complications and the offspring's long-term effects of preconceptional and gestational exposure to SIS.

Maternal (MTPS) and paternal (PTPS) transgenerational prenatal stress using the chronic variable stress (CVS) model

As discussed in the previous section, the uterine tissue collection's main limitation and lack of estrus cycle analysis remain in the CVS models of chapters 5 and 6. Collecting uterine tissues right after labour represents a significant challenge. It would require an exclusive study with a primary goal of assessing stress-induced uterine modifications of genes of interest and adverse pregnancy outcomes. The findings of this study instead reflect the long-lasting uterine transcriptome programmed by maternal

and ancestral CVS, increasing the AL and predisposing the offspring to future health and pregnancy complications. To better understand the inflammatory and endocrine changes occurring at the uterine and placental levels and fetal programming of the offspring due to CVS, leukocyte tissue composition and placental 11β-HSDs enzymatic activities should have been measured.

The CVS paradigm using restraint and SIS is considered a mild psychological and psychosocial stress [295,332], attempting to mimic human stressors in rodents. However, the human perception and the types of stress in our daily lives are considerably different and likely hard to emulate. A perfect animal model to reproduce all the complex daily life events, replicate how humans perceive stress, and induce preterm birth and other diseases (e.g. non-communicable diseases) does not exist. In this context, rats were chosen because of their relatively short pregnancy duration, cost, the possibility of having genetic homogeneity, and shared similarities with humans. Animal models remain the preferable approach to understanding the TG effects of PNMS. It does not rule out the reproductive tissue's microenvironment where biochemical mediators and physiological mechanisms regulate fetal programming of health and disease early in life [529] (e.g., extracellular vesicles [530]) when compared to *in vitro* studies. Additional constraints include the ethical concerns of studying stress during pregnancy and early life, especially after the solid evidence of the life-long consequences and TG effects of stress in future generations. Other confounding effects comprise the offspring's post-weaning behavioural tests conducted in the MTPS and PTPS models, which may serve as an additional source of stress.

Few studies focus on the inflammatory and endocrine changes in the uterus during pregnancy and parturition, particularly the effects of stress on uterine homeostasis, labour, and fetal programming of the offspring's uteri, limiting the conclusions of the MTPS and PNMS studies. This is rather unexpected, given that labour onset encompasses a series of culminating events to change the uterus and cervix's biochemical, morphological, and physiological status to deliver the baby safely.

Knowing that the objective of the proposed studies includes assessing stress-induced pregnancy outcomes and reproductive tissues programming by stress, we did not measure the testicular expression of spermatogenesis-specific genes or plasma testosterone levels in males. Also, plasma CORT levels were only measured in the adult offspring on P110, but not during the gestation of the dams and at the time of conception in males. Finally, testicular tissues were snap-frozen for future gene expression and

protein abundance analyses. This unfortunately hindered the oxidative stress analysis via DHE staining and sperm collection, as well as miRNA and DNAm analysis.

7.4 Future Directions

Filling some of the gaps in our prenatal CVS study

Knowing that PNMS affects the normal physiology of reproductive tissues [152,331], several other routes can be investigated to clarify the mechanisms involved in stressed-induced damage. In future studies, histological assessments of fresh uteri and testes of stressed rats should be pursued since it provides evidence of stress-related injuries. The testis has been shown to produce constitutive immunoregulatory cytokines such as IL-4, IL-10, and TFG-β [531]. Also, uterine expression of IL-10 was upregulated in rats exposed to psychological stress and IL-1β injections late in pregnancy [331]. Analysis of immunoregulatory cytokines in the testes and uteri of stressed animals would help elucidate these tissues' inflammatory profiles. Furthermore, the evaluation of the stress effects on immune cells and identification of the cells producing pro-inflammatory cytokines could be done with tissue immune cell composition and localization analysis.

The data presented in this dissertation successfully shows how increased stress load can impact future offspring's inflammatory and endocrinal regulation and either overwhelm or improve their resilience. Other stress and endocrine markers could be explored in both reproductive tissues, including apoptotic factors, spermatogenesis-specific genes, oxidative stress indicators, and other antioxidant enzymes' expression and activities. Furthermore, apoptosis of Leydig cells and trophoblasts, testosterone, estrogen, and progesterone levels, androgen receptor expression in the testes, testis and uterine morphology, structure and weight, as well as sperm parameters could be analyzed. Epigenomic and transcriptomic studies of female and male reproductive tissues and cells, including the testis, epididymis, sperm, uterus, and placenta are important tools to reveal novel stress biomarkers.

Early-life stress effects can impact the long-term structure and function of several organ systems by altering developmental processes including, but not limited to, the HPA axis [532], central receptors [533], enzymatic activity in the brain [533], peptide expression [313], behaviour [532], etc. Investigating the effects of SIS and CVS on the brain and behaviour of stressed mothers and offspring would also

show other areas of susceptibility and potential targets for interventions. Previous studies indicated that the adverse effects of prenatal stress on behavioural outcomes might be explained by tissue inflammation [427]. Thus, we can assess whether the immunological changes in reproductive tissues of stressed pregnant dams can impact offspring behavioural outcomes through fetal inflammation of the brain and other organs (e.g., liver, lung, guts, and kidneys). Understanding the GC-immune coordination is important. Therefore, examining metabolic hormones, receptors, HPA axis, and inflammatory mediators should also be measured.

Another aspect to investigate would be the gut-microbiome-brain axis, whereby the microbiota has a leading role in controlling the axis, brain, and behaviour, shaping later health outcomes [534]. Moreover, early-life gut colonization is essential for the maturation of the gut immune and metabolic systems and influences later in life disease risks, such as metabolic disorders, allergies, and asthma [535].

Long-term exposure to enriched environment therapy has been shown to reverse stress by inducing habituation to the novel environment [457]. Although we used long-term enrichment from post-weaning until the delivery of pups, we did not observe acclimation to the stress. The enriched environment intervention used in the MTPS study promoted stress-like effects on the markers of stress selected in the uterus. Future studies could test whether cumulative enrichment effects using a MG paradigm would produce different results in the uteri of stressed animals and improve the adverse effects of CVS. In addition to the analysis of enriched environment contribution to reproductive health, behavioural and brain outcomes are expected to be significantly changed by the introduction of enriched housing.

SIS study implications for research practice

The SIS study has human applications. The timely discoveries of this dissertation allow the translation of this study to human research, taking advantage of the global social isolation imposed by the COVID-19 pandemic. Given the current worldwide controlled number of infections and hospitalizations, lockdown measures were lifted (Fall 2022). Therefore, prospective studies of infants and children born to pregnant women exposed to higher levels of social isolation during pregnancy would enable researchers

to investigate the long-term effects of SIS. Non-pharmacological enrichment feasible strategies could improve life-long health outcomes of infants exposed SIS *in utero.* Some possible approaches to improve resilience and reduce stress load in infants/children include early-life bonding when parental leave is available, singing and talking to the child, baby/child massage, exposure to music, engaging together in various activities, and allowing play dates to increase social interactions. Cognitive behavioural therapy can also improve the mental health outcomes of prenatally stressed children [536]. Therapy can be combined with 30 min physical exercises before the session since it was shown to improve the effects of therapy on mental health [537]. Retrospective studies could be designed to investigate the impacts of SIS on different gestational ages.

Epigenetic assessments contribute to identifying biomarkers of exposure and disease [59]. By targeting these biomarkers or underlying physiological pathways with preventative and therapeutic interventions, it might be possible to generate efficient pharmacological therapies for disease prevention or enable epigenetic-based diagnostic approaches. Thus, maternal and cord blood and placental tissue collection would enable multi-omics assessments in these tissues to examine potential new stress biomarkers. Increasing evidence points toward the exploitation of multi-omics data integration that provides valuable insights into complex human biology and a more robust analysis of the whole-tissue pool of epigenetic marks, transcripts, proteins, and metabolites, and analysis of the genome [538]. These relatively new technologies present exciting and promising notions into the mechanistic details of health and diseases, including the preterm birth syndrome [539].

How can we early diagnose preterm birth in pregnant women?

In the context of multi-omics analyses, machine learning algorithms could predict preterm birth in human cohorts using blood and urine samples by identifying the transcriptomic and proteomic profiles of these samples [540,541]. The development of new algorithms using multi-omics profiles might be a powerful tool to diagnose preterm birth early, considering the multifactorial aspects of the syndrome and the inherited difficulties of measuring various potential biomarkers in maternal blood. The discoveries of more specific preterm birth biomarkers can also guide scientists toward a more efficient development of pharmacological therapeutic interventions for preterm birth.

Knowing women at risk of preterm birth, how can we treat them effectively?

As extensively covered in this dissertation, inflammation has a central role in the labour process. Pro-inflammatory mediators are upregulated in the blood [542] and placenta of women undergoing preterm birth and pPROM [543]. Moreover, transcriptomic analyses revealed upregulation of inflammatory and immunological pathways in chorio-amnion membranes from severe preterm fetuses [544]. There is a gap in understanding how to delay or prevent preterm birth with pharmacological approaches effectively. Based on these findings, researchers have developed novel pharmacological therapies to inhibit inflammatory pathways or molecules to delay or prevent preterm birth. The TLR4 antagonist +-naloxone is being tested in animal models since it inhibits TLR activation by DAMPs and PAMPs early in the inflammatory cascade. In mouse models of systemic inflammation, +-naloxone effectively delayed preterm birth and improved offspring health [545,546]. Other therapeutic options currently under analysis by our group involve an allosteric modulator of the IL-1 receptor that inhibits some signals while preserving parts of the downstream signalling pathways. Differently from the anti-IL-1 drugs currently approved, IL-1 allosteric modulators do not interfere with all signalling pathways, including the NF-κB, which is involved with immune vigilance and cytoprotection during pregnancy [547]. Rytvela (or 101.10) administration to mice has been shown to delay preterm birth, reduce fetal inflammation, and pro-inflammatory cytokines and pro-labour genes in the myometrium of a murine model of sterile inflammation induced by IL-1β, lipoteichoic acid, and LPS [547,548]. Finally, the efficacy of Rytvela in ameliorating the effects of PNMs on inflammatory dysregulation of the offspring can be investigated in a TG model where mice are exposed to CVS in a TG and MG fashion.

7.5 Conclusion

The series of PNMS studies included in this dissertation shows the effects of maternal stress on adverse pregnancy and offspring health outcomes. Our primary finding is that SIS and CVS paradigms induced tissue reprogramming of uterine markers of stress and inflammation across generations of rats. The transmission of stress-induced phenotypes is complex and generation-, sex-, stress-, and biomarker-

specific. The offspring exposed to ancestral stress exhibited either adaptive responses to stress or maladaptive outcomes that may influence disease risk in the offspring.

The results presented in this dissertation are novel and demonstrate a link between preconceptional and gestational SIS and shorter gestational lengths in the exposed parental generation. Furthermore, CVS induces TG transmission of inflammatory and stress traits in reproductive tissues through maternal and paternal lineages exposed to ancestral stress. These modifications are maintained across generations and remain present in the F3 offspring that did not experience direct effects of stress.

The beneficial effects of environmental enrichment have been investigated in a rat cohort of maternal TG prenatal stress triggered by CVS. Subjecting stressed F1 female offspring to enriched housing induced uterine expression patterns similar to stressed animals. The effects of enriched environment are unique and transgenerationally transmitted across generations, modifying offspring health outcomes.

Finally, future studies using novel technologies and pharmaceutical interventions would assist in identifying mechanisms through which uterine inflammatory and endocrine dysregulation occur because of prenatal stress. Finding an effective therapy to reduce the effects of prenatal stress and predictive stress biomarkers that can be translated to humans may improve maternal and child health over multiple generations.

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Appendix

Appendix A

Table A9.1: Health outcomes comparison between three types of prenatal maternal stress models [152,331].

Outcome	Generations				Stress Model
	F0	F1	F2	F3	
Gestational length (h)	▼	ns	ns	N/A	**SIS**
	ns	**▼ TG, MG**	**▼ TG, MG**	N/A	**Single-hit**
	Increased variation in gestational length	N/A	-	-	**Two-hit**
Maternal weight (g)	ns	ns	ns	ns	**SIS**
	▼ GD21	**▼ MG**	**▼ MG**	N/A	**Single-hit**
	▼ GD11-18	N/A	-	-	**Two-hit**
Pup weight (g) on P1	N/A	**▼ TG, MG ♂ ♀**	**▼ MG ♀/TG, MG ♂**	**▲ MG ♀/TG ♂**	**SIS**
	N/A	ns	ns	**▼ TG, MG**	**Single-hit**
	N/A	**▼ two-hit ♂ ♀**	-	-	**Two-hit**
Blood glucose (mmol/L) on GD18	ns	**▼ TG**	ns	**▼ MG**	**SIS**
	ns	ns	**▲MG**	ns	**Single-hit**
	ns	ns	-	-	**Two-hit**
CORT (ng/mL)	ns	**▲ TG, MG**	ns	-	**SIS**
	ns	ns	▲MG	ns	**Single-hit**
	ns	ns	-	-	**Two-hit**
Adverse health outcomes	**Moderate disinterest in breeding**				**SIS**
	-				**Single-hit**
	Resorption, preterm, and post-term delivery				**Two-hit**

▲: increase; ▼: decrease; ns: non-significant; N/A: not applicable. The dash depicts data not covered in the study. Single-hit stress study: used psychological stressors such as forced swimming and restraint to stress rats in a single generation (TG stress paradigm) or across four generations (MG stress paradigm) [152]. Two-hit stress study (or, Two-stress study): used a combination of psychological stressors and

intraperitoneal injections of IL-1β to stress rats [331]. Green colour: data from the current study; off-white colour: data from the single-hit study [152]; grey colour: data from the two-hit study [331].

Table A2: Uterine gene expression comparison between SIS and two-hit stress models [331]. Uterine gene expression was not measured in the one-hit stress model of restraint and forced swimming [152].

Gene Expression	Generations				Stress Model
	F0	F1	F2	F3	
Il1a	ns	ns	ns	ns	**SIS**
	ns	**▼ two-hit**	-	-	**Two-hit**
Il1b	**▲ TG, MG**	**▼ TG, MG**	**▼ TG, MG**	**▼ TG**	**SIS**
	▲ two-hit	ns	-	-	**Two-hit**
Il1r1	ns	ns	ns	**▲MG**	**SIS**
	ns	ns	-	-	**Two-hit**
Crh	ns	ns	ns	**▼ MG**	**SIS**
	▲ two-hit	▼ two-hit	-	-	**Two-hit**
Crhr1	**▼ TG, MG**	**▲MG**	**▲TG**	ns	**SIS**
	▲ two-hit	**▼ two-hit**	-	-	**Two-hit**
Crhr2	ns	ns	ns	**▲MG**	**SIS**
	ns	**trend to ▼ two-hit**	-	-	**Two-hit**
Hsd11b2	ns	**▲TG**	ns	ns	**SIS**
	ns	ns	-	-	**Two-hit**

▲: increase; ▼: decrease; ns: non-significant; N/A: not applicable. The dash depicts data not covered in the study. Single-hit stress study: used psychological stressors such as forced swimming and restraint to stress rats in a single generation (TG stress paradigm) or across four generations (MG stress paradigm) [152]. Two-hit stress study (or, Two-stress study): used a combination of psychological stressors and intraperitoneal injections of IL-1β to stress rats [331]. Green colour: data from the current study; off-white colour: data from the single-hit study [152]; grey colour: data from the two-hit study [331].

Appendix B

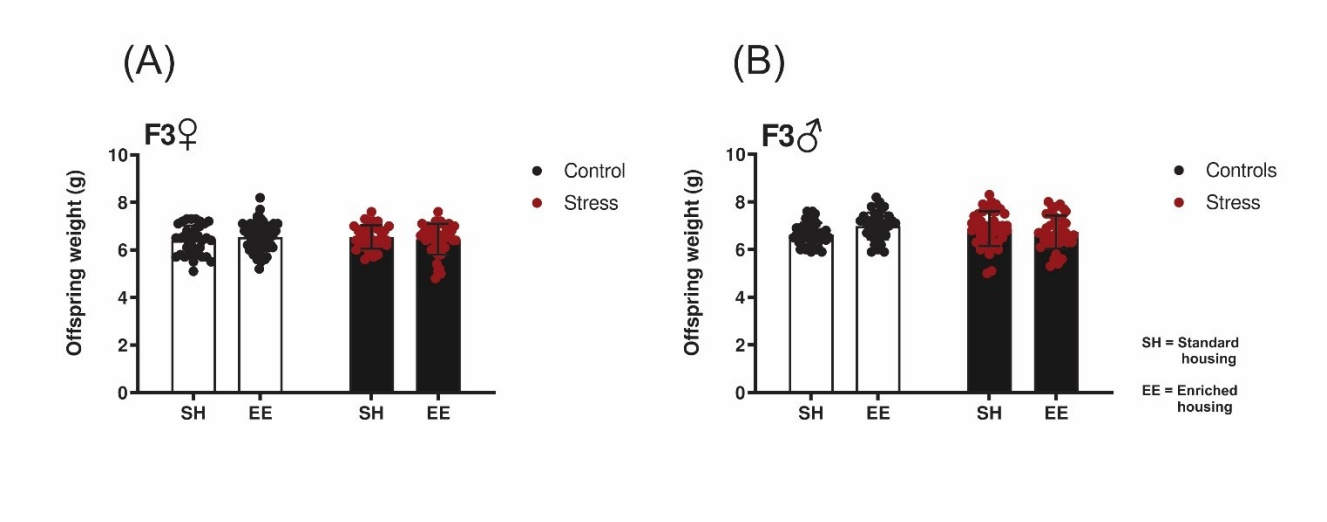

Figure B9.1: Offspring weights remained unchanged between treatment and housing groups in the F3 offspring for both (A) females and (B) males ($p > 0.05$). Data are compared to F2C, mean ± SEM. N = 35-65 (females, A); 38-66 (males, B). Data were analyzed with Kruskal-Wallis test.

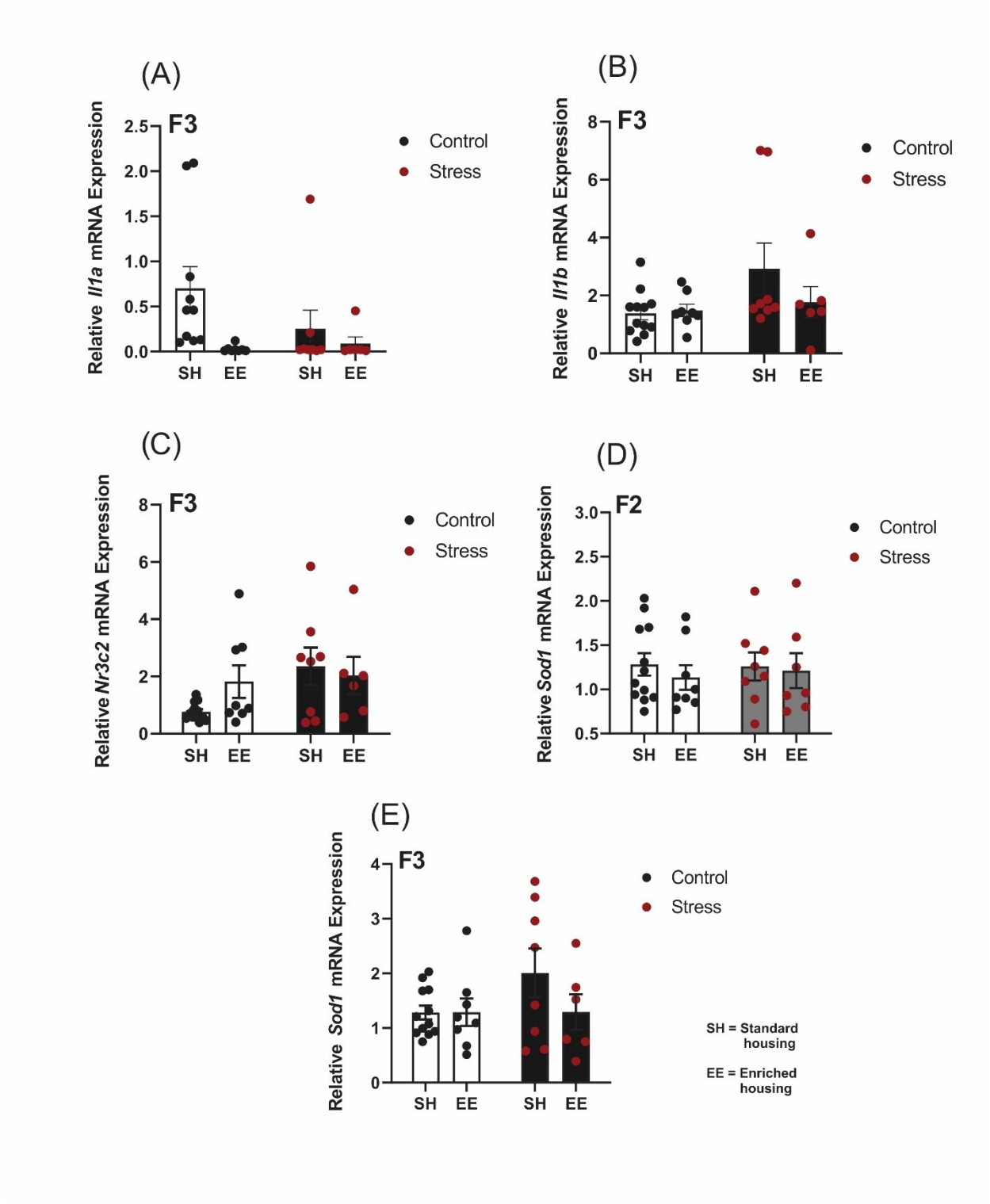

Figure B9.2: The analysis of the effects of housing and treatment revealed a non-significant interaction for inflammatory and stress markers of dams exposed to CVS and enriched housing ($p > 0.05$). Data are compared to F0C, mean ± SEM. N = 6-12 per group. Data were analyzed with a two-way ANOVA test.

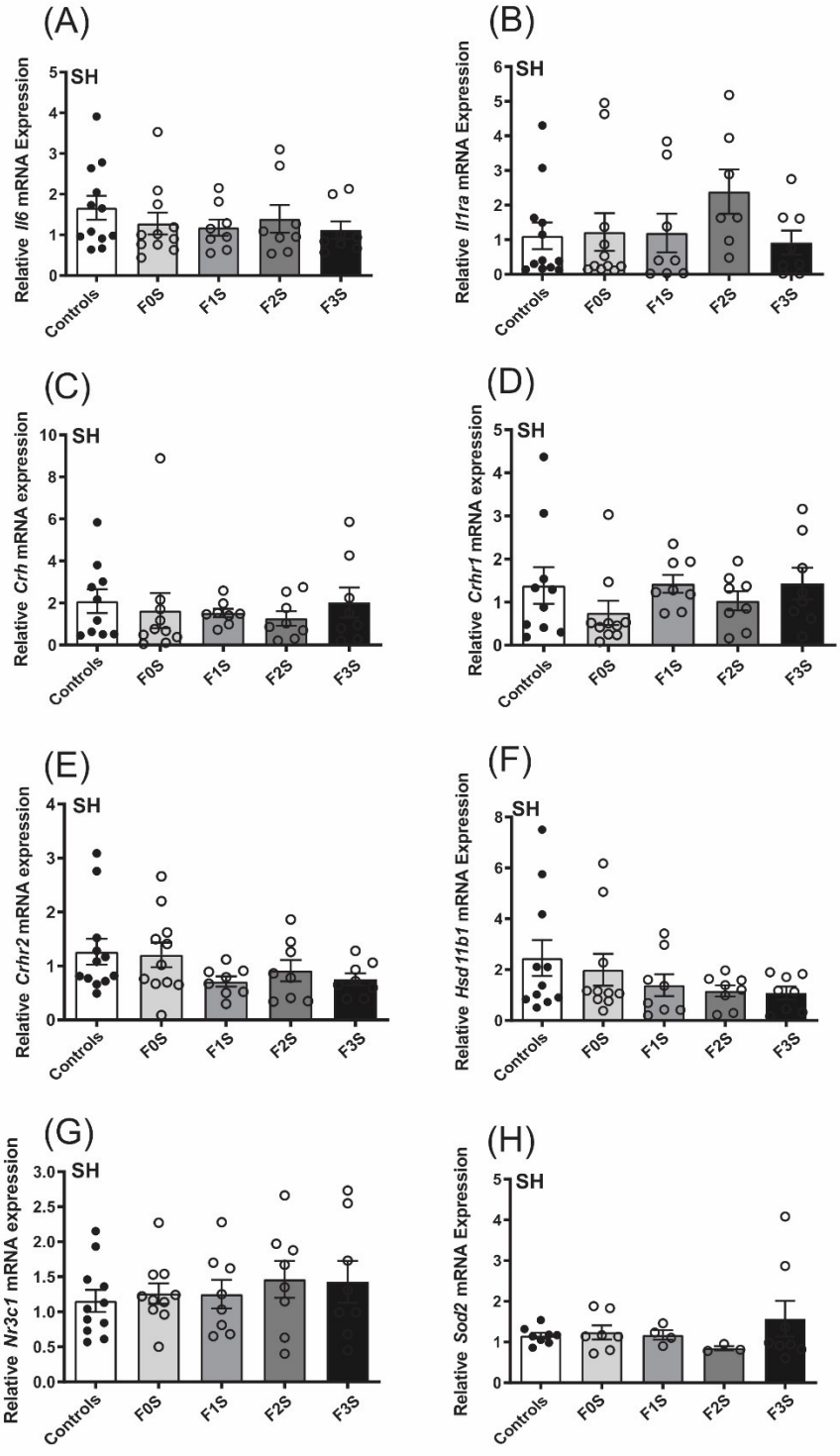

Figure B9.3: Uterine inflammatory and stress markers expression were unchanged in dams exposed to CVS under standard housing ($p > 0.05$). Data are compared to F0C, mean ± SEM. N = 3-12 per group. Data were analyzed with a one-way ANOVA.

Appendix C

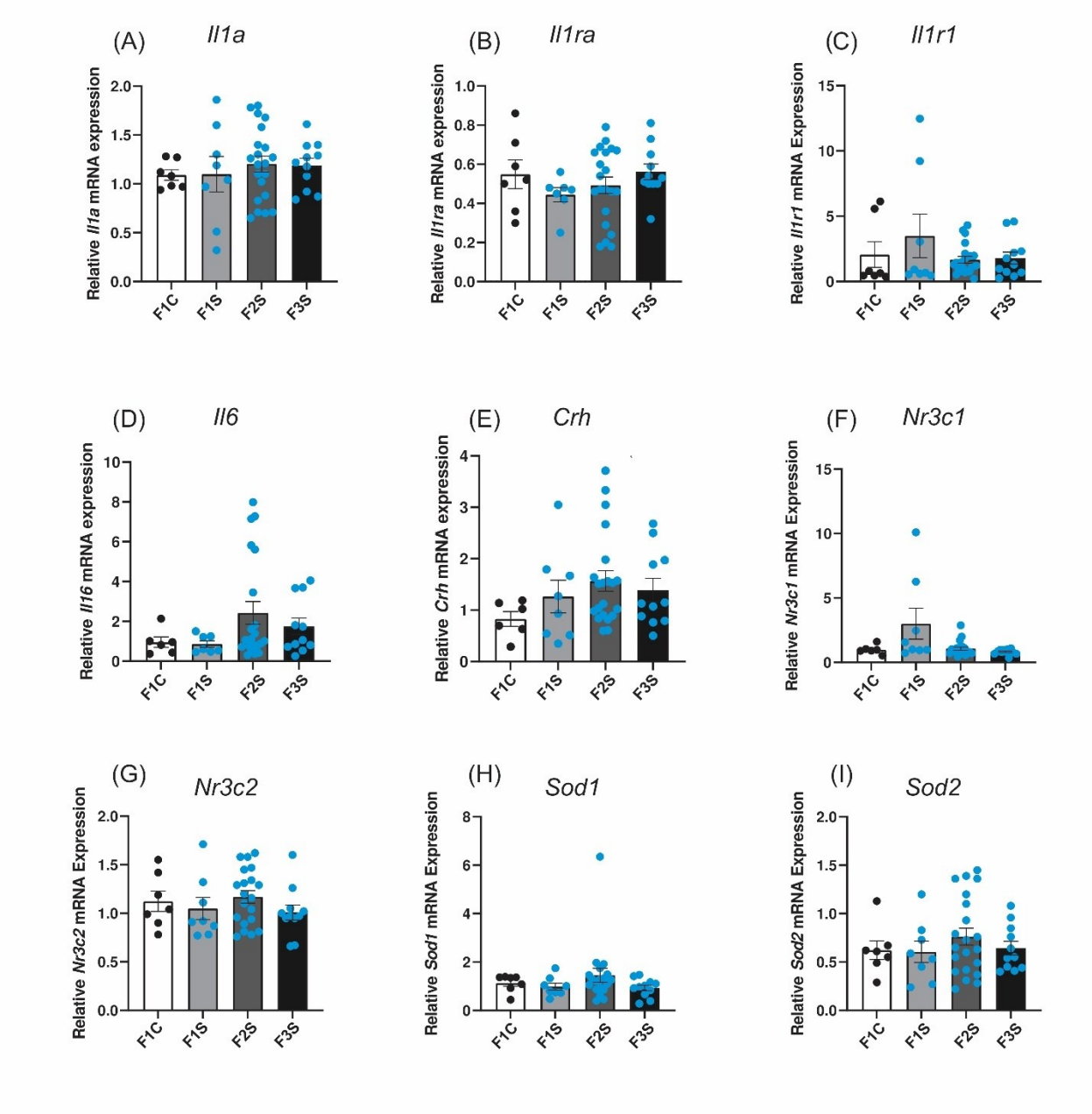

Figure C4: (A-I) Testicular tissue expression of inflammatory and stress markers was unchanged in animals exposed to CVS ($p > 0.05$). Data were analyzed with the Kruskal-Wallis test. Mean ± SEM, N = 7-21.

Appendix D

Nayara Gabriela Lopes <nayaraga@ualberta.ca>

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		Publication Type	e-Journal
Date	01/01/1983	URL	http://arjournals.annualreviews.org/loi/immunol
Language	English		
Country	United States of America		

REQUEST DETAILS

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NEW WORK DETAILS

Title	Method, degree and lineage of prenatal maternal stress: effects on inflammatory and stress markers pro le of reproductive tissues and pregnancy outcomes	Institution name	University of Alberta
		Expected presentation date	2022-12-07
Instructor name	Nayara Antunes Lopes		

ADDITIONAL DETAILS

Order reference number	N/A

The requesting person / organization to appear on the license	Nayara Antunes Lopes

REUSE CONTENT DETAILS

Title, description or numeric reference of the portion(s)	Figure 3: Schematic of the feedback regulation of the interrelated systems forming the neuroendocrine-immune network.	Title of the article/chapter the portion is from	General introduction
		Author of portion(s)	Nayara Antunes Lopes
		Issue, if republishing an article from a serial	N/A
Editor of portion(s)	N/a	Publication date of portion	2023-01-13
Volume of serial or monograph	N/a		
Page or page range of portion	1-55		

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