DISSERTATION

Stress during pregnancy leads to long-term consequences in the offspring

Submitted by

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ABSTRACT

STRESS DURING PREGNANCY LEADS TO LONG-TERM CHANGES IN OFFSPRING

Neuropsychiatric disorders encompass a wide range of conditions that affect neurological health and brain function and lead to disabilities worldwide. Such disorders include, but are not limited to, Major Depressive Disorder, schizophrenia, and anxiety disorders. Risk factors for developing neuropsychiatric disorders are multifaceted and can range from genetic predisposition, lifestyle, and environmental influences. Exposure to maternal stress is one type of environmental factor that can lead to changes in brain function and signaling pathways and increase susceptibility for related diseases. Maternal stress encompasses a diverse array of environmental stimuli, ranging from acute traumatic events to chronic or day-to-day life stressors. Maternal stressors, experienced by pregnant women, lead to overexposure of stress hormones in the developing fetus and impact short- and long-term neurological health the offspring. These studies evaluated developmental, neuroendocrine, and behavioral outcomes in offspring exposed to different models of maternal stress.

Chapter 1 provided a brief history of stress, the development of the hypothalamic-pituitary-adrenal axis that regulates the stress response, and maternal-fetal interactions in stress regulatory systems and related behaviors. Chapter 2 evaluated several models of maternal stress, maternal high fat diet, maternal caloric restriction, maternal exposure to synthetic glucocorticoids. Although there were vast discrepancies between each type of maternal stress, one similarity was an activated immune response with elevated maternal cytokines. Therefore, Chapter 3 characterized a model of maternal immune activation using a toll-like receptor agonist, Resiquimod, that increased maternal and fetal cytokines, produced

delayed developmental milestones and stress-related behavioral impairments in prepubertal (social-like) and adult (social-like, depressive-like, anxiety-like) offspring. Because these behavioral phenotypes are partially regulated by the paraventricular nucleus of the hypothalamus (PVN), Chapter 4 examined the neuroendocrine stress response and blood-brain barrier of the PVN. Data showed altered stress response accompanied by impaired blood-brain barrier integrity in the PVN of the adult offspring exposed to maternal injection of Resiquimod. Taken together, Chapters 2, 3, and 4 suggest maternal stress led to negative developmental, behavioral, and cellular pathologies indicative of neuropsychiatric-like disease. By teasing apart these specific programming mechanisms, we can better diagnose and treat progression of neuro-related disorders.

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CHAPTER 1:
INTRODUCTION[1]

"If it works the first time, it'll never work again"
-Dr. Bob Handa

Humans and animals respond to environmental perturbations with a stress response that allows physiological adaptation to the stressor and maintain homeostasis. A major component of the homeostatic response is the hypothalamic-pituitary-adrenal (HPA) axis, an intricate, yet robust, neuroendocrine mechanism that mediates the effects of stressors by regulating physiological processes, such as metabolism, immune responses, and the autonomic nervous system [1-3]. HPA axis signaling begins in the paraventricular nucleus of the hypothalamus (PVN). Neurons in the PVN synthesize and secrete the hypothalamic releasing factor, corticotropin releasing hormone (CRH). The release of CRH to the hypothalamo-hypophyseal portal vasculature, a capillary system that connects the median eminence with the anterior pituitary gland, controls the release of the anterior pituitary hormone, adrenocorticotrophic hormone (ACTH) into the general circulation. In turn, ACTH acts upon the adrenal cortex to cause the synthesis of glucocorticoid (GC) hormones, of which cortisol is the predominate form in humans (corticosterone in rats and mice). GC hormones, in addition to adrenomedullary epinephrine, are important for regulating a variety of physiological functions including behavioral, cardiovascular, metabolic, and immunologic functions. Importantly, the HPA axis robustly responds to

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environmental perturbations that might threaten homeostasis. Thus, disruption of the secretion of adrenal hormones, whether too high or too low, can lead to stress-related pathologies.

There are critical developmental stages that must be reached to ensure proper function of the HPA axis and appropriate behavioral and physiological stress-responses in adulthood. The HPA axis begins to develop in around mid-gestation in the fetus with changes occurring into postnatal life. Prenatal exposure of the offspring to excess maternal GCs hormones alters neural, vascular, and neuroendocrine mechanisms. Fetal overexposure to maternal GCs could be caused by environmental stimuli in the maternal environment, such as maternal stressors (i.e. nutritional, immune, psychological stressors) Maternal stress can influence fetal development and lead to abnormal physiological function in adulthood, thereby increasing risk for adult diseases, such as mood disorders or cardiometabolic disorders[4].

In this chapter, we discuss the HPA axis as the essential regulator of various physiological responses to stressors. We also examine the role of sex steroid hormones during fetal and early development into adulthood that result in the sexually dimorphic responses of the neuroendocrine function in adults. The effect of environmental perturbations, such as maternal stress, on stress circuitry and its role in increased susceptibility to stress-related neuropathologies in adulthood is also addressed.

The physiology of stress and central regulation of the HPA axis

It is well established that animals and humans respond to threats to their welfare by activating neurons that control neuroendocrine and autonomic responses. The neuroendocrine response of the HPA axis is characterized by the secretion of GCs from the adrenal cortex. Circulating GCs act on a variety of tissues to mobilize energy stores, induce lipolysis and proteolysis, potentiate vasoconstriction driven by the ANS, suppress reproduction, and alter stress-related

behaviors, to allow homeostasis[5]. It is important that most of the physiological responses to acute elevations in GCs that occur following stressors, such as enhanced cognition and metabolism and inhibition of immune function are beneficial, as they permit the 'fight or flight' response. By contrast, although some benefits to chronic stress exist, chronic activation of the HPA axis has deleterious effects on immune, cardiovascular, metabolic and neural functions and may decrease the resilience of neurons and glia to subsequent insults[6-8], resulting in increased risk for a number of diseases. Whether these are direct or indirect effects of GCs remains to be determined.

Negative Feedback Circuitry

The HPA axis is governed by a GC-dependent negative feedback system that is essential for termination of the stress response. Normal HPA function is highly influenced by dose and duration of GC exposure[9]. For example, adrenalectomy decreases GC secretion, which increases PVN neuropeptide expression and secretion in both basal and stress-induced states[10, 11]. Negative feedback can also act at the level of the PVN, the anterior pituitary, and indirectly via brain regions that project to the PVN[12].

Since circulating GCs can bind to both GC receptor (GR) and mineralocorticoid receptor (MR), both receptors are involved in the negative feedback regulation of the HPA axis. MRs have a greater affinity for corticosterone (cortisol in humans)[13] and consequently, they are predominantly bound during low or basal secretion of corticosteroid[13]. Adrenalectomy increases basal CRH and ACTH levels, suggesting that a decrease in circulating corticosterone removes the negative feedback signal [14], whereas corticosterone replacement at doses that bind MR selectively, returns ACTH levels to baseline[15]. Moreover, the hippocampus (HIPP), a primary site for HPA regulation during basal states, expresses MR at elevated levels. MR antagonists administered directly to the rat HIPP elevate basal ACTH and corticosterone levels

like those seen after adrenalectomy[16]. Studies using transgenic mice that overexpress forebrain MR show reductions in the corticosterone response to restraint and decreases in anxiety-like behaviors[17]. Together, such data suggest that the ratio of MR:GR are as influential as absolute levels for regulating stress-induced HPA axis activity and stress-related behaviors.

In contrast to MR, GR has a lesser affinity for corticosterone and is thought to be the primary target for negative feedback when GC levels are elevated[13]. GRs remain mostly unoccupied during the basal state but are quickly occupied after a stress-induced increase in circulating GCs[13]. This supports the hypothesis that GR activation regulates the return of HPA activity to baseline following high amplitude secretion of corticosteroids after an acute stressor. Like MR, GR is highly expressed in the HIPP, as well as in the PVN and adenohypophysis[18]. Initiation of HPA axis negative feedback occurs via GR expressing neurons in the HIPP and the hypothalamus[19], following stress-induced elevations in corticosterone. The importance of GR in negative feedback regulation is further demonstrated using a transgenic mouse model. Selective knockout of forebrain GR causes an increase in basal and stress-induced corticosterone levels[20], further implicating GC binding sites in the forebrain. In comparison, Wei et al.[21] showed that the overexpression of forebrain GR does not alter basal ACTH or corticosterone levels, indicating that the forebrain GR is not the only player in regulating the baseline activity of HPA axis. Selective disruption of GR in the PVN increased CRH immunoreactivity in the PVN, with corresponding increases in levels of plasma ACTH and corticosterone, supporting the hypothesis that GR is involved in negative feedback[22]. Furthermore, GR has been reported to be absent in the suprachiasmatic nucleus, suggesting an alternate circadian-like feedback mechanism where GC influences HPA axis activity as discussed previously.

Limbic pathways regulate HPA axis

PVN neurons receive important information from a variety of limbic structures, including the Bed Nucleus of Stria Terminalis (BNST), a group of related subnuclei that directly project to the parvocellular PVN[23, 24]. BNST neurons express androgen receptor (AR) and estrogen receptor (ER)[25], and play a crucial role in gonadal steroid regulation of HPA function. Most of these neurons are GABAergic and their activity can be enhanced by collateral CRH afferents[26]. The BNST also contains CRH neurons that project to the PVN and contain ARs[27]. Lesion studies show that BNST GABAergic neurons inhibit CRH mRNA levels and inhibit the corticosterone responses to stress as does treatment with androgens[28-30]. However, not all BNST neurons are inhibitory since selective lesions of the anterior or lateral BNST can decrease ACTH secretion[31]. Nonetheless, the BNST represents an important modulating region that is GR- and gonadal steroid hormone sensitive.

Other limbic regions known to modulate stress responsive HPA axis activity include the HIPP, prefrontal cortex, amygdala (AMY), and lateral septum. These regions do not directly innervate the PVN but relay through areas such as the BNST or peri-PVN[8]. The glutamatergic projections from HIPP and prefrontal cortex are translated to inhibitory actions on the HPA axis via the GABAergic nature of these relays[32]. By contrast, AMY to BNST and peri-PVN projections are GABAergic. Thus, reducing the inhibitory tone on the HPA axis from GABAergic signals from BNST and peri-PVN regions is an effective mechanism to increase HPA axis activity[33].

Inputs from the brainstem regulate HPA axis

Direct inputs arising from the brainstem are essential to integrating HPA reactions to systemic stressors. Projections originating from noradrenergic and adrenergic neurons in the nucleus of

the solitary tract (NTS), locus coeruleus, and the ventrolateral medulla that innervate the parvocellular PVN have been identified [34, 35]. In the mouse, CRH neurons of the medial PVN receive noradrenergic innervation from A2 adrenergic cell groups of the NTS[36, 35]. The co-expression of alpha(1) and alpha(2) receptors in medial parvocellular CRH neurons[37] allows norepinephrine to rapidly increase *crh* mRNA[12]. Alpha(1) adrenergic receptors are responsible for the stimulatory effects of norepinephrine[37], whereas alpha(2) adrenergic receptors, specifically alpha(2A) and alpha (2C), are essential in inhibition of norepinephrine release at the presynaptic membrane[38].

Another circuit that strongly influences HPA responses to stress are projections from the median and dorsal raphe nuclei. Serotonergic fibers to the parvocellular PVN [39] stimulate the HPA axis. Serotonin (5-HT) 2C receptors have been implicated in 5-HT-induced activation of the HPA axis[40, 41]. However, 5-HT1A receptors have also been shown to increase ACTH secretion[42] and knockout of 5-HT1b receptors causes a 50% reduction in the diurnal rise in plasma corticosterone[43]. Restraint-induced elevations in ACTH and corticosterone can be increased by blocking 5-HT7 receptors[44], while 5-HT can inhibit GABAergic synaptic transmission at the PVN[45] providing evidence that the effect of 5-HT on PVN neurons vary depending on where the afferents terminate.

Neurodevelopment of the HPA axis

Morphology and development of the paraventricular nucleus (PVN)

The PVN, located in the ventral forebrain along the third ventricle, plays a critical role in regulating physiological processes, such as stress response, autonomic function, and hormone secretion. The PVN houses three functional neuronal types: parvocellular, neurosecretory magnocellular, and long-projecting neurons. These neurons are characterized by their unique

electrophysiological properties[46-48]. Parvocellular neurons display small low threshold depolarizations, which allow for rapid stress signaling and activation of the stress response. Dysregulation to parvocellular neurons could contribute to pathologies for psychiatric or endocrine disorders[49]. Magnocellular neurons do not display low-threshold potentials, but are characterized by a distinct return to baseline after a depolarizing stimulus[50]. Magnocellular neurons control homeostatic processes, including water balance or lactation/parturition. Consequences of impaired signaling of these neurons could include diabetes insipidus or issues with pregnancy or lactation[51]. Long-projecting neurons generate large low-threshold depolarizations and are important for the integration of stress signals to the brainstem to influence cardiovascular function, immune system activity, and energy metabolism. Disruption to these neurons could result in altered impaired autonomic function, contributing to conditions such as high blood pressure or metabolic syndromes[49].

Neurosecretory parvocellular neurons send their axons to the external zone of the median eminence to regulate secretion of releasing factors [e.g., corticotropin releasing hormone (CRH), thyrotropin releasing hormone] into the hypothalamo-hypophyseal portal vasculature to control the secretion of corresponding anterior pituitary hormones[52, 53]. An *anterior* parvocellular division extends from the rostral boundary of the PVN to the rostral boundary of the medial magnocellular division, just lateral to the periventricular area[49]. The *medial* parvocellular division lies lateral to the periventricular area and medial to the medial magnocellular division. Neurons in the anterior and medial parvocellular groups project to the median eminence or other hypothalamic and extrahypothalamic regions.

Neurosecretory magnocellular neurons project to the neurohypophysis to regulate secretion of oxytocin (OT) and arginine vasopressin (AVP) directly into the general circulation[54]. While magnocellular neurons are distributed into two distinct areas in the rat PVN, these areas are less distinguished in the mouse PVN. In the rat, the *medial* magnocellular division lies

anteromedially within the PVN and has mostly OT-expressing neurons. The *lateral* magnocellular division is comprised of AVP-expressing neurons that are surrounded by a loop of OT neurons. In the mouse, the magnocellular neurons are divided into four parts: anterior magnocellular, medial magnocellular, medial zone of the posterior magnocellular, and lateral zone of the posterior magnocellular part. OT-expressing neurons are located in the anterior magnocellular, medial magnocellular division, and medial zone of the posterior division. AVP-expressing neurons are found along the lateral edge of the medial magnocellular division and medial zone of the posterior division[49]. *Long-projecting* neurons send their axons to brainstem and spinal cord regions to control autonomic and somatosensory function. These neurons project to caudal medullary regions, such as NTS and rostral ventrolateral medulla, or spinal autonomic control centers, including the intermediolateral cell column[55].

The hypothalamus is derived from the antero-ventral neuroectoderm during early development[56]. Mapping of gene expression along the rostral-caudal axis shows that the early hypothalamic primordium differentiates into the floor plate, basal plate, and alar plate. The dorsal-most portion of the alar plate gives rise to the PVN and supraoptic nucleus and is identified by the expression of *Brn-2, Otp, and Sim1* genes and the absence of *Dlx*, *Arx*, *Gad67*, *Isl1*, and *Vax1* genes that are found in the subregion immediately below the PVN[56].

The transcription factor, *Brn-2* (POU-homeodomain protein BRIN-2), is endogenously expressed in both parvocellular and magnocellular neurons[57]. *Brn-2* null mutations in rodents show a failure to differentiate between CRH parvocellular neurons and OT and AVP magnocellular neurons, suggesting it is necessary for terminal differentiation of these hypothalamic cells[58].

The homeobox gene, *Otp,* transcribes a transcription factor that helps regulate differentiation and maturation of the neurosecretory PVN neurons expressing TRH, AVP, and OT. In mice, the

induction of a missense mutation in the *Otp* gene causes acute onset obesity and increased anxiety, phenotypes that have similarly been shown to be modulated by AVP and OT. Moreover, *Otp* seems to be necessary for regulating transcriptional activity of PVN neurons[59].

The *Sim1* transcription factor acts as another key regulatory gene of the PVN, encoding a protein that also regulates AVP, TRH, and OT expression, as well as CRH and somatostatin [57]. *Sim1* knock-out mice show severe loss of AVP, TRH, CRH, OT, and somatostatin neurons and rarely survive to adulthood [60], while heterozygous mice display early obesity, hyperinsulinemia, hyperphagia and hyperleptinemia, phenotypes that are associated with PVN neurosecretory neurons[60]. The Sim1 protein has been shown to dimerize with Aryl hydrocarbon receptor nuclear translocator 2 (ARNT2), which is thought to differentiate PVN and SON neurons. *Brn2*, a downstream target of the Sim1/ARNT2 dimer, also mediates Sim1 function. *Brn2* promotes the expression of AVP, OT, and CRH in the PVN, and decreased numbers of these cell phenotypes are seen in *Brn2* knock-out mice[58].

While the PVN is functionally conserved between species, from humans to rodents, the structure of it differs slightly. In the human, the PVN is subdivided into five subnuclei: the magnocellular subnucleus, parvicellular nucleus, dorsal subnucleus, posterior subnucleus, and anterior parvicellular subnucleus. In contrast, the PVN in rats consists of distinct magnocellular and parvocellular [61], but much less differentiated in the mouse[49, 62]. The magnocellular subnucleus and dorsal subnucleus in the human correspond to the magnocellular neurons in the rodent PVN and the parvicellular and posterior subnuclei are homologues to the same in humans[63]. Interestingly, the PVN in the human begins anterior to the fornix, forms ventrally and dorsally to the fornix, and extends into the posterior hypothalamus. In the rodent, the PVN sits medial to the fornix and ends rostral to the posterior hypothalamus[63]. Despite similarities in general function and pattern of chemo-architecture of the PVN, there are other interspecies

differences in the cyto-architecture and anatomical location that need to be considered when using rodents as model species for human studies.

Development of the blood-brain barrier of the PVN

The blood-brain barrier (BBB) is a critical interface that protects the central nervous system (CNS) from harmful toxins and other circulating compounds in the blood. Several cell types make up the BBB to maintain its integrity, including endothelial cells with tight junctions, astrocyte endfeet, and pericytes. Astrocytes regulate surrounding neuronal function and blood vessel activity by regulating the junctions between endothelial cells. Astrocyte end feet also help maintain proper ion concentration. Pericytes influence proper tight junction and astrocyte end feet distribution, stability, and structure of blood vessels. Pericytes can also modulate diameter and blood flow of capillaries and help clear tissue debris and toxins from the CNS[64], including Alzheimer's amyloid-β toxin in Alzheimer's Disease mice[65]. The BBB in the PVN unique because it is 3-5 times more vascularized than any surrounding area[66]. Vascularization of the PVN occurs during fetal development, around embryonic day (E) 9.5 in a mouse (~4-7 gestational weeks in humans)[67, 68]. Around E11-14 (7 gestational weeks in humans), angiogenic sprouting begins to occur from the perineural vascular plexus[69]. Around E13.5 (~6-7 gestational weeks in humans), the fetal BBB becomes capable of clearing wastes and toxins from the brain while keeping important metabolites in[69]. The development of the BBB continues postnatally with the help of upregulation of tight junction proteins, astrocytes, and pericytes. Improper development of the BBB can lead to infiltration of harmful substances from the periphery, potentially leading to downstream consequences on neurological health.

Morphology and development of the pituitary gland

The pituitary gland functions in response to releasing factors from the hypothalamus. The pituitary gland is divided into two structures: the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary)[18]. The adenohypophysis makes up 80% of the pituitary gland and houses specialized hormone-producing cells that synthesize and secrete several hormones, including, but not limited to, growth hormone GH, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone, prolactin, and ACTH. These hormones target diverse types of tissues to mediate several physiological processes in response to stress[70, 71]. Annexin-1 (formerly known as lipocortin 1) is another protein that exists in the anterior pituitary. While it is not directly involved in the synthesis of hormones discussed above, it is an important regulator of their secretion through inhibitory pathways.

The adenohypophysis can be further divided into the pars distalis, pars intermedia and the pars tuberalis. The pars distalis is composed of chromaffin and chromophobe cells and is where most hormone synthesis occurs. The pars tuberalis is an extension of the pars distalis and houses epithelial cells and the hypophyseal portal vessels that connect the anterior pituitary to the hypothalamus. The pars intermedia, found between the pars distalis and neurohypophysis, secretes products produced by the proopiomelanocortin (POMC) gene, particularly melanocyte-stimulating hormone [18].

In contrast to adenohypophysis, neurohypophysis is directly connected to the hypothalamus by axonal projections of magnocellular neurons originating from either the PVN or SON. The posterior pituitary stores OT and AVP synthesized by these neurons and secretes them into the general circulation in response to various hypothalamic releasing factors. OT is needed for lactation, while AVP is involved in regulation of osmotic balance [72, 6]. Peptide hormones synthesized in the SON and PVN travel along axons to their terminals in the posterior pituitary

where they are released into the general circulation in response to signals from their hypothalamic cell bodies.

The development of the pituitary gland is complex, yet unique because of its dual origin. In humans, during the fourth week of gestation, cells of the oral part of the ectoderm begin to thicken to form the hypophyseal placode[73]. The hypophyseal placode elongates to form Rathke's pouch. At six-eight weeks of development, the base of Rathke's pouch is separated from the oral epithelium. Rapid proliferation of cells of the anterior wall of the pouch forms the anterior lobe of the pituitary, (pars distalis) and slower proliferation of cells of the posterior wall give rise to the intermediate lobe or pars intermedia. By contrast, a specific part of the ventral diencephalon located dorsal to Rathke's pouch, gives rise to the infundibulum from which posterior pituitary originates[74].

The development of the pituitary gland is mediated by several cellular transcription factors. *Sonic hedgehog,* expressed in the oral ectoderm, and *bone morphogenic protein 4* and *fibroblast growth factor 8,* found in the ventral diencephalon, are all important signaling genes that initiate cellular proliferation of pituitary cells. These genes have also been shown to effect expression of transcription factors that contribute to differentiation of specific pituitary lineages, however specific mechanisms are not yet known[73]. Furthermore, the transcription factor, *Tpit,* is critical for the expression of the POMC gene. POMC is expressed in corticotrophs, the first pituitary cell type to terminally differentiate (about E12.5 in the mouse)[75]. A deficiency of *Tpit* blocks terminal differentiation, but not commitment to the corticotroph lineage[75]. Somatotrophs, lactotrophs, and thyrotrophs are differentiated through the influence of transcription factors, *Prop1* and *Pit-1 while* gonadotrophs require *GATA-2* and *SF1* signaling molecules for terminal differentiation. Although these terminal cell lineages are found in the pituitary gland, the existence of a common ancestral precursor pool is unclear [76, 71].

Morphology and development of the adrenal gland

The adrenal gland of adult mammals is surrounded by a fibrous capsule and is composed of two regions with differing embryological origins[77]. While the adrenal medulla, responsible for catecholamine production, derives from neuroectoderm, the steroid-hormone producing adrenal cortex has an embryonic origin from the adrenogonadal primordium[78].

The adrenal medulla is composed of chromaffin cells that secrete epinephrine and norepinephrine following sympathetic stimulation. They can be considered as a grouping of modified postganglionic neurons that are directly innervated by preganglionic neurons from the central nervous system[77]. Thus, the adrenal medulla is a vital component of the ANS and responds very rapidly to stressors, releasing epinephrine and norepinephrine into the bloodstream to affect heart rate, blood pressure, metabolism, and others[79, 80]. These hormones are classically involved in the 'fight or flight' response. The effects of epinephrine and norepinephrine on various physiological systems are emphasized by changes noted in patients with pheochromocytoma, a catecholamine secreting neuroendocrine tumor of the adrenal chromaffin cells[81]. Symptoms include sweating, heart palpitations, markedly elevated blood pressure, nausea, tremors and weight loss[82].

Histologically, the adrenal cortex is composed of three zones. The outer zona glomerulosa produces aldosterone, which participates in water and mineral balance through its actions on the kidney and colon[83]. The intermediate zona fasciculata is the thickest region of the adrenal cortex and synthesizes corticosteroids (primarily cortisol in the human, corticosterone in most rodents) and androgens. Similarly, the innermost zona reticularis also synthesizes adrenal androgens[84]. Of note, dehydroepiandrosterone (DHEA) is the most abundant circulating adrenal androgen in adult humans, while these are low in adult rats and mice[85]. Both the zona

reticularis and fasciculata are regulated by ACTH and in the absence of ACTH, these zones atrophy, while following chronic ACTH stimulation, these zones hypertrophy[86].

The adrenal cortex is derived from mesoderm and is dependent upon several transcription factors such as *steroidogenic factor-1* and *dosage-sensitive sex reversal-adrenal hypoplasia - 1*[87]. Deletion of either of these genes results in the absence of adrenocortical development in mice[87, 76, 88]. During human gestation, an inner fetal adrenal zone makes up the bulk of the adrenal gland, and an adult-like 'definitive' zone, a group of small tightly packed cells is also present[89, 90]. The human fetal adrenal responds to ACTH, but because of the absence of the 3-hydroxysteroid dehydrogenase enzyme, fetal adrenal mainly produces DHEA and DHEA sulfate[91]. These fetal adrenal steroids serve as precursors of maternal placental estrogens. The definitive zone is the major producer of fetal cortisol in response to ACTH stimulation.
By contrast, the developing rodent adrenal is quiescent. It is questionable whether the rodent adrenal has a fetal adrenal zone *per se,* although some studies indicate a transient fetal adrenal zone based on the presence of fetal adrenal enhancer elements[92]. While the adult cortex of rodents increases in size from late gestation through puberty, the fetal zone cells disappear gradually and accumulate along the boundary with the adrenal medulla[93]. However, even after the adult zones are developed, the adrenal gland of the rodent fetus does not yet express aldosterone synthase nor does it respond to stimulation by increasing mineralocorticoid or GC synthesis[94].

Development of the HPA axis during early life

During pregnancy, the stress response of the fetus is immature and relies heavily on inputs from the maternal and placental systems[95]. During late gestation, the fetus becomes capable of secreting its own CRH and ACTH in response to maternal stress, resulting in its own corticosterone production [95]. Basal levels of corticosterone during this time are similar to those

of adults[96], suggesting functional HPA axis activity. From P4 to P14, basal corticosteroid levels drop, accompanied by decreased ACTH and corticosterone production in response to stress[97]. This period is known as the “stress hypo-responsive period” (SHRP)[98, 99]. During this time, expression of GR and MR mRNA are significantly increased[98]. Accompanied by the lower levels of corticosterone, these changes are thought to dampen the HPA axis responses. Stress exposure during the SHRP induces a slight increase of expression of c-Fos mRNA in the PVN but does not influence ACTH or peripheral corticosterone secretion[100]. Such data suggests adrenal insensitivity may also play a role in the SHRP, but further mechanisms have not yet been determined.

Recent studies show that the SHRP can be maintained through the influence of maternal care [101-104]. Maternal care, quantified through observations of pup licking and maternal arch-backed nursing, are highly correlated with each other [105]. Dams categorized by levels of maternal care show causal relationship to epigenetic reprogramming that alters negative feedback sensitivity through changes in DNA methylation and histone modifications[106, 107, 102]. Several studies suggest this may be related to the transcription factor, nerve growth factor-inducible protein A (NGFI-A) which binds to a promoter region on exon 1-7 of *GR*[108] consisting of two CpG dinucleotides on opposing ends of the response element that are methylated on the day of birth. Offspring that received high maternal care show higher amounts of a demethylation of 5’ CpG sites in the NGFI-A binding region in adulthood[109]. Offspring that received low levels of maternal care displayed no change in methylation. Point mutation studies indicate that the NGFI-A binding strength and gene expression is determined by the presence of a methyl group at the 5’ CpG site, where a mutated 5’ CpG site resulted in increased transcription of *NR3C1,* the gene encoding GR [97]. These studies support the actions of low maternal care to program increased GR expression and increase feedback sensitivity of adult offspring[110, 108].

Additional studies show decreased corticosterone and ACTH responses to acute stress in adulthood of high maternal care-exposed offspring. Such data further supports epigenetic reprogramming of the 5' CpG site. A genome analysis of chromosome 18 containing *NR3C1* found that varying amounts of maternal care correlated with changes in protocadherin loci [101] which regulate development of the CNS [101, 97] thereby implicating maternal care during the SHRP for proper brain development. However, it is important to note that the promoter region on exon 1-7 of *GR* only accounts for about 1% of all GR mRNA transcripts in the HIPP. Reports examining promoter region on exon 1-7 of *GR* methylation found that upregulation of NGFI-A did not alter stress-induced activation of the promoter region on exon 1-7 of *GR* transcription or total expression of GR[111]. Consistent with this, Witzmann et al. [112] showed low methylation levels at the 5' CpG site following acute stress, further indicating that NGFI may not actually drive the promoter region on exon 1-7 of *GR* transcription nor play a role during the acute stress response[112]. Thus, while epigenetic reprogramming has been shown to be altered through maternal care, specific mechanisms in which this occurs are still unclear.

Maternal separation during fetal development is another variable that influences HPA axis development and adult patterns of stress-reactivity[113, 114, 104]. Prolonged separation from the dam is associated with a hyperactive HPA axis and increased anxiety- and depressive-like behaviors in adult offspring[108]. In contrast, brief separation increases maternal attentiveness to pups, resulting in better attenuation of the stress-response[115]. Rodent studies have demonstrated that these changes are partially a consequence of alterations in the dopaminergic system since prolonged maternal separation caused decreased dopamine uptake associated with changes in dopamine transporter expression [116]. This is thought to lead to increased stress-induced dopamine activity resulting in a hyperactive HPA axis. 5-HT signaling has also been shown to be altered by maternal separation with decreased metabolism in the AMY and enhanced concentrations of 5-HT and associated metabolites in the dorsal raphe nucleus and

cingulate cortex[117]. Long-term consequences include altered function of 5-HT receptors and transporters, as well as decreased expression of 5-HT receptor subtypes in the prefrontal cortex and hypothalamus[118]. These changes correlate with increased anxiety- and depressive-like behaviors, suggesting that a signaling pathway linking the dopaminergic and serotonergic systems with stress responses exists, however, specific mechanisms have yet to be elucidated.

Paternal influences on the stress axis of adult mice have also been reported. Males exposed to 6 weeks of chronic variable stress prior to breeding had offspring of both sexes with reduced HPA axis activation to acute restraint in adulthood[119]. This correlated with gene expression changes, such as OT or AVP changes[120], in the PVN and BNST of offspring suggesting the possibility of epigenetic reprogramming through the male lineage. Studies have also investigated paternal retrieval and grooming effects in offspring. Testosterone levels were decreased in offspring from rats with increased paternal retrieval, as was AVP expression in the BNST, and this correlated with reduced aggressiveness in social interaction tests, such as the resident-intruder test[121]. Such data suggests an important hormonal link between paternal care, testosterone levels, and aggression. AVP immunoreactivity in the PVN was also found to be increased with reduced paternal care [119], correlating with increased stress-induced corticosterone secretion, further linking paternal care and HPA axis responses. Nonetheless, specific mechanisms of how paternal transmission to the offspring occurs have yet to be elucidated.

The development of HPA axis at puberty

Puberty is a unique developmental event, influenced largely by the maturation of the hypothalamic-pituitary gonadal axis, which is responsible for gonadal maturation and adult hormone secretory patterns[122]. Some reports also suggest that this represents a second critical period for organizational actions of gonadal hormones that further sculpt the HPA axis

into its adult-like characteristics[123]; [see review [124]for more thorough analysis of age-dependent changes in the HPA axis].

Importantly, HPA axis reactivity is significantly greater prior to puberty than following puberty. Rat studies in males show increased and prolonged stress-responsive release of ACTH and GC prepubertally in comparison to post-pubertal animals[125]. Similarly, the stress-induced activity of CRH neurons in the prepubertal PVN is greater than that of adults, demonstrating that the prolonged prepubertal pattern of corticosterone and ACTH may be driven by increased hypothalamic CRH synthesis[125] and altered by the onset of male puberty. These findings indicate prenatal exposure to GCs impair negative feedback in prepubertal males[125].

Studies in pre-pubertal male rodents show elevated HPA activation, with increases in CRH activation following restraint in comparison to adults, indicating that PVN CRH neuron activity changes across puberty[123]. Following a corticosterone injection, Romeo and McEwen[123] showed increased GR expression in regions of the brain, such as HIPP, AMY, and prefrontal cortex, in adolescents compared to adults. This observation further indicated that puberty represents a critical period during development that renders the brain more vulnerable to environmental perturbations and increases risk to HPA-related neuropathologies[125]. The changes in the HPA axis do not appear to be the consequence of pubertal rises in testosterone[126]. However, because the initial increase of gonadotropin-releasing hormone secretion and kisspeptin occurs near the onset of puberty, one possibility is that changes in the HPA axis observed across puberty are preprogrammed developmental events that are independent of changes in gonadal hormones[126].

Some reports further suggest that the pubertal rise in estradiol may also play a role in shaping the adult HPA axis. Studies in pre-pubertal females show an inhibitory effect of estradiol on stress induced HPA axis function, while estradiol treatment in post-pubertal females show a

stimulatory effect of estradiol during the acute stress response[127]. Further, regardless of whether females were ovariectomized before or after puberty, administration of estradiol consistently elevated basal and stress-induced GC secretion, as well as GC pulse amplitude and frequency [127]. Data suggests there is a reversal effect of estradiol on HPA axis function during puberty where estradiol is inhibitory prior to puberty and stimulatory post-puberty, implying an estradiol-independent mechanism in the development of the HPA axis during puberty in adult females[127].

Maternal-fetal interactions on the HPA axis

The development of the fetal brain begins *in utero* and is highly malleable to influences in the maternal environment. Fetal exposure to maternal stressors alters fetal development, leading to long-lasting outcomes on neurological and behavioral health in adulthood. Because the PVN of the HPA axis is a key player in stress response, dysregulation of PVN function is highly linked to many stress-related diseases in adults, such as Major Depressive Disorder (MDD), heart disease, and metabolic syndromes[128]. Many studies further indicate sex differences for these disorders arise during prenatal development and can be influenced by adverse events in the maternal environment (Figure 1.1). For instance, females are twice as likely to develop MDD, while males are two-fold more susceptible than premenopausal females to heart attacks and stroke. Interestingly, the comorbidity of MDD and cardiometabolic disorders is twice as high in women compared to men[128].

Maternal stress leads to increased endogenous GCs that can cross the placental interface. A key mediator of fetal exposure to maternal elevations in GCs is the placenta. The placenta is enriched with the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which

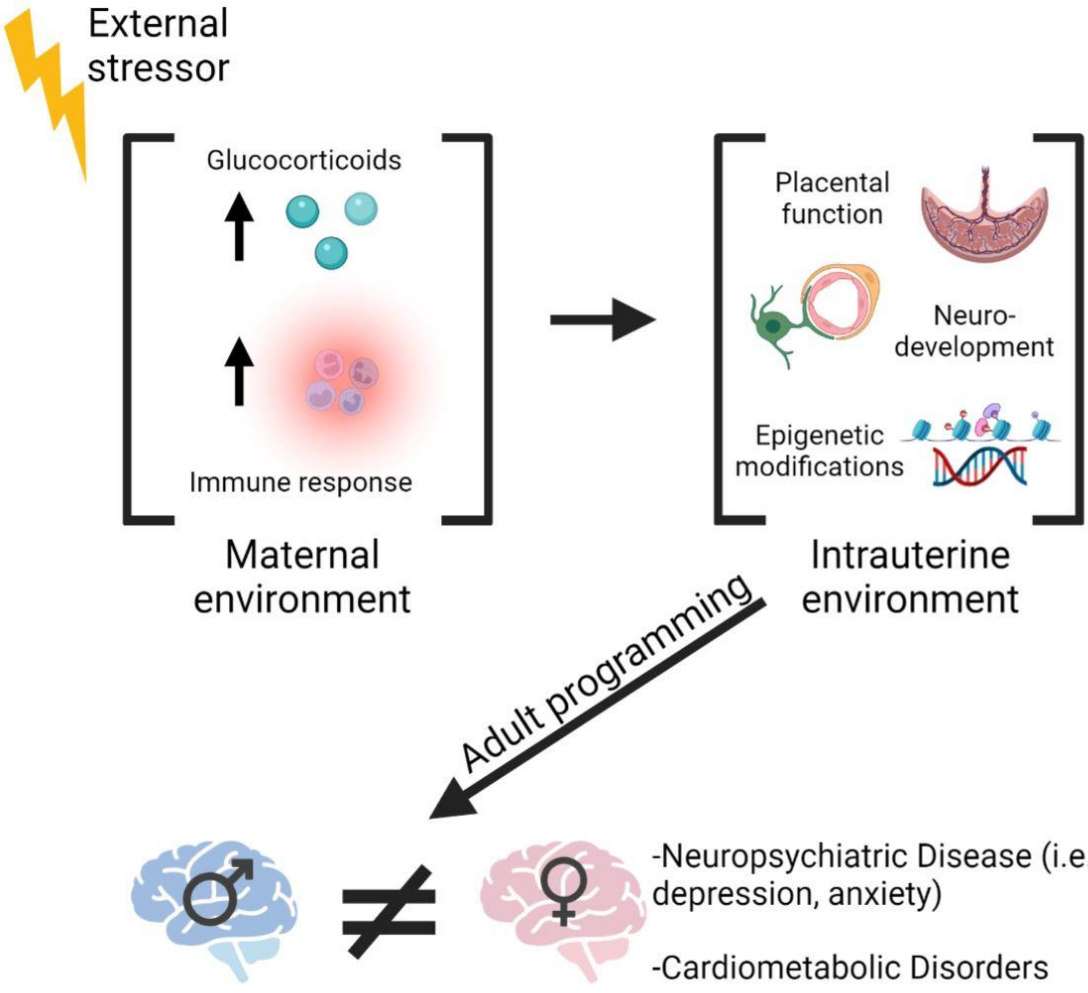

Figure 1.1 Maternal stress can lead to changes in the maternal and intrauterine (fetal) environments, resulting in adult programming and higher risk for adult neuropsychiatric disorders. Environmental perturbations in the maternal environment causes increased GC production and maternal immune response with increased pro-inflammatory cytokine release. These changes in the mother can influence placental function, neurodevelopment, and epigenetic modifications in the developing fetus with long-term consequences in brain health.

oxidizes GCs into inactive 11-keto derivatives. Synthetic GCs, including dexamethasone (DEX), are widely used in the clinic in women at risk for preterm labor. DEX is not oxidized by 11β-

HSD2, allowing it to freely bypass the placental interface to the fetus. Previous studies in the lab used DEX as a GC analog to stimulate the stress response in pregnant rodents and examine longitudinal effects on offspring. Male and female offspring of rodent dams prenatally treated with DEX exhibit decreased body weights, and sex-dependent changes in neuroendocrine stress response, and stress-related behavior impairments[129, 130]. However, more recent information suggests administration of synthetic GC, DEX, is not an ideal model of maternal stress. DEX escapes inactivation at the placental-fetal interface by the enzyme 11β-HSD2 and binds to GR at a much higher affinity and potency than endogenous GCs, such as cortisol or corticosterone[131-133]. DEX does not simulate a normal physiological stress response as other stressors, such as nutritional and immune stress, making it an insufficient model of maternal stress.

Maternal Nutritional Stress

Maternal nutrition is a critical intrauterine environmental factor that influences fetal programming. Proper brain development of the fetus requires much of the available nutrients and energy stores during gestation. The main source of energy comes from the breakdown of carbohydrates into glucose. Therefore, insufficient intake of proper nutrients during pregnancy can increase risk for metabolic and neurodevelopmental disorders in offspring through alterations in neural cell proliferation, differentiation, migration, and cell death programming[134, 135]. Growing evidence additionally shows maternal diet alters fetal gene expression through epigenetic changes, such as histone modifications and DNA methylations[136]. These epigenetic modifications can contribute to the remodeling of neural circuities and signaling in the brain. Therefore, it is important to examine the effect of maternal malnutrition, both under- and over-nutrition, on brain, endocrine, and metabolic dysfunction.

Common animal models of maternal undernutrition include caloric and protein restriction. Caloric restriction (CR) models, relevant to cases of famine, does not change the composition of macronutrients in the diet but does allow for different severities of undernutrition from mild (15-30% restriction) to moderate (50% restriction) to severe (70%) restriction of total calories[137]. Mild restriction does not appear to influence the birth weight of pups, while moderate and severe CR models result in smaller pups [138, 139]. In rodent studies, maternal CR also resulted in higher expression of GR in the offspring hypothalamus, a hyperactive HPA stress response, and stress-related behavioral symptoms postnatally[138, 140]. Taken together, data suggests a potential correlation between restricted caloric diet, smaller offspring, and dysregulation in stress circuitry. Insufficient macronutrient intake during pregnancy also influences neurodevelopment[141]. Protein is one of the more expensive macronutrients to obtain, especially in developing countries. Therefore, many maternal diets consist of poor-quality fats and carbohydrates and low protein intake. In rodent studies, the low protein diet is 8% protein compared to the normal 20% protein levels [142]. Offspring from low protein diets are born small with less weight gain into adulthood, suggesting significant metabolic programing by the maternal diet[139]. On the other hand, maternal overnutrition models examine patterns of the “Western diet,” typically comprised of junk food or food high in fats. In high fat diet (HFD) studies, rodents are fed a chow that is 60% fat (lard, soy) while control diets consist of about 7-20% fat [143]. Dams placed on a HFD are found to produce larger offspring at birth with metabolic changes (i.e., weight gain, glucose) [144].

While the paradigm in under- and over-nutrition models differ, these maternal malnutrition models produce shared neuroendocrine and behavioral phenotypes in the offspring. These pathologies include altered stress response circuitry and stress-related behaviors. Such behaviors include anxiety- and depressive-like, social impairments, and cognitive and memory dysfunction, pathologies indicative of adult neuropsychiatric disorders[134].

Maternal immune activation

Models of maternal immune activation (MIA) provide evidence of neurodevelopmental programming that can increase risk for neuropsychiatric diseases, including MDD, schizophrenia, autism spectrum disorders (ASD), and cardiometabolic syndromes. Cytokines are important for proper fetal brain development, such as neuron proliferation and migration, synaptogenesis, and vascular formation. Abnormally high levels of cytokines and other inflammatory mediators cross the placental barrier and exert programming effects on the fetal brain, disrupt important neural or vascular mechanisms, and increase risk for neuropsychiatric disease. Clinical evidence demonstrates elevated pro-inflammatory cytokines are found to be linked to MDD. Higher levels of maternal serum IL-6 was found to alter white matter structural changes in the limbic cortex of offspring within the first year[145] and impair development in the AMY of newborns with sensory and cognitive behavioral phenotypes found as early as 2-years old[146]. A more longitudinal study demonstrated offspring exposed to elevated pro-inflammatory cytokines *in utero* presented with changes in hypothalamic neural responses and greater sensitivity to stress in adulthood[103]. Taken together, data suggest prenatal exposure to elevated cytokines can significantly alter important brain regions that are important for stress response and stress-related behaviors. Improper development of these areas can further increase risk for pathologies of MDD and other mood-disorders.

Rodent models are a useful tool to examine the effect of MIA on neurodevelopment at a more neurobiological and mechanistic level. We can use animal models to look at molecular and cellular changes and evaluate associated behavioral and physiological pathologies. The two main types of MIA involve stimulating the maternal immune system with a toll-like receptor (TLR) agonist, such as a bacterial pathogen (lipopolysaccharide, LPS) or viral mimetic [Polyinosinic: polycytidylic acid (poly I:C), Resiquimod (RQ)]. A significant limitation of using LPS or Poly I:C is that the data is not fully translatable to humans since they only activate

certain components of bacterial or viral responses through TLR 4 and TLR3 pathways, respectively. TLR4 is expressed in microglia, a resident immune cell of the brain in rodents but not humans[147] and the promotor sequence and mechanistic pathways of TLR3 in rodents is not conserved with humans[148]. Despite this, Poly I:C has still been one of the most widely used viral molecules to investigate how inflammatory conditions in the maternal environment are linked to higher risk of neuropsychiatric disease in offspring. Numerous reports show exposure to poly I:C during mid-gestation leads to several neurodevelopmental changes in offspring, including disrupted neurogenesis, higher microglial activation, impaired sociability and cognition, and depressive- and anxiety-like behavioral phenotypes[149-152].

However, many viruses associated with neuropsychiatric disease in adults (i.e. SARS-CoV-2, influenza) are single-stranded RNA molecules that activate TLR7[153]. Therefore, I used a TLR7 agonist, RQ, in my studies as a more translatable mouse model of MIA to humans. TLR7 is especially interesting because its gene is located on the X-chromosome and can escape X-inactivation, leading to higher expression in females. The sex-dependent nature of many neuropsychiatric disorders that are linked to MIA further emphasizes the importance of understanding the role of TLR7 activation during pregnancy on offspring development.

As discussed earlier, the central relay station for HPA axis is the PVN. The PVN is sexually dimorphic and regulates neuroendocrine and behavioral functions liked to stress-related pathologies. Therefore, improper development and/or regulation of the PVN could increase susceptibility to stress-related disease. The PVN is protected from toxins and harmful compounds from the circulatory system by its BBB. Improper function of the BBB in the PVN can lead to detrimental consequences to neuronal health and stress regulatory systems. Previous studies in the lab further demonstrate maternal stress with elevated GCs can actually impaired the integrity of the BBB in the PVN and lead to depressive-like phenotypes in offspring mice[154]. Other studies show similar phenotypes with increased permeability of the BBB in

other brain regions (i.e. HIPP, prefrontal cortex, nucleus accumbens) and behavioral phenotypes with prenatal exposure to maternal stressors[155, 156, 69]. However, there are less reports examining maternal immune stress and brain vascular development as a mechanism for stress-behavioral phenotypes. Therefore, the next chapters of my thesis will examine how maternal stressors lead neurodevelopmental programming of prepubertal to adult (post-pubertal offspring).

I hypothesize maternal stress leads to changes in PVN neuroendocrine function, BBB changes, and stress-related pathologies in offspring. Given the vast literature on sex differences in pathologies for neuropsychiatric disorders, I also hypothesize these changes might occur in a sex-selective manner. In my studies, I first evaluated several models of maternal stress (maternal HFD, maternal CR, maternal exposure to synthetic GCs). Although there were vast discrepancies between each type of maternal stress, one similarity was an activated immune response with elevated maternal cytokines. Our collaborators at Mass General Hospital, Dr. Jill Goldstein's group, also demonstrated in a longitudinal human study that increased pro-inflammatory cytokines led to changes in brain activity in stress-related regions. Therefore, my next aim was to characterize a model of MIA to recapitulate the cytokine profiles of Dr. Jill Goldstein's group using a TLR7 agonist, evaluate developmental milestones and stress neuroendocrine function. My last aim was to examine stress-related behaviors, including social-, anxiety- and depressive-like behaviors in prepubertal and post-pubertal offspring. Because these behaviors are pathologies of a dysregulated stress response system, I then determined if there were structural changes to the central regulator of the stress response, the BBB of the PVN of adult offspring.

CHAPTER 2:
EVALUATING DIFFERENT MODELS OF MATERNAL STRESS ON STRESS-RESPONSIVE SYSTEMS IN PREPUBERTAL MICE[1]

Maternal adversity during pregnancy influences neurodevelopment in human and model animal offspring[128] [157]. Adversity can result from stressors coming from many different directions ranging from environmental to nutritional and physiological to immune (e.g., infection). Most stressors result in fetal overexposure to GCs [corticosterone in rodents, cortisol in humans] that have been directly linked to long- and short-term negative impacts on neurological health of offspring[5]. Normally, the placenta protects the fetus from circulating GCs via the enzyme, 11β-HSD2. However, when there are sustained high levels of GCs, 11β-HSD2 cannot keep up the conversion of active to inactive GCs, leading to fetal overexposure[158, 159]. Neuropsychiatric diseases postulated to have fetal origins are diverse and include such things cardiovascular disease, obesity, affective disorders, and metabolic and immune disorders[128].

Responses to stress are driven by components of the HPA axis. The neuroendocrine pathways and feedback loops of the HPA axis result in the stimulated release of GCs from adrenal glands[160]. As physiological modulators, GCs stimulate adaptative changes to physiological demands resulting from external stressors and regain homeostasis. Regardless of the nature of maternal stress, one common critical impact is HPA axis activation and the release GCs that can influence developing fetuses. A frequently used model for evaluating fetal stress has been to bypass the complicated nature of the stressors and simply increase fetal GC stimulation [161]. Given there is questionable access of endogenous GCs across placentas, studies often

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administer the synthetic GC, DEX during periods of gestation. Evidence suggests that there may be negative long-term consequences of prenatal DEX exposure. Male and female offspring of rodent dams prenatally treated with DEX exhibit decreased body weights, sex-dependent changes in neuroendocrine and autonomic stress responses, and stress-related behavioral impairments (e.g., social-/anxiety-like behavior) [162-168]. However, prenatal DEX is a complicated model because DEX inhibits multiple aspects of the immune system and MIA is an important aspect of maternal stress. The current study investigated other approaches to promote inflammation that could influence the maternal-fetal environment[139] and compare them to fetal DEX exposure.

Two frequent models of maternal stress include nutritional stress by HFD or CR [143, 169]. These exposures have been linked to behavioral alterations indicative of attention deficit hyperactivity disorder (ADHD), ASD, anxiety, and depression[143, 170, 134]. In rodents, HFD during pregnancy increases anxiety-like behaviors in adolescent males and females. Inflammatory co-activators of the immune-stress axis (e.g., IL-6, NFκB, CD11b) were additionally increased in the HIPP and AMY with HFD in male and female juvenile offspring. These data indicate maternal nutritional stress alters neurobehavioral and immune responses in rodent offspring, but not all necessarily in a consistent manner between and even within the same models[171]. Many prenatal CR studies in rodents show increased anxiety-like behavior in male and female adult offspring [172, 173], while others show decreased anxiogenic behavior in adult offspring [174, 175] or no changes in anxiety-like behavior with improved, memory performance, novelty-seeking and locomotor activity in adult male offspring [176].

Sex differences in the onset of neurodevelopmental disorders may arise due to the impact of gonadal steroid hormones, sex chromosomes or other gene-dependent mechanisms[177, 178]. In rodents, many studies focus on behavior disorders after puberty, and there are less data available during the early adolescent periods when behavioral symptoms of adult

neuropsychiatric disorders may begin to develop [179]. The current study examines prepubertal juvenile (28 days of age) male and female mice to determine whether sex differences develop prior to the emergence of significant pubertal gonadal steroids. The experiments in the current study directly compare the prenatal exposure to DEX model to nutritional stress models; maternal HFD and maternal CR. The effects of these modes of maternal stressors were examined to evaluate HPA stress responsiveness and stress-related behaviors in in prepubertal male and female mice (P21-28). Specifically, we measured social- and anxiety-like as our stress-related behavioral outputs since these disorders are thought to have fetal origins influenced by maternal stress.

Methods

Maternal stress models

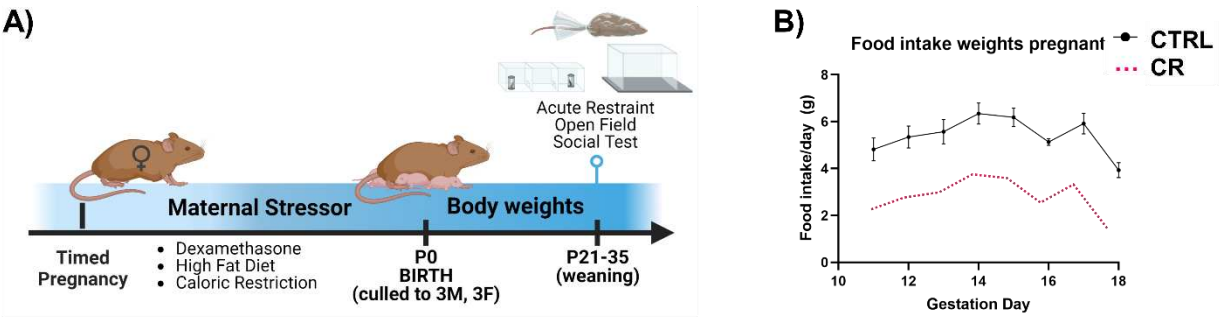

Figure 2.1. Experimental timeline. (A) Pregnant females were randomly assigned to one of the following types of maternal stress model: injection of dexamethasone (DEX; gestation day 15-18), high fat diet (HFD; gestation day 0 to parturition), or caloric restricted diet (CR). (B) The CR diet was 50% of the average food intake of the control (CTRL) females from gestation day 11 to parturition. N = 6-8dams/treatment, 1-2 mice/sex used for each outcome.

Timed-pregnant C57BL/6N females were exposed to one of the following types of maternal stress described below (N = 8 dams/treatment and control group for each maternal stressor; each model of maternal stress had its own control group). All litters were culled to 3 females and 3 males at birth. These methods maintained controlled litter size of 6 and prevent variability in

nutritional differences from biasing data. One to two offspring of each sex per dam was used in each treatment group, with a total of 7-9 mice/sex in each group. Offspring were weaned at postnatal day (P) 21 -28 to begin testing for anxiety-like and social-like behaviors, followed by acute physical restraint to examine corticosterone responses to stress (Figure 2.1). Pups were weighed weekly until euthanasia. Mice were housed with *ad libitum* access to food (unless otherwise stated) and water and on a 12:12 light: dark cycle (lights on at 06:00 and off at 18:00). Restraint, blood collections, and behavior testing were performed during 09:00 and 15:00 to avoid diurnal elevations in plasma corticosterone. Behavior assays were performed from least stressful to most, ending with restraint. All subject animals were gonadally intact at the time of testing. All mice were euthanized by inhalation of 30-70% carbon dioxide delivered in a sealed chamber until breathing ceased, consistent with Colorado State University's Institutional Animal Care and Use Committee and AVMA Euthanasia Guidelines. This was followed by exsanguination by intracardial perfusion with phosphate saline buffer and 4% buffered paraformaldehyde to fix tissues or decapitation using sharp scissors according to AVMA approved methods. All procedures were approved by Colorado University Lab Animal Resources and Institutional Animal Care and Use Committee Guidelines.

Dexamethasone - Timed-pregnant females treated with DEX dissolved in 25% beta-cyclodextrin in 0.9% saline (Subcutaneous injection; 0.4 mg/kg) or vehicle (VEH) (25% beta-cyclodextrin in 0.9% saline; control group) on gestation day (GD)15-18[180, 165].

High Fat Diet (HFD) - Timed-pregnant females placed on control diet (Envigo TD.2919) or 60% HFD (Envigo TD.06414) from beginning of pregnancy (GD0) to parturition[181]. Females were switched back to control diet after birth to specifically tease out behavioral outputs due to nutritional stress *during* gestation without the confounding variable of the rearing/lactation period.

Caloric Restriction (CR) - Timed-pregnant females underwent CR compared to controls from GD11 to parturition[182]. Daily food intake was measured in control pregnant dams (in grams) and then CR dams received 50% of the measured amount (Figure 1). Control dams were allowed *ad libitum* access to food. Both groups were allowed *ad libitum* access to water. All dams were placed back on *ad libitum* access to food and water at parturition to parse out effects due to effects *during* gestation without potentially altering behavioral outputs that can occur if the mother was kept caloric restricted throughout lactation. Cannibalism of neonate pups was also tracked, with no significant differences between caloric restricted and control mothers.

Behavioral Assays

Social Interaction Test

Subjects were placed in a 3-chamber apparatus with an empty wire-mesh cage on opposing ends as previously described[6]. Subject were then allowed to habituate in the apparatus. After 10 minutes, an unfamiliar age- and sex-matched stimulus mouse was placed under a wire-mesh cage. The opposing wire-mesh cage remained empty. To examine social discrimination behavior of juvenile offspring, we measured the time spent investigating stimulus mice versus empty wire-mesh cage. The cups and 3-chamber apparatus were cleaned with 70% ethanol and dried prior to and following each test. All behavior trials were video-recorded, and analysis and animal position tracked by Ethovision software (Noldus Information Technologies). Sociability was analyzed by examining the duration and frequency of visits the subject mice spent investigating the wire-mesh cage containing the novel stimulus mice. Social interaction test results were analyzed statistically using a two-way ANOVA (maternal stress X offspring sex) using GraphPad Prism (GraphPad Software, La Jolla, CA).

Open Field

Activity was assessed by placing an experimental mouse in the center of a circular open field area made of Plexiglass (height = 30 cm, radius = 20 cm; area = 1.26m^2) as in previous studies[183]. Mice were left undisturbed for 10 minutes and returned to their home cage following the test. Tests were performed on one mouse at a time. The arena was washed with 70% ethanol and water and dried to eliminate odors between each subject. The time spent in the center ring and total distance traveled were measured. The total time spent in the outer ring (closer to the wall of the arena) was also measured, demonstrating the same output in anxiety-like behavior trends as the time spent in the center ring (data not shown).

Corticosterone measurements and acute restraint

The offspring underwent 20-minute acute restraint inside a spatially constricted tube with 60-minute recovery in their home cage. Restraint is considered a mild psychological stress. The animal was not completely immobilized, but movement was restricted in restraint tube. A breathing hole was located at the end of the tube where the nose reaches. The animal had no more than a centimeter of movement on all sides. There were also holes along the lateral sides of the restraint tube for more adequate ventilation. All restraint stress was performed between 09:00 and 14:00 to avoid diurnal elevations in corticosterone. Tail blood was collected at three timepoints during restraint: 0-minutes (prior to restraint), 20-minutes immediately after acute restraint, and 60-minutes after released from restraint and allowed to recover in home cage. About 15 microliters of blood will be collected to obtain at least 10 microliters of plasma for enzyme-linked immunosorbent assay. After the 60-minute recovery in home cage, the animal was anesthetized by inhalation of 30-70% carbon dioxide delivered in a sealed chamber until breathing is ceased and euthanized by intracardial perfusion[68]. Plasma corticosterone levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) per manufacturer's

guidelines (Arbor Assays, Ann Arbor, MI; cat no. K014-H1; Limit of detection 7.7 pg/mL mean intra-assay CV = 8.5%). Briefly, tail vein blood (collected at the 3 timepoints described above) was placed into chilled tubes with 0.5M EDTA and aprotinin (4mg/mL; Sigma-Aldrich, St. Louis, MO), then centrifuged in a Beckman J6 centrifuge at 2000 rpm at 4 ºC for 10 minutes. After the plasma was separated, it was stored at -20º C until assayed. At the beginning of the assay, plasma samples (5uL plasma per sample well, every sample was run in a duplicate) were prepared with Dissociation Reagent to dissociate the corticosterone from corticosteroid binding globulin. 50uL of each plasma sample was used (every sample was run in a duplicate). A standard curve was prepared from increasing dilutions (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.063, and 19.531 pg/mL) of corticosterone. DetectX Corticosterone Conjugate and DetectX Corticosterone Antibody were added to each well. Tetramethylbenzidine solution was next added to each well. The optical density of each sample was determined with a wavelength of 450 nm in Azure biosystems Ao microplate reader (Azure Biosystems, Inc, Dublin, CA). To calculate the concentration of corticosterone, the duplicate optical density readings for each standard and sample were averaged. A standard curve was generated using the online tool from “MyAssays” through Arbor Assays. The sample concentrations were calculated from the %B/B0 curve and multiplied by the dilution factor to obtain the neat sample values.

Statistical analysis

Results are presented as means ± SEM. Data were analyzed using Prism (GraphPad Software Inc., La Jolla, CA). For each significant three-way ANOVA, *post hoc* comparisons were made using Tukey’s method for the comparison of all groups *vs.* the control group for multiple comparisons. Results for corticosterone assay were analyzed statistically by three-way ANOVA (Treatment X restraint x sex) using GraphPad Prism (GraphPad Software, La Jolla, CA). Bonferroni’s correction factor was used for post-hoc analysis. Significance was set at $p < 0.05$.

Results

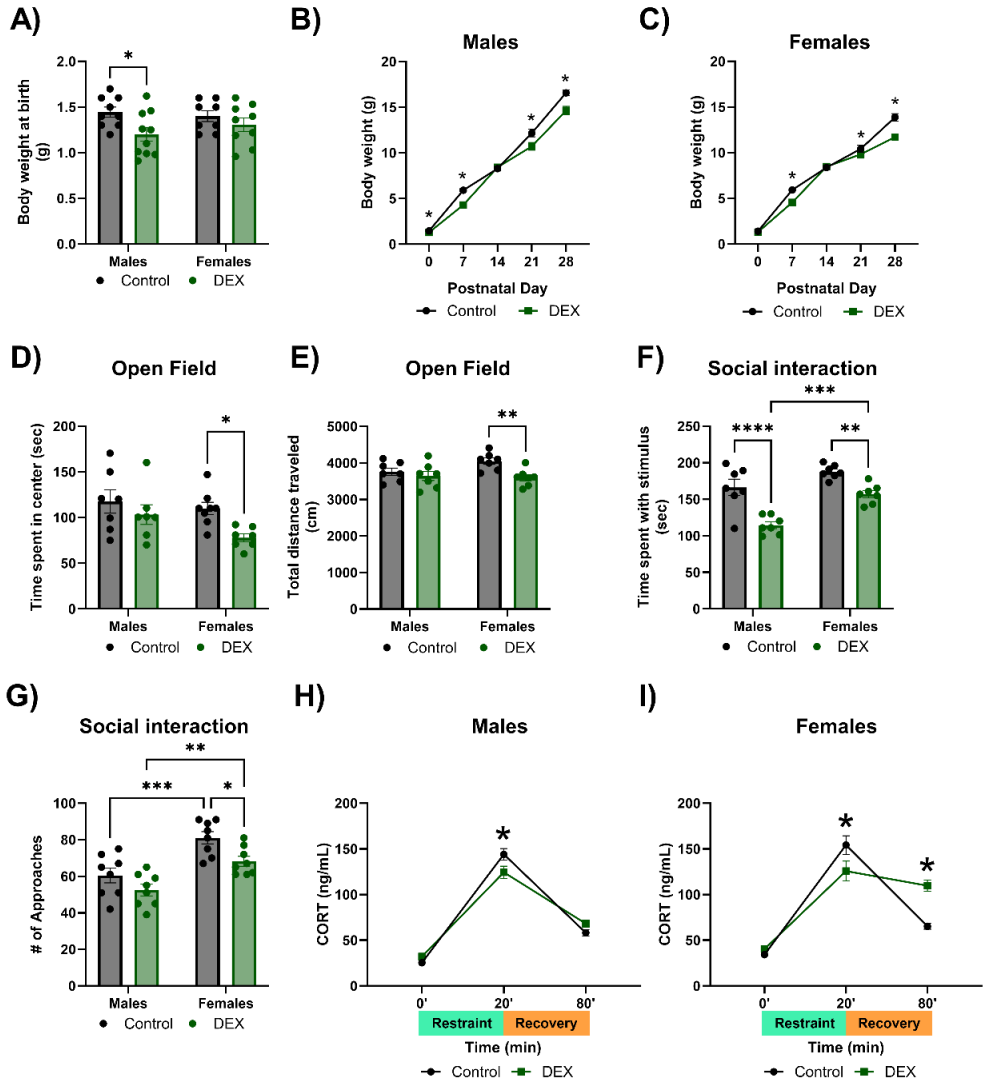

Figure 2.2. Maternal dexamethasone (DEX) resulted in altered body weights, behavior, and stress response in prepubertal male and female offspring. (A) Neonatal body weights at postnatal day 0 (P0) were decreased in males, but not females, exposed to prenatal DEX. Over time (B) males and (C) females demonstrated decreased body weights from P7-P28. Open field assay revealed increased anxiety-like behavior in females as measured by (D) time spent in the center and (E) total distance traveled. No changes were seen in males. Social-like behavior was impaired in offspring. Male and female pups exhibited decreased (F) time spent with and (G) and frequency of visits to stimulus mouse. Acute stress response was decreased in (H) males and (I) females exposed to prenatal DEX immediately after 20-minutes of restraint as measured by plasma corticosterone. Prenatal DEX females further exhibited prolonged increases of corticosterone at the 80-minute timepoint (60-minutes after removal from restraint), indicating delayed negative feedback to HPA axis stress response. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 DEX vs. Control.

Maternal treatment with dexamethasone

There were several characteristic changes in juvenile male and female offspring from mothers treated with DEX during the last 4 days of gestation. Weekly assessment of body weights in offspring from treated mothers indicated a decrease related to maternal DEX that was noted selectively in males (Figure 2.2A-C) [$F(1, 32) = 6.002$, $P = 0.02$]. Using an open field assay to assess anxiety-like behavior, 2-way ANOVA revealed female, but not male offspring, from mothers exposed to prenatal DEX, exhibited less time spent in the center ring and total distance traveled compared to controls (Figure 2.2D-E; $p < 0.01$). This implies increased anxiety-like behavior selectively in female offspring of mothers exposed to DEX during gestation. Social behavior was impaired in offspring of mothers treated with DEX [$F(1, 28) = 9.591$, $P = 0.004$] in both sexes (Figure 2.2F-G). Post-hoc analysis revealed male ($p < 0.01$) and female ($p < 0.01$) offspring of mothers treated with DEX spent less time investigating stimulus mice. Prenatal DEX offspring also exhibited a lower frequency of visits to stimulus mice compared to control offspring ($p < 0.01$). Plasma corticosterone levels as a measure of HPA axis stress responsiveness altered by prenatal DEX treatment in males [$F(2, 36) = 269.1$, $P < 0.0001$] and females [$F(2, 39) = 116.1$, $P < 0.0001$]. Peak levels were lower after 20-minutes of acute restraint stress in prenatal DEX exposed males ($p < 0.05$) and females ($p < 0.05$) compared to control offspring (Figure 2.2H-I). Interestingly, plasma corticosterone showed prolonged elevations 60-minutes after restraint in female offspring from DEX treated mothers versus controls ($p < 0.001$).

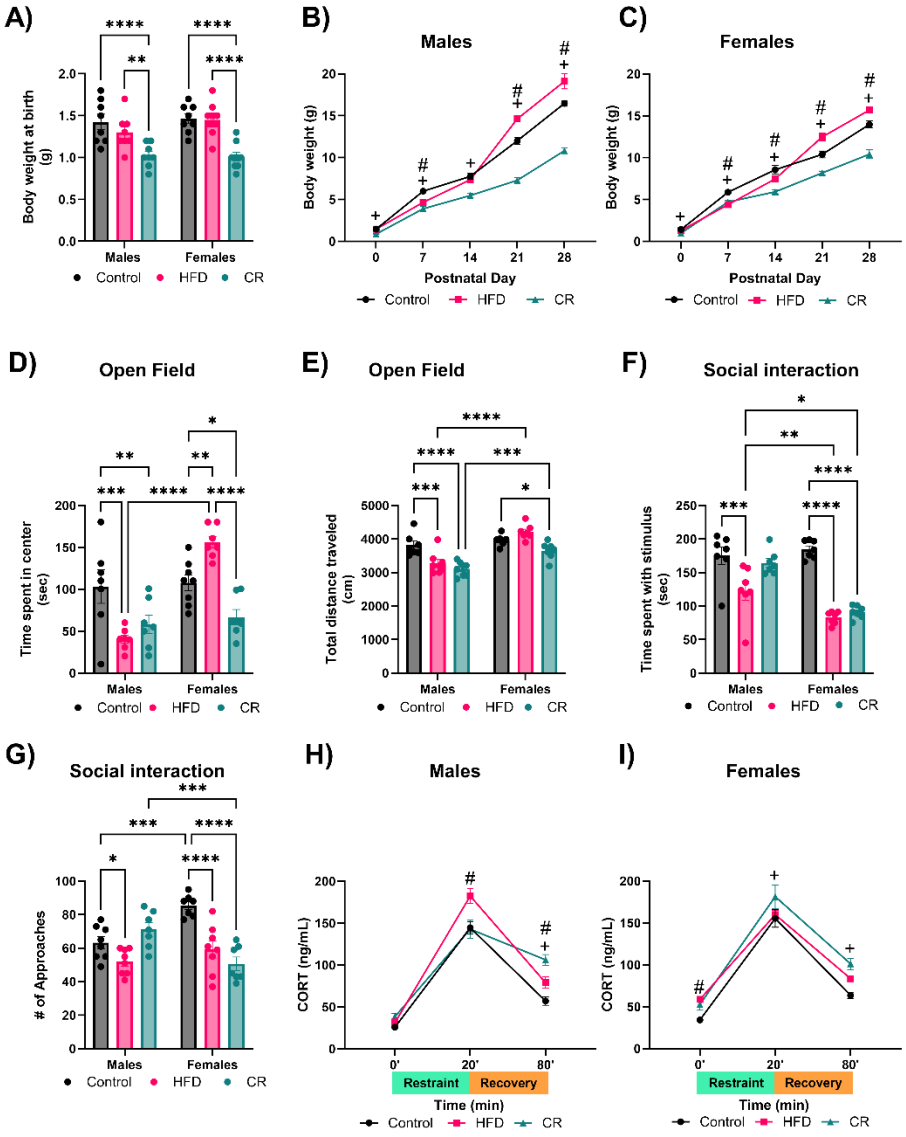

Figure 2.3. Maternal dietary manipulation resulted in altered body weights, behavior, and stress response in prepubertal male and female offspring. (A) Neonatal body weights were unchanged in high fat diet (HFD) but decreased in caloric restriction (CR) pups. (B) Male and (C) female offspring exposed to maternal HFD were heavier, while CR offspring were smaller. HFD and CR males showed higher anxiety-like behavior in open field assay, as measured by (D) time spent in center and (E) total distance traveled. CR females exhibited higher anxiety-like behavior with less (D) time spent in center and (E) total distance traveled. Social behavior was impaired in HFD males and females and CR females with decreased (F) time and (G) frequency of visits to stimulus mouse. Plasma corticosterone was higher in (H) HFD males after 20-minutes of restraint. HFD and CR males both demonstrated prolonged elevations of corticosterone after 60-minutes recovery. (I) HFD females showed increased baseline corticosterone levels while CR females had elevated levels after 20-minutes of restraint and 60-minutes recovery. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 vs. Control. #HFD vs. Control. +CR vs. Control.

Maternal Dietary Manipulations (HFD and CR)

Offspring from mothers on HFD did not show significant differences in neonatal body weights compared to controls. Body weights of CR male [$F(1, 104) = 163.6$, $P < 0.0001$] and female [$F(2, 93) = 79.21$, $P < 0.0001$] offspring (Figure 2.3A-C) were significantly lower than both control and HFD groups. After weaning, juvenile pups exhibited alterations in anxiety-like and social-like behaviors (Figure 2.3D-G). In open field assays, offspring maternal dietary manipulation increased anxiety-like behavior measured by time spent in center ring [$F(2, 37) = 8.391$, $P < 0.0001$] and total distance traveled [$F(2, 38) = 15.27$, $P < 0.0001$]. Post-hoc analysis revealed HFD and CR males showed less time spent in center ring ($p < 0.01$) and less total distance traveled ($p < 0.01$) compared to controls (Figure 3D-E). In contrast, female offspring from mothers fed HFD showed lower anxiety-like behavior with greater duration of time spent in center ring ($p < 0.0001$) while female offspring from CR mothers demonstrated less time spent in center ($p < 0.0001$ vs. Control) and distance traveled ($p < 0.05$ vs. Control). Such data suggest more anxiety-like behavior with maternal exposure to CR. Social interaction testing additionally revealed effects of diet [$F(2, 39) = 13.09$, $P < 0.0001$]. Male ($p < 0.01$) and female ($p < 0.0001$) offspring from mothers fed HFD exhibited impaired social behavior with less time investigating the stimulus mouse compared to controls (Figure 2.3F-G). Interestingly, only female offspring from mothers fed HFD showed fewer visits to stimulus mice (Figure 2.3F-G). Male offspring from mothers fed HFD also spent increased time investigating the empty cage ($p<0.001$) with more visits to the empty cage ($p<0.005$) while females did not when compared to their same-sex controls (Figure 2.3F-G). Comparatively, female offspring from CR mothers, but not male offspring showed impaired social behavior with less investigatory behavior ($p < 0.0001$) and number of visits ($p < 0.0001$) to stimulus mice (Figure 2.3F-G). Maternal HFD and CR further elevated acute stress response in female [($F(2, 56) = 11.56$, $P < 0.0001$) and male [$F(2, 52) = 9.175$, $P < 0.0004$] offspring. Stress-induced plasma corticosterone levels were

elevated at baseline in female offspring of HFD mothers ($p < 0.05$) compared to controls. After 20-minutes acute restraint stress, plasma corticosterone was higher only in male offspring from HFD mothers ($p < 0.001$) compared to controls. Similarly, corticosterone continued to be elevated in a male-biased manner after 60-minutes recovery in offspring from HFD mothers compared to controls (Figure 2.3H-I). In female offspring of CR mothers, plasma corticosterone was higher after 20-minutes restraint compared to controls ($p < 0.05$) and was still elevated in CR and HFD male ($p < 0.0001$ vs. controls) and female ($p < 0.001$ vs. controls) offspring after 60-minutes.

Discussion

The current study examined effects of three types of maternal stressors on male and female offspring assessed in pre-pubertal mice. Behaviors were examined prior to puberty because of the potential hormone dependence of the onset of neuropsychiatric disorders. Behaviors or

Summary Table 2.1. Brief description of changes in male (♂) and female (♀) offspring exposed to dexamethasone (DEX), caloric restriction (CR), or high fat diet (HFD) stress during pregnancy. Acronyms: Increase (↑), Decrease (↓), No change (-).

Maternal Treatment	DEX		CR		HFD	
	♂	♀	♂	♀	♂	♀
P0 body weight	↓	---	↓	↓	---	---
Anxiety-like (OF)	---	↑	↑	↑	↑	↓
Social	↓	↓	---	↓	↓	↓
Basal CORT at 0'	---	---	---	↑	---	↑
CORT after 20' acute stress	↓	↓	---	---	↑	↑
CORT after 60' recovery	---	↑	↑	↑	↑	---

biomarkers that might show sex differences prior to the emergence of significant hormone secretion at puberty could be helpful for predicting the onset of disorder since many neuropsychiatric disorders are diagnosed during early juvenile years in humans [184]. The results demonstrate birth weight, weight gained over time, stress response, and stress-related behaviors (anxiety- and social-like behaviors) were altered differentially with some similarities depending on the model of maternal stress exposure given the different timings (early, middle, late gestation), type (GC, metabolic), and duration (part- or entirety- of gestation) of exposure (Summary Table 2.1).

Prenatal DEX Exposure

Fetal exposure to the synthetic GC, DEX, altered development of the offspring. In agreement with other studies in rodents, body weights were lower in offspring treated with DEX during late gestation[129]. In the current study there was more anxiety-like behavior using an open field behavior assay with less time spent in center ring and total distance traveled selectively in female offspring. This agrees with other studies that demonstrated adult female offspring to be more susceptible than males after prenatal exposure to GCs[165]. Specifically, timing of fetal exposure to elevated GCs has been implicated in sex differences in the developing offspring. In rats, earlier exposure has demonstrated male-specific effects on the HPA axis stress response, while exposure later in gestation has shown greater effects in females[185, 186]. Levels of baseline corticosterone and ACTH were found to be elevated, accompanied by greater anxiety- and depressive-like behaviors, in selectively females exposed to late gestation GCs [187, 165]. Interestingly, both males and females prenatally exposed to DEX exhibited social impairments in the social interaction test. Many reports suggest prenatal GC exposure by treatment significantly influences brain regions involved in the HPA axis stress response, including AMY and HIPP [188] [189].These regions are also involved in stress-related outputs, including cognitive, sociability, and memory function. Several studies demonstrate high expression of

mineralocorticoid and GC receptors, making regions, such as the AMY and HIPP, susceptible to excess exogenous GCs, such as DEX[188]. Prenatal exposure to DEX could influence the development of these areas in the brain, by altering receptor function or morphology. Future studies will need to be conducted to better examine these mechanisms. Furthermore, in the current study, female mice, but not males, exhibit dysregulation of HPA axis reactivity with sustained elevation in plasma corticosterone following acute stress. Another study that administered prenatal DEX to pregnant mice dams during mid-late gestation (GD11-17) reported greater corticosterone stress response in female offspring from DEX-treated mothers [154]. Even though the stressor began earlier in gestation, DEX was administered throughout the end of late gestation, which seems to be a critical timepoint of fetal development for sex-dependent effects. These sex differences in stress responses and related behaviors could potentially be used as early indicators to better predict the emergence of affective disorders after puberty. There is, however, a significant limitation of the use of DEX as a maternal stressor in that it is an incomplete model of stress. Injection of a synthetic GC mimics one aspect of a stress response but it can potentially down-regulate other aspects of a normal stress response. Therefore, we explored additional models of maternal stress, i.e., under- and over-nutrition during pregnancy.

Prenatal Nutritional Stress (HFD, CR) Exposure

In the current study, nutritional stress during pregnancy altered body weights, anxiety- and social-like behavior, and HPA axis reactivity (Summary Table 1). Animal models of maternal nutritional stress impacted fetal neural programming, leading to increased risk for developmental disorders, including early onset of ASD- and ADHD-like symptoms and those that emerge later, such as anxiety- and depressive-like symptoms [143, 134]. Offspring from clinically obese mothers have increased risk for severe ADHD and ASD symptoms pre-pubertally [190, 134, 191] while those exposed to nutrient deficiencies are two times more

susceptible in developing schizophrenia later in adulthood[192, 134] . Rodent models of maternal obesity commonly use a HFD during pregnancy to mimic diets of Western societies. Studies have shown maternal HFD produces offspring that exhibit increased anxiety-like behavior in adult males and females. In the current study, juvenile males selectively showed greater levels of anxiety-like behavior in open field, suggesting male offspring are more susceptible to behavioral changes caused by a maternal HFD than females. This male-dependent effect is consistent with previous findings indicating maternal HFD programs the HPA axis and increases anxiety-like behavior in male offspring[193, 135]. Such findings suggest males at higher risk for anxiety-like disorders with exposure to HFD during pregnancy when the dietary manipulation was throughout the pregnancy (and differs from female selective DEX treatment effect that was limited to GD15-18 or maternal CR from GD11-parturition). Future studies need to be done to determine whether this effect is influenced more by male gonadal sex hormones (androgens) or sex chromosomes [194]. In mice, testes develop around E12.5 [195]and testosterone levels rise more in males than females by E16[196, 197]. Sex chromosome affects could occur at any point. Social-like behavior was also impaired in male and female offspring[198, 144]. In agreement with our results, other studies also showed impaired social interaction in mouse offspring exposed to maternal HFD {Kang, 2014 #6195}{Sgritta, 2019 #3048}. One study correlated such social deficits with increased proinflammatory cytokines in the brain and a female-specific increase in microglial activation [199]. There is growing evidence that proinflammatory cytokines, such as interleukin (IL)-1β and Tumor Necrosis Factor (TNF) α, are associated with altered cognitive and social function [200, 201]. Higher levels of cytokines in the brain could be a result of greater microglia activity, but more experiments would be necessary to tease this effect out.

Fetal exposure to maternal HFD elevated HPA axis stress reactivity, altering social-like and aggressive-like behaviors in offspring [198, 144]. The current study examined HPA axis function

in response to an acute restraint stressor. Data show the HPA axis stress response was greater in offspring exposed to maternal HFD with a delayed return to baseline GC levels. Such effects could be explained by other studies that demonstrate maternal HFD increases basal corticotropin releasing hormone expression in the hypothalamus, the main neuropeptide that responds to stressful stimuli to trigger the HPA axis cascade and increase GC secretion [140]. Studies are needed to pinpoint specific mechanisms by which a maternal HFD programs HPA axis in offspring and how these programming effects lead to social- and anxiety- like impairments.

Under-nutrition models can range from mild restriction of food intake (10-15%) to more moderate restricted food intake (50-75%) [138]. The current study consisted of more moderate restricted food intake (50%) over the last week of pregnancy, but still resulted in increased social/anxiety-like symptoms and a hyperactive stress response in offspring. Interestingly, maternal CR resulted in social deficits only in female offspring. This could be associated with higher basal corticosterone levels in females, suggesting an overactive HPA axis at baseline. In another experiment, maternal CR led to hyperactivation of the HPA axis in both dams and offspring (Peixoto Martins et al. 2023). Hyperactivation of the stress axis in dams could robustly influence maternal behavior, where poor maternal care can negatively impact neural development in offspring and lead to increased anxiety- and social-like behaviors. While the current results are in agreement with several maternal feeding restriction studies [172, 202, 174, 169], other studies show maternal CR (undernutrition during pregnancy) results in decreased in anxiety/social-like behavior in prepubertal offspring [138]). These differing results could be explained by varying restricted food intake regimens, such as restriction during all of gestation, from parturition throughout lactation, or during both gestation and lactation[172, 202, 175]. Another reason for varying results could be that many studies investigate behavioral changes in older adult offspring exposed to CR during fetal life. Juvenile, pre-pubertal mice can exhibit

different behavior than adult post-pubertal mice, such as altered social- or anxiety-like and locomotor behavior in social interaction test and open field assays[179, 203]. Many mice studies that examine neuropsychiatric-like symptoms are in adults. However, the current study demonstrates the feasibility of mimicking diagnoses of neuropsychiatric-like symptoms in juvenile mice, emphasizing the importance of behavioral testing early on in preclinical models.

Summary

Extensive evidence shows that perturbations to fetal environments cause long-term consequences to HPA axis physiology, increasing risk for related disorders in adulthood. The idea that GC responses to different stressors might serve as a common stimulus across stress paradigms is insufficient, given that different modes of prenatal stress may produce differential effects as we showed in our study. Opposite nutritional stressors produced similar outcomes for anxiety-like behavior in both sexes, social-like behavior in females, and a hyperactive adrenal stress response in males. The timing and duration of exposure to maternal stressor also heavily influences the outputs because several organs and tissues develop at different critical periods during gestation [204]. Such programming effects can lead to sex-dependent behavioral and neuroendocrine outcomes due to the timing of development and maturation of sex gonads (and subsequent gonadal steroid production) and sexual dimorphic regions of the brain [205]. Studies suggest maternal insults during certain critical periods during fetal development influences sex-specific effects on HPA axis development, altering stress response and related behaviors. One critical period of HPA axis development occurs during late gestation in male rodents, when the rodent is exposed to a robust surge of testosterone, which masculinizes and defeminizes its brain. Fetal exposure to maternal stressors during this time could alter the testosterone surge, leading to improper HPA axis development and function in postnatal life. However, more studies

are required to tease apart specific mechanisms involved in the testosterone surge in the male fetus and maternal stress. Moreover, the fetal HPA axis begins to function independently from the mother's during late gestation in rodents. The fetus secretes its own CRH and ACTH when exposed to maternal stress, leading to corticosterone production. Clinically, DEX is used to treat female fetuses diagnosed with congenital adrenal hyperplasia, an autosomal recessive disorder that leads to higher syntheses of cortisol and adrenal androgens. DEX is administered to female fetuses with this syndrome to inhibit adrenal androgen production and minimize consequences[206]. DEX can also suppress fetal CRH synthesis and secretion from the hypothalamus and consequently reduce downstream signals to the anterior pituitary for ACTH production and release through negative feedback mechanisms[207]. These indirect consequences of DEX are important to consider when evaluating long-term consequences in offspring exposed to prenatal DEX. Accompanied by fluctuating steroid hormones (androgens and estrogens) during this period, sex-dependent alterations to neural circuits and morphologies in brain regions involved in HPA axis function could develop.

Of note in the current study, the 3 treatments were overlapping in their timing, and different in their duration. HFD was from the beginning of the pregnancy, CR from E11-birth, and DEX from E15-18. Additionally, perturbations in the postnatal environment also play a role in susceptibility for neuropsychiatric disorders. This addresses the idea of a "dual hit" hypothesis, where the compounding effects of both a pre- and postnatal stressor (e.g. during lactation prior to weaning) increases risk for disease later in life even more [208-210]. In the current study, the offspring were not cross fostered at birth and potential maternal behavior changes were not evaluated.

One common theme among the three models presented here (DEX, CR, HFD) is that there are consistent reports demonstrating their role in activating the maternal and fetal immune responses ([211, 4, 212, 154, 213]. Prenatal adversity in humans is shown to lead to elevated

GCs and cytokines in the maternal environment[4]. These alterations in the maternal environment can lead to changes in the fetus (impaired brain function, neuronal cell death, irregular hormone and cytokine levels, etc.,) that may serve as fetal antecedents for neurological disorders in adulthood [4]. By focusing more granularly on the immediate physiological impact of different stressors on developing fetuses (e.g., hormones, cytokines), future studies may be able to parse out the different longer-term outcomes of prenatal stress models in rodents and their relationship to outcomes in humans.

CHAPTER 3:
MATERNAL IMMUNE ACTIVATION WITH TOLL-LIKE RECEPTOR 7 AGONIST DURING MID-GESTATION ALTERS PRE- AND POST-PUBERTAL DEVELOMENTAL MILESTONES AND BEHAVIOR[3]

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Introduction

Models of MIA provide evidence of neurodevelopmental programming that can increase susceptibility for a several diseases with fetal origins, including, but not limited to, MDD, schizophrenia, ASD[4]. Interestingly, a large body of research suggests sex differences in risk factors for neuropsychiatric disorders arise during fetal development and are influenced by stressors in the maternal environment. For instance, females are twice as predisposed as males for developing MDD while males have a higher likelihood to present with ASD[214, 163, 157, 215]. Factors that could account for these sex differences range from organizational and activational effects of gonadal hormones (androgens, estrogens), sex chromosomes, and developing sexual dimorphic brain regions and neurocircuits [216-218].

Stressors in the maternal environment impact fetal programming and can include physical stressors, immune sources, as well as psychological stress. A common output of such maternal stressors is fetal overexposure to elevated maternal stress hormones (e.g., epinephrine or GCs) that may alter immune function by changing the secretory profiles of multiple cytokines[4, 219, 103]. When these abnormalities in the maternal stress-immune system occur during middle to late gestation, the development of brain circuits [219, 103, 173, 167] and vasculature[68, 154]

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can be highly impacted in sex-biased ways. Important brain circuitries include those that regulate stress response, mood, autonomic function, and metabolism[220]. Dysregulation among these circuits can increase risk for associated neuropsychiatric and autonomic disorders later in life.

Behavioral symptoms of these disorders caused by the maternal stress-immune response may develop during early adolescence[179, 221]. Early adolescence is defined as prior to puberty when the offspring have not yet been exposed to robust levels of fluctuating gonadal steroid hormones. At puberty, androgens and estrogens may still exert organizational effects on neural circuits and brain morphology (see reviews [222-224, 3] for more in-depth descriptions). Because gonadal hormones are demonstrated to play a large role in the sex-dependence of disease (e.g., MDD, cardiovascular disease, other mood disorders), it is important to consider developmental symptoms of disorder prior to and after the onset of pubertal steroid hormone release[5] in the context of MIA.

The maternal stress-immune response has been studied in human and model animals in paradigms defined as MIA and related to developmental origins of neuropsychiatric disease (schizophrenia, ASD, MDD) [225, 103]. Clinical and epidemiological studies in humans demonstrate how peripheral and neural immune responses of the mother correlate with a predisposition of individuals for psychiatric disorders with hypothesized developmental origins[226]. Alterations in offspring responses range from cellular (e.g., brain microglial activation) to physiological (e.g., peripheral/central cytokines that influence development of neural circuitries or programming of the stress-immune system) to behavioral (e.g., social, anhedonia). One unique longitudinal study in humans found a positive association between exposure to elevated maternal pro-inflammatory cytokines *in utero* and an imbalance of anti- and pro-inflammatory cytokines in offspring. Adult offspring (40-50 years of age) of mothers with elevated serum cytokines during fetal development presented with more diagnoses of stress-

related disorders [103], suggesting a role for the maternal immune environment in greater risk for adult disease.

Clinical studies link elevated maternal pro-inflammatory cytokines to neurodevelopmental consequences and diagnoses for MDD later in life. Higher maternal pro-inflammatory cytokines, such as IL-6 and IL-17A, are shown to cause white matter damage in the neonatal brain associated with MDD (see reviews [227, 228, 226] for more information). These maternal cytokines cross the placental-fetal barrier (and fetal BBB) and are found in increased levels in fetal blood and brain samples[227]. Fetal exposure to abnormal levels of maternal cytokines is demonstrated to change functionality and connectivity between brain regions in a sex-dependent manner (hypothalamus, HIPP). These brain regions are important regulators of the stress response and stress-related behavior (anxiety, depression, sociability, cognition) and dysregulation to the regions are demonstrated to influence susceptibility to MDD and other mood disorders in a potentially sex-dependent manner[4, 226].

Two common rodent models of MIA use pathogen mimics such as bacteria derived LPS and double-stranded viral construct, Poly I:C[229, 4]. An alternative is to use small molecule agonists of TLRs that these constructs stimulate[230]. Stimuli mimicking infections alter maternal serum cytokines, including IL-6, TNFα, and IL-10 in rodent studies[231, 186], as well as humans[103]. Maternal injection of Poly I:C impaired stress signaling at the level of the hypothalamus and HIPP in adult male and female offspring[232]. Hyperactive HPA axis stress reactivity was accompanied by deficits in social-like behaviors[232]. This occurred in a sex-dependent manner in some studies where social-like behavior is altered in only juvenile males exposed to MIA[233] where other studies demonstrate social deficits in adult male and female offspring[234-236].

The current MIA study used a TLR7/8 agonist, RQ. Many viruses associated with neuropsychiatric disease in humans (i.e. SARS-CoV-2, influenza) are single-stranded RNA structures that activate TLR7[231], making it a critical TLR to consider in MIA. The TLR7/8 signaling pathway is similar between rodents and humans[237] and is highly implicated in autoimmunity in humans. Epidemiological studies demonstrate a positive correlation between maternal autoimmune infection and mental/mood disorders[238-241]. Rodent studies show activation of TLR7/8 leads to increased monocyte, T and B cell, and macrophage trafficking into the brain and leads to stress-related behavioral phenotypes later on[242, 243]. Chronic exposure to TLR7/8 agonist produces greater levels of anti-nuclear antibodies, indicative of pathologies associated with autoimmune disease and immune dysregulation[244]. Mouse studies showed deletion of TLR7 reduces progression of autoimmune of disease [245, 246], suggesting overactivation of TLR7 could lead to higher susceptibility to neuroimmune disorders, The role of TLR7/8 signaling been studied for host immune responses, but the role of TLR7/8 in neurodevelopment has only recently been examined[247, 231]. More general MIA has been demonstrated to induce maternal and fetal inflammatory responses with altered cytokine and chemokine levels [247, 231]. Whether changes in offspring are due to direct effects on the developing embryo by RQ, downstream effects of cytokines from the mother that cross the placental interface, or by another immune pathway of mother or fetus remain to be tested.

Current data demonstrate that offspring of mothers injected with a TLR7/8 agonist display less social behavior in female, but not male mice. The gene for TLR7 is located on the X-chromosome and escapes X-inactivation in females, consequently leading to higher expression of TLR7 in females[248] [249]. In contrast, TLR3 and 4 are located on autosomes and are not directly regulated by sex-selective expression [148, 147]. Because females are twice as likely to develop some neuropsychiatric disorders than males (e.g., MDD), the current study addresses the effects of a TLR7/8 agonist in a MIA paradigm in the context of sex differences. RQ was

administered during mid-pregnancy to trigger inflammatory responses in mother and fetus. Developmental milestones and social-, anxiety-, and anhedonia-like behaviors of juvenile and adult offspring to MIA were assessed.

Methods

Mice

Adult C57BL/6N female mice (6-8 weeks old) were monitored daily by vaginal lavage for 1 week to identify estrous cyclicity. Females on day of proestrus were mated with adult males (8 weeks old) and removed the following day to their own cage. This day was noted as GD0. Upon successful pregnancy, females were injected with RQ (HY-13740, MedChemExpress; s.c. 2mg/kg body weight) dissolved in phosphate buffer saline (PBS; 1mg/mL) or PBS (VEH saline] on GD 12.5. One cohort of RQ or VEH treated pregnant females (n = 6-8 dams per group) were euthanized 60-hours post-injection on GD 15. This collection time point was chosen to detect cytokine responses in the developing fetus. Maternal and fetal trunk blood was collected into chilled 0.5M EDTA tubes and stored at -20°C until assayed for cytokines. Fetuses were collected by removal of uterine horns from euthanized pregnant dams. Fetuses were placed immediately over ice and dissected one at a time under a dissecting microscope (Evident – Olympus SZ51 Stereomicroscope). Fetal trunk blood was collected, and sexes were determined by identification of the presence of undescended testes (males) or no testes (females) in the abdominal region using the dissecting microscope.

The second cohort of RQ or VEH treated pregnant females (n = 8-10 dams per group) was allowed to proceed through parturition noted as P0. Sex of neonates was determined on P0, and all litters were then culled to 6 pups (3 male:3 female; randomized selection of pups for each sex). Litters were culled to equal numbers of males and females to reduce variability of

maternal behavior toward one sex (usually males) and litter-size to optimize pup growth and development [250]. The offspring were weaned, and group-housed on P21. Behavior testing was performed in P21 (anxiety- and social-like behavior) and P52 (anxiety-, social-, and anhedonia-like) offspring. Of note, mice were assessed at either ages P21 *or* P52, but not both timepoints. These age groups were selected to examine behavioral phenotypes both prepubertal (P21) and post-pubertal (P52). One pup of each sex from each litter was used in all studies to avoid a litter effect. Tails of neonatal pups were marked with sharpie and then ear clipped at P7 for identification. Behavior testing was performed during 09:00 and 15:00 to avoid diurnal elevations in plasma corticosterone and were run and scored blindly to treatment conditions. Behavior assays were performed on different days to allow animals to recover from one test to the next. Tests were run from least stressful to most (open field, social interaction test, sucrose preference test). These behavioral assays were chosen for their predictive validity of modeling depression in rodents [251]. Mice were housed with *ad libitum* access to food and water and on a 12:12 light: dark cycle (lights on at 06:00 and off at 18:00). All mice were euthanized by inhalation of 30-70% carbon dioxide delivered in a sealed chamber until breathing ceased, consistent with Colorado State University's Institutional Animal Care and Use Committee and American Veterinary Medical Association Euthanasia Guidelines. This was followed by decapitation using sharp scissors according to American Veterinary Medical Association approved methods. All procedures were approved by Colorado University Lab Animal Resources and Institutional Animal Care and Use Committee Guidelines.

Developmental Milestones

During early neonatal to juvenile life (P0-P42), pups were assessed for developmental milestones. Body weights of male and female offspring were recorded weekly until euthanasia. Eye opening was assessed from P8-P18 (until both eyes of all pups had opened) in male and female offspring. Surface righting reflex was assessed by placing P7 mice in supine position on

a flat surface. Mice were scored by the time taken to become surface-right. Each mouse was tested 3 consecutive times and the median for each mouse was calculated. Pubertal milestones were additionally assessed (P15-P35): vaginal openings (females) and first estrous (females). Males were handled at the same times to insure comparable contact by sex.

Open Field Assay

Mouse activity was assessed by placing one mouse in the center of a circular open field area made of Plexiglass (radius = 20 cm; height = 30 cm). Mice were left undisturbed for 10 minutes and returned to their home cage following the test. Tests were performed one mouse at a time. The arena was washed with 70% ethanol and water and dried between each subject. The time spent in the center of the area (radius = 6.67 cm) was measured, where the more time spent in the center ring indicated lower anxiety-like behaviors. Number of entries into center ring and total distance traveled (locomotion) were also measured.

3-Chamber Social interaction test

Prepubertal (juvenile, P21) and adult (post-pubertal, P52) male and female offspring were placed in a 3-chamber apparatus with an empty wire-mesh cage on opposing ends as previously described[6]. Subject were allowed to habituate in the apparatus for 10 minutes. After 10 minutes of habituation, the subject mouse was briefly removed from the apparatus while an unfamiliar age- and sex-matched stimulus mouse was placed under a wire-mesh cage. The side of the stimulus mouse in the 3-chamber apparatus was alternated between tests to control for any potential side preference. The stimulus mice were reused (always age- and sex- matched of subject mouse). The opposing wire-mesh cage remained empty. The subject mouse was returned to the testing chamber and investigatory behavior of the stimulus mouse and empty wire-mesh cage was measured to

examine social discrimination of subject mouse. The cups and 3-chamber apparatus were cleaned with 70% ethanol and dried prior to and following each test. All behavior trials were video-recorded, and animal positions (head of the animal) were tracked by Ethovision software (Noldus Information Technologies). Behavioral analysis of video recordings was performed using Ethovision software (Noldus Information Technologies). Sociability was analyzed by examining total time spent with stimulus mouse (active sniffing of the stimulus mouse cage), frequency of visits to stimulus mouse (measured in bouts of active sniffing of the stimulus mouse), and the duration the subject mouse spent investigating the wire-mesh cage containing the novel stimulus mouse relative to the empty cage.

This was calculated as follows:

$$\frac{(time\ spent\ investigating\ stimulus\ mouse)}{(time\ spent\ investigating\ stimulus\ mouse)+(time\ spent\ investigating\ empty\ cage)}.$$

Sucrose Preference Test

The sucrose preference test was performed in the home cage of the adult mice (P52) for 6 consecutive days. Sucrose preference tests were not performed in juveniles because some offspring (particularly those exposed to MIA) were not large enough to reach the bottles in the cage hopper. On day 1, single-housed mice were housed in their home cage with 2 bottles containing filtered tap water. On day 2, bottles were replaced with a bottle filled with filtered tap water or 2% sucrose in filtered tap water. Both bottles were weighed before prior to placing in the cage topper. On day 3, weights of bottles were recorded. Difference in bottle weight indicated liquid consumption over the last 24 hours. Bottles were refilled, weighed, and put back into the cage topper, but on the alternate side to avoid side place preference. On day 4, bottles were weighed,

refilled, weighed again, and placed back into the cage topper (again on the alternate side). This process was repeated until 6 days had passed. A normalized sucrose consumption [percent sucrose water consumed / total liquid consumption (sum of water + sucrose water)] was calculated for each day. The normalized sucrose consumption values were averaged over the 6-day period.

Cytokine ELISA

Blood was collected from mice on E15 and placed into chilled tubes with 0.5M EDTA and aprotinin (4mg/mL; Sigma-Aldrich, St. Louis, MO) then centrifuged in a Beckman J6 centrifuge at 2000 rpm at 4 ºC for 10 minutes. After the plasma was separated, it was stored at -20º C until assayed with Mouse Custom 6-Plex ELISA kit for following cytokines: IL-6, TNF-α, IL-10, and IL17A (AimPlex Biosciences, Inc; cat #: T2C0620610K). Standards were prepared in a serial dilution according to Aimplex user manual. Capture beads were added to each well. Samples and standards were added to appropriate wells, followed by biotinylated and streptavidin antibodies. ELISA plate was read using a flow cytometer at the Molecular Core on Colorado State University's campus. Standard dose recovery 70-130%, intra-assay covariance <10%, and inter-assay covariance <20%.

Statistical Analysis

Data were analyzed using Prism (GraphPad Software Inc., San Diego, CA) with results presented as means ± standard error of mean. Significance was set at $p < 0.05$. Statistical significance for developmental milestones (body weights, eye openings, surface righting reflex), fetal plasma cytokines, and behavioral assays was determined by 2-Way ANOVA to examine effect of sex (males vs. females) and treatment (RQ vs. VEH). Tukey's multiple comparisons test was used for post-hoc analysis where appropriate. For statistical analysis of maternal

cytokines, vaginal openings of females, first day of estrous in females, and sex ratio of litters, 2-group ANOVAs were used to analyze by treatment (RQ vs. VEH).

Results

The maternal cytokine profile was altered after RQ injection during pregnancy

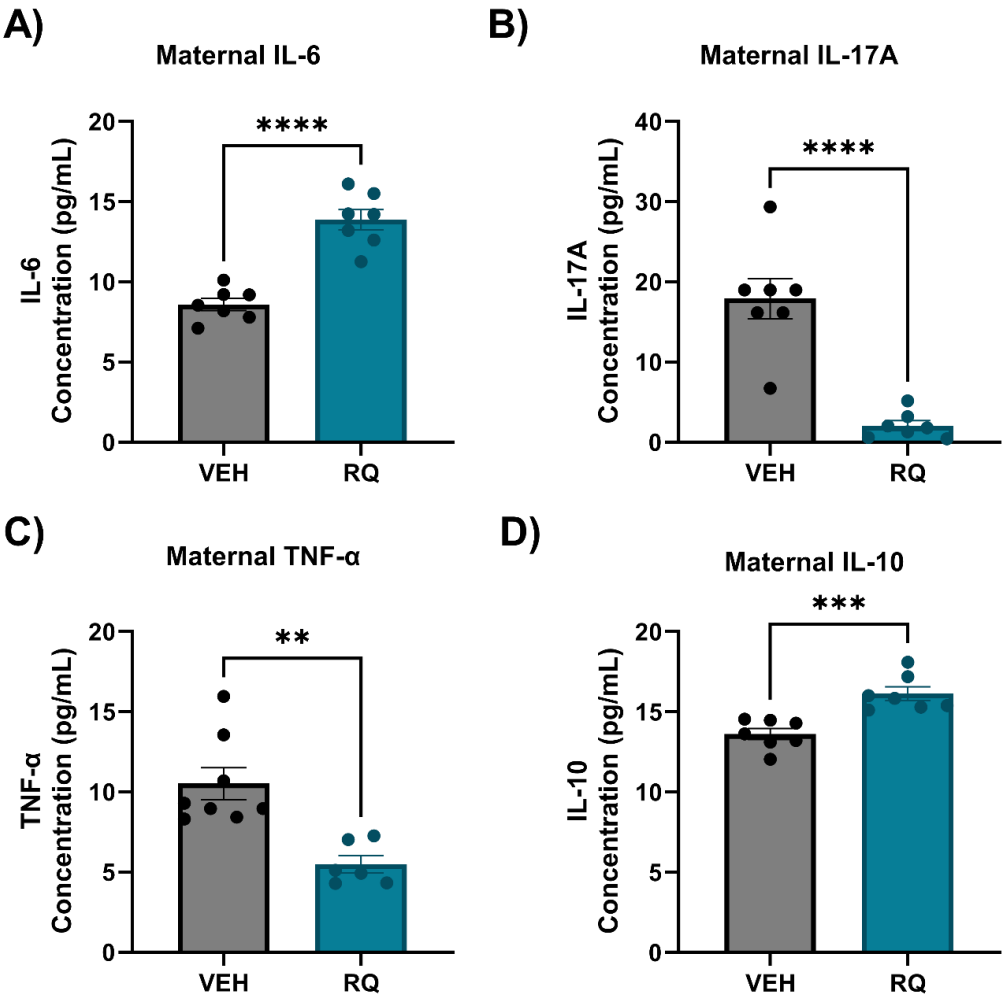

Figure 3.1. Resiquimod (RQ) exposure during mid-gestation altered maternal serum cytokine profiles 60-hours post-injection. RQ increased (A) IL-6 and decreased (B) IL-17A and (C) TNFα. RQ exposure further increased (D) IL-10. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 7-9/group.

Serum cytokines in the mother were altered 2.5 days (G15) following RQ injection on G12.5. 2-group ANOVA test revealed pro-inflammatory cytokine IL-6 (Figure 3.1A) was increased in RQ-

exposed maternal serum [F (1, 12) = 51.39, P = 0.0011] while TNFα [F (1, 12) = 16.45, P = 0.0016] and IL-17a [F (1, 12) = 37.37, P = 0.0001] were decreased (Figure 3.1B-C). Anti-inflammatory cytokine IL-10 was increased [F (1, 12) = 21.59, P = 0.0006] following RQ injection (Figure 3.1D).

Peripheral fetal immune response was changed after maternal RQ injection

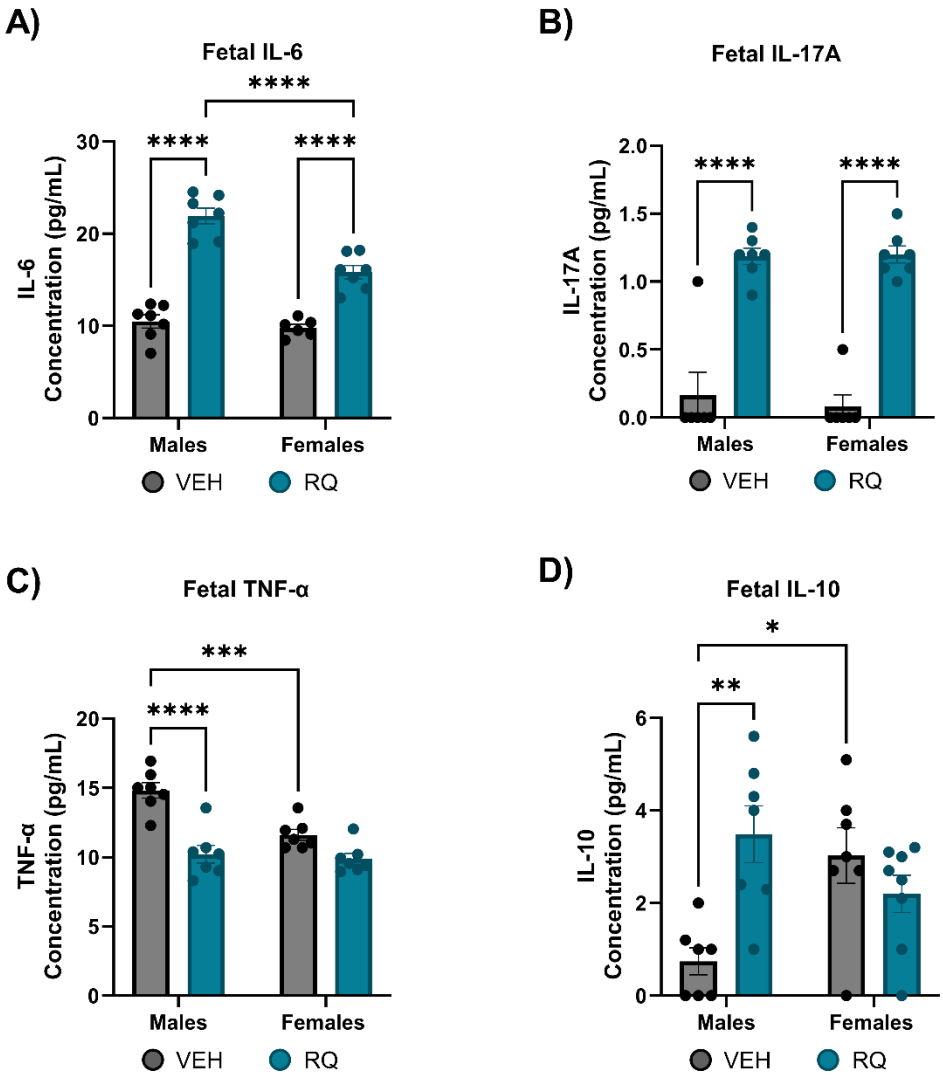

Figure 3.2. Resiquimod (RQ) exposure during mid-gestation (GD12.5) altered fetal serum cytokine levels 60-hours post-injection. (A) IL-6 and (B) IL-17A were increased in RQ male and female fetuses, while (C) TNFα was decreased in RQ males. (D) IL-10 was only increased in RQ male, but not female fetuses. RQ = Resiquimod. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 7-9/group.

Fetal serum cytokines were altered after maternal RQ injection. 2-Way ANOVA showed pro-inflammatory cytokine IL-6 [F (1, 23) = 148.5, P = 0.0013] (Figure 3.2A) was elevated in male (p = 0.0001) and female (p = 0.0021) fetuses exposed to maternal RQ. Tukey's post-hoc analysis further showed lower plasma IL-6 in RQ females than RQ males (0.0001). IL-17a was also

greater [F (1, 22) = 120.1, P = 0.00014] in male (p = 0.001) and female (p = 0.0012) fetuses exposed to maternal RQ versus their VEH controls, while TNFα was less [F (1, 24) = 39.17, P = 0.0002] in RQ males (p = 0.0001) but not females (Figures 3.2B-C). 2-Way ANOVA revealed an effect of prenatal RQ In anti-inflammatory cytokine IL-10 [F (1, 25) = 13.21, P = 0.0013]. Post-hoc analysis demonstrated IL-10 was lower in RQ males versus VEH males (p = 0.0035) and in VEH female fetuses compared to VEH male fetuses (p = 0.0.0167) (Figure 3.2D).

Developmental milestones were impaired offspring after maternal injection of RQ

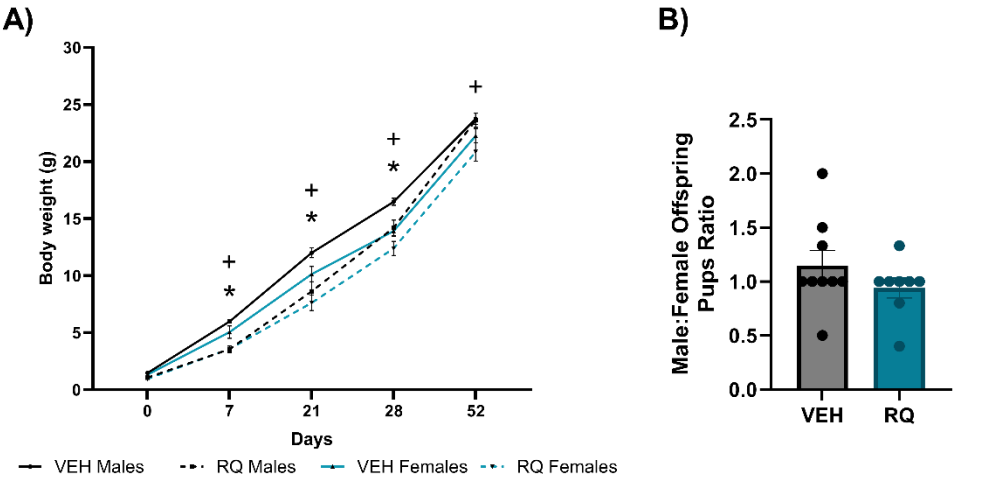

Figure 3.3. Exposure to MIA lead to decreased body weights (A) in males at P7, 21, and 28 indicated by (*) and females at P7, 21, 28, and 52 indicated by (+). (B) RQ exposure did not alter number of male:female offspring ratio in litters. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 8-12/group.

Developmental measurements were examined in offspring exposed to MIA. 2-Way ANOVA at each time-point showed body weights were reduced in offspring by exposure to maternal RQ treatment [F (3. 141) = 26.3, P = 0.0001] in males at P7 (p = 0.0180), P21 (p = 0.0001), and P28 (p = 0.0360) and females at P7 (p = 0.023), P21 (p = 0.0091), P28 (p=0.031), and P52 (p = 0.019) (Figure 3.3A-E). Female:male sex ratio was not influenced by RQ treatment [F (1, 15) = 1.43, P = 0.2507] (Figure 3.3F). Surface righting reflex in P7 pups was not altered by RQ treatment (Figure 4A), indicating no motor reflex impairments caused by exposure to maternal

RQ injection. Offspring of mother injected with RQ opened their eyes 3-5 days later than their VEH counterparts [F (1, 36) = 401.7, P=0.0011] (Figure 3.4B). RQ males opened their eyes

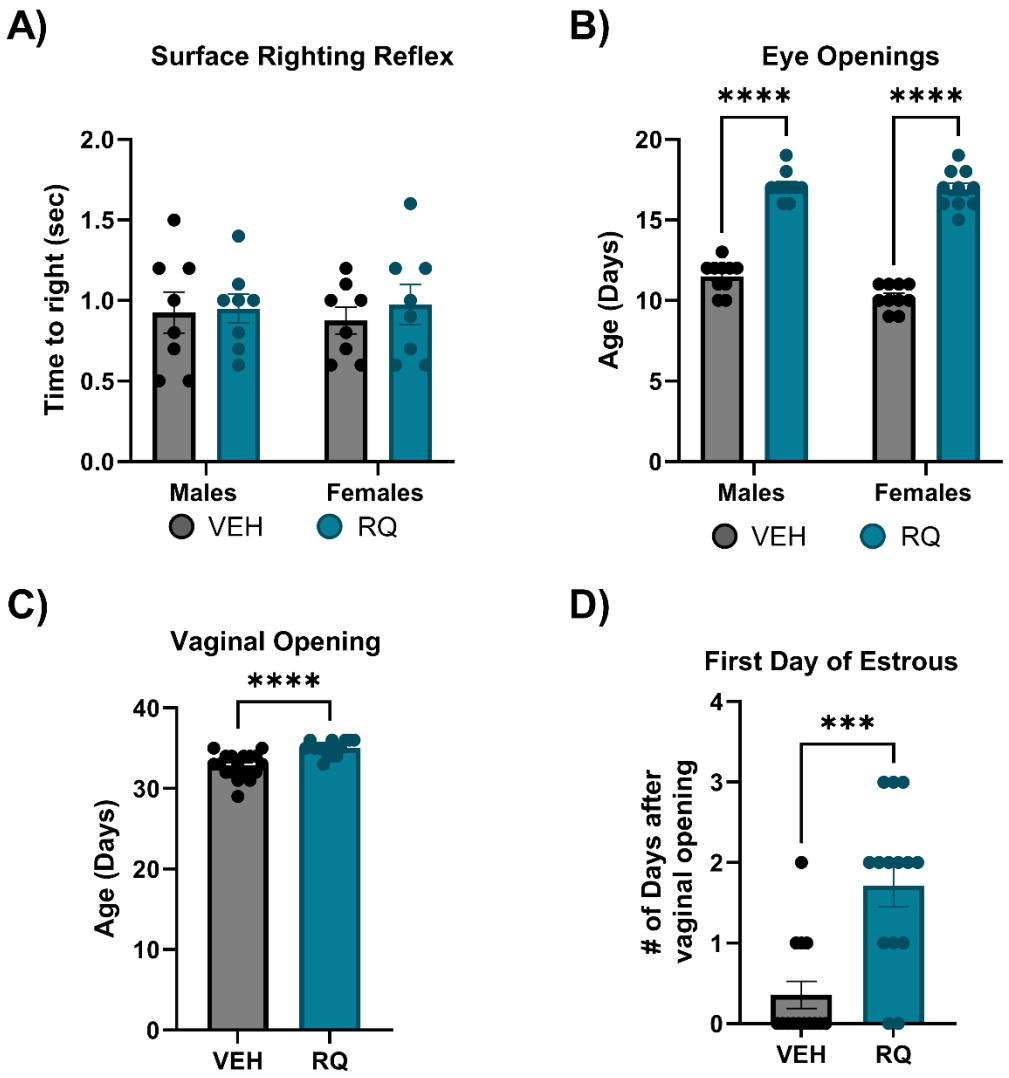

Figure 3.4. Developmental milestones were delayed in offspring exposed to maternal RQ injection. While (A) Surface righting reflex was unchanged, (B) fetal RQ exposure caused slower eye openings in male and female offspring. (C) Maternal RQ treatment also delayed vaginal openings and (D) first day of estrous following vaginal opening in females, indicative of delayed pubertal development. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 11-15/group.

around P16 while VEH opened their eyes around P17 (p = 0.0001; Figure 3.4B). Females from mothers injected with RQ opened their eyes around P17, while VEH females opened eyes at P9 (p = 0.00011) (Figure 3.4B). RQ-female offspring showed delayed vaginal openings around P35

whereas VEH female offspring opened three days earlier on P32 [F (1, 30) = 28.58, P = 0.00012] (Figure 3.4C). First estrous was delayed by almost 2 days in RQ versus VEH females [F (1, 26) = 18.55, P = 0.0002]. (Figure 3.4D).

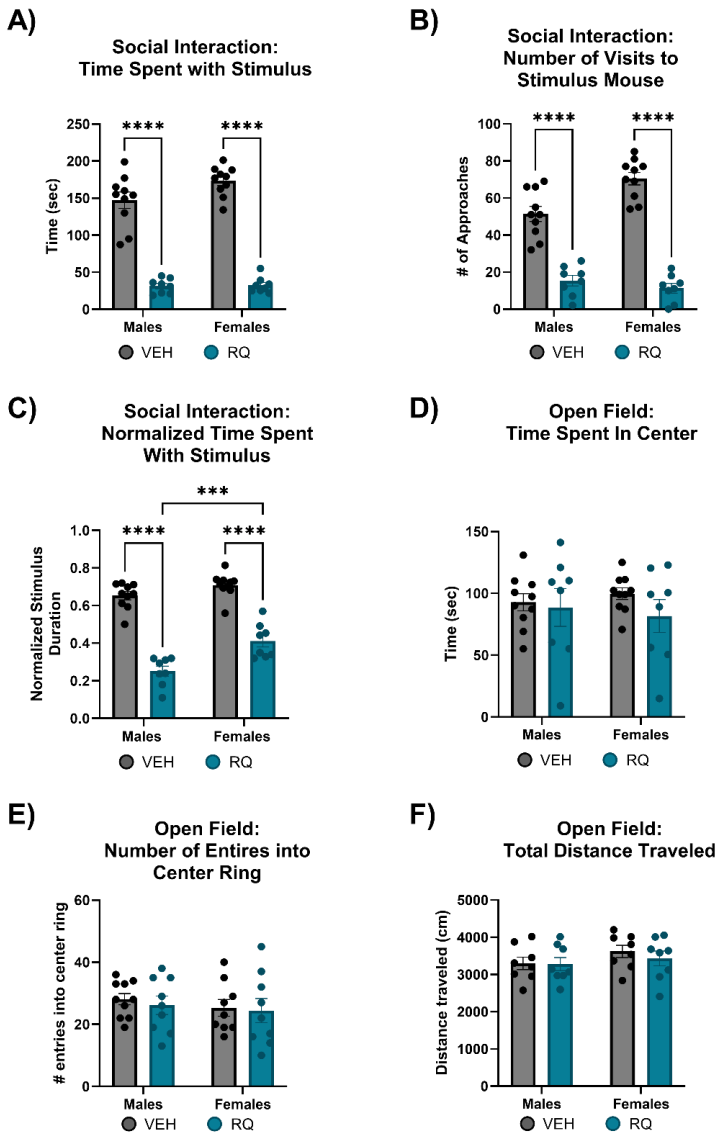

Figure 3.5. Social- and anxiety-like behavioral phenotypes in juvenile offspring were assessed in social interaction and open field tests. Social-like behavior was lowered in juvenile males and females prenatally exposed to RQ, with (A) less time spent with stimulus, (B) reduced number of visits to stimulus mouse, and (C) normalized time spent with stimulus in 10-minute social interaction test. Anxiety-like behavior was not altered by RQ treatment in open field assay with no changes in (D) time spent in the center, (E) number of entries into center ring, or (F) total distance traveled. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 8-10/group.

Social-like behavior was lower in juvenile offspring after maternal RQ injection

Juvenile offspring displayed less social-like behavior in 3-chamber social interaction tests with prenatal exposure to RQ. 2-Way ANOVA showed a main-effect of RQ with less time spent investigating stimulus mouse [F (1, 32) = 295.5, P = 0.0001] in male (p =0.00011) and female (p = 0.0001) offspring (Figure 3.5A). Offspring of mothers injected with RQ also showed ~50% fewer visits (Figure 3.5B) to stimulus mice [F (1,32) = 190.8, P = 0.0001] in males (p = 0.0001) and females (p = 0.00013). Data also showed an effect of RQ [F (1, 32) = 195.5, P = 0.0001], with lower normalized time spent with stimulus mouse (Figure 3.5C; time spent investigating stimulus/total time spent investigating stimulus and empty cage) in males (p = 0.00011) and females (p = 0.0001) compared to VEH. Post-hoc analysis demonstrated RQ females spent more normalized time with stimulus mouse than RQ males (p = 0.0008). No change was found in anxiety-like behavior as assessed by open field testing (time spent in center ring, number of entries into center ring) (Figures 3.5D/E). Total distance traveled in open field was unchanged as well (Figure 3.5F) suggesting locomotion was not disrupted.

Social- and anxiety-like behavior was altered sex-specifically in post pubertal mice after maternal RQ injection

The social interaction test revealed (~20% in males, ~10% in females) social-like behavior in offspring from mothers injected with RQ. 2-way ANOVA showed less time spent with stimulus mouse in RQ-males and females [F (1, 28) = 86.64; P = 0.0001] (Figure 3.6A), indicating impaired social-like behavior in offspring of mothers treated with RQ. Tukey's post-hoc analysis additionally revealed VEH and RQ females spent more time with the stimulus mouse than their VEH (p = 0.0088) and RQ (p = 0.0207) male counterparts. Similarly, the normalized time spent investigating stimulus mouse (Figure 3.6C; time spent investigating stimulus/total time spent investigating stimulus and empty cage) was lower in RQ offspring ([F (1, 28) = 15.18, P =

0.0006], males p = 0.009, females p = 0.012). The number of visits to stimulus mouse was not significantly different by treatment (Figure 3.6B). Only RQ males exhibited greater anxiety-like behavior in open field assay [F (1, 28) = 8.597; P = 0.0066] with less time spent in the center of the arena (Figure 3.6D) than VEH males (p = 0.011). No change was seen in number of entries into center ring or total distance traveled (Figures 3.6E/F).

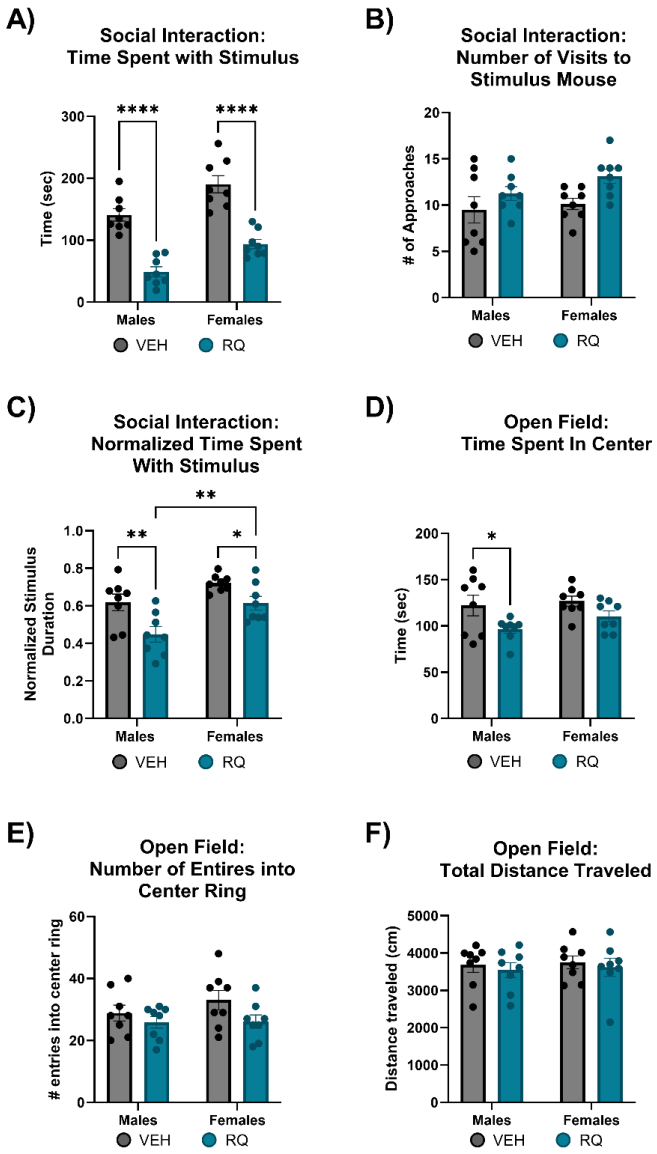

Figure 3.6. Social- and anxiety-like behavioral phenotypes were assessed in adult offspring in social interaction and open field tests. Social-like behavior was lower in adult males and females prenatally exposed to RQ**,** with (A) less time spent with stimulus, (B) no change in number of visits to stimulus mouse, and (C) less normalized time was spent with stimulus. Anxiety-like behavior was reduced by RQ treatment in 10-minute open field assay by (D) time spent in the center ring in a male-dependent manner. No change was seen in (E) number of entries into center ring or (F) total distance traveled in open field test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 8-10/group.

Anhedonia-like behavior was greater in a female-dependent manner in adults after maternal RQ injection

Sucrose preference tests were used to assess anhedonia-like behavior in adult male and female offspring (Figure 3.7). 2-way ANOVA revealed differences by RQ treatment selectively in females exposed to MIA [F (1, 25) = 11.5; P = 0.0023]. Post-hoc analysis showed RQ females showed 20% less normalized sucrose intake compared to VEH controls (p = 0.0101), suggesting a female-dependent change in anhedonia-like behavior by RQ exposure. No differences were seen in males.

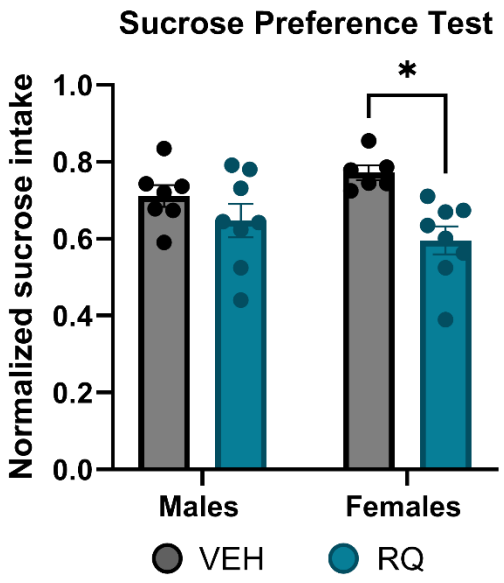

Figure 3.7. Sucrose preference test revealed more anhedonia-like behavior in female mice exposed to MIA. RQ females had decreased normalized sucrose intake compared to VEH females. No change in males. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 6-9/group.

Discussion

The current study used an MIA paradigm with the TLR7/8 agonist RQ to examine alterations in juvenile and adult developmental milestones and behavior. RQ was administered to timed-pregnant mice on GD12.5. Two days later (GD15), maternal and fetal pro- and anti-

inflammatory cytokines were altered (Figures 1 and 2) confirming the effectiveness of the immune stimulus during pregnancy. Offspring of mothers injected with RQ had delayed developmental milestones (birth weights, body weights, eye openings, female vaginal openings). Additionally, the offspring of mothers injected with RQ had impaired social-like behavior as juveniles and again as adults. There was evidence for greater anxiolytic-like behavior in adult RQ males, but greater anhedonia-like behavior in adult RQ females. Such behavior phenotypes (anxiety-like, social-like, and anhedonia-like) are often assayed in rodent models (see review [251]for more in-depth discussion) for potential predictive validity relative to humans. The current findings suggest a significant activation of the maternal immune system by RQ, with downstream consequences in delayed early development and impaired neuropsychiatric-like behavior in juvenile and adult offspring. Such findings could additionally provide context for underlying sex differences influenced by fetal antecedents, increasing susceptibility for adult disorders through immune mechanisms.

Plasma maternal and fetal cytokine levels were altered after RQ injection at E12.5

Mid-gestation injection of RQ resulted in greater levels of plasma maternal cytokines, IL-6 and IL-10, and lower levels TNFα and IL-17 suggesting successful activation of maternal inflammatory responses. These data agree with other MIA studies with TLR7/8 activation in mice, with similar increases in maternal cytokines, IL-6 and IL-10, to help recruit immune cells, produce more inflammatory cytokines[247, 231]. Maternal increases in pro-inflammatory cytokines, such as IL-6 and TNFα, have been suggested previously to lead to dysregulated fetal brain development and increased risk for neurodevelopmental disorders [252-254]. However, while TLR7/8 activation in some studies increased TNFα [231], the pregnant dams in this study exhibited lower levels of TNFα. Differences in cytokine profiles could have occurred because the previous study (C57BL/6 mice) collected and measured serum samples four hours after RQ injection (2mg/kg) on E12.5[231]. In contrast, we collected serum two and a half days (60 hours)

following injection based on the idea that it would take more than a few hours for changes in cytokines in mother and fetuses to effect brain development. It is unclear whether unmetabolized RQ crosses the placental-fetal barrier to directly exert effects on the developing embryo. Our study used pregnant rodents, where pregnancy itself exerts an immune response to the host and could have a compounding effect in the presence of RQ[255]. Additional studies need to be done to better understand the pharmacokinetic time-course of RQ, particularly in the lens of maternal infection and different fetal stages of development.

Maternal exposure to RQ not only resulted in cytokine changes in the mother, but also in the fetal offspring, with greater levels of IL-6 and IL-17A in males and females and IL-10 in males. Many reports indicate MIA alters these fetal cytokine profiles, especially IL-17 and IL-6[256], predisposing them to neuropsychiatric disorders in adulthood[257]. Changes in fetal cytokines that increase risk factors for disease range from the balance of pro- and anti- inflammatory cytokines (i.e., IL-6:IL-10 ratio) to cytokines that act on glial cells (e.g., astrocytes, microglia)[248, 201, 258]. Cytokines and microglia function are shown to be dependent on gonadal hormones, which could account for sex differences in the immune response of the fetus to the same maternal insult [259]. Other prenatal sex-specific mechanisms that occur prenatally, such as neurogenesis, cellular migration and proliferation, could also lead to numerous sex differences in the response to MIA[260]. Clinical studies show pregnant women exhibit elevated proinflammatory cytokine production with a female fetus versus male[261, 262]. Maternal estrogens are shown to exert anti-inflammatory effects through a neuroprotective mechanism, which can greatly affect the neuroimmune balance of the fetal-placental environment (refer to reviews [261, 262] for more in-depth information). More specific research of the role of fetal sex signaling in maternal immune function are required.

The interaction of fetal GCs and cytokines can increase susceptibility to disease with neurodevelopmental origins through the neuro-immune stress axis[103, 226]. A recent study in

humans evaluated maternal inflammatory cytokines and found similar increases in serum IL-6 and IL-10 in mothers exposed to adversity, or immune exposure, during pregnancy. Offspring were followed into adulthood (age 55), where they exhibited lower levels of circulating TNFα and impaired hypothalamic and memory function[226]. Increases in fetal pro-inflammatory cytokines during brain development could provide an early biomarker for later progression of neurodevelopmental diseases.

Developmental milestones of offspring were delayed after maternal injection of RQ

In the current study, mice prenatally exposed to maternal immune stress exhibited developmental delays in body weight and eye opening[233, 150]. Delays in these milestones have been associated with slower cognitive development during adolescence that could be predictive of MDD later in life [263, 264]. There was some concern over the magnitude of the eye-opening effect, and therefore it was repeated in an additional small cohort of mice resulting in confirmation of the developmental delay. Additionally, RQ females showed later vaginal opening with delayed first day of estrous. This is consistent with other maternal stress studies in rats and mice, where females exposed to stress during pregnancy exhibited later vaginal openings, longer estrous cycles, and decreased fertility[248, 249, 242]. By contrast, one MIA study using the viral mimetic, Poly I:C, resulted in earlier vaginal openings and first estrous in female pups[264]. While timing of immune exposure matched our current study (mid-gestation GD12.5), Poly I:C is recognized by TLR3. Both TLR3 and 7, pattern recognition receptors (PRR), are both located inside endosome compartments but have different signaling cascades. TLR3 is activated by double stranded RNA and signals interferon regulatory factor 3, which *prevents* translocation of nuclear factor kappa b (NF-kB) to inhibit viral inflammation. TLR7 signals myeloid differentiation primary response 88 , an adaptor protein for inflammatory pathways that *promotes* NF-kB translocation and downstream proinflammatory cytokine production[231, 243]. While both pathways participate in immune responses, differences in

secretion of downstream interferon factors could play a role in onset of puberty in each study. TLR7 additionally escapes X inactivation, with increased expression in females. This could result in more robust immune signaling cascades, further influencing the delay of puberty we found in our females exposed to MIA[248, 249, 242]. Additional research is necessary to establish clear sex-selective mechanisms of MIA by TLR7 activation on offspring development.

Decreased social- and anxiety-like behaviors in offspring of RQ injected mothers

In the current study, maternal injections of RQ during mid-gestation led to social- behavioral deficits in adolescents and adults. Our findings show less social behavior in prepubertal and adult male and female offspring prenatally exposed to RQ with less time spent with and fewer visits to stimulus mice. This indicates that social impairments detected early could be predictive of long-term social deficits [179]. Other MIA studies in rodents indicate that where poly I:C viral infection causes social behavioral impairments in female and/or male offspring, they are often associated with early symptoms of ASD-like- and anhedonia-like behaviors later in life [265, 233, 247, 215]. One study in rats demonstrated behavioral deficits in social play and ultrasonic vocalizations in prepubertal and post-pubertal rat offspring with mothers exposed to Poly I:C during mid-gestation (G15)[266, 234]. Another study in mice found developmental delays and impaired social-like behavior in adult (P35-45) offspring exposed to fetal Poly I:C (GD12.5) [233]. Such data suggest a correlation between exposure to MIA, developmental delay, and symptoms of cognitive dysfunction later in life[267]. Interestingly, neither adolescent RQ male or female offspring in the current study demonstrated changes in anxiety-like behavior in open field assays. In adulthood, RQ-exposed males exhibited greater levels of anxiety-like behavior compared to VEH, but this was not observed for females. Anxiety-like behavior is important to consider when examining symptoms indicative of MDD because these phenotypes are often found to be comorbid in rodent studies, along with other phenotypes such as social interaction behavior, sleep-wake disturbances, and metabolic changes[268]. There is also high comorbidity

between MDD and anxiety disorder in humans[128], another reason to track this comorbidity in the mouse model.

There are many types of maternal adversity and they have varied outcomes for offspring. A recent MIA study used a different TLR7 agonist, imiquimod, with different results. They reported a male-biased reduction in anxiety-like behavior in open field tests[247]. Two major differences between studies are the timing and number of imiquimod injections along with the immune receptor pathways activated by the drug. In the current study, pregnant dams received a single dose on GD 12.5, while females in the other study received three doses, on GD 12.5, 14.5, and 16.5. While Imiquimod activates TLR7 by T cell recruitment (T helper cell 17, Th17), analog, RQ, activates both TLR7 and TLR8 immune pathways, is 10-fold more potent in inducing the Th17 immune pathway[269], and could account for the different behavioral outcomes. Not only are they used slightly differently in the inflammatory responses they produce, but the timing of exposure is likely critical (e.g., middle of gestation significantly different compared to later gestation)[270, 4, 103, 271, 272]. In a preliminary test of this hypothesis using RQ injections at E15 compared to E12.5, decreased birth weights and weight gain with behavioral outputs were found. There was lower social-like behavior in prepubertal males and higher anxiety-like behavior in prepubertal males and females(data not shown). Such data suggest time-dependent change in behavioral phenotypes where additional studies will be important to parse these effects out further. There are many factors to consider relative to effects of exposure timing[270, 273] as well as pharmacokinetics of exposure to immune stressors to try and correlate treatments with behavioral symptoms and associated sex differences later in life.

Human studies further demonstrate a role for MIA in neurological changes in offspring that lead to sex-dependent behavioral phenotypes[274], such as anxiety and depressive behaviors, and cognitive and learning deficits. Other changes induced by MIA in humans include epigenetic modifications (i.e. gene methylation), reduced cell proliferation and neurogenesis, and

neurochemical changes in the brain with changes in dopaminergic and serotonergic systems[275] [276]. Evidence also suggests males are more susceptible to immune stress early in fetal development while female fetuses are more negatively impacted by immune insults later during pregnancy. Similarly, recent functional MRI data link elevated pro-inflammatory cytokines in maternal sera and reduced connectivity of stress-related brain regions in offspring in a sex-selective manner[103]. Such findings indicate a maternal immune cytokine response may significantly influence brain activity in human offspring, but more specific sex-selective mechanisms need to be better understood (see reviews [277-279] for more discussion regarding sex-specific and time-dependent mechanisms).

More anhedonia-like behavior in adult female offspring from mothers injected with RQ

Many reports indicate immune stress during pregnancy can increase susceptibility to MDD in offspring in adulthood, with females at higher risk than males[4]. Our findings agree, where prenatal exposure to maternal RQ led to greater anhedonia-like behavior in a female-biased manner. Normally, a mouse will prefer drinking sweetened sugar water over regular water with the assumption that this is hedonic behavior. However, mice showing anhedonia-like behavior (i.e., females from mothers injected with RQ in this study) have 15% lower sucrose water intake[221]. Sex-dependent effects of maternal RQ injection could be influenced by timing of exposure to stressor relative to critical periods of gestation that lead to altered programming of sexually dimorphic brain regions (e.g. AMY, preoptic area, hypothalamus, dentate gyrus of the HIPP), such as invoking neurogenesis or cell death, cell migration, or phenotypic differentiation and wiring[280, 260, 281-284]. Potential brain regions that might be affected include AMY, HIPP, prefrontal cortices, and the PVN, each with important regulatory circuits involved in stress and mood disorders, metabolism, and autonomic function[285, 286, 4, 283]. Improper development of these areas would increase risk for sex-biased disorders, including MDD and other comorbid diseases (e.g., cardiovascular disease, metabolic disorders, obesity, etc.)[287,

173]. Specifically, the PVN is a central regulator for stress responses, neuroendocrine, and stress-related behavior outputs, such as anxiety- and anhedonia-like symptoms[288]. Therefore, additional studies that examine PVN function in the context of our maternal immune stress model may be useful for better understanding of the pathophysiology of the stress-immune axis and related diseases.

Conclusions

The current study examined pre and post pubertal behaviors following prenatal MIA in mice. Maternal RQ altered serum cytokine profiles in maternal and fetal compartments, and then subsequent developmental milestones, and behavioral phenotypes in offspring. Maternal behaviors after birth were not evaluated in this study and reports suggest maternal care can influence behavioral and neuroendocrine responses in the offspring (e.g., anxiety- and anhedonia-like phenotypes and HPA axis stress reactivity[289, 109, 102]). Future studies are needed to determine if there is an effect of RQ–- MIA on maternal behavior with potential consequences in the offspring.

Fetal origins of neuropsychiatric disorders, such as MDD, are implicated to be influenced by maternal stressors during pregnancy, resulting in dysregulation of the HPA axis stress response and impaired stress-related behaviors [159]. Since neuroinflammation is associated with vascular, cardiometabolic, and mood disorders, the HPA-immune axis has more recently been implicated in models of maternal immune stress studies[4]. The central regulator of the stress response and related disorders is the hypothalamus. Changes in neural activity by maternal stressors could play a role in the development of the PVN during fetal life[162, 165]. Changes in neural programming by MIA could be due to disruption of structures in the PVN, such as GABAergic responses[290, 163, 291, 292], BBB integrity[68, 154], or even glial function[293]. Critical studies of brain structure will be needed to examine more specific pathophysiological

characteristics to better understand the origins of increased susceptibility to affective disorders later in life.

CHAPTER 4:
MATERNAL IMMUNE ACTIVATION BY TOLL-LIKE RECEPTOR 7 AGONIST INCREASED SUSCPETIBILITY TO BLOOD-BRAIN BARRIER LEAKAGE IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS IN ADULT MICE

Introduction

Fetal brain programming brain is heavily influenced by the physiological environment of the mother[130]. Stressors ranging from psychological to immune insults lead to elevated maternal GCs and inflammatory cytokines[294, 8, 295]. Stimulation or inhibition of the maternal immune system during middle to late gestation can lead to dysregulation of fetal brain circuitry, behavior, and cerebral vasculature[128, 103, 5]. Such phenotypes increase risk for associated mood and autonomic disorders, including MDD and cardiometabolic disease[214, 163, 160, 166]. MDD is sex-selective, with 2x higher incidence of MDD in females[173, 5], while sex differences in heart disease show twice the incidence in males than females[296].

The PVN is a sexually dimorphic nucleus in the brain and the central regulator of the HPA axis [6, 7]. The PVN is a nexus for the integration of inputs from other brain regions[2], and plays a key role in the central response to environmental stressors. Dysregulation of PVN development or circuitry negatively alters the stress response and increase susceptibility for stress-related disorders later in life in rodent and human studies[297, 193, 219]. In this study, we examined the acute neuroendocrine stress output in adults, indicative of potential HPA stress axis changes when mice exposed to MIA (MIA). In previous experiments, we demonstrated MIA driven by agonizing TLR7 during mid-gestation leads to an increase in peripheral immune response in mother and fetus (Sheng & Tobet, 2023 JNE). MIA offspring exhibited developmental delay and stress-related behavioral symptoms, including social-, anxiety-, and anhedonia-like, in a sex-dependent manner. Given that these behavioral phenotypes can be regulated by PVN neurons,

we wanted to investigate cellular changes, such as vasculature impairment, in this region[68, 154].

The PVN is 2-3 times more densely vascularized than surrounding regions in the brain[298, 67]. The blood-brain barrier (BBB) offers protection from potentially harmful peripheral compounds and is comprised of endothelial cells connected by tight junctions, astrocytic end-feet, and pericytes. Previous studies in the lab showed overexposure to fetal GCs during development disrupted components of the BBB in the PVN with lowered blood vessel density in juvenile mice[68]. In other mice exposed to excess GCs during fetal development and examined as adults, there was greater expression of astrocytes in females, higher expression of pericytes in males, and elevated anhedonia-like behavior[154], suggesting a potential relationship between impaired BBB integrity in the PVN and mood behavioral phenotypes in adulthood. Studies in humans have associated BBB disruption with MDD and other neurological diseases[299, 155], driven by weaker tight junctions between endothelial cells[300] and alterations in astrocytes surrounding the capillary endothelia[301, 302]. Microglia are important regulators of central immune function and may indicate locations of impaired BBB function and elevated vascular leakage in disease[303, 258]. Several reports indicate higher microglial activation in rodent offspring of mothers exposed to TLR 3/7 activation by examining changes in morphology and secreted compounds (i.e. chemokines and cytokines) in areas of tissue damage or infiltration of harmful substances [271, 304, 305, 151, 152]. The current study focused on long term consequences of maternal Resiquimod (RQ) injection (TLR7 agonist) on BBB integrity in the PVN of offspring in adulthood.

There are an increasing number of studies that show BBB leakage in offspring from MIA mothers in brain regions such as prefrontal cortex and HIPP[306, 303, 307], however, none have investigated the influence of MIA on BBB function in the PVN where the vascular density significantly greater. The current experiments examined the neuroendocrine stress response

and BBB function in the PVN of offspring of mothers injected with the viral mimetic, TLR7 agonist RQ. Sex differences in vasculature were assessed because of sexual differences in the PVN and because TLR7 is found on the X-chromosome and escapes X inactivation.

Methods

Mice

Adult female C57BL/6N female mice (6-8 weeks old) were monitored daily by vaginal lavage for 1 week to identify estrous cyclicity. Females on day of proestrus were time-mated with adult male (8 weeks old) and removed the following day to their own cage. This day was noted as E0. Upon successful pregnancy, females were injected with RQ (HY-13740, MedChemExpress; s.c. 2mg/kg body weight) dissolved in phosphate buffer saline (0.05M PBS) or VEH (PBS) on E12.5. Pregnant females were allowed to parturition and noted as P0 (Figure 4.1A). Sex of neonates was determined on P0 and all litters were culled to 6 pups (3 male, 3 female; randomized selection of pups for each sex) to avoid litter sex bias[250]. Mice were housed with *ad libitum* access to food and water and on a 12:12 light: dark cycle (lights on at 06:00 and off at 18:00). All mice were euthanized by carbon dioxide delivered in a sealed chamber at a fill rate of 30 – 70% chamber volume per minute until breathing ceased, consistent with Colorado State University's Institutional Animal Care and Use Committee. This was followed by exsanguination by intracardial perfusion with fluorescein isothiocyanate (FITC; 1ug/mL) phosphate saline buffer and 4% buffered paraformaldehyde to fix tissues according to American Veterinary Medical Association approved methods. FITC is a small, 496kDa molecule, which binds to open amine sites in blood vessels to allow visualization of blood vessels. All procedures were approved by Colorado University Lab Animal Resources and Institutional Animal Care and Use Committee Guidelines under protocol #1567.

Acute restraint and plasma corticosterone assays

Adult male and female mice were restrained inside a plastic 50 mL conical for 20-minutes with restricted movement followed by 60-minutes of recovery. The 50 mL conical was a spatially constricted tube with a breathing hole on one end and holes along the lateral sides for increased ventilation. After the 60-minutes of recovery in the home cage, the animal was killed by inhalation of isoflurane in a sealed chamber until breathing was ceased. Animal was euthanized by intracardial perfusion as previously described[68]. Restraint stress was performed between 09:00 and 14:00 to avoid diurnal elevations in corticosterone. Cardiac blood was collected and placed into chilled 0.5M EDTA/aprotinin tubes (4mg/mL; Sigma-Aldrich, St. Louis, MO). Blood was centrifuged in a Beckman J6 centrifuge at 2000 rpm at 4 ºC for 10 minutes. Separated plasma was stored at -20º C until assayed. Plasma corticosterone levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) per manufacturer's guidelines (Arbor Assays, Ann Arbor, MI; cat no. K014-H1; Limit of detection 7.7 pg/mL mean intra-assay CV = 8.5%). 5uL of plasma samples (run in duplicates) were combined with dissociation reagent provided with the ELISA kit (Catalog # X058. This allowed dissociation of the corticosterone from corticosteroid binding globulin. The optical density of each sample was determined at a wavelength of 450 nm in Azure biosystems Ao microplate reader (Azure Biosystems, Inc, Dublin, CA). The optical density readings for the standards and samples were used to calculate the concentration of corticosterone. A standard curve was generated using the online tool from "MyAssays" through Arbor Assays. The sample concentrations were calculated from the %B/B0 curve and multiplied by the dilution factor to obtain the neat sample values. Values were analyzed with GraphPad Prism (v10, La Jolla, CA) by 3-Way ANOVA to examine the effect of prenatal RQ treatment X sex X restraint. Šídák's multiple comparisons post-hoc analysis was performed, where appropriate.

Immunolabeling for blood vessels (FITC), astrocytes (GFAP), and microglia (IBA-1)

Mice were intracardially perfused with FITC; (1ug/mL) in PBS (pH 7.4) at a rate of 4mL/min for 5 minutes. This was followed by perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were dissected and post-fixed in 4% paraformaldehyde overnight followed by immersion in 0.05M PBS until processing. Immunohistochemistry methods were followed as previously described[68, 154]. Briefly, brains from male and female mice were sectioned coronally at 50 µm with a vibrating microtome (Leica VT1000S). Free-floating sections were collected in 0.05M PBS, followed by treatment with 0.1M glycine to neutralize unreacted aldehydes. Sections were then incubated in 0.5% sodium borohydride prior to being placed in blocking serum [0.5% Triton X-100 (Tx), 1% hydrogen peroxide, 5% normal goat serum (NGS)]. Sections were then incubated in primary antisera for 48 h at 4°C against glial fibrillary acidic protein (GFAP; 1:250, RRID AB_10013382; Z0334, Dako) for astrocytes and ionized calcium binding adaptor molecule 1 (IBA-1; 1:1000, 0.1mg/mL, Cat# 019-19741, Wako) for microglia. After 48 hours, all sections were washed at room temperature in 0.05M PBS with 1% NGS and 0.02% Tx. The tissue was incubated in secondary antiserum containing Cy3 conjugated anti-rabbit (1:500), 1% NGS, and 0.32% Tx for 2 hours and washed in 0.05M PBS. Sections were mounted onto SuperFrost slides and cover-slipped with Aqua-Poly/Mount prior to imaging.

Imaging and Analysis

FITC was detected using a 505/530 nm emission filter on a confocal microscope (Carl Zeiss LSM880) with an Axiocam 503 mono camera. GFAP and IBA-1 were imaged with Cy3, detected with a 585/615 emission filter. Images were acquired for the PVN and two control regions [cerebral cortex (CX) and lateral hypothalamus (LH)]. All images were taken in 10 µm z-stacks with two optical sections for every 1 µm using a 40x/0.95 Corr M27 (Plan-Apochromat) oil immersion objective. Images were analyzed by an investigator blinded to treatment groups. For

FITC extravascular leakage, z-stack images were compiled in FIJI (ImageJ, v1.54f) using a maximum intensity z-projection. The mean gray area of a 2.97 x 2.97 µm selection (area = 8.793µm^2) inside (intravascular FITC) and outside (extravascular FITC), directly adjacent to the selection on the inside of the blood vessel, was measured. The ratio of extravascular FITC to intravascular FITC was then determined by dividing the mean gray area of the outside selection by that of the inside adjacent selection to account for differences in quality of the perfusion. This ratio was taken 12 times for each image and averaged for the single output value per image. For GFAP and IBA-1 in proximity to FITC (blood vessels), images of FITC-labeled blood vessels and GFAP-immunoreactivity (-ir) or IBA-1-ir were independently z-projected to max intensity and thresholded in FIJI (GFAP threshold to 20% intensity, IBA-1 threshold to 10% intensity). The colocalization plug-in was used to determine the percent of colocalization of GFAP or IBA-1-ir with proximity (within 2 pixels = 1.18µm) of FITC-labeled blood vessels. The number, area size, and total percent immunoreactivity of IBA-1 positive cells was also measured using Analyze Particles on thresholded images in ImageJ. Total percent area immunoreactivity was also measured for GFAP-labeled cells. FITC leakage analysis and immunolabeled protein values were statistically analyzed with GraphPad Prism (v10, La Jolla, CA) by 3-Way ANOVA to examine the effect of prenatal sex x RQ treatment X restraint. Šidák correction factor was used for multiple comparisons post-hoc analysis.

Results

Plasma corticosterone measurements indicative of impaired HPA axis stress reactivity in adult mice offspring

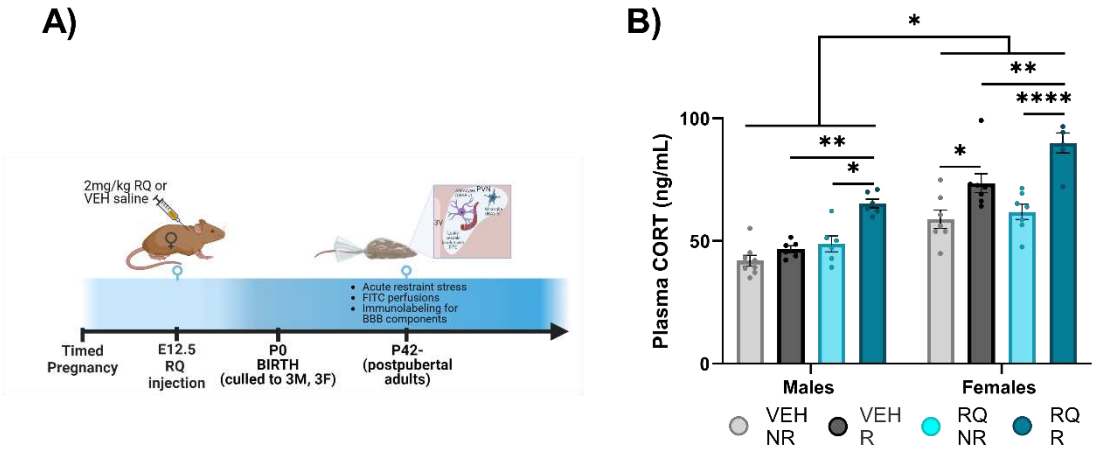

Figure 4.1. Experimental timeline and plasma corticosterone (CORT) measurements indicative of impaired HPA axis stress reactivity in adult mice offspring. (A) Experimental timeline. (B) In males, plasma CORT was highest in RQ-R adult offspring compared to the other groups. Plasma CORT was greater in restraint/recovery groups in adult female offspring (VEH-R/RQ-R vs. NR) and overall greater with prenatal RQ treatment (RQ-NR/RQ-R vs. VEH). HPA = Hypothalamic-Pituitary-Adrenal, VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint + recovery, CORT = corticosterone. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 6-9/group.

Plasma CORT levels were altered in adult offspring of RQ injected mothers (Figure 4.1B). 3-way ANOVA (Sex x RQ treatment x Restraint) revealed an effect of prenatal RQ treatment [F (1, 46) = 25.16, P = 0.0001], and restraint/recovery [F (1, 46) = 51.17, P = 0.0001]. Šidák multiple comparisons test further revealed higher levels of plasma CORT in RQ males that underwent restraint/recovery compared to VEH males (RQ-R vs. VEH-R p = 0.0032). RQ-R males showed prolonged levels of plasma CORT after recovery compared to RQ-NR (p = 0.0129). In contrast, CORT levels in VEH-R males returned to baseline levels seen in VEH non-restrained controls. Females showed elevated levels of CORT in restraint/recovery groups compared to no restraint (VEH-NR vs. VEH-R p = 0.0131, RQ-NR vs. RQ-R p = 0.0001). RQ-R females additionally

showed higher CORT after restraint/recovery than VEH-R females (p = 0.0064). 3-way ANOVA also revealed an effect of Sex [F (1, 46) = 83.05, P = 0.0001] between restraint/recovery groups within the same prenatal treatment group with higher levels of CORT in females across all groups compared to males (P = 0.0193).

The blood-brain barrier permeability in the PVN was impaired in adult offspring of RQ injected mothers

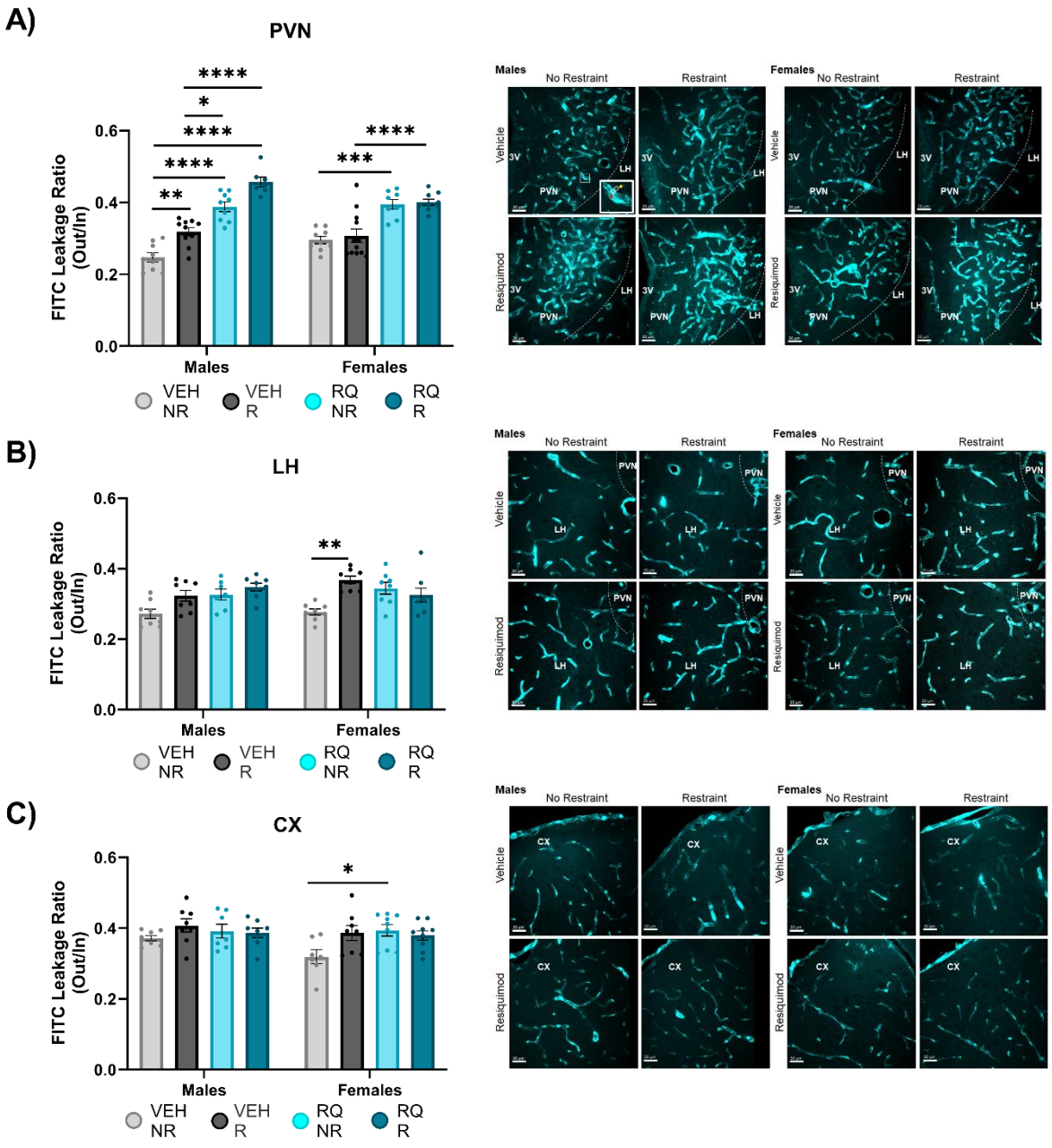

Figure 4.2. The blood-brain barrier permeability in the PVN was impaired in adult offspring exposed to MIA, indicated by FITC leakage in the (A) PVN, (B) LH, and (C) CX. Representative images to the right of each graph. The enlarged image in the bottom right corner of Male VEH-NR in (A) shows how the FITC ratio out (yellow arrow): in (red arrow) was measured. Mean gray area of the extravascular FITC (8.793µm^2 selection) was divided by the mean gray area of the intravascular FITC (8.793µm^2 selection) to obtain FITC leakage ratio. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, LH = Lateral Hypothalamus, CX = Cortex. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented sin +/- SEM. N = 8-10/group.

Leakage was indicated by a higher ratio of extravascular to intravascular FITC leakage (mean gray area intensity). A 3-way ANOVA (Sex x RQ treatment x Restraint) test showed greater leakage from FITC-labeled blood vessels in the PVN of males and females exposed to prenatal RQ treatment [F (1, 65) = 144.8, P = 0.0001] (Figure 4.2A). In males, Šidák's post-hoc analysis showed higher FITC leakage with RQ treatment in both NR and R groups (VEH-NR vs. RQ-NR p = 0.0001, VEH-R vs. RQ-R p = 0.0001). RQ females also displayed greater FITC leakage in the PVN of VEH-NR vs. RQ-NR (p = 0.00011) and VEH-R and RQ-R (p = 0.0001) groups. A 3-way ANOVA test additionally revealed an effect of restraint [F (1, 65) = 16.21, P – 0.0002] in males (VEH-NR vs. VEH-R p = 0.0045, RQ-NR vs. RQ-R p = 0.0190). There was no effect of restraint in females in the PVN. Leakage from FITC-labeled blood vessels was specific to the PVN. Two control regions (LH, CX) showed little leakage between groups. In the LH, VEH females that had undergone restraint/recovery [F (1, 55) = 6.551, P = 0.0133] had higher FITC leakage (p = 0.0164) (Figure 4.2B). In the CX, only RQ-NR females showed greater leakage from FITC-labeled vessels compared to VEH-NR with Š'dák's post-hoc analysis test (p = 0.0282) (Figure 4.2C).

Astrocyte localization to blood vessels and total immunoreactivity in the PVN in offspring of RQ-injected mothers

Astrocytic localization (labeled by GFAP) with blood vessels was altered in RQ treated animals. The percent of FITC-labeled blood vessel coverage by GFAP immunoreactivity was measured in PVN (Figure 3A). 3-way ANOVA (Sex x RQ treatment x Restraint) revealed an effect of prenatal RQ treatment in the PVN [F (1, 51) = 43.47, P = 0.0001]. Males show lower percent of blood vessel coverage by GFAP in RQ treated animals without restraint with Š'dák's post-hoc analysis test (RQ-NR vs. VEH-NR p = 0.0451). In females, percent of FITC-labeled blood vessels by GFAP was less in RQ no restraint and restraint groups compared to VEH groups in the PVN (RQ-NR vs. VEH-NR p = 0.0002, RQ-R vs. VEH-R p = 0.0006).

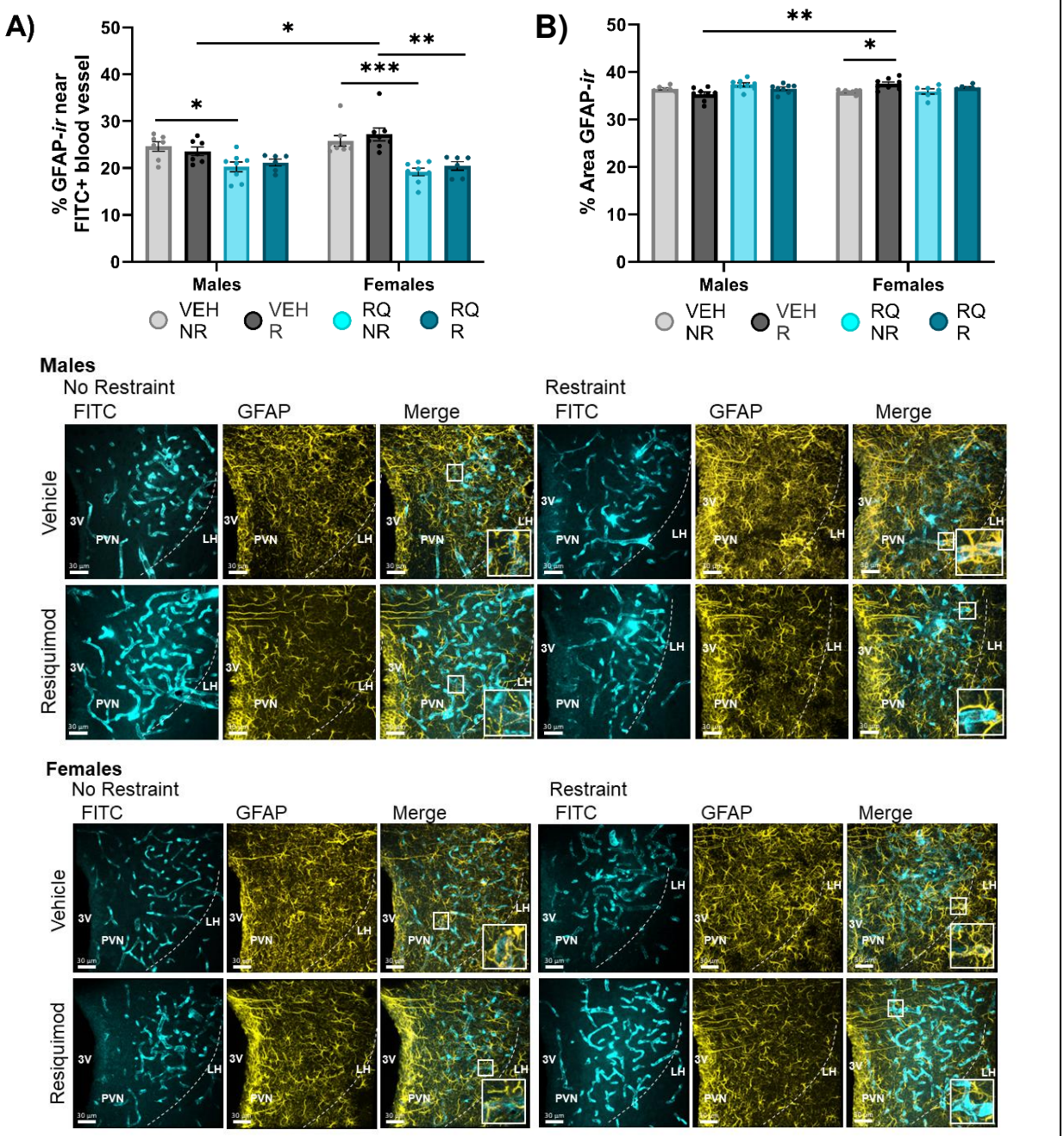

Figure 4.3. Astrocyte localization to blood vessels and total immunoreactivity in the PVN were altered with prenatal RQ treatment. (A) GFAP-labeled astrocyte coverage of PVN blood vessels was lower in male and female adult mice exposed to prenatal RQ. (B) The total immunoreactivity denoted by "% Area GFAP-*ir*" was higher in VEH-R females compared to no restraint counterparts. Representative images below. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, 3V = 3rd ventricle, F = Female, M = Male. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 6-8/group.

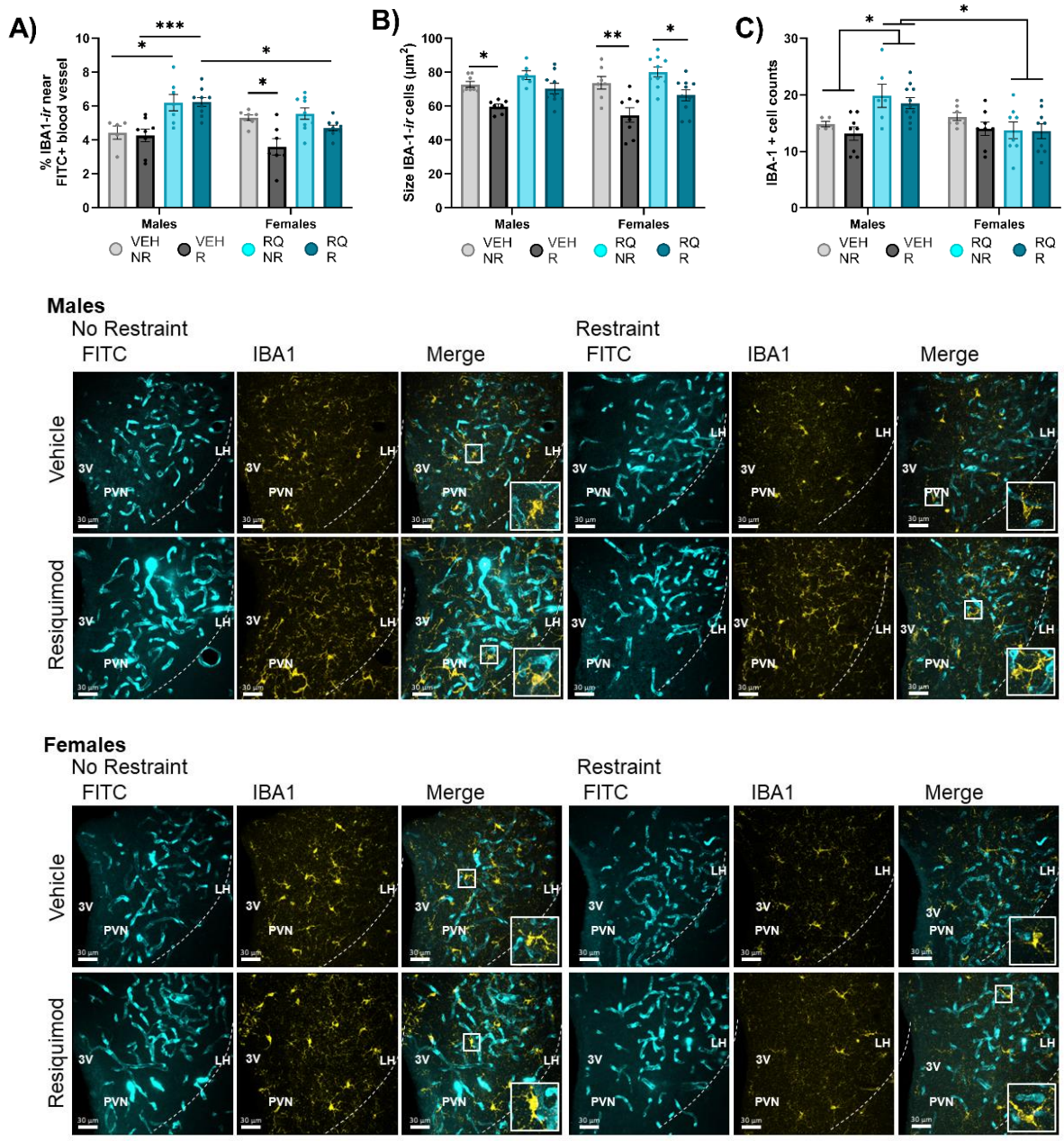

Figure 4.4. Exposure to prenatal RQ changed microglial localization and other properties, including (A) Proximity of microglia to blood vessels (within 1.18µm), (B) average size of IBA-1-labeled microglia cells, and (C) the number of IBA-1-labeled cells in the PVN. Representative images below. VEH = vehicle, RQ = Resiquimod, NR = no restraint, R = restraint, PVN = Paraventricular Nucleus of the Hypothalamus, 3V = 3rd ventricle, F = Female, M = Male. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. Error bars represented in +/- SEM. N = 8-10/group.

Microglia localization (immunoreactive IBA-1) with blood vessels, size, and number was altered in offspring of RQ injected mothers. The percent of FITC-labeled blood vessel coverage in proximity (within 2 pixels = 1.18 µm) to IBA-1 positive cells was measured in the PVN (Figure 4A). A 3-way ANOVA test revealed an effect of prenatal RQ [F (1, 53 = 27.14, P = 0.0001] and restraint [F (1, 53) = 7.710, P = 0.0076]. In males, there was more IBA-1 immunoreactive cells in proximity to blood vessels in RQ non-restraint and restraint groups compared to VEH as showed by Šidák's post-hoc analysis test (RQ-NR vs. VEH-NR p = 0.0254, RQ-R vs. VEH-R p = 0.0008). There was no effect of RQ treatment in females but there was less immunoreactive IBA-1 in proximity to vessels in VEH-treated female animals with restraint (VEH-R vs. VEH-NR p = 0.0146). A 3-way ANOVA also showed a sex difference in offspring of RQ injected mothers [F (1, 53) = 4.141, P = 0.0469] with more immunoreactive IBA-1 in proximity to FITC-labeled vessels in RQ-R males than RQ-R females (p = 0.0117). The average area (μm^2) of IBA-1 immunoreactive cells (Figure 4C) was lower with restraint in certain groups [F (1, 57) = 36.44, P = 0.0001]. Š'dák's post-hoc analysis test showed smaller IBA-1 immunoreactive cells in the PVN of VEH-R vs. VEH-NR of males (p = 0.0483) and females (p = 0.0014). RQ females that underwent restraint also displayed smaller average area of immunoreactive IBA-1 in the PVN compared to no restraint group (RQ-R vs. RQ-NR p = 0.0114). A 3-way ANOVA analysis further revealed an effect of prenatal maternal RQ injection in males only [F (1, 54) = 4.423, P = 0.0401] with more IBA-1- immunoreactive cells in the PVN in the RQ-treated groups compared to VEH (Figure 4D). 3-way ANOVA also found an effect of Sex x RQ treatment [F (1, 54) = 13.47, P = 0.0006] with more IBA-1 labeled cells in the PVN of RQ-treated males than females regardless of restraint (male RQ-R vs. female RQ-R p = 0.0272, male RQ-NR vs. female RQ-NR p = 0.0276 by Š'dák's).

Discussion

The current study examined the neuroendocrine stress response and BBB integrity in the PVN of adult offspring mice from mothers injected with the TLR7/8 agonist RQ. A two-hit stress model was tested to evaluate if exposure to fetal immune stress could predispose offspring to be more sensitive to a second adult stressor in adulthood. Data suggest HPA axis stress activity was impaired in offspring of RQ injected mothers. The BBB integrity in the PVN was compromised with greater leakage and changes in astrocytes and microglia in offspring of RQ injected mothers. There was no change in BBB integrity in control regions (cerebral motor cortex and LH) indicating this effect was PVN-selective. Because the PVN plays a critical role regulating homeostasis and the stress response, dysregulation to its BBB could alter neuronal signaling and act as a potential mechanism for increased risk of adult neuropsychiatric disease.

Offspring of RQ injected mothers displayed delays in acute-stress induced HPA axis negative feedback in adulthood

The PVN of the hypothalamus acts as the central regulator of the HPA axis stress response. PVN receives signals in relation to stressful stimuli from the environment and sends signals to the periphery to stimulate the release of GCs. Data from this study indicates that maternal injection of RQ leads to hyperactive HPA axis with impaired negative feedback following acute stress in adult offspring. A dysregulation of the neuroendocrine stress response in adulthood may lead to abnormal levels of stress hormones and associated pathologies. These findings align with previous studies in the lab and from other groups that showed fetal overexposure to exogenous GCs (DEX)[308, 309, 165, 154, 130], maternal HFD[310, 198, 171, 311, 140, 130] and maternal CR[172, 202, 130] led to impaired HPA axis function and stress-related behavioral phenotypes (Sheng and Tobet, 2023 JNE). Perturbing the maternal environment can influence brain development, acting as a potential fetal origin driving adult risk for neuropsychiatric

disorders, including MDD and cardio metabolic disease[159, 312, 193, 140, 104, 135]. Many reports suggest disruption in the PVN, the central regulator of the HPA axis, as a common pathway associated with these disorders[313-315, 128]. In the current study, fetal exposure to maternal RQ injection led to impaired stress induced HPA axis function and identified specific cellular changes (i.e., related to BBB) that could be causing improper function to this PVN circuitry.

Although the PVN is an important region of stress regulation, there are other components of the HPA axis involved in negative feedback, such as at the level at the anterior pituitary, extra-hypothalamic regions (i.e., HIPP). PVN neurons may not be the only contributors to an altered stress response and other pathways to glucocorticoid release may involve inputs or outputs outside the PVN. Such pathways may include activation of the sympathetic nervous system where catecholamines are released by the adrenal medulla and stimulate the adrenal cortex to produce cortisol[8], circadian rhythms regulated by the SCN[316], and low blood glucose levels that trigger the adrenal glands to release glucocorticoids[160]. Therefore, plasma levels of corticosterone alone do not provide direct evidence that the PVN is involved in the changes in stress response. Measures of CRH and/or ACTH will be needed to conclude the full role of maternal RQ injection on HPA axis acute stress circuitry in offspring.

Offspring of RQ injected mothers displayed greater leakage from blood vessels in the PVN

The BBB protects the brain by preventing harmful compounds in the circulatory system from infiltrating into the CNS[156]. In mice, the BBB starts to develop embryonically at ~E11.5 and continues to mature through early postnatal life [68, 317, 318]. Maternal stress can perturb BBB formation and lead to downstream influx of toxins and harmful immune cells (e.g., inflammatory T cells) into the brain. Reduced BBB integrity driven by maternal immune stress has been linked with increased susceptibility to neuropsychiatric pathologies, including MDD and ASD, among

others[319, 303, 320, 321]. While many maternal immune studies on BBB permeability have focused in cortical, hippocampal, or cerebellar regions and their downstream influence over adult neuropsychiatric disease incidence, the unusually dense vasculature of the PVN led to the hypothesis that the BBB in the PVN of the hypothalamus might be particularly important[306, 321]. In the present study, BBB leakage by FITC was increased offspring of RQ injected mothers in the PVN of adult males and females. FITC leak was selective to the PVN, with no change in leakage in control regions. This effect was exacerbated with acute restraint stress in males. Such results demonstrate males may be more susceptible to BBB leakage in the PVN following a stressor in adulthood if previously exposed to excess maternal immune stimulation during prenatal development.

Astrocytes are an important component of a functional BBB. In the current study, adult offspring of RQ injected mothers showed lower total astrocytes and coverage of GFAP-labeled astrocytes over blood vessels in the PVN. Reduced coverage of astrocytes surrounding blood vessels of the PVN correlates with increased permeability, as suggested by higher leakiness found of FITC in the adjacent extravascular areas. Astrocytic endfeet are shown to mediate water flow through a bi-directional regulatory channel, aquaporin 4[322, 302]. Uptake (glucose transporter; GLUT4) and efflux (phospho-glycoprotein) transporters are also found on the astrocytic endfeet[323]. Disruption in the function of these transporters could alter trafficking of glucose and other metabolites across the BBB[321]. The current data show that paracellular FITC leaks into extravascular space in the PVN in adult offspring of RQ injected mothers. This effect could be a consequence of the fewer total astrocytes and reduced astrocytes near blood vessels in the adult offspring. Mechanisms that influence astrocyte coverage of the blood vessels could be programmed during early development. These could include mutations in or epigenetic modifications of genes that regulate proper astrocyte differentiation and function of astrocyte end-feet or altered signaling pathways, which are involved in BBB development and could result

in poor astrocyte-vascular relationship. Overall, these findings indicate changes in astrocytes lead to a leaky BBB in the PVN in adulthood, a potential pathological symptom for neuropsychiatric disease. In addition to astrocyte endfeet, the BBB is comprised of endothelial cells and perivascular pericytes along with endothelial cells linked through tight junctions. Previous studies in the lab demonstrated prenatal GC exposure led to dysfunction in pericytes [154], while other MIA studies show disruption in endothelial cell and tight junction protein and mRNA expression[155, 307, 69]. Future studies will be needed to determine if maternal injection of RQ has a similar effect on BBB components in the PVN of offspring.

Prenatal RQ treatment led to sex-selective changes in microglia in the PVN.

Microglia are resident immune cells in the CNS and have been implicated in driving inflammatory effects after MIA from fetal life to adulthood [324, 201, 325, 326, 304, 151]. Rodent studies demonstrate a link between elevated pro-inflammatory cytokines in response to maternal immune stress, altered microglial phenotypes postnatally and adulthood [324, 201], and increased stress-related behavioral phenotypes (depressive-, anxiety- and social-like impairments). In a parallel study with RQ injected mothers, there were maternal and fetal peripheral immune responses seen as elevated serum inflammatory cytokines (IL-6, IL-17a) (Sheng & Tobet, 2023 JNE). The present study extends previous findings by examining changes in anatomic localization and morphology of microglia in offspring of RQ injected mothers selectively in the PVN. The data showed microglia were closer to blood vessels in the PVN of RQ-males (assessed as coverage) than VEH males. Females did not show an effect of maternal RQ-injection on microglial coverage of PVN blood vessels but did have lower microglial coverage on PVN blood vessels following restraint stress. These data suggest sex-selective programming of microglial location in the PVN. In a study by others, pregnant mice treated with the TLR7 agonist, imiquimod, resulted in greater expression of microglial markers, including chemokines (*Ccl2, Ccl6, Cxcl10*) and pro-inflammatory cytokines (*Tnf-α, IL*-6),

indicating increased microglial activation[247]. This study, however, did not assess sex as a variable, a crucial factor when investigating microglia in the context of sex-selective neuropsychiatric disorders.

Many studies demonstrate maternal immune stress leads to morphological changes in adult microglia, with retracted processes and a smaller, more “amoeba-like” shape[327]. The decrease in size induced by restraint in VEH males and all females in the current study could suggest the PVN microglia react quickly to stress. Other evidence suggests activated microglia might disrupt BBB function and could be a cause for increased leak in the PVN[327]. Microglial size did not change with restraint in RQ-males, indicating less functional microglia caused by exposure to maternal injection. Moreover, activated microglia often migrate to “injured” sites, phagocytose harmful compounds or cells in a region, and secrete inflammatory cytokines to recruit additional innate immune cells to help repair damaged tissue and neurons[258]. Data in the current study demonstrate impaired integrity of the BBB of the PVN, suggesting an increased need for microglia to help “repair” leaky vascular tissue by releasing vascular growth factors and phagocytose harmful compounds from the periphery. Changes to microglia function near or around blood vessels could alter vascular and brain development and contribute to stress-related behavioral phenotypes (Sheng & Tobet, 2023 JNE). A study using CX3CR1 deficient mice, a receptor on microglia, showed reduced microglial density in the HIPP with weak synaptic function, cognitive defects, social impairments, and improper HPA axis stress response[328]. The current study supports these findings linking impaired microglial properties in the PVN to changes in neuroendocrine stress response.

Conclusions

In summary, the current study demonstrated maternal injection with RQ led to neuroendocrine dysfunction that was selective for PVN. Improper PVN function was associated with sex

selective differences in vasculature integrity, and alterations in astrocyte and microglial cell interactions with blood vessels. A previous study showed that maternal injection with RQ led offspring to have more stress-related behavioral phenotypes linked to HPA axis dysregulation. Taken together, the data leads to the hypothesis that maternal immune stress leads to changes in neuroendocrine PVN signaling and BBB integrity, potentially influencing susceptibility to neuropsychiatric pathologies.

CHAPTER 5:
DISCUSSION

"It'll get done because it needs to"
– Dr. Taben Hale

To those who have made it to this point in this [*insert final page number here*] page document, whether you read every single word, the title of each chapter, or simply skipped to this chapter, much appreciation – and hopefully, at least 5 of those that are reading this are my stellar committee members who have had to put up with me these past 5 years. Thank you again for the push and inspiration needed to make it to this point.

This section of the thesis might honestly be the most challenging part yet. How does one concisely sum up five years of procrastinating, accidentally offending my committee member (s) (Apologies Stu, we all really do think the vest/button up shirt is a good look! Sorry, Brent. I know you are not as old as a typewriter.), research successes, and utmost failures in one chapter (And the procrastination continues, as I am sitting here writing this nonsense rather than an informative discussion)?

But here goes… … …..

Introduction

Maternal stress encompasses numerous environmental stimuli, ranging from traumatic events to chronic or day-to-day life stressors (i.e., emotional death of a loved one, daily traffic, or even the COVID-19 pandemic). Such stressors in pregnant women result in overexposure to GCs that are demonstrated to impact short- and long-term neurological health in the offspring. In a population-based study conducted across 11 states from 1990-1995, 64% of pregnant women reported experiencing at least one stressful life event during gestation. In 2010, 78% of a

second cohort of women reported experiencing such an event during pregnancy[329, 4]. More recently, stress levels were examined in pregnant women during the COVID-19 pandemic, with 80% of subjects reporting to have felt overly stressed (Centers for Disease Control, 2021). Overall, 65%-70% of all pregnant women experience one or more stressful life events, increasing risk for adverse outcomes in the fetus. Because of the prevalence of maternal stress in our society, it is important to understand its impact on the offspring. Developmental programming by maternal stress can cause permanent neurological changes in offspring and increase likelihood of adult diseases, including pathologies of MDD (i.e. negative mood, decreased motivation, anhedonia), schizophrenia, social disorders, and cardiometabolic disorders[330].

The HPA axis, the body's main stress response, is a complex system of neuroendocrine signaling pathways and feedback loops working to maintain homeostasis. The HPA axis receives signals from several brain regions that differ significantly by sex, including the HIPP, AMY, ventromedial prefrontal and orbitofrontal cortices, and hypothalamus[331]. These brain areas develop and function differently in males versus females due to dense expression of GC and gonadal steroid receptors[5]. Sex differences in the programming of such areas are becoming more well studied to better understand underlying pathologies in stress-related disorders.

The overarching goal of my dissertation was to examine the effect of stress during pregnancy on sex-dependent programming of the brain in the offspring. The manuscript detailed in Chapter 2[130] details various models of maternal stress and how they influence stress-responsive systems, like the HPA, in prepubertal mice. The study compared three types of stressors, prenatal overexposure to GCs (DEX), maternal HFD, and maternal CR. Prenatal exposure to DEX lowered neonatal body weights, social interaction, and lead to a hypoactive neuroendocrine stress response. Maternal CR also showed smaller offspring, lowered social

interaction in males and females, and increased anxiety-like behavior and hyperactive stress response in male offspring. Conversely, maternal HFD led to greater weight gain in offspring with lowered anxiety-like behavior in females. Many of the developmental, neuroendocrine, and behavioral outcomes were quite varied with each model of maternal stress. However, one commonality that was overall model-, age- and sex-independent was the robust reduction in social-like behavior. Such result indicate the importance of the prenatal environment in the development of a behavioral phenotype shared across a broad spectrum of neuropsychiatric and neurodevelopmental disorders, including schizophrenia, ASD, social, cognitive, and mood related outcomes. Many studies examine “the social brain” in the context of MIA, with gene expression changes in brain regions that regulate social-like behaviors, such as the HIPP[332], AMY [333], and dorsal striatum[247]. In future studies, it would be interesting to examine these same brain regions in mice prenatally exposed to RQ to determine the extent to which this model of MIA alters social and cognitive function.

Of note, the mice evaluated in this study were prepubertal. Many mouse studies that investigate mechanisms of neuropsychiatric disease begin behavioral testing in adults because older mice make for easier handling and assessment of complex cognitive and behavioral function. However, adult assessments miss valuable information on developmental milestones and prepubertal behavior[179, 221]. In humans, behavioral symptoms of neuropsychiatric disease can be clinically diagnosed during infancy and early adolescence. Therefore, it is important to test behavioral phenotypes in mice studies to parallel the timing of onset of these pathologies in humans. If we can determine predispositions or biomarkers sooner, therapeutic intervention can occur earlier on and prolong or even prevent development of disease.

An extensive line of literature demonstrates fetal antecedents leads to long-term effects on HPA axis physiology. Data described in Chapter 2 suggests varied models of prenatal stress produce varied effects. For instance, opposing nutritional stressors (CR and HFD) showed similar anxiety-like behavior in males and females, but different social-like behavior phenotypes and neuroendocrine stress response depending on the type of nutritional stress. Administration of DEX produced differing outcomes in offspring compared to either CR or HFD, further suggesting the GC response is not a common pathway in all types of maternal stress. One common theme among the three models is that consistent data in the literature implicates their role in activating maternal and fetal immune responses[163, 4, 212]. In addition to modulating maternal stress responses, the HPA axis also regulates immune responses (i.e. inflammatory cytokine/chemokine release, microglial activation) to environmental or inflammatory stimuli during pregnancy[4, 128]. More recent epidemiological reports have linked MIA and cytokine exposure with mood and cardiometabolic disorders[103, 241]. Given the long history of animal models of maternal immune stress, the goal of my thesis project was to investigate this in rodents. Viral Poly I:C (TLR3 agonist) and bacterial LPS (TLR4 agonist) are two common modes of MIA. My studies described in Chapter 3 used an MIA protocol that activates TLR7, a conserved viral response pathway between mice and humans. While TLR7 signaling has been studied for immune responses in the host, few reports examine activation of TLR7 in neurodevelopment. TLR7 is also found to be expressed higher in females as it is encoded on the X chromosome and can escape X-inactivation[248]. Therefore, this study assessed sex differences in mouse offspring with maternal exposure to the TLR7 agonist RQ, during mid-gestation. Results showed RQ induced changes in serum maternal cytokine levels and sex-dependent changes in fetal cytokines.

One caveat of this study is that cytokine levels in the brain were not measured, which would have provided better evidence of a fetal neuroimmune response following RQ exposure. Serum

levels were measured at a single timepoint, 60 hours post injection. It would be important to do a time-dependent study with serum and neural tissue samples taken at various timepoints following the administration of RQ. This would allow us to gain a better idea of more immediate effects of TLR7 agonism on both peripheral and central cytokine responses in maternal and fetal environments. These studies could also help tease apart specific mechanisms or signaling pathways involved in the maternal and/or fetal response to RQ. Does RQ stimulate an immune response in the mother, increase maternal cytokines which cross the placental-fetal interface, affecting the fetus? Conversely, does RQ act directly on the fetus by crossing the placental barrier and binding TLR7 receptors in the fetal environment (peripheral or central), thereby exerting an immune response? These questions could be addressed by examining TLR7 expression in the fetal brain at the time of injection (E12.5). If fetal TLR7 is absent, we could hypothesize it is more likely RQ activates inflammatory pathways in the mother with downstream effects on the developing fetus. However, this does not preclude an off-target effect of RQ, or an effect requiring longer time to activate. As Stu always says:

"The presence of absence does not always mean the absence of presence"

– Stu

There is a possibility TLR7 is simply undetectable at the stage of development we are looking since the mucosal immune system in the mouse is not fully developed until post weaning (about 21 days of age). Other reports further demonstrate TLR7 is detectable as early as E13, half a day before the pregnant dams in our studies receive the injection. Such information would suggest it is more of the downstream effects of the maternal and placental inflammatory response that influences the fetal response[334].

Another finding described in Chapter 3 is that offspring of mothers injected with RQ were smaller, with developmental delays, later puberty onset in females, and changes in behavior

pre- and post-pubertally. Social interaction was lower in juvenile and adult offspring. However, only adult males exhibited more anxiety-like behavior and adult females showed greater anhedonia-like behavior. Interestingly, these behaviors only arose in post-pubertal mice, after they had been exposed to endogenous gonadal sex steroids through puberty. Such data point towards gonadal hormones (such as androgens and estrogens) exerting organizational effects on sexually dimorphic brain regions that control these stress-related pathologies. It is also interesting to note that the gonads of the mice embryos are forming around the same time of RQ injection (E12.5)[335]. Disruption in this important process could lead to altered gonadal development and gonadal hormone secretions, influencing puberty onset and sex-selective mechanisms in adults. It would be interesting to investigate the possibility of impaired development of gonads during these embryonic stages to determine if these could help explain the changes in development and behavior in RQ-offspring.

Several studies demonstrate the influence of maternal care on offspring physiological and behavioral development. In rodent studies, maternal care refers to the behavior of the dam (i.e., tactile licking, grooming, arched back nursing) in nourishing and protecting their litter before offspring are weaned (usually around P21). Poor maternal care has been shown to lead to epigenetic and behavioral consequences. These include lower protein and mRNA expression levels of hippocampal GR, dysfunctional neuroendocrine function of the HPA axis, and more anxiety- and social-like behavioral phenotypes[105, 289, 109, 107, 336]. Mouse studies show disruptions in maternal care of mice exposed to MIA with Poly I:C with lower licking and grooming[337], impaired nest-building[232], and reduced pup retrieval[338]. Preliminary data (unpublished) from the RQ study described in Chapters 3 demonstrate lowered maternal care with longer pup retrievals in dams exposed to RQ during gestation. While these findings were not followed up on, it is possible that the poor maternal care in RQ-treated dams influenced

behavioral outcomes in offspring. Cross-fostering in future RQ studies could help tease apart the effect of maternal behavior and prenatal RQ treatment on pup neurodevelopment.

Chapter 4 more closely examines sex-dependent effects of prenatal RQ on the BBB of the PVN that could be playing a role in the stress-related behavior symptoms demonstrated in Chapter 3. Previous findings in the lab were some of the first to demonstrate that the BBB in the PVN is 3-5 times more vascularized than any surrounding brain region[339, 67]. The BBB of the PVN protects its neuronal population from the influx of harmful compounds in the blood and regulates trafficking of molecular compounds to and from the periphery. The BBB is composed of multiple cell types, including endothelial cells sharing tight junctions, endothelial wrapping pericytes, and astrocytic end feet. Vascularization of the PVN occurs during fetal development. At birth, PVN vasculature is anatomically similar to neighboring forebrain areas. In the second postnatal week in mice and rats, PVN vasculature becomes significantly denser[339, 67]. It is not known what drives this increased angiogenesis unique to the PVN but is a critical interface between the circulatory system and brain. Several studies suggest impaired integrity of the BBB can lead to infiltration of harmful substances from the periphery (i.e., immune cells, toxins) into the brain, resulting in altered neuronal circuitry and/or function[340, 321]. Additional evidence indicate dysfunction of the BBB is result of fetal exposure to maternal stress, a symptom becoming more common in the pathologies of neuropsychiatric disease[307].

Chapter 4 of my dissertation determined the effect of maternal RQ injection on the integrity of the BBB in the PVN of adult offspring. Maternal RQ injection resulted in dysregulation of the neuroendocrine stress response, potentially influenced by impaired integrity of the PVN vasculature. We also examined control regions (cerebral motor cortex and LH) but found this effect was specific to the PVN. Male and female adult mice with fetal exposure to RQ showed greater leak in the PVN with less astrocyte endfeet coverage around the blood vessels. Under healthy conditions, astrocyte endfeet wrap surround vessels to regulate permeability and

passage of water and certain molecular compounds[321]. Lower astrocyte coverage could be reason for greater leak seen in the PVN of these mice. Unfortunately, other components of the BBB were not examined, such as pericytes, endothelial cells, or tight junctions. These cell types could provide further insight into how or where the BBB is disrupted in offspring exposed to maternal immune stress.

Microglia are the resident immune cells of the CNS. In rodents, many MIA experiments show a heightened neuroimmune response with more activated microglia that secrete pro-inflammatory cytokines to maintain a healthy environment for surrounding neurons and vasculature. Given my previous data noted in Chapter 3 showing higher levels of pro-inflammatory cytokines in RQ-treated animals, I wanted to determine microglia changes in the context of MIA using RQ. Observations were focused in the PVN because we wanted to investigate microglial changes under stressful conditions in relation to the BBB, where the data showed maternal RQ injection reduced BBB integrity. Differences in microglia properties were found, with changes in size, number, and proximity to blood vessels with maternal infection and by sex. Future work to measure a neuroimmune response would be to quantify expression of inflammatory markers (cytokines, chemokines, or immune cells) in the PVN. Many of these are secreted by microglia, and even astrocytes, and could help tease apart the role of these nonneuronal cells in the BBB and immune function in the brain.

Final thoughts

The impact of stress on neurological health begins before we take our first breath. A long line of literature in humans and animals provides evidence that perturbations to the maternal environment affects brain development of the offspring. Exposure to these maternal stressors – whether they be infection, nutritional, metabolic, or psychological – increases the risk of mood disorders, heart disease, and even metabolic dysfunction in adulthood. Now, one may ask, how

can a single event during pregnancy, no matter the flavor of stress, program the fetal brain so robustly to result in comorbidity of such disorders? Depending on the time of development, many vital organs, including the brain and heart, are forming. Cells are migrating, proliferating, and differentiating into their final forms. If any single or multiple of these mechanisms are altered, another pathway or circuit changes to compensate for what was lost. The effects of such developmental outcomes could be hugely consequential down the road.

Downstream effects of maternal stress on offspring may even go back to the idea of the “two-hit” hypothesis. Many reports indicate exposure to fetal stress predisposes an individual to be more affected by stressors during early postnatal or even later life stages, which then raises susceptibility to onset of stress-related disease. Ideally, studies in this field will help to uncover early biomarkers or behavioral symptoms of stress-related disease. My studies are unique to the field of maternal stress and its role in mood disorders and comorbid diseases. The use of animals in the same animal housing facility and suite on the same timed-breeding protocol between models allowed me to directly compare the neuroendocrine regulation of maternal stress in a well-controlled and robust fashion. Few MIA studies use a TLR7 agonist in the context of mood and other neuropsychiatric disorders. The more we understand how different immune signaling pathways are stimulated and how these mechanisms then program fetal and postnatal brain development, the better chance we have at diagnosing these disorders sooner and everyone can live a happier, healthier life. 😊

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LIST OF ABBREVIATIONS

11β-HSD2	11-β hydroxysteroid dehydrogenase type 2
5-HT	Serotonin
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
AMY	Amygdala
AR	Androgen receptor
ARNT2	Aryl hydrocarbon receptor nuclear translocator 2
AVP	Arginine vasopressin
BBB	Blood-brain barrier
BNST	Bed n. of the stria terminalis
CNS	Central nervous system
CR	Caloric restriction
CRH	Corticotropin releasing hormone
DEX	Dexamethasone
DHEA	Dehydroepiandrosterone
E	Embryonic day
ER	Estrogen receptor
FITC	Fluorescein Isothiocyanate
GC	Glucocorticoid
GD	Gestational day
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
HIPP	Hippocampus
HFD	High fat diet
HPA	Hypothalamic-pituitary-adrenal
IBA-1	Ionized calcium binding adaptor molecule 1
IL	Interleukin
LH	Lateral hypothalamus
LPS	Lipopolysaccharide
MDD	Major depressive disorder
MIA	Maternal immune activation

MR	Mineralocorticoid receptor
NF-κB	Nuclear Factor Kappa-B
NGFI-A	Nerve growth factor-inducible protein A
NTS	Nucleus of solitary tract
OT	Oxytocin
P	Postnatal day
PFC	Prefrontal cortex
Poly I:C	Polyinosinic: polycytidylic acid
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
RQ	Resiquimod
SHRP	Stress hypo-responsive period
Th17	T-helper cell 17
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VEH	Vehicle