Sex Differences in the Neuroimmune Modulation of Learning and Memory

by

Caitlin K. Posillico

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Doctoral Committee:

      Associate Professor Natalie C. Tronson, Chair
      Professor Jill Becker
      Professor Martin Sarter
      Assistant Professor Joanna Spencer-Segal

Caitlin K. Posillico

ckpos@umich.edu

ORCID iD: 0000-0001-5764-0517

Dedication

This one's for me.

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Abstract

The neuroimmune system is a specialized immune system in the brain crucial for both responding to illness and injury as well as regulating normal neural function and behavior. As such, it is perhaps not surprising that activation of the neuroimmune system results in significant impairments in synaptic plasticity and learning and memory mechanisms. In fact, neuroimmune dysregulation has been implicated in memory- and cognitive-related disorders including Alzheimer's disease, Post-Traumatic Stress disorder, and most recently long-COVID, a series of long-lasting cognitive impairments caused by the virus SARS-CoV-2. There are known sex differences in the neuroimmune response to various pathogens, and because the neuroimmune system is at the convergence of pathological and normal function, the immune cells and signaling mechanisms involved are well-poised to modulate memory processes differently in males and females which may contribute to sex differences in the prevalence or severity of memory-related disorders. Here, we aimed to investigate the interaction of neuroimmune and memory processes in both males and females using central administration of a viral mimic, polyinosinic:polycytidylic acid (poly I:C), in C57BL/6N mice. Poly I:C is synthetic, double-stranded RNA that stimulates several cell types involved in mounting an immune response in the brain including astrocytes, microglia, and neurons, making it an excellent tool for studying broad-based neuroinflammation. Poly I:C treatment induced significant inflammation in the hippocampus of both sexes. Males had a greater magnitude of response than females for cytokines IL-1alpha, IL-1beta, IL-6, IL-10, IFNalpha, TNFalpha, CCL2, and CXCL10. Additionally, while both males and females showed increased expression of the anti-viral Type I

interferon beta, only males showed increased anti-viral Type I interferon alpha, highlighting a potentially important sex difference in the anti-viral response to poly I:C. We used a T-maze task and a contextual fear-based memory task to determine the effects of neuroinflammation on learning and memory mechanisms. Pre-training poly I:C did not impair learning in the T-maze task. In contrast, pre-training poly I:C disrupted learning of contextual fear conditioning in both males and females, and analysis of cFos levels revealed significant sex differences in hippocampal activation during context fear conditioning training with poly I:C on board. Together, these findings suggest that a similar behavioral deficit induced by poly I:C in males and females involve sex-specific molecular and signaling mechanisms of learning and memory. To further investigate this, we targeted Type I interferon signaling because of the sex difference in Type I interferon induction we found previously and the capacity for Type I interferons to modulate synaptic plasticity mechanisms. We found that inhibiting Type I interferon receptors prior to treatment with poly I:C attenuated the poly I:C-induced learning deficits in males, and we did not find the same effect in females. This suggests that Type I interferons play a more important role in modulating learning in males compared with females, and Type I interferon signaling is a potential target for understanding sex differences in biological mechanisms of memory impairment induced by neuroimmune activation.

Chapter 1:

Introduction

The neuroimmune system is necessary for regulating normal neuronal functions under healthy conditions in addition to detecting and responding to injuries and infections during illness (Marin & Kipnis, 2013). Consequently, neuroimmune activation disrupts important cognitive processes including learning and memory (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Donzis & Tronson, 2014; Marin & Kipnis, 2013; McAfoose & Baune, 2009). Aberrant neuroimmune function has been implicated as both a cause and consequence of debilitating memory-related disorders such as Alzheimer's disease and Post-Traumatic Stress disorder (Krstic & Knuesel, 2013; Pace & Heim, 2011; Z. Wang & Young, 2016), and notably, the risk for developing either disorder is more than double in women compared with men (Henderson & Buckwalter, 1994; Laws, Irvine, & Gale, 2018). Despite evidence that memory processes, the neuroimmune system, and biological sex are intricately linked, we do not know precisely *how* neuroimmune activation interacts with mechanisms of learning and memory and how this is similar or different in males and females.

Sex differences in the activation of the immune and neuroimmune systems have been reported in various contexts. In the periphery, females have a greater inflammatory response compared with males (Klein & Flanagan, 2016), while in the brain, male-derived cortical astrocytes have a significantly greater reaction to inflammatory insults compared with immune cells from females (Loram et al., 2012; Santos-Galindo, Acaz-Fonseca, Bellini, & Garcia-Segura, 2011). We have also previously found sex differences in the magnitude, time course, and

pattern of inflammation in the brain after stimulating the immune system in the body (Speirs &

Tronson, 2018). These sex differences in immune function may differentially impact regions of

the brain responsible for normal cognition, learning, and memory – such as the hippocampus –

which may explain why there is a sex bias in memory-related disorders.

Inflammation in the brain has become a front-runner for an underlying causal mechanism

of dementias, including Alzheimer's disease, characterized by severe cognitive and memory

impairments (Heneka et al., 2015). Additionally, we know that COVID-19 of today's pandemic

presents with both significant neuroinflammation and cognitive dysfunction (B. Liu, Li, Zhou,

Guan, & Xiang, 2020; Wan et al., 2020; H. Zhou et al., 2020). According to the Alzheimer's

Association, 50 million people world-wide suffer from Alzheimer's disease and other dementias,

and chronic neuroimmune dysfunction from long-COVID could very well cause these numbers

to increase in the near future. My dissertation research investigates how related

neuroinflammation impacts memory and whether distinct mechanisms are at play in males and

females so that we may have a better understanding of associated risk and resilience factors for

each sex in the context of health and disease.

1.1 The Neuroimmune System: An Overview

For many years, the brain was considered to be immune-privileged – that it lacked the

necessary machinery, so to speak, to mount an immune response. Scientists noted the presence of

a blood-brain barrier, a missing classically draining lymphatic system, and sparse presence of

any antigen-presenting cells. These findings, combined with studies that showed transplanted

tissue into the brain parenchyma failed to mount a sizeable rejection response (Medawar, 1948;

Widner & Brundin, 1988), all pointed to a missing immune system in the brain (Louveau, Harris,

& Kipnis, 2015). While the brain is absolutely a privileged and complex organ, it is now well-

established that it does, in fact, have a unique immune system called the neuroimmune system.

Research from the past few decades has unveiled both a glymphatic and meningeal lymphatic

system in the brain for draining (Iliff & Nedergaard, 2013; Iliff et al., 2012; Louveau, Smirnov,

et al., 2015; Yankova, Bogomyakova, & Tulupov, 2021), that antigens from the central nervous

system do, in fact, mount immune responses (Cserr, Harling-Berg, & Knopf, 1992; Kida,

Pantazis, & Weller, 1993), and there is extensive crosstalk between specialized immune cells in

the neuroimmune system with the peripheral immune system that have complex effects on brain-

behavior interactions (Dantzer, 2018; Louveau, Harris, et al., 2015; Reardon, Murray, & Lomax,

2018).

1.1.1 Microglia and Astrocytes

Key players of the neuroimmune response include microglia and astrocytes, which are

both capable of being stimulated by and responding to various molecules released by infected or

damaged cells termed alarmins (Ransohoff & Brown, 2012). Microglia are known as the resident

immune cells of the brain. During embryonic development, the ectoderm germ layer gives rise to

the central nervous system, including neurons, astrocytes, and oligodendrocytes. Early on, even

before they were named, microglia were identified to be a unique cell type in the brain that likely

derived from a different germ layer, but where they came from remained a mystery for decades

(Ginhoux, Lim, Hoeffel, Low, & Huber, 2013; Ginhoux & Prinz, 2015). Recent work now

supports that microglia arise from extraembryonic yolk sac macrophages and migrate into the

brain during early development in both humans and non-human animals (Chan, Kohsaka, &

Rezaie, 2007; Ginhoux et al., 2013).

Once established in the brain, microglia play a major role in neural development *via*

synaptic pruning. Specifically, microglia actively survey the microenvironment and detect

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changes in synaptic activity where they preferentially engulf less active synapses through phagocytosis (Favuzzi et al., 2021; Hong, Dissing-Olesen, & Stevens, 2016; Paolicelli et al., 2011; Schafer et al., 2012). Interestingly, there are sex differences in the number of microglia in different brain regions during the early postnatal stage of rodents, where males at postnatal day 4 have significantly more microglia than females in the hippocampus and amygdala (Schwarz, Sholar, & Bilbo, 2012). Recent work expanded on this and showed that on postnatal days 0 (day of birth), 2, and 4, male microglia in the amygdala are more phagocytic than female microglia and specifically engulf more newborn astrocytes in males than in females (VanRyzin et al., 2019). This results in fewer astrocytes in the amygdala of juvenile males and directly corresponds to the normal appearance of sex differences in social play behavior at this juvenile timepoint (VanRyzin et al., 2019). Together, these data show the importance of microglia synaptic pruning and phagocytic activity during development for cognition and behavior later in life.

Microglia continue their role in actively monitoring and sampling the microenvironment after development as well. In this surveying state, microglia have long, thin, ramified processes that extend from a smaller cell body and move around to physically monitor the extracellular space. They express innate immune receptors that allow them to sense invading pathogens and cellular debris (Carpentier, Duncan, & Miller, 2008; Kigerl, de Rivero Vaccari, Dietrich, Popovich, & Keane, 2014; Ransohoff & Brown, 2012). Upon detecting of an alarmin, their processes begin to retract and become thicker, their cell body increases in size, and in this state, microglia can use phagocytosis to engulf the infected cell, bacterium, or cellular debris and digest it (Karperien, Ahammer, & Jelinek, 2013; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Stence, Waite, & Dailey, 2001). In addition, microglia can also signal to other immune cells to

induce neuroinflammation and aid in the response to the immune stimulant. Afterwards, microglia return to a ramified morphology and continue their role in surveillance.

Astrocytes are a type of macroglia, and scientists have argued over the years about the ratio of glia to neurons in the human brain. Some studies found that glia vastly outnumber neurons, others report a roughly equal ratio, and others still have reported that the ratio of glia to neurons changes depending on the brain region being studied (Herculano-Houzel, 2014; Keller, Erö, & Markram, 2018; von Bartheld, Bahney, & Herculano-Houzel, 2016). Nevertheless, researchers long held the assumption that astrocytes were simply nutritionally supportive cells of the central nervous system and reacted to damaged or diseased tissue *via* astrogliosis, with little attention to whether this had any regulatory or communicative function. It is now well-established that astrocytes play a much more critical role in neural communication and synaptic plasticity mechanisms which will be discussed later in further detail. In addition, astrocytes help to regulate blood-brain barrier permeability, and in disease states, astrocytic dysfunction is associated with a weakened blood-brain barrier and increased vulnerability of the central nervous system to further damage (Abbott, Rönnbäck, & Hansson, 2006; Alvarez, Katayama, & Prat, 2013; Cabezas et al., 2014; Sofroniew & Vinters, 2010). Similar to microglia, astrocytes also express innate immune receptors capable of detecting and responding to invasive pathogens or tissue damage, and reactive astrocytes may also change in morphology upon stimulation (Farina, Aloisi, & Meinl, 2007; Kielian, 2006; L. Li, Acioglu, Heary, & Elkabes, 2021; Sofroniew, 2014). Reactive astrocytes and astrogliosis have been shown to have both helpful and harmful effects depending on the method of activation, and they have also been implicated in the pathophysiology of diseases (Liddelow & Barres, 2017; Ludwin, Rao, Moore, & Antel, 2016; Pekny & Pekna, 2014; Sadick & Liddelow, 2019).

1.1.2 Activating the Neuroimmune System via Innate Immune Receptors

Astrocytes, microglia, and even neurons express pattern-recognition receptors that are activated by pathogen- and danger-associated molecular patterns found on bacteria, viruses, and damaged tissue (Kigerl et al., 2014). The promiscuity of pattern-recognition receptors is a crucial function of the innate immune system to detect anything foreign or "non-self" that has the potential for harm. One prominent family of pattern-recognition receptors is the Toll-like receptor (TLR) family which, when activated, stimulate and potentiate the immune response to A) promote the clearance of infected cells, bacteria, and debris, B) recruit additional immune cells to the site of activation, and C) activate the adaptive immune system to create a cellular memory of the invasive pathogen or insult for the future (Akira & Takeda, 2004; Kawasaki & Kawai, 2014; Mishra, Mishra, & Teale, 2006).

Toll-like receptors were the first class of pattern-recognition receptors to be identified and have been well-characterized as a result. Different Toll-like receptor subtypes are sensitive to different classes of bacteria, viruses, and other pathogen- and danger-associated molecular patterns. Toll-like receptors can be found on both the cell surface membrane as well as intracellularly on organelles. Cell surface Toll-like receptors include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, and intracellular Toll-like receptors include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 (Kawasaki & Kawai, 2014; Mishra et al., 2006). Different immune cells express different subsets of Toll-like receptors, creating an army of immune cells that can protect the host against a variety of potential insults.

Extensive studies have focused on the cell surface Toll-like receptor TLR4 which can be activated by the endotoxin lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria (Lu, Yeh, & Ohashi, 2008). In the periphery, injections of intraperitoneal LPS are often

used to model sepsis due to the massive levels of inflammation LPS can induce in a short period of time (Deng et al., 2013). In the brain, TLR4 is predominantly expressed on microglia. Thus, here, LPS is used to implicate microglia in cell-type specific effects of neuroinflammation in health and disease.

Intracellular Toll-like receptors are crucial for responding to pathogens that have made their way inside cells, which can happen *via* phagocytosis and endocytosis. As such, intracellular Toll-like receptors are known for being activated by nucleic acids, including RNA and DNA viruses (Blasius & Beutler, 2010). In the brain, TLR3 is predominantly expressed by astrocytes as well as microglia and neurons (Kielian, 2006). TLR3 is specifically activated by double-stranded RNA viruses and the synthetic viral mimic, polyinosinic:polycytidylic acid (poly I:C), though some studies have shown TLR3 activation by single-stranded RNA viruses including West Nile virus and respiratory syncytial virus (RSV) as well as double-stranded DNA viruses such as herpes simplex virus as well (Blasius & Beutler, 2010; M. Matsumoto & Seya, 2008). In addition, double-stranded RNA and DNA is an intermediate product of viral replication that occurs inside infected cells (Louten, 2016), making TLR3 activation an important player in the innate neuroimmune response to viruses.

1.1.3 Cytokines Sound the Immune System Alarm

Upon activation, Toll-like receptors initiate the production and release of immune signaling molecules called cytokines (Akira & Takeda, 2004; Liu & Ding, 2016). Cytokines act as a language of the neuroimmune system whereby immune cells including microglia, astrocytes, and even neurons, have the capability of both releasing inflammatory cytokines and receiving signals from them through cytokine receptors. In the periphery, significant immune stimulation results in the production and circulation of massive levels of cytokines throughout the body, and

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this acts as an alarm signal to recruit immune cells to action to fight off an infection or injury (Chousterman, Swirski, & Weber, 2017; Sriskandan & Altmann, 2008). Cytokines can then stimulate the vagus nerve and signal to the brain that the immune system has been activated (Dantzer, 2018). In turn, the cells of the neuroimmune system begin production and release of their own cytokines which induce fever responses and adaptive sickness behaviors to aid in battling the infection (Dantzer et al., 2008; Kelley et al., 2003).

Over 200 cytokines have been recognized, many with overlapping functions, and they are divided into different families based on shared structures and functions. Prominent cytokine families include the interleukins (ILs), interferons (IFNs), chemokines, tumor necrosis factors (TNFs), and colony stimulating factors (CSFs). As with Toll-like receptors, different immune cells produce different families of cytokines and under different inflammatory conditions. Notably, cytokines can exhibit a wide range of functions, and even the functions of an individual cytokine depend on the context in which it was produced and the cell type it later activates (Cavaillon, 2001). That being said, many researchers have classified functions of specific cytokines most often seen highly upregulated during inflammation, major depression, and cancer, including IL-1β, IL-6, and TNFα (Dinarello, 2006; Elenkov & Chrousos, 2002; Glauser, 2012; Taher, Davies, & Maher, 2018). Here, these cytokines tend to increase inflammation and immune reactions (Glauser, 2012; J. M. Zhang & An, 2007). In contrast, other cytokines including IL-4, IL-10, and IL-13, tend to act to downregulate the inflammatory response and maintain homeostasis (J. M. Zhang & An, 2007). The regulation of cytokines in health and disease is crucial because while acute increases in cytokines during infection are adaptive and helpful for eliminating infections, chronic elevations in cytokines, particularly in the absence of any lingering infection, can contribute to cognitive impairments and neuropsychiatric disorders

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(Bilbo & Schwarz, 2012; Dantzer et al., 2008; Kelley et al., 2003; Marin & Kipnis, 2013; Ray, 2016).

1.1.4 Sex Differences in Neuroimmune Activation

Various levels of sex differences have been noted in immune and neuroimmune function and response to inflammatory insults. In the periphery, females have a greater immune response compared to males, as measured by levels of circulating cytokines and chemokines (Klein & Flanagan, 2016). We have found that peripheral immune stimulation using lipopolysaccharide in mice induces multi-faceted sex differences in the pattern, time course, and magnitude of cytokine levels in the hippocampus, where females show a more rapid onset and resolution of cytokine levels compared to males (Speirs & Tronson, 2018). By contrast, studies on inflammation initiated in the brain show sex differences in the opposite direction. For example, a comprehensive review by Guneykaya and colleagues found that microglia from male mouse brains have a higher capacity and potential to respond to stimuli that would result in a higher magnitude reaction to neuroinflammation in males relative to females (Guneykaya et al., 2018). Microglia from aged male mice are more capable of phagocytosis of neuronal debris under neuroinflammatory conditions relative to aged female microglia (Yanguas-Casás, Crespo-Castrillo, Arevalo, & Garcia-Segura, 2020). Similarly, male-derived astrocytes from cortical brain tissue in rodents have a much greater cytokine response to stimulation relative to astrocytes from female tissue (Astiz, Acaz-Fonseca, & Garcia-Segura, 2014; Loram et al., 2012; Santos-Galindo et al., 2011). Given the direct and indirect roles of astrocytes, microglia, and cytokines in modulating neuronal activity and synaptic plasticity mechanisms, sex differences in cytokine responses in the brain likely have important functional consequences on sex-specific immune modulation of learning and memory (Donzis & Tronson, 2014; Tronson & Collette, 2017).

1.2 Open Communication Between Neurons, Astrocytes, and Microglia is Important for Synaptic Plasticity and Learning and Memory

1.2.1 The Tripartite Synapse

Experience-dependent synaptic plasticity in the hippocampus is a core feature of mechanisms of learning and memory. The presynaptic neuron, postsynaptic neuron, and surrounding astrocytes comprise what is known as the tripartite synapse (Araque, Parpura, Sanzgiri, & Haydon, 1999). Here, communication between neurons is regulated and modulated by the activity and bidirectional communication with astrocytes (Anderson & Swanson, 2000; Haydon, 2001; Noriega-Prieto & Araque, 2021), and this communication has particularly important implications for mechanisms of synaptic plasticity and learning and memory.

Glutamatergic signaling is necessary for initiation and maintenance of hippocampal long-term potentiation and depression *in vitro* (Katagiri, Tanaka, & Manabe, 2001; Neyman & Manahan-Vaughan, 2008), *in vivo* (Heynen, Quinlan, Bae, & Bear, 2000; Naie & Manahan-Vaughan, 2004; Stäubli et al., 1994), and for learning and memory in both rodent models (Aultman & Moghaddam, 2001) and humans (Stanley et al., 2017). Insufficient stimulation of glutamatergic N-Methyl-D-aspartic acid (NMDA) receptors in the rodent hippocampus can be insufficient to induce long-lasting synaptic plasticity *in vitro* (Bliss & Collingridge, 2013; Fox, Russell, Wang, & Christie, 2006; W. Y. Lu et al., 2001), while too much glutamate can result in seizure activity and cell death due to excitotoxicity (Petr et al., 2015; K. Tanaka et al., 1997). As such, it is extremely important that levels of glutamatergic activity are tightly regulated in the synapse. Glutamate transporter 1 (GLT-1) is a high-affinity glutamate transporter that accounts for more than 95% of excess hippocampal glutamate uptake (Tanaka et al., 1997). While many cell types express glutamate transporters, astrocyte-specific expression makes up approximately

80% of total GLT-1 expression (Furness et al., 2008; Petr et al., 2015). Importantly, mobilization of these transporters into and out of the astrocytic membrane is specifically sensitive to levels of glutamate and neuronal activity under normal, healthy conditions (Al Awabdh et al., 2016; R. A. Swanson et al., 1997). Notably, studies using a single-prolonged stress model of Post-Traumatic Stress disorder (PTSD) in male rodents found increased glutamate concentrations and decreased expression of astrocytic glutamate transporters coupled with enhanced fear memory expression and impaired fear memory extinction (Feng et al., 2015; Yamamoto et al., 2009). Administration of fibroblast growth factor 2, a mitogen produced by astrocytes and important for hippocampal neurogenesis and neuronal activation (Kirby et al., 2013), alleviated memory impairments by restoring function in astrocytic glutamate transporters (Feng et al., 2015; Xia et al., 2013). Astrocytic control of glutamate has also been implicated in Alzheimer's disease (Vincent, Gasperini, Foa, & Small, 2010). Taken together, astrocytic control of glutamate has important implications for fine-tuning mechanisms of experience-dependent synaptic plasticity and memory in health and disease.

In addition to glutamate uptake, astrocytes also release glutamate and other neurotransmitters crucial for synaptic plasticity in a process known as gliotransmission (Hamilton & Attwell, 2010). These actions augment long-lasting synaptic plasticity between pre- and post-synaptic rodent neurons in addition to increasing neuronal synchrony *in vitro* (Angulo, Kozlov, Charpak, & Audinat, 2004; Carmignoto & Fellin, 2006; Fellin et al., 2004). For example, changes to intracellular calcium levels in astrocytes stimulate the release of glutamate, D-serine, and adenosine 5'-triphosphate (ATP), among others (Montana, Malarkey, Verderio, Matteoli, & Parpura, 2006; Parpura et al., 1994). Both D-serine and glutamate are required for NMDA receptor activation, and astrocytic contributions of both ligands have been shown to bind

to postsynaptic neuronal NMDA receptors as well as extra-synaptic NMDA receptors in rat slice preparations (Fellin et al., 2004; Yang et al., 2003). Importantly, dysregulation of astrocytic calcium in male mice was found to reduce astrocytic coverage of neuronal synapses and subsequently impair both spatial and contextual fear memory (Tanaka et al., 2013). Astrocytes, therefore, are critical for synaptic plasticity, and dysregulation of astrocyte functions can have a detrimental impact on learning and memory.

1.2.2 Microglia Supplement Neurons and Astrocytes at the Tripartite Synapse

Microglia are the resident immune cells of the brain and, like astrocytes, also play important roles in neuronal communication, synaptic plasticity, and memory mechanisms. Like astrocytes, microglia receive signals from activated nearby neurons as a supplement member of the tripartite synapse. Microglia express a chemokine receptor, CX3CR1 that binds to fractalkine CX3CL1 released by neurons (Hughes, Botham, Frentzel, Mir, & Perry, 2002). Several *in vitro* rodent studies show that this communication acts to inhibit microglia activation and maintain microglia in a surveying state (Bjornevik et al., 2022; Ransohoff, Liu, & Cardona, 2007) as well as suppress inflammatory cytokine production (Zujovic et al., 2000; Zujovic, Schussler, Jourdain, Duverger, & Taupin, 2001). Interestingly, disruption of CX3CL1-CX3CR1 communication can either improve (Maggi, 2011; Reshef, Kreisel, Beroukhim Kay, & Yirmiya, 2014) or impair (Bachstetter et al., 2011; Rogers et al., 2011) synaptic plasticity and memory in rodents. These contradictory results may be due to the inverted U-shaped relationship between neuroinflammation and cognitive processes including learning and memory, where lower magnitude, acute neuroimmune activation improves memory and higher magnitude changes with either elimination or massive activation of the neuroimmune system impair memory (Goshen et al., 2007; Yirmiya & Goshen, 2011).

Microglia and neurons also communicate in the hippocampus *via* the interleukin (IL) cytokine IL-33. In adult male and female mice, Nguyen and colleagues showed that IL-33 is expressed in neurons in an experience-dependent manner, and microglial detection of IL-33 triggers engulfment of the extracellular matrix at the synapse to allow for dendritic spine remodeling, increased synaptic plasticity, and increased memory acuity (Nguyen et al., 2020). Although necessary for bouts of experience-dependent synaptic plasticity, chronic instability of the extracellular matrix is detrimental for homeostatic processes that support neuronal activity and cognition. Specifically, the perineuronal nets that stabilize the extracellular matrix were found to be significantly decreased in both sexes of the 5xFAD mouse model of Alzheimer's disease as well as in the postmortem brain tissue of men and women with Alzheimer's disease relative to controls (Crapser et al., 2020). This study implicated microglial engulfment of the perineuronal nets as a key contributing factor to these findings. Taken together, the ability of microglia to modulate the stability of the synapse is highly important in the context of both health and disease.

Microglia also detect synaptic ATP, an important modulatory signaling molecule released by both activated neurons and astrocytes. A recent study by Badimon and colleagues showed that microglial processes are directed to activated synapses *via* ATP detection. In response, microglia locally produce adenosine that activates the neuronal A1R adenosine receptor to suppress neuronal excitability (Badimon et al., 2020). Thus, microglia, together with astrocytes, are key players for regulating learning and memory processes due to their extensive abilities to modulate synaptic plasticity through neuronal activity-dependent mechanisms.

1.3 Neuroimmune Modulation of Synaptic Plasticity and Learning and Memory

In the periphery, cytokines help to recruit additional immune cells to action in a coordinated response to fight off the invading pathogen or clear up tissue damage and signal to the brain. In the brain, cytokines induce adaptive sickness behaviors, depressive-like behaviors, and disruption of cognitive processes including learning and memory in both rodents and humans (Dantzer et al., 2008; Marin & Kipnis, 2013; Raison, Capuron, & Miller, 2006; Yirmiya & Goshen, 2011). Interestingly, cytokines such as tumor necrosis factor α (TNFα) (Santello, Bezzi, & Volterra, 2011; Stellwagen, Beattie, Seo, & Malenka, 2005), interleukins (IL) IL-1β (Huang, Smith, Ibáñez-Sandoval, Sims, & Friedman, 2011; Viviani et al., 2003), IL-6 (D'Arcangelo et al., 2000), and IL-33 (Nguyen et al., 2020), and interferons (IFNs) IFNα, IFNβ, and IFNγ (Costello & Lynch, 2013; Mendoza-Fernández, Andrew, & Barajas-López, 2000; P. J. Zhu et al., 2011) can modulate synaptic function by altering membrane receptor trafficking, neuronal signaling, extracellular matrix remodeling, and/or neurotransmitter release at both excitatory and inhibitory synapses (Pribiag & Stellwagen, 2013). For example, TNFα has been shown to play a critical role in synaptic scaling, which increases or decreases cell-wide sensitivity to incoming signals based on prolonged heightened or suppressed neuronal activity (Beattie et al., 2002; Stellwagen et al., 2005). Studies have shown that astrocytes detect these changes in neuronal activity, produce TNFα, and TNFα signals back to neurons to change surface expression of GluR1-containing glutamatergic AMPA receptors (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen & Malenka, 2006). Additionally, manipulation of the interleukin family of cytokines can significantly impair *in vitro* hippocampal long-term potentiation (Ross, Allan, Rothwell, & Verkhratsky, 2003; H. Schneider et al., 1998) and memory (Goshen et al., 2007) in rodents. The effects of individual cytokines can be both cell and

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brain region specific, and it is important to keep this in mind when discussing implications of neuroimmune activation on neuronal functions and mechanisms of synaptic plasticity.

Studies in male rats showed that microglia activation contributes to dysregulated synaptic plasticity during inflammatory events, and preventing it ameliorates these effects (Riazi et al., 2015). *In vivo* imaging of neurons in awake male mice showed that microglial contact at neuronal synapses increased synaptic activity and neuronal synchrony important for experience-dependent synaptic plasticity (Akiyoshi et al., 2018). These effects were blocked when microglia were activated by lipopolysaccharide. Viruses including West Nile virus and Zika virus have also been shown to cause microglial engulfment of synapses that result in hippocampal-dependent memory impairment in both sexes (Garber et al., 2019; Vasek et al., 2016), and this mechanism of synapse elimination is also postulated in the pathophysiology of Post-Traumatic Stress disorder (Enomoto & Kato, 2021). These findings contribute to a growing consensus that mechanisms of synaptic communication and plasticity can be modulated by neuroimmune activation, placing the interaction of neurons, astrocytes, and microglia at the crux of health and disease in the context of learning and memory.

1.4 Hormonal Modulation of Learning and Memory: A Focus on Estrogen

Estrogen receptors are located in brain regions critical for learning and memory, including the hippocampus, medial prefrontal cortex, and amygdala – among others – in both male and female brains (Brailoiu et al., 2007; Hazell et al., 2009; Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998; Shughrue, Lane, & Merchenthaler, 1997). In normally cycling females, the ovaries produce a major source of circulating estrogen, but estrogen can also be produced locally in the brains of both sexes when the enzyme aromatase converts testosterone into estradiol (Hojo et al., 2004; Ooishi et al., 2012; Schlinger & Arnold, 1991; Simpson & Davis,

2001). Importantly, aromatase has been found in the hypothalamus, hippocampus, amygdala, and cerebral cortex with specific localization in both the soma and presynaptic axon terminals of neurons (Hojo et al., 2004; Saldanha, Remage-Healey, & Schlinger, 2011). Thus, estrogens are a well-poised tool to study the mechanisms underlying modulation of memory in both males and females.

Estrogen deprivation *via* ovariectomy in females or *via* aromatase inhibition in males has been shown to impair both recognition and spatial memory (Lu et al., 2019; Luine, Richards, Wu, & Beck, 1998; Tao et al., 2020; Xu & Zhang, 2006). Pre-training deprivation of estrogen may affect confounding variables such as motivation and attention during learning (McGaugh, 1973; McGaugh, 2000). As such, several research groups have studied post-training modulation of estrogen quite extensively as well and have shown marked enhancements of recognition and spatial memory with exogenous estradiol treatments into the dorsal hippocampus immediately post-training in both female and male rodents (Frick & Kim, 2018; Jacome et al., 2016; Koss, Haertel, Philippi, & Frick, 2018; Luine & Frankfurt, 2012; Luine, Serrano, & Frankfurt, 2018; Packard, 1998; Sheppard, Koss, Frick, & Choleris, 2018; Tuscher, Fortress, Kim, & Frick, 2015; Tuscher et al., 2016; Tuscher, Taxier, Schalk, Haertel, & Frick, 2019). Tuscher and colleagues (2019) also found that exogenous estradiol in the medial prefrontal cortex immediately post-training significantly enhanced memory consolidation in the same tasks. Thus, estrogen has a significant effect on learning and memory consolidation, and these effects are not state-dependent or confounded by manipulations during learning phases.

While much of the work on the role of estrogen in learning and memory has used spatial and object recognition tasks, there is some evidence that estrogen also modulates other types of hippocampal-dependent memory, including fear-based memories (Zeidan et al., 2011). In

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contextual fear conditioned extinction training, female rodents trained in high-estrogen estrous

phases showed enhanced fear extinction compared with both females in low-estrogen phases as

well as males (Chang et al., 2009; Jasnow, Schulkin, & Pfaff, 2006; Milad, Igoe, Lebron-Milad,

& Novales, 2009). This estrogen-mediated memory enhancement has also been shown in women

that underwent fear conditioning and extinction training during high- and low-levels of

circulating hormones throughout their menstrual cycle (Zeidan et al., 2011). Likewise, women

using hormonal contraceptives that suppress endogenous levels of estradiol have shown

significant impairment in fear memory extinction tasks, and this holds true for naturally-cycling

women in low-estrogen phases as well (Wegerer, Kerschbaum, Blechert, & Wilhelm, 2014).

Notably, estrogen is also important for proper fear extinction in males, where aromatase

inhibitors administered immediately after fear extinction training resulted in deficits in fear

extinction recall (Graham & Milad, 2014).

1.4.1 Estrogen Modulates Mechanisms of Synaptic Plasticity

It is clear that estrogen is required for in-tact hippocampal-dependent memory, but how

does estrogen mediate these memory mechanisms? Estrogens modulate neuronal activity and

long-term synaptic plasticity mechanisms in both males and females (Frick, Tuscher, Koss, Kim,

& Taxier, 2018; Hyer, Phillips, & Neigh, 2018; Woolley, 2007). Despite that females have an

additional source of ovarian estrogen, the local production of estrogen by aromatase in both

sexes plays a more significant role in these mechanisms of neuronal modulation specifically (Lu

et al., 2019; Luine et al., 2018; Wang et al., 2018). Recent work by Lu et al. (2019) created a

forebrain neuron-specific knockout of aromatase to examine the role of neuron-derived estradiol

on long-term potentiation and memory in both male and female mice. They found that a 65-70%

decrease in aromatase in the CA1 region of the hippocampus and cortex resulted in impairments

of long-term potentiation and disruptions of spatial, recognition, and contextual, but not auditory, fear memory in both sexes. Importantly, these effects were seen in both in-tact and ovariectomized females, suggesting a crucial role for neuron-derived estradiol in these memory tasks, not just estradiol produced by the ovaries.

In the hippocampus, both males and females express the estrogen receptors (ER) ERα, ERβ, and G protein-coupled estrogen receptor 1 (GPER1), with greater expression of GPER1 compared with both ERα and ERβ in both sexes (Brailoiu et al., 2007; Hutson et al., 2019). Interestingly, expression of ERα and ERβ is restricted to different subcellular locations of excitatory neurons, and expression levels in both neurons and astrocytes are modulated by levels of hormones, revealing notable sex differences and differences across the estrous cycle (Mitterling et al., 2010). Early rodent studies showed estradiol increased neuronal excitability of hippocampal glutamatergic synapses in both sexes (Teyler, Vardaris, Lewis, & Rawitch, 1980; Wong & Moss, 1992), though more recent studies revealed that modulation of pre- and post-synaptic mechanisms is *via* different estrogen receptors in each sex (Oberlander & Woolley, 2016; W. Wang et al., 2018). Taken together, these findings suggest that sex differences in expression levels and/or subcellular locations of ERα and ERβ may have important functional implications for sex differences in mechanisms of long-lasting synaptic plasticity.

Memory modulation by estrogen is not simply about whether hippocampal-dependent memories can be made stronger or weaker in males or females. Rather, these data highlight the complex, network-based, and multi-modal components of what is necessary to consider during acquisition and consolidation of long-term memories. Such an expanded view is especially important when considering things such as the therapeutic use of estrogen treatments during fear

extinction therapy for Post-Traumatic Stress disorder, hormone replacement therapy during perimenopause, or gender-affirming hormone therapy for those with gender dysphoria.

1.5 Rationale and Specific Aims

The neuroimmune system is a specialized immune system in the brain critical for both regulating normal neural function and behavior as well as responding to illness and injury (Marin & Kipnis, 2013). Extensive evidence shows that dysregulation of neuroimmune processes impairs cognitive behaviors including learning and memory. The neuroimmune system is capable of such distinct functions due to the precise extracellular location of two innate immune cells, astrocytes and microglia, at neuronal synapses and the communication between all three cell types under both healthy and inflammatory conditions (Cerbai et al., 2012; Posillico, 2021; Scholz & Woolf, 2007). Astrocytes, neurons, and microglia all express TLR3 (Kielian, 2006), which is stimulated by the double-stranded viral mimic polyinosinic:polycytidylic acid (poly I:C), making poly I:C an excellent tool to disrupt neuron-glia communication and study the effects of broad-based neuroinflammation on learning and memory (M. Matsumoto & Seya, 2008). The neuroimmune system can be activated directly by invading pathogens at leaky places in the blood-brain barrier and indirectly from vagal signals from the periphery and infiltrating activated macrophages (Dantzer, 2018). To avoid the complexities of indirect activation, we used central administration of poly I:C into the ventricles of the brain. It is important to keep in mind that this is not a translationally relevant model of disease or infection. Rather, this method allows us to ask how neuroimmune activation and neuroinflammatory processes modulate mechanisms of learning and memory in each sex more directly and specifically.

There are significant sex differences in immune and neuroimmune system activation as well as sex differences in hippocampal neurogenesis, intracellular signaling cascades, and

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transcription during hippocampal-dependent memory formation (Chow, Epp, Lieblich, Barha, & Galea, 2013; Gresack, Schafe, Orr, & Frick, 2009; Koss & Frick, 2017; Tronson & Keiser, 2019; Yagi & Galea, 2019). While some male-specific mechanisms of memory have been identified and characterized, the historical exclusion of females in this research has made female-specific memory mechanisms elusive and not explained by estrogen alone (Tronson, 2018; Tronson & Keiser, 2019). This severe gap in literature has important implications for health and disease, as sex differences in memory are also evident both qualitatively and quantitatively in behavior (Andreano & Cahill, 2009; Loprinzi & Frith, 2018). Given the vast evidence of sex differences at all levels from cells to behavior, it is highly possible that signal transduction of neuroimmune and memory processes happens *via* sex-specific mechanisms. The experiments of this dissertation research were designed with this overarching question in mind, and the results herein significantly contribute to the fast-growing, important literature on sex differences in psychoneuroimmunological mechanisms in health and disease.

1.5.1 Aim 1: Establish the Neuroimmune Activation Profile in the Hippocampus Following Central Administration of Poly I:C

Neuroimmune cells including astrocytes and microglia cause inflammation in the brain by releasing signaling molecules called cytokines and chemokines. The increase of these cytokines and chemokines is associated with sickness behaviors, depressive-like behaviors, and disruption of cognition such as learning and memory, all of which recruit the hippocampus (Dantzer et al., 2008; Marin & Kipnis, 2013; Raison et al., 2006; Yirmiya & Goshen, 2011). While sex differences in immune activation have been reported, they are highly context dependent (i.e., whether looking at endpoints in the periphery or the brain, whether using *in vivo* or *in vitro* methodology, etc.). Additionally, many of these studies only examine a select few

cytokines and chemokines including IL-1β, IL-6, and TNFα, despite that many other families of cytokines are also implicated in cognitive dysfunction. Therefore, the goal of Aim 1 was to describe a broader set of cytokines and neuroimmune cell activation markers induced by direct neuroimmune activation from intracerebroventricular administration of poly I:C in both males and females.

Poly I:C induced weight loss and a fever response in both males and females which recovered within 48 hours. In the hippocampus, we found significant increases in several inflammatory cytokines and chemokines of both sexes, including IL-1α, IL-1β, IL-6, IFNβ, TNFα, CCL2, and CXCL10. These cytokines peaked 4 hours after poly I:C treatment with the exception of IFNβ, an anti-viral Type I interferon, which showed peak levels 2 hours earlier. Males tended to show a greater magnitude of cytokine response compared with females. Additionally, we found sex differences in the gene expression of IFNα, another anti-viral Type I interferon, such that only males showed significant increases in IFNα expression, but not females. These results indicate that not only are there are sex differences in the magnitude of response to poly I:C, there are sex differences in the expression of important anti-viral interferons which might have significant implications for how the neuroimmune response to poly I:C impacts learning and memory processes.

1.5.2 Aim 2: Determine the Effects of Neuroimmune Activation on Learning and Memory Processes in Males and Females

The neuroimmune system is required for learning and memory (Goshen et al., 2007; Marin & Kipnis, 2013), and dysfunction of this system causes significant cognitive impairments (Dantzer et al., 2008; Donzis & Tronson, 2014; Marin & Kipnis, 2013; McAfoose & Baune, 2009). Specifically, activation of the immune system by both viruses and bacteria disrupts

hippocampal-dependent memory processes (Czerniawski & Guzowski, 2014; Goshen et al., 2007; Shaw, Commins, & O'Mara, 2001; Vasek et al., 2016). Given the significant sex differences in magnitude of neuroimmune activation and induction of anti-viral Type I interferons following central administration of poly I:C found in Aim 1, the goal of Aim 2 was to determine whether this translated to functional sex differences in neuroimmune modulation of memory.

We found that both males and females treated with poly I:C were capable of learning how to complete an escape-motivated T-maze task, but pre-training poly I:C disrupted learning strategies in multiple memory systems differently in each sex. We also found that while both sexes were vulnerable to disruptions of learning or encoding of hippocampal-dependent context fear conditioning, only males were vulnerable to neuroimmune disruptions of fear memory consolidation. Further, pre-training poly I:C resulted in sex- and subregion-specific effects on neuronal activation in the hippocampus, suggesting a similar disruption of learning in both sexes occurs *via* distinct hippocampal mechanisms.

1.5.3 Aim 3: Identify Intracellular Memory Mechanisms Modulated by Neuroimmune Activation in Males and Females

Neuroimmune activation, including that from poly I:C, alters the expression of glutamate receptors and transporters (Randall, Vetreno, Makhijani, Crews, & Besheer, 2019). Additionally, signaling from anti-viral Type I interferons IFNα and IFNβ can individually modulate mechanisms of synaptic plasticity such as long-term potentiation *via* distinct interactions with glutamate receptors (Costello & Lynch, 2013; Mendoza-Fernández et al., 2000). These data are significant because glutamatergic signaling is necessary for hippocampal memory formation (Katagiri et al., 2001). Given that we found sex differences in the expression of IFNα (Aim 1)

and found that neuroinflammation disrupts learning in both sexes but *via* distinct hippocampal mechanisms (Aim 2), the goal of Aim 3 was to test whether poly I:C altered glutamatergic signaling *via* sex-specific Type I interferon-induced changes in glutamate receptors and transporters that underlie context fear learning deficits.

We found that poly I:C increased protein levels of phosphorylated STAT3, a transcription factor known to be activated by the cytokine IL-6, in both sexes. Inhibition of the anti-viral Type I interferon receptor prior to administration with poly I:C significantly blunted both IL-6 expression and phosphorylated STAT3 in the hippocampus of both sexes. Additionally, Type I interferon receptor inhibition attenuated the poly I:C-induced learning deficit in context fear conditioning in males but did not have the same effect in females. Collectively, these data suggest that Type I interferons may have sex-specific functions in the neuroimmune modulation of hippocampal-dependent learning and memory processes and warrant further research to better describe the precise mechanisms at play.

Chapter 2:
Sex Differences and Similarities in the Sickness and Neuroimmune Responses to Central Administration of Poly I:C

2.1 Abstract

The neuroimmune system is required for normal neural processes, including modulation of cognition, emotion, and adaptive behaviors. Aberrant neuroimmune activation is associated with dysregulation of memory and emotion, though the precise mechanisms at play are complex and highly context dependent. Sex differences in neuroimmune activation and function further complicate our understanding of their roles in cognitive and affective regulation. Here, we characterized the physiological sickness and inflammatory response of the hippocampus following intracerebroventricular (ICV) administration of a synthetic viral mimic, polyinosinic:polycytidylic acid (poly I:C), in both male and female C57BL/6N mice. We observed that poly I:C induced weight loss, fever, and elevations of cytokine and chemokines in the hippocampus of both sexes. Specifically, we found transient increases in gene expression and protein levels of IL-1α, IL-1β, IL-4, IL-6, TNFα, CCL2, and CXCL10, where males showed a greater magnitude of response compared with females. Only males showed increased IFNα and IFNγ in response to poly I:C, whereas both males and females exhibited elevations of IFNβ, demonstrating a specific sex difference in the anti-viral response in the hippocampus. This suggests that Type I interferons are one potential node mediating sex-specific cytokine responses and neuroimmune effects on cognition. These findings highlight the importance of using both

males and females and analyzing a broad set of inflammatory markers in order to identify the precise, sex-specific roles of neuroimmune dysregulation in neurological diseases and disorders.

2.2 Introduction

The neuroimmune system is responsible for surveying the microenvironment and responding to illness, injury, and infection. It is also required for physiological and behavioral responses to infection (Dantzer, 2004; Hart, 1988) as well as normal, non-immune neural processes (Marin & Kipnis, 2013; Rizzo et al., 2018; Yirmiya & Goshen, 2011) including synaptic plasticity and memory formation (Barrientos, Frank, Watkins, & Maier, 2012; Pribiag & Stellwagen, 2014). There is significant evidence for sex differences in immune responses in the periphery (Klein & Flanagan, 2016; Klein & Huber, 2010), but there is limited literature on similar findings in adult brains. Knowing whether such sex differences occur in similar magnitudes and directions in the neuroimmune system is important for understanding exactly how neuroimmune dysregulation impacts cognition, and contributes to psychiatric and neurological disorders, in both sexes.

Fever responses to infections are highly conserved and thought to be an adaptive physiological response to illness, as they are generally associated with increased rates of survival (Kluger, 1986; Kluger, Ringler, & Anver, 1975). Additionally, behaviors including lethargy, immobility, decreased food consumption, and decreased sociability typically occur along the same time course as fever and are also conserved across human and animal species (Hart, 1988, 2010; Shattuck & Muehlenbein, 2015). Studies have found that these collective behaviors, termed sickness behaviors, are a coordinated and adaptive response of the host that act to conserve and redirect metabolic energy towards fighting off invading pathogens (Johnson, 2002; Kelley et al., 2003). However, long-lasting sickness behaviors without the presence of an active

infection can be maladaptive and are associated with neurodegenerative diseases and mental health disorders (Dantzer et al., 2008).

Illness, injury, or aseptic triggers of the innate immune system – either bacterial endotoxins (e.g., lipopolysaccharide, LPS) or viral mimics (e.g., polyinosinic:polycytidylic acid, poly I:C) – cause activation of neuroimmune cells, including microglia and astrocytes, and rapid production of cytokines in the brain (Ransohoff, 2009; Ransohoff & Brown, 2012). The initial increase in cytokines is important for coordinating the neuroimmune response as well as the induction of adaptive sickness behaviors (Kelley et al., 2003). Due to key roles in peripheral inflammation, the cytokines IL-1β (Barrientos, O'Reilly, & Rudy, 2002; Goshen et al., 2007; Yirmiya, Winocur, & Goshen, 2002), IL-6 (Sparkman et al., 2006; Trapero & Cauli, 2014; Weaver et al., 2002), and TNFα (Feuerstein, Liu, & Barone, 1994; Lindbergh et al., 2020; Strieter, Kunkel, & Bone, 1993) have been the focus of much of the research of neuroimmune function (Donzis & Tronson, 2014). More recently, other cytokines, including interferons (Bekhbat & Neigh, 2018; Blank et al., 2016), CCL2 (Westin et al., 2012; J. Xu et al., 2017), and CXCL10 (Bajova, Nelson, & Gruol, 2008; Blank et al., 2016; Bradburn et al., 2018) also play critical roles in modulation of behavior, cognition, and affective states, suggesting that many cytokines – likely far beyond this short list – play important roles in these processes.

Various sex differences in immune and neuroimmune activation have been reported. Females have a greater peripheral immune response compared with males (Klein & Flanagan, 2016). In contrast, neuroimmune cells *in vitro*, including astrocytes derived from male cortical tissue, are shown to have significantly greater reaction to inflammatory insults compared with female-derived cells (Loram et al., 2012; Santos-Galindo et al., 2011). However, data on whether and how neuroimmune activation differs between males and females in adult brains *in vivo* is

quite limited. Our lab has identified sex differences in the magnitude, time course, and pattern of

cytokines activated in the hippocampus following peripheral LPS (Speirs & Tronson, 2018), and

in the long-lasting impact of LPS on hippocampal function (Tchessalova & Tronson, 2020).

Activation of immune signaling in the hippocampus has been implicated in disorders of affect

and cognition, many of which show sex-biases in prevalence and outcomes (Bekhbat & Neigh,

2018; Dantzer et al., 2008; Neigh, Bekhbat, & Rowson, 2018). Thus, sex differences in

neuroimmune responses, specifically within the hippocampus, may be a contributing factor to

sex differences in neural and cognitive processes and disorders. As such, it is imperative to

understand exactly how neuroimmune activation impacts such processes in both sexes for health

and disease.

The bulk of studies aimed at understanding neuroimmune activation and its behavioral

sequelae thus far have used the gram-negative bacterial shell and Toll-like receptor 4 (TLR4)

agonist LPS. Viral illnesses also trigger changes in behavior, cognition, and emotional states, and

significant sex differences have been observed in the context of viral infections as well (Barna,

Komatsu, Bi, & Reiss, 1996; Klein & Flanagan, 2016; Klein & Huber, 2010). These findings

have been propelled to the forefront of research during the current COVID-19 pandemic (Klein

et al., 2020; Takahashi et al., 2020). Given that viruses act through distinct Toll-like receptors,

their impact is likely mediated by a different, albeit overlapping, pattern of cytokine activation

compared with LPS or bacterial triggers. Further, due to its relevance for disease states, many *in

vivo* studies of neuroimmune function use a peripheral immune challenge. Here, neuroimmune

activation is primarily driven by peripheral immune signals that infiltrate the brain (Watkins,

Maier, & Goehler, 1995). This complicates the interpretation of whether sex differences in

cytokine levels observed in the brain are due to indirect effects based on sex differences in peripheral immune response, or to direct effect of sex differences in neuroimmune function.

In this study, we aimed to characterize physiological and behavioral sickness responses and identify a broader set of inflammatory cytokines induced in the hippocampus by direct neuroimmune stimulation *via* central administration of poly I:C in both males and females. We focused on the hippocampus because elevation of hippocampal cytokines is associated with both disruption of memory processes (Barrientos et al., 2002; C. Cunningham et al., 2009; Czerniawski & Guzowski, 2014; Marin & Kipnis, 2013; McAfoose & Baune, 2009; Vasek et al., 2016; Yirmiya et al., 2002) and increased depression-like behaviors (Koo & Duman, 2009; M. Tang, Lin, Pan, Guan, & Li, 2016). Within the hippocampus, we focused on cytokines and chemokines that have previously been implicated in cognitive and affective dysfunction, including the commonly studied IL-1β, IL-6, IL-10, and TNFα (Dantzer, 2009; Koo & Duman, 2008), as well as IL-4 (Gadani, Cronk, Norris, & Kipnis, 2012; Nolan et al., 2005), IL-2 (Koo & Duman, 2009; Petitto, McCarthy, Rinker, Huang, & Getty, 1997), CXCL10 (Bradburn et al., 2018; Gruol, 2016), and CCL2 (Gruol, 2016). Additionally, we measured virus-specific responses (IFNα and IFNβ; (Teijaro, 2016)) and measures of generic microglial and astrocyte activation (CD11b and GFAP; (Akiyama & McGeer, 1990; Eng & Ghirnikar, 1994)).

We demonstrate that poly I:C induces fever, weight loss, and changes in mRNA expression and protein levels of cytokines, chemokines, and markers of glial activation across a 24-hour period in both sexes. Interestingly, poly I:C disrupted social behaviors in females, but not males. Further, only IFNα and IFNɣ showed male-specific patterns of activation after central poly I:C administration, and many cytokines and chemokines showed a greater magnitude increase in males compared with females. Whether these sex differences in neuroimmune

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activation contribute to sex differences in modulation of cognition and affect and subsequent prevalence of memory- and mood-related diseases and disorders is an important area of research for our ongoing studies.

2.3 Methods

2.3.1 Animals

99 male and female 8–9-week-old C57BL/6N mice from Envigo (Indianapolis, IN) were used in these experiments. For all experiments, mice were individually housed in standard polypropylene mouse cages with *ad libitum* access to food and water in a room with maintained temperature, pressure, and humidity under a 12:12-hour light:dark cycle. All mice had at least one week of acclimation to the colony room prior to any manipulations. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

2.3.2 Stereotaxic Surgeries

Bilateral guide cannulae (PlasticsOne, Roanoke, VA) targeting the lateral ventricles were implanted using standard stereotaxic methods (KOPF, Tujunga, CA) at the following coordinates relative to Bregma: ML: +/- 1.00 mm, AP: 0.30 mm, DV: -2.50 mm. Animals were administered a pre-surgical analgesic (5 mg/kg Carprofen, subcutaneous) and anesthetized for surgery using an intraperitoneal injection of 250 mg/kg of Avertin (2,2,2-tribromoethanol) which maintained a surgical plane of anesthesia for the duration of the craniotomy. Bilateral holes were drilled into the skull at the above coordinates, and guide cannulae were implanted using dental cement. Animals were given a second dose of Carprofen (5 mg/kg, subcutaneous) 24 hours after surgery

to maintain a total of 48 hours of analgesia. Mice were monitored daily for 10 days post-operative and were given at least 2 weeks to recover from surgery prior to use in experiments.

2.3.3 Poly I:C Administration

Polyinosinic:polycytidylic acid (poly I:C; Cat. No. P1530; Sigma-Aldrich, St. Louis, MO) was prepared according to the manufacturer's instructions and sterile-filtered using a 0.22 μm filter prior to administration. For intracerebroventricular (ICV) administration, we infused 20 μg of poly I:C (2 μL of 10 μg/μL poly I:C) (X. Zhu, Levasseur, Michaelis, Burfeind, & Marks, 2016) or an equal volume of 0.9% sterile saline *via* the implanted guide cannula under brief isoflurane anesthesia.

2.3.4 Sickness Responses

To confirm the efficacy of the ICV dose of poly I:C, and the specific poly I:C used here (McGarry et al., 2021), poly I:C-induced physiological measures of sickness in males and females were assessed. Body weights and rectal temperatures (RET-3; Physitemp, Clifton, NJ) were measured at 2, 4, 6, 12, 24, and 48 hours following ICV administration of poly I:C ($n = 10$ male; $n = 9$ female) or sterile saline ($n = 10$ male; $n = 8$ female; Figure 2.2A). Visual measures of sickness (piloerections, squinted eyes, hunched posture, and low responsivity) were assessed throughout (Hart, 1991). No changes in overt sickness behaviors were observed for any experiment (data not shown).

Statistical Analysis of Sickness Responses

Analysis of body weight and temperature changes in response to poly I:C was completed using a mixed repeated-measures ANOVA, using time post-infusion as the within-subjects factor and treatment and sex as the between-subjects factors with Greenhouse-Geisser corrections for

sphericity. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method. Any outliers were identified as samples outside the range of 2 standard deviations from the group mean.

2.3.5 Social Preference Test

We tested measures of social behavior using a social preference test. For this test, we used a three-chamber apparatus (76 cm x 21.5 cm x 30.5 cm L x W x H) in which the two end chambers contained a wire corral in the center for exploration, and the center chamber was empty and considered neutral. In the social preference test, mice ($n = 20$ male; $n = 14$ female) were habituated to the apparatus with empty corrals for 10 minutes 24 hours prior to the start of the test. On the day of testing, males and females were treated with either ICV poly I:C ($n = 10$ male; $n = 7$ female) or sterile saline ($n = 10$ male; $n = 7$ female), and 4 hours later were placed into the apparatus in which a "stranger mouse" of the same strain, age, and sex was contained in one corral, and a toy mouse was contained in the opposite corral as a novel object. Animals were given 10 minutes to freely explore the apparatus, and behavior was recorded using a video camera and EthoVision XT Ver. 19 computer software (Noldus Information Technology Inc.; Leesburg, VA). Time spent actively interacting with the corrals was hand-scored by two individuals blind to treatment groups, and preference for the stranger mouse was calculated as follows:

$$\left(\frac{time\ exploring\ stranger\ mouse\ (s)}{time\ exploring\ stranger\ mouse + novel\ object\ (s)} \right) \times 100\%$$

Statistical Analysis of Social Preference

Social preference data were analyzed using a two-way ANOVA with treatment and sex as factors. Significant interactions were followed up using post-hoc tests with Bonferroni corrections for *a priori* comparisons of saline- vs. poly I:C-treated groups within sex, and effect sizes were calculated using the partial eta squared method. We expected that saline-treated groups would show a significant preference for the stranger mouse. To test this, we ran one-sample, one-tailed t-tests of the saline-treated groups within each sex against a mean of 50% preference, which represents the null hypothesis that there is no preference for the stranger mouse. To test whether poly I:C-treated groups showed a preference for either the stranger mouse or novel object, we ran one-sample, two-tailed t-tests of the poly I:C-treated groups within each sex against a mean of 50% representing the null hypothesis that there is no preference for either the stranger mouse or novel object. We corrected p-values of t-tests for multiple comparisons using a Bonferroni correction.

2.3.6 Characterization of the Acute Hippocampal Neuroimmune Response

We used RNA and protein endpoints to examine induction of cytokines and glial activation markers in the hippocampus. Males and females were treated with either poly I:C ($n = 22$ male; $n = 24$ female) or sterile saline ($n = 8$/sex) and brains were collected 0.5 hours ($n = 5$ male; $n = 6$ female), 2 hours ($n = 6$/sex), 4 hours ($n = 5$ male; $n = 6$ female), and 24 ($n = 6$/sex) hours later. All animals were transcardially perfused with 0.1 M phosphate buffer to remove circulating blood from the brain. Both hemispheres of dorsal hippocampus tissue were collected in separate RNase-/DNase-free, sterile microcentrifuge tubes and immediately flash frozen. All samples were stored at -80°C before tissue processing.

Quantitative Real-Time PCR

One hemisphere of dorsal hippocampal tissue per mouse was processed for gene expression analysis using quantitative real-time PCR. Frozen samples were homogenized, and messenger RNA (mRNA) was extracted under sterile, RNase-free conditions (PureLink RNA Mini Kit; Cat. No. 12183020; Invitrogen, Carlsbad, CA). RNA quality was assessed using gel electrophoresis, and UV spectroscopy was used to assess RNA purity (A260/280 > 1.80) and quantity (BioSpectrometer Basic; Eppendorf, Hamburg, Germany). Any genomic DNA in the sample was removed using DNase treatment, and 800 ng of cDNA was synthesized from each mRNA sample (QuantiTect Reverse Transcriptase Kit; Cat. No. 205314; Qiagen, Hilden, Germany). Any samples that did not have a high enough concentration of RNA to make 800 ng of cDNA were removed from further analyses ($n = 3$ male; $n = 5$ female). Relative gene expression was measured using Power SYBR Green PCR Master Mix (Cat. No. 4368702; Applied Biosystems, Foster City, CA) in 10 µL reactions (ABI 7500 real-time PCR system; Cat. No. 4351105; Applied Biosystems).

We measured expression of four commonly used housekeeping genes: *18s*, *gapdh*, *hprt1*, and *rplp0* (all QuantiTect Primer Assays: *18s* Cat. No. QT02448082, *gapdh* Cat. No. QT01658692, *hprt1* Cat. No. QT00166768, *rplp0* Cat. No. QT00249375; Qiagen). We analyzed the relative expression of the following genes of interest: *ccl2*, *cd11b*, *cxcl10*, *gfap*, *ifnα*, *ifnβ*, *ifnγ*, *il-1α*, *il-1β*, *il-6*, *il-10*, and *tnfα*. The gene primer for *il-1α* was a QuantiTect Primer Assay (Cat. No. QT00113505; Qiagen). The sequences for the remaining gene primers can be found in Table 2.1 and were ordered through Integrated DNA Technologies and diluted to 0.13 µM to be used for PCR. All Qiagen primers were diluted as per the manufacturer's instruction.

Housekeeping Gene Stability Analysis

To control for the transcriptional activity of the samples being analyzed, we confirmed the stability of four housekeeping genes (*18s*, *gapdh*, *hprt1*, and *rplp0*). While many studies use common housekeeping genes such as GAPDH or HPRT1, it is less common for authors to report that their chosen housekeeping gene is indeed stable across experimental groups or tissues prior to use in analyses. Thus, we confirmed the stability of our housekeeping genes using a combination of four techniques to ensure the most reliable quantification of gene expression in our studies. First, we assessed the variability of the candidate genes by measuring the standard deviation of the raw quantification cycle (Cq) values from all samples (Figure 2.1A). We found that *18s* had the largest standard deviation of Cq values (1.540), followed by *gapdh* (0.527), *rplp0* (0.225), and *hprt1* (0.151; Figure 2.1B). By this approach, *rplp0* and *hprt1* showed the greatest stability compared to *18s* and *gapdh*, with *hprt1* exhibiting the lowest variability.

Second, we employed a comparative ΔCq approach in which the standard deviations of the differences in Cq values (ΔCqs) between all possible pairs of candidate genes were compared (Silver, Best, Jiang, & Thein, 2006) (Figure 2.1C). From highest to lowest variability, the genes ranked as follows: *18s* (1.609 average standard deviation), *gapdh* (0.911), *rplp0* (0.764), and *hprt1* (0.753). Again, this method indicated that the most variable genes were *18s* and *gapdh* while the most stable genes were *rplp0* and *hprt1*, and this is most apparent when considering the lowest ΔCq standard deviation from this method was from the *rplp0* and *hprt1* comparison at 0.206 (Figure 2.1D).

The third method we employed was that developed by Vandesompele and colleagues, which calculated the average pairwise variation of one candidate gene with all other candidate genes (Vandesompele et al., 2002). We used R packages ReadqPCR and NormqPCR (Perkins et al., 2012) to calculate *M* stability values, as depicted in Figure 2.1E. Consistent with the previous

methods, *hprt1* and *rplp0* were the most stable of the candidate genes, with the lowest pairwise variability, *M* value, of 0.206.

Fourth, and last, we used a model-based stability analysis approach developed by Andersen et al., an algorithm called NormFinder (v5) (Andersen, Jensen, & Ørntoft, 2004). This method protects against identifying two genes *via* the pairwise approach that might be misinterpreted as being the most stable if they are coregulated. Using this method, again, *hprt1* and *rplp0* were found to be the most stable genes with the lowest expression stability values (Figure 2.1F). However, NormFinder resulted in *rplp0* having the lowest stability value of 0.326, indicating that the model-based approach identified *rplp0* as the most stable gene.

Together, these methods identified the two most stable candidate housekeeping genes as *hprt1* and *rplp0*. Vandesompele et al. (Vandesompele et al., 2002) posits that using the geometric mean of multiple housekeeping genes results in more accurate expression levels of genes of interest. We calculated the geometric mean of the Cq values from *hprt1* and *rplp0* to be used in the $2^{-\Delta\Delta Cq}$ method for calculations of relative expression for our target genes.

Statistical Analysis of mRNA Gene Expression

For each PCR reaction, the quantification cycle (Cq) was determined, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression of each gene. Any samples with abnormal amplification curves, melt curves, and/or melt peaks across replicates were removed from analyses ($n = 1$/sex). Any outliers were identified as samples outside the range of 2 standard deviations from the group mean and excluded from analyses.

Baseline sex differences in relative gene expression (qPCR) were assessed by evaluating the male and female saline-treated groups. To directly and meaningfully compare these two

groups in the PCR analysis, the male saline-treated group was normalized to the female saline-treated group and analyzed using independent, two-sample t-tests.

To appropriately analyze sex differences in relative gene expression (qPCR) across the 24-hour time course, we normalized each group to its respective same-sex saline-treated group to control for any sex differences in gene expression at baseline and used two-way ANOVA tests using treatment and sex as factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

Multiplex Assays

The second hemispheres of dorsal hippocampal tissue were processed as previously described using low-detergent RIPA buffer sonication (Speirs & Tronson, 2018). Milliplex magnetic bead panel assays (CCL2, CXCL10, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, and IL-10; Millipore Sigma, Burlington, MA) were used as per manufacturer's instructions. Cytokine concentrations were calculated as pg/mg of hippocampal tissue *via* Luminex software. Only samples that showed readable bead counts according to the Luminex software were included in the analyses.

Statistical Analysis of Protein Levels

Baseline sex differences in protein levels from multiplex assays were analyzed with independent, two-sample t-tests comparing the saline-treated groups. To analyze changes in protein levels from poly I:C across the 24-hour time frame, we used two-way ANOVA tests using treatment and sex as factors. Significant main effects and interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and

effect sizes were calculated using the partial eta squared method. Any outliers were identified as samples outside the range of 2 standard deviations from the group mean and excluded from analyses.

2.3.7 Data Visualization and Statistical Software

Data visualization and statistical analyses were completed using R 3.6.2 (R Core Team, 2019) with the following packages: dplyr (v0.8.5; (Wickham, Francois, Henry, & Muller, 2020)), tidyr (v1.0.2; (Wickham & Henry, 2020)), rstatix (v0.5.0; (Kassambara, 2020)), DescTools (v0.99.34; (Signorell et al., 2020)), sjstats (v0.17.9; (Ludecke, 2020)), ReadqPCR and NormqPCR (Perkins et al., 2012), ggplot2 (Wickham, 2016), gridExtra (v2.3; (Auguie, 2017)), pheatmap (v1.0.12; (Kolde, 2019)), and viridis (v0.5.1; (Garnier, 2018)).

2.4 Results

2.4.1 Central Administration of Poly I:C Induces Sickness Responses

Poly I:C Results in Fever and Weight Loss in Both Sexes

Both females and males showed physiological responses to poly I:C. Whereas both saline- and poly I:C-treated animals showed changes in weight across the 48-hour period (Figure 2.2B, main effect of Time: $F(3.13, 96.92) = 28.899$, $p < 0.001$, $\eta^2_p = 0.482$), poly I:C caused weight loss in both sexes (main effect of Treatment: $F(1, 31) = 8.781$, $p = 0.006$, $\eta^2_p = 0.221$; trend towards a Time x Treatment interaction: $F(3.13, 96.92) = 2.476$, $p = 0.064$, $\eta^2_p = 0.074$). Specifically, males and females treated with poly I:C lost significantly more weight than the saline-treated animals at the 12- ($p = 0.004$) and 24-hour ($p = 0.022$) timepoints. By 48 hours post-treatment, the weights of poly I:C-treated animals had recovered and were no longer different from those of saline-treated animals ($p = 1.00$; Figure 2.2B).

In both males and females, poly I:C caused significant increases in body temperature relative to the saline-treated group (Figure 2.2C; main effect of Treatment: $F(1, 31) = 23.759$, $p < 0.001$, $\eta^2_p = 0.434$; Time x Treatment interaction: $F(4.6, 142.62) = 11.635$, $p < 0.001$, $\eta^2_p = 0.273$). Post-hoc tests revealed that body temperature began to increase 2 hours following poly I:C ($p = 0.068$), remained elevated at the 4- ($p < 0.001$) and 6-hour ($p < 0.001$) timepoints, and recovered to saline-treated body temperatures by 12 hours post treatment (all $p = 1.00$; Figure 2.2C). These data, and the similarity of febrile response in males and females, are consistent with previous studies using ICV (X. Zhu et al., 2016) or systemic (Flannery, Henry, Kerr, Finn, & Roche, 2018) poly I:C.

Poly I:C Decreases Social Interaction in Females Only

As expected, saline-treated females showed a preference for the stranger mouse in the social preference test (Figure 2.2D; $t(6) = 2.401$, $p = 0.053$). However, contrary to what we expected, saline-treated males did not show any stranger preference during this task ($t(9) = -1.514$, $p = 0.918$). Neither poly I:C-treated groups showed preference for the stranger mouse or novel object in this task (females: $t(6) = -2.146$, $p = 0.151$; males: $t(9) = 0.557$, $p = 0.591$).

Poly I:C disrupted preference for a novel, stranger mouse in females, but not males (Figure 2.2D; Sex x Treatment interaction: $F(1, 30) = 7.862$, $p = 0.009$; $\eta^2_p = 0.208$). Specifically, poly I:C decreased stranger preference in females ($p = 0.016$) but did not have any effect in males ($p = 0.396$). Notably, this effect of poly I:C in females was not due to a change in total exploration of the corrals during the test (Figure 2.2E; no effect of Treatment: $F(1, 30) = 0.17$, $p = 0.683$). Overall, males showed less interaction with the corrals compared with females (Figure 2.2E; main effect of Sex: $F(1, 30) = 4.58$, $p = 0.041$), though this effect was relatively weak ($\eta^2_p = 0.132$).

2.4.2 Gene Expression of Hippocampal Cytokines in Response to Poly I:C is Greater in Males Compared with Females

Glial Activation Markers

Poly I:C treatment significantly increased expression of both *cd11b* and *gfap*, although this appeared to be true only at the 24-hour timepoint (Figures 2.3A2 and 2.3B2, respectively; *cd11b* main effect of Treatment: $F(4, 42) = 12.96$, $p < 0.001$, $\eta^2_p = 0.552$; *gfap* main effect of Treatment: $F(4, 42) = 12.992$, $p < 0.001$, $\eta^2_p = 0.553$). Sex did not affect the response of either *cd11b* or *gfap* to poly I:C (no Sex x Treatment interactions: *cd11b*: $F(4, 42) = 0.684$, $p = 0.607$; *gfap*: $F(4, 42) = 0.923$, $p = 0.460$).

Interleukins

Poly I:C caused increased expression of *il-1α*, *il-1β*, and *il-6* in both males and females (Figures 2.3C2, 2.3D2, and 2.3E2, respectively; main effects of Treatment: *il-1α*: $F(4, 42) = 9.784$, $p < 0.001$, $\eta^2_p = 0.482$; *il-1β*: $F(4, 42) = 9.512$, $p < 0.001$, $\eta^2_p = 0.475$; *il-6*: $F(4, 42) = 22.28$, $p < 0.001$, $\eta^2_p = 0.680$). In males, expression began to increase at the 2-hour timepoint following poly I:C treatment for *il-1α* ($p = 0.015$; Figure 2.3C2), *il-1β* ($p = 0.057$; Figure 2.3D2), and *il-6* ($p = 0.029$; Figure 2.3E2), showed peaks at the 4-hour timepoint ($p < 0.001$ for all), and decreased to saline-treated levels by 24 hours ($p = 1.00$ for all). Each of these genes also showed an overall greater expression in males than females (main effects of Sex: *il-1α*: $F(1, 42) = 6.398$, $p = 0.015$, $\eta^2_p = 0.132$; *il-1β*: $F(1,42) = 6.695$, $p = 0.013$, $\eta^2_p = 0.137$; *il-6*: $F(1, 42) = 21.1$, $p < 0.001$, $\eta^2_p = 0.334$), and a significantly greater magnitude of response in males compared with females (Sex x Treatment interactions: *il-1α*: $F(4, 42) = 3.103$, $p = 0.025$, $\eta^2_p = 0.228$; *il-1β*: $F(4, 42) = 4.288$, $p = 0.005$, $\eta^2_p = 0.290$; *il-6*: $F(4, 42) = 15$, $p < 0.001$, $\eta^2_p = 0.588$).

Post-hoc tests revealed for all three genes, males exhibited an even greater response at only the

4-hour timepoint compared with females ($p < 0.05$ for all). Notably, the peak *il-1α* and *il-1β*

expression in males was roughly 3-fold higher than that of the peak female expression for these

cytokines, and the *il-6* peak expression in males was more than 10-fold higher than that of

females (Figures 2.3C2, 2.3D2, and 2.3E2, respectively).

Males showed greater *il-10* gene expression across all timepoints compared with females

(Figure 2.3F2; main effect of Sex: $F(1, 39) = 25.642$, $p < 0.001$, $\eta^2_p = 0.397$). Additionally, poly

I:C significantly increased gene expression of *il-10* in males, but not females (Sex x Treatment

interaction: $F(4, 39) = 3.304$, $p = 0.02$, $\eta^2_p = 0.253$). Specifically, male expression of *il-10* at the

4-hour timepoint following poly I:C was significantly greater than that of saline-treated controls

($p = 0.001$), and this was also greater than the 4-hour expression in females ($p = 0.001$; Figure

2.3F2).

Interferons

Both *ifnα* and *ifnγ* showed a similar response pattern to poly I:C, whereby males treated

with poly I:C exhibited a significant acute increase in gene expression of both cytokines, but

females did not show the same response (Figures 2.3G2 and 2.3I2, respectively; *ifnα*: main effect

of Treatment: $F(4, 42) = 5.007$, $p = 0.002$, $\eta^2_p = 0.323$; Sex x Treatment interaction: $F(4, 42) =$

3.35, $p = 0.018$, $\eta^2_p = 0.242$; *ifnγ*: main effect of Treatment: $F(4, 40) = 4.698$, $p = 0.003$, $\eta^2_p =$

0.32; Sex x Treatment interaction: $F(4, 40) = 4.178$, $p = 0.006$, $\eta^2_p = 0.295$). Specifically, 4 hours

after poly I:C treatment, males showed significantly elevated expression compared to the saline-

treated controls (*ifnα: p = 0.001, ; ifnγ: p = 0.0001*), and this was greater in magnitude than the

4-hour timepoint in females (*ifnα: p* = 0.014; *ifnγ: p* = 0.001; Figures 2.3G2 and 2.3I2,

respectively). Female *ifnα* and *ifnγ* did not respond to poly I:C treatment at any timepoint.

In contrast, *ifnβ* showed a transient increase in both males and females, and there were no

sex differences in magnitude of expression increase (Figure 2.3H2; main effect of Treatment:

$F(4, 42) = 4.855$, $p = 0.003$, $\eta^2_p = 0.316$; no Sex x Treatment interaction: $F(4, 42) = 1.297$, $p =$

0.287). Unlike all other cytokines examined in this study, peak expression appeared to be at the

2-hour timepoint, and expression began decreasing again by 4 hours post-treatment. The

magnitude increase was also notable, with a 100-fold increase in females and a 300-fold increase

in males.

Tumor Necrosis Factor Alpha

Gene expression of *tnfα* increased in response to poly I:C, males had significantly higher

expression than females overall, and males showed a greater magnitude of response compared

with females (Figure 2.3J2; main effect of Treatment: $F(4, 42) = 6.407$, $p = 0.0004$, $\eta^2_p = 0.379$;

main effect of Sex: $F(1, 42) = 10.1$, $p = 0.003$, $\eta^2_p = 0.194$; Sex x Treatment interaction: $F(4, 42)$

$= 4.117$, $p = 0.007$, $\eta^2_p = 0.282$). Post-hoc tests showed that males 4 hours post-treatment had

significantly greater expression than those treated with saline ($p < 0.001$), and this was again

greater than the 4-hour peak expression in females ($p = 0.001$; Figure 2.3J2).

Chemokines

Poly I:C significantly increased the expression of both *ccl2* and *cxcl10* in males and

females, with a peak increase in expression at 4-hours post-infusion (Figures 2.3K2 and 2.3L2,

respectively; main effects of Treatment: *ccl2*: $F(4, 41) = 25.47$, $p < 0.001$, $\eta^2_p = 0.713$; *cxcl10*:

$F(4, 42) = 87.37$, $p < 0.001$, $\eta^2_p = 0.893$).

Expression of both *ccl2* and *cxcl10* was greater overall in males compared with females (Figures 2.3K2 and 2.3L2, respectively; main effects of Sex: *ccl2*: $F(1, 41) = 44.55$, $p < 0.001$, $\eta^2_p = 0.521$; *cxcl10*: $F(1, 42) = 92.79$, $p < 0.001$, $\eta^2_p = 0.688$); and males showed a markedly greater magnitude of response than did females for both chemokines (Sex x Treatment interactions: *ccl2*: $F(4, 41) = 20.96$, $p < 0.001$, $\eta^2_p = 0.672$; *cxcl10*: $F(4, 42) = 60.51$, $p < 0.001$, $\eta^2_p = 0.852$).

Remarkably, male *ccl2* expression following poly I:C peaked at nearly 450-fold greater expression than that of saline-treated males, and this is compared to a roughly 20-fold increased peak in females (Figure 2.3K2). Similarly, *cxcl10* expression in males peaked at nearly 500-times that of saline-treated males, while female *cxcl10* expression peaked at just over 40-times greater than saline-treated females (Figure 2.3L2). These massive increases in gene expression are reflected in the strong effect sizes noted for the interaction effect above. Post-hoc tests confirmed that the male 2- and 4-hour timepoints post-treatment showed significantly greater gene expression of both *ccl2* and *cxcl10* than saline-treated males (Figures 2.4K2 and 2.4L2, respectively; $p < 0.001$). Additionally, both the male 2- and 4-hour timepoints of both genes proved to be significantly greater than the 2- and 4-hour timepoints in females, respectively (Figures 2.3K2 and 2.3L2, respectively; $p < 0.01$).

2.4.3 Cytokine Protein Levels in Males and Females After Poly I:C

Interleukins

IL-1α, IL-1β, IL-4, and IL-6 significantly increased following ICV poly I:C administration in both males and females (Figures 2.4A, 2.4D, 2.4C, 2.4E, respectively; main effects of Treatment: IL-1α: $F(4, 51) = 3.523$, $p = 0.013$, $\eta^2_p = 0.216$; IL-1β: $F(4, 51) = 5.721$, p

$= 0.001$, $\eta^2_p = 0.31$; IL-4: $F(4, 51) = 5.146$, $p = 0.001$, $\eta^2_p = 0.288$; IL-6: $F(4, 51) = 10.298$, $p <$ 0.001, $\eta^2_p = 0.447$). In all cases, protein levels increase to a peak 4 hours following poly I:C, similar to the effects seen in mRNA expression.

Both IL-4 and IL-1β also exhibited a main effect of sex such that protein levels of these cytokines, regardless of timepoint, were significantly higher in females compared with males (Figures 2.4C and 2.4D, respectively; IL-4: $F(1, 51) = 11.03$, $p = 0.002$, $\eta^2_p = 0.178$; IL-1β: $F(1, 51) = 114.226$, $p < 0.001$, $\eta^2_p = 0.691$).

No interactions of sex and treatment were found for any of the interleukin cytokines examined here (Figures 2.4A, 2.4B, 2.4C, 2.4D, 2.4E; IL-1α: $F(4, 51) = 0.446$, $p = 0.775$; IL-2: $F(4, 51) = 0.987$, $p = 0.423$; IL-4: $F(4, 51) = 0.982$, $p = 0.426$; IL-1β: $F(4, 51) = 0.513$, $p = 0.726$; IL-6: $F(4, 51) = 1.779$, $p = 0.148$).

Neither IL-2 nor IL-10 showed any effects of poly I:C treatment in either sex (Figures 2.4B and 2.4F, respectively; no main effects of Treatment: IL-2: $F(4, 51) = 1.498$, $p = 0.217$; IL-10: $F(4, 51) = 1.122$, $p = 0.357$). However, females had overall higher levels of IL-10 than did males (Figure 2.4F; main effect of Sex; $F(1, 51) = 20.27$, $p < 0.001$, $\eta^2_p = 0.284$).

Interferons

Unlike mRNA expression, IFNγ protein levels did not change following poly I:C administration in either sex (Figure 2.4G; no main effect of Treatment: $F(4, 52) = 1.93$, $p = 0.119$). However, IFNγ protein levels were higher in females relative to males (Figure 2.4G; main effect of Sex: $F(1, 52) = 150.64$, $p < 0.001$; $\eta^2_p = 0.743$). This was consistent with mRNA expression data where saline-treated females also showed significantly higher expression of *ifnγ* at baseline than did males (see Figure 2.3I2).

Chemokines

Both CCL2 and CXCL10 were significantly increased in the hippocampus by ICV poly I:C and in different ways in males and females (Figures 2.4H and 2.4I, respectively; CCL2: main effect of Treatment: $F(4, 46) = 18.517$, $p < 0.001$, $\eta^2_p = 0.617$; Sex x Treatment interaction: $F(4, 46) = 3.381$, $p = 0.017$, $\eta^2_p = 0.227$; CXCL10: main effect of Treatment $F(4, 52) = 14.54$, $p < 0.001$, $\eta^2_p = 0.528$; Sex x Treatment interaction: $F(4, 52) = 2.796$, $p = 0.035$, $\eta^2_p = 0.177$).

In males, CCL2 levels increased earlier (at 2 hours) post-infusion than females (male saline vs 2hr $p = 0.014$; female saline vs 2hr $p = 1.00$; Figure 2.4H). For CXCL10, females took longer for protein levels to begin to decrease as compared to the time course in males, with females still showing the massive elevation at 24 hours post-infusion as they did at 4 hours (Figure 2.4I).

Notably, CCL2 and CXCL10 levels showed the most substantial increases out of all cytokines measured in protein analysis in the hippocampus. CCL2 levels induced by poly I:C peaked at approximately 4 times that of the saline-treated animals in females and nearly 8 times that of saline-treated males (Figure 2.4H). For CXCL10 levels rose roughly 16-fold in females, and 12-fold in males after poly I:C administration (Figure 2.4I).

2.4.4 Baseline Sex Differences in mRNA Expression and Protein Levels of Select Hippocampal Immune Molecules

Understanding baseline differences in neuroimmune gene expression and protein levels is essential for understanding sex differences in neuroimmune activation. We found that several cytokines and other immune markers showed greater than 2-fold higher levels at baseline (in saline-treated mice) in females compared with males, and in both gene expression and protein. In contrast, none of the markers examined here were higher in males than in females in either

mRNA or protein levels at baseline. This is notable given that we observed the opposite pattern in activation, where males showed stronger poly I:C-induced activation of many cytokines.

Markers with Significantly Higher Baseline Levels in Females Compared with Males

mRNA expression of *il-1α* exhibited a trend towards greater baseline expression in females (Figure 2.3C1; $t(12) = 2.006$, $p = 0.068$), and *il-6* showed a significantly higher level in females compared with males (Figure 2.3E1; $t(11) = 3.079$, $p = 0.01$, 95% CI [0.182, 1.062]). However, these gene expression differences were not reflected at the level of protein (Figures 2.4A and 2.4E).

In contrast, although *il-1b* and *il-10* showed no difference in gene expression between the sexes (Figures 2.3D1, 2.3F1, respectively; *il-1β*: $t(12) = 1.365$, $p = 0.197$; *il-10*: $t(9) = 1.480$, $p = 0.173$), females had significantly higher protein levels of both IL-1β and IL-10 than males (Figures 2.4D and 2.4F, respectively; IL-1β: $t(13) = 4.275$, $p = 0.001$, 95% CI [5.682, 17.291]; IL-10: $t(13) = 2.236$, $p = 0.044$, 95% CI [0.672, 39.314]).

Two interferons (IFN), *ifnα* and *ifnγ*, also showed higher relative mRNA expression levels in females compared with males (Figures 2.3G1 and 2.3I1: IFNα: $t(12) = 5.546$, $p = 0.0001$, 95% CI [0.441, 1.01]; IFNγ: $t(11) = 2.995$, $p = 0.012$, 95% CI [0.259, 1.694]). Likewise, protein levels of IFNγ were higher in saline-treated groups compared with males (Figure 2.4G; $t(14) = 6.475$, $p < 0.001$, 95% CI [6.534, 13.006]).

Expression of chemokine *ccl2* also showed higher levels of both baseline gene expression (Figure 2.3K1; $t(12) = 3.287$, $p = 0.006$, 95% CI [0.259, 1.279]), and protein levels (Figure 2.4H; $t(12) = 2.751$, $p = 0.018$, 95% CI [12.798, 110.318]) in females compared with males.

Neuroimmune Markers with No Sex Differences in Baseline Levels

Neither the microglial activation marker *cd11b* nor the astrocyte activation marker *gfap* showed sex differences in gene expression in the saline-treated groups (Figures 2.3A1 and 2.3B1, respectively; *cd11b*: $t(12) = 0.723$, $p = 0.483$; *gfap*: $t(12) = 1.603$, $p = 0.135$).

Levels of IL-2 and IL-4 protein did not differ between males and females (Figures 2.4B and 2.4C, respectively; IL-2: $t(12) = -0.832$, $p = 0.420$; IL-4: $t(13) = 0.489$, $p = 0.633$); nor were there any differences in *tumor necrosis factor (tnf)α* gene expression (Figure 2.3J1; $t(12) = 1.585$, $p = 0.139$). Finally, CXCL10 did not differ between the sexes in either mRNA (Figure 2.3L1; $t(12) = -0.923$, $p = 0.374$) or protein (Figure 2.4I; $t(14) = 0.548$, $p = 0.592$).

2.4.5 Summary of mRNA and Protein Data

Overall, hippocampal mRNA expression and protein levels of most of the cytokines and chemokines examined in this experiment responded to central administration of poly I:C in both males and females. We found significant sex differences in baseline mRNA expression and protein levels of several cytokines, where females showed greater basal levels than males. In addition, we found the magnitude of mRNA expression increases was greater in males than females. Protein data showed this to be true only for 2 chemokines, CCL2 and CXCL10.

The heatmaps shown in Figure 2.5 indicate that most of the immune signaling molecules affected in the immediate phase following poly I:C treatment peaked at 4 hours for both mRNA expression (Figure 2.5A) and protein levels (Figure 2.5B) and returned to levels of saline-treated animals by 24 hours post-infusion.

2.5 Discussion

Here we demonstrated that after central administration of poly I:C sufficient to induce acute physiological sickness responses (fever, weight loss) in both sexes, male and female mice

showed acute hippocampal cytokine and chemokine elevations, as measured by both mRNA expression and protein levels, that followed the time course of fever. Interestingly, mRNA gene expression of *il-1α*, *il-1β*, *il-6*, *il-10*, *ifnα*, *tnfα*, *ccl2*, and *cxcl10* and protein levels of CCL2 and CXCL10 showed a stronger response in males compared with females. Further, gene expression of *il-10*, *ifnα*, and *ifnγ* increased in males only.

Sickness behaviors including increased sleep, decreased food intake, and decreased social interaction are adaptive responses that act to save and direct metabolic energy to physiological responses such as mounting a fever and stimulating the immune system to fight off infections. We did not find visible signs of sickness such as piloerections, squinted eyes, or hunched backs in males or females during any of our experiments, in contrast to what has been shown previously during sickness in many animal species, including rodents (Hart, 1991). However, these measures do not always signal underlying immune or neuroimmune activation or infections (Lenczowski et al., 1999). Instead, we found that central administration of poly I:C did induce a significant fever response and weight loss in both males and females. Additionally, we found that poly I:C treatment significantly decreased sociability in females during a social preference test, as expected. We did not find an effect of poly I:C on social behavior in males; however, we were not able to show social preference in saline-treated males, making it difficult to conclude that poly I:C had no effect here. The three-chamber sociability task adapted for this study was originally developed using juvenile male C57BL/6 mice as the experimental mice and adult male conspecifics as the strangers (Nadler et al., 2004). Juvenile males partake in a significant amount of social behavior (Cox & Rissman, 2011), and it has been shown that singly housing male mice for 30 days at either 1- or 3-months of age can result in significantly greater fighting tendencies during adulthood than socially housed males (Crawley, Schleidt, & Contrera, 1975). Thus, it is

possible that the experimental conditions used here fostered slightly greater aggressive-like behaviors in our adult males, making social behavior more difficult to assess. It has been suggested that performing these tasks in a completely novel environment (i.e., without habituation) may decrease the level of aggression between rodents in social-based tasks (Toth & Neumann, 2013), as well as using a juvenile male conspecific as the stranger (Kogan, Franklandand, & Silva, 2000; Thor & Holloway, 1982). Future studies will also consider additional measures of sociability than the social preference score reported here to get a more comprehensive assessment of the effect of neuroimmune activation on sociability for both males and females.

Poly I:C treatment in both sexes resulted in a significant and transient increase in hippocampal gene expression and protein levels of most, but not all, cytokines and chemokines measured, including IFNβ, IL-1α, IL-1β, IL-6, TNFα, CCL2, and CXCL10. That administration of an immune stimulant, including viral mimics such as poly I:C, induces a neuroimmune response is not new; however, most of the previous studies on poly I:C used peripheral administration (C. Cunningham, Campion, Teeling, Felton, & Perry, 2007; Fortier et al., 2004; Kamerman, Skosana, Loram, Mitchell, & Weber, 2011; Murray et al., 2015). As such, multiple, indirect mechanisms are likely involved in causing inflammation in the brain (Watkins et al., 1995). Peripheral administration of poly I:C, specifically, was found to induce neuroinflammation through a separate and distinct pathway than central administration (X. Zhu et al., 2016). Thus, ICV poly I:C administration is one way to study sex differences and similarities in the neuroimmune response to a TLR3 agonist without initial interference from sex-specific peripheral response. Additionally, we extend previous work to include a broader set of cytokines and chemokines, including CCL2 and CXCL10, and Type I interferons that

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typically respond to viruses. Given evidence of mechanistic complexities governing

neuroimmune activation, particularly from stimulants such as poly I:C, and given that there are

hundreds of cytokines with important roles in the immune system and neural function, it is

critical to begin looking beyond IL-1β, IL-6, and TNFα and more strongly consider implications

of such limits in experimental design for the field of psychoneuroimmunology.

Males and females differ in immune responses, and the direction of these differences

depends on whether one is looking in the periphery (Klein & Flanagan, 2016) or the brain

(Loram et al., 2012; Santos-Galindo et al., 2011); and whether the immune challenge itself is

systemic or brain-specific. We found that mRNA gene expression of *il-1α*, *il-1β*, *il-6*, *il-10*, *ifnα*,

tnfα, *ccl2*, and *cxcl10* and protein levels of CCL2 and CXCL10 in the hippocampus showed a

stronger response in males compared with females. A greater magnitude of cytokine and

chemokine response in males is consistent with previous findings that male-derived astrocytes

have a greater reaction to inflammatory insults compared with females (Astiz et al., 2014;

Chistyakov et al., 2018; Loram et al., 2012; Santos-Galindo et al., 2011).

Poly I:C is recognized by microglia, astrocytes, and neurons *via* Toll-like receptor 3

(TLR3) (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Kielian, 2006; M. Matsumoto & Seya,

2008). The interaction of these 3 cell types is crucial in mediating inflammatory responses

(Cerbai et al., 2012; Scholz & Woolf, 2007). Given that TLR3 shows much greater expression in

astrocytes relative to microglia (Bsibsi, Ravid, Gveric, & Van Noort, 2002), we speculate that

the reaction of astrocytes in males may be driving the sex differences in magnitude gene

expression response of cytokines following poly I:C. The astrocyte activation marker, GFAP,

and the microglial activation marker, CD11b, did not increase until 24 hours after poly I:C

treatment and did not show sex differences. However, this does not absolve astrocytes or

microglia from the acute response to poly I:C. Specifically, Norden and colleagues found that cytokine gene expression from both astrocytes and microglia preceded increases in astrocyte and microglial activation markers, (GFAP and Iba1, respectively), and that these activation markers similarly did not show reliable increases until the 24-hour timepoint (Norden, Trojanowski, Villanueva, Navarro, & Godbout, 2016). Further work is needed to understand how neuroimmune cells, and in particular astrocytes, drive sex differences in cytokine response to poly I:C.

We observed that for most cytokines examined here, males showed a greater response to poly I:C than did females. Whereas others have reported increases in select inflammatory markers following poly I:C treatment, these studies used either only used male (Fortier et al., 2004; Kamerman et al., 2011) or female rodents (C. Cunningham et al., 2007; Murray et al., 2015). To the best of our knowledge, this is the first direct comparison of hippocampal cytokines in males and females as a consequence of poly I:C. Whether the greater magnitude in male response to poly I:C indicates greater neuroprotection or vulnerability to cognitive dysfunction is yet to be determined. It is also important to note that we did not find identical patterns of cytokine data in both the mRNA and protein analyses. While we did find that several cytokines had greater basal expression in females for both mRNA and protein, we did not show the same greater magnitude response of male cytokines in our protein data. Several studies have reported a poor correlation between mRNA expression and protein levels of the same endpoints (De Sousa Abreu, Penalva, Marcotte, & Vogel, 2009; Koussounadis, Langdon, Um, Harrison, & Smith, 2015; Vogel & Marcotte, 2012). A number of factors may be at play in this discrepancy, including: 1) differences in the sensitivity of mRNA and protein extraction and measurement techniques, 2) differences in the timing of mRNA and protein synthesis, and 3) differences in

post-translational modifications for certain endpoints. Here, we collected tissue for mRNA and protein analyses simultaneously, though one study found a delay of 1-4 hours between peaks in mRNA expression and corresponding protein endpoints (Israelsson et al., 2020). Additional factors such as mRNA and protein half-lives and protein-level buffering might be playing a role in our contrasting data (Buccitelli & Selbach, 2020). Importantly, what does it mean for there to be such a stark difference between mRNA expression and protein levels of the cytokines we examined here for neuroimmune function and potential impacts on cognitive processes? Perhaps it is simply a result of differences in methodologies and technological sensitivities. Perhaps, alternatively, we captured significant sex differences in post-translational modifications or protein-level buffering, where males have much more of this to control levels of proteins from mRNA products than females. Whether these or other explanations are the cause of this are still unknown, and this is an ongoing and important question for the field of (neuro)immunology as a whole.

A critical question, arising from our observation of greater baseline mRNA expression and protein levels of cytokines and chemokines in females relative to males, is what is the biological relevance of these differences, and how do they relate to activated neuroimmune states? One possibility is that females mount a greater immune response to help clear viral loads and recover faster (Barna et al., 1996; Channappanavar et al., 2017; Klein, 2012; vom Steeg & Klein, 2016), and also start out with greater immune activity that allows them to reach necessary activation states faster than males. Perhaps females do not need to have as strong of an activated response because they already have "more players in the game". This layer of nuance for understanding sex differences in immune/neuroimmune function adds to the broader notion that sex differences are not just about which sex has a stronger response, but that the type and pattern

of response matters (Donzis & Tronson, 2014; Speirs & Tronson, 2018), together with the context (e.g., dose, type of challenge, method of administration, timing, hormonal states (Coelho, Cruz, Maba, & Zampronio, 2021; Klein, 2012; Klein & Flanagan, 2016; Speirs & Tronson, 2018)) all contribute to the complexity of understanding sex differences and their functional implications. Future work will need to address whether and how sex differences in the cytokine and chemokine basal levels or activation in response to immune challenge result in modulation of neural function and contribute to sex-biases in neurological and psychiatric disease.

Of particular note, we observed a sex-specific pattern of expression of the interferon family of cytokines in the hippocampus. Specifically, males showed increases in IFNα, IFNβ, and IFNγ, but females only showed a significant response in IFNβ. This is consistent with previous findings that showed increased gene expression of IFNβ, but not IFNα, in females in response to peripheral poly I:C, though this study did not measure these effects in males for comparison (Murray et al., 2015). Type I interferons, IFNα and IFNβ, are key to the anti-viral response of the immune system and, as such, are known to respond to viral stimulants including poly I:C (Costello & Lynch, 2013; Scumpia, Kelly, Reeves, & Stevens, 2005; Seth, Sun, & Chen, 2006; Stetson & Medzhitov, 2006; Teijaro, 2016). Consistent with our data, in which IFNβ showed an early peak expression levels, Type I interferon activity is responsible for inducing inflammatory cytokines such as IL-6 and TNFα (Fortier et al., 2004; Murray et al., 2015). Additionally, interferon signaling from poly I:C treatment also results in altered glutamatergic signaling (Costello & Lynch, 2013; Scumpia et al., 2005), which is critical for hippocampal memory formation (Katagiri et al., 2001). One caveat is that we only measured IFNα and IFNβ gene expression. Nevertheless, other studies have demonstrated a correspondence of increased IFNβ gene expression and modulation of memory in females

(McGarry et al., 2021; Murray et al., 2015). Thus, given that males show increased expression of both IFNα and IFNβ in the hippocampus following poly I:C whereas females only induce IFNβ, together with the roles of IFNβ in learning and memory, interferon-related signaling is likely key for understanding sex differences in virus, or virus-like, modulation of memory and cognition.

This study characterizes the neuroimmune and sickness responses to central administration of poly I:C, and we observed sex-specific patterns of hippocampal cytokine transcription and translational responses. Specifically, we identified Type I interferons as one potential node mediating sex-specific cytokine responses and neuroimmune effects on synaptic plasticity and cognition. Additionally, the magnitude of response of cytokines such as CCL2 and CXCL10 highlight the importance of future work incorporating a more comprehensive set of inflammatory markers using multiple endpoints. Neuroimmune activation is known to play a role in cognitive deficits and affective dysregulation in diseases such as Alzheimer's disease and other dementias (Krstic & Knuesel, 2013), Post-Traumatic Stress disorder (Pace & Heim, 2011; Z. Wang & Young, 2016), depression (Bekhbat & Neigh, 2018; Hodes, Kana, Menard, Merad, & Russo, 2015), and now also COVID-19 (H. Zhou et al., 2020). Given the sex/gender biases in prevalence, severity, and/or survival outcomes, identifying sex-specific neuroimmune responses will provide novel targets for personalized prevention and treatment of these diseases.

2.6 Table and Figures

Table 2.1 *Primer sequences used for real-time PCR*

Gene Target	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')	NCBI Reference Sequence
ccl2	CCACAACCACCTCAAGCACT	AAGGCATCACAGTCCGAGT	NM_011333.3
cd11b	CGTGAATGGGGACAAACTGAC	GCACTGAGGCTGGCTATTGA	NM_008401.2
cxcl10	TCCATCACTCCCCTTTACCCA	TGGCTTGACCATCATCCTGC	NM_021274.2
gfap	AAACCGCATCACCATTCCTG	CCCGCATCTCCACAGTCTTTA	NM_010277.3
ifnα	AGAGAAGAAACACAGCCCCT	AGCACATTGGCAGAGGAAGA	NM_010502.2
ifnβ	GCTCCAAGAAAGGACGAACAT	GGATGGCAAAGGCAGTGTAA	NM_010510.1
ifnγ	GTCAACAACCCACAGGTCCA	CGACTCCTTTTCCGCTTCCT	NM_008337.4
il-1β	TGCCACCTTTTGACAGTGATG	GCTCTTGTTGATGTGCTGCT	NM_008361.4
il-6	GAGACTTCCATCCAGTTGCCT	TCATTTCCACGATTTCCCAGAG	NM_001314054.1
il-10	CTGGACAACATACTGCTAACCG	AATGCTCCTTGATTTCTGGGC	NM_010548.2
tnfα	ACCCCTTTACTCTGACCCCTT	ACTGTCCCAGCATCTTGTGT	NM_001278601.1

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Figure 2.1 *Housekeeping gene stability analysis*

(A) Distribution of the quantification cycles (Cq) for housekeeping genes *18s*, *gapdh*, *hprt1*, and *rplp0***,** with **(B)** associated standard deviations. **(C)** Distribution of the difference of Cq values (ΔCq) between pairs of housekeeping genes, and **(D)** the associated standard deviations. **(E)** Stability values calculated using gene ratio method by Vandesompele et al., 2002, which uses stepwise elimination of lowest stability (highest *M* value) to rank gene stability. **(F)** Stability values calculated using a model-based approach by Andersen et al., 2004 which measures expression variation such that highest stability results in the lowest Rho value. **(G)** Summary of results from each of the four methods of housekeeping gene stability are shown.
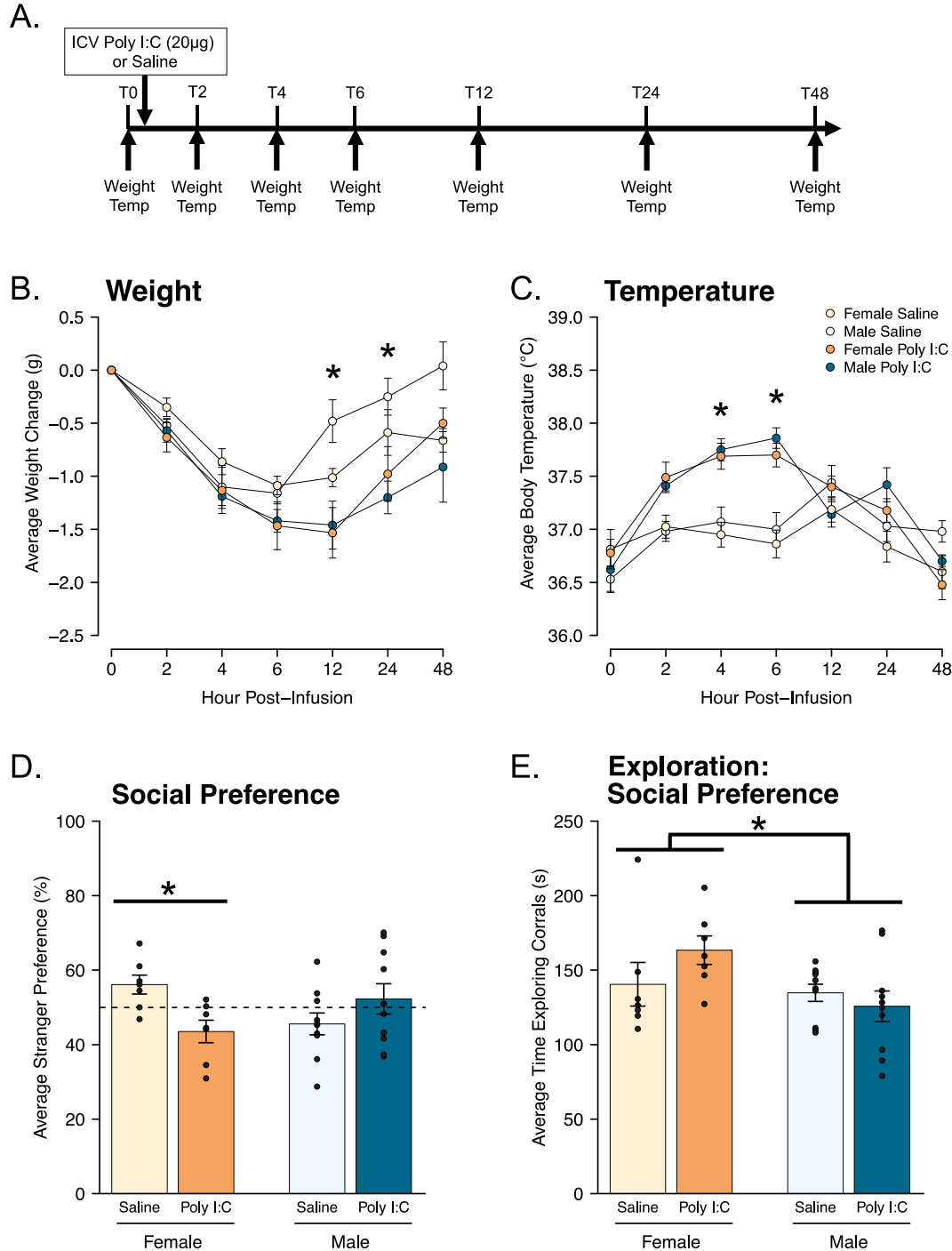
Figure 2.2 *Analysis of sickness behaviors following poly I:C administration*

(A) Timeline of body weight and temperature measurements following poly I:C or sterile saline administration. **(B)** Average weight change from baseline (Hour Post-Infusion = 0) prior to treatment. **(C)** Average body temperature as measured *via* rectal thermometer. **(D)** Average preference for a stranger mouse compared to a novel object. Dashed line drawn at 50%. **(E)** Total time spent interacting with corrals during social preference test. * above points on a line graph (B and C) indicate a significant difference ($p < 0.05$) between poly I:C- and saline-treated groups following mixed repeated-measures ANOVA; * above a horizontal line (D) indicates a significant post-hoc comparison ($p < 0.05$) following a significant interaction in a two-way ANOVA; * above a bracket (E) indicates a significant main effect ($p < 0.05$) following a two-way ANOVA. Error bars represent standard error of the mean.

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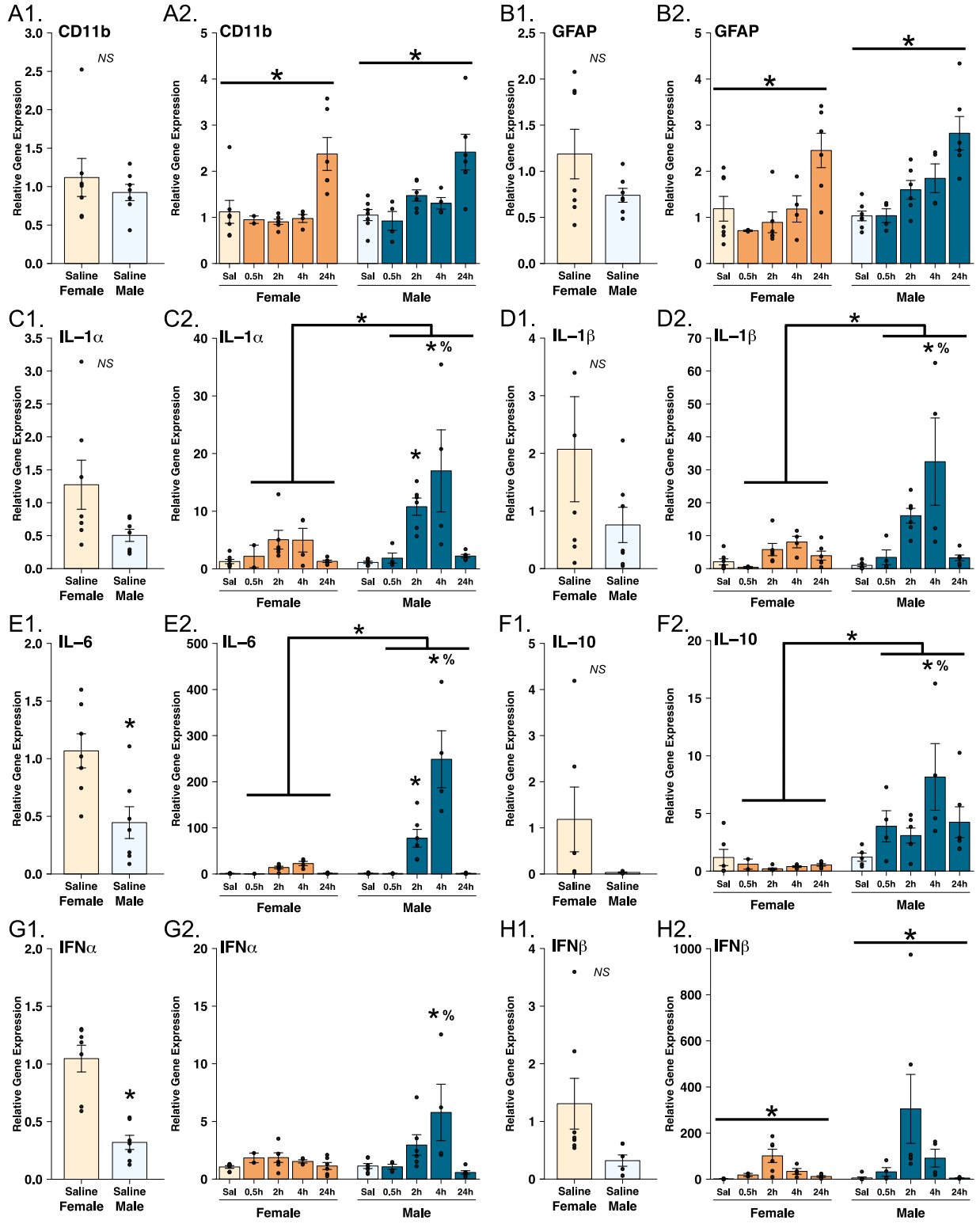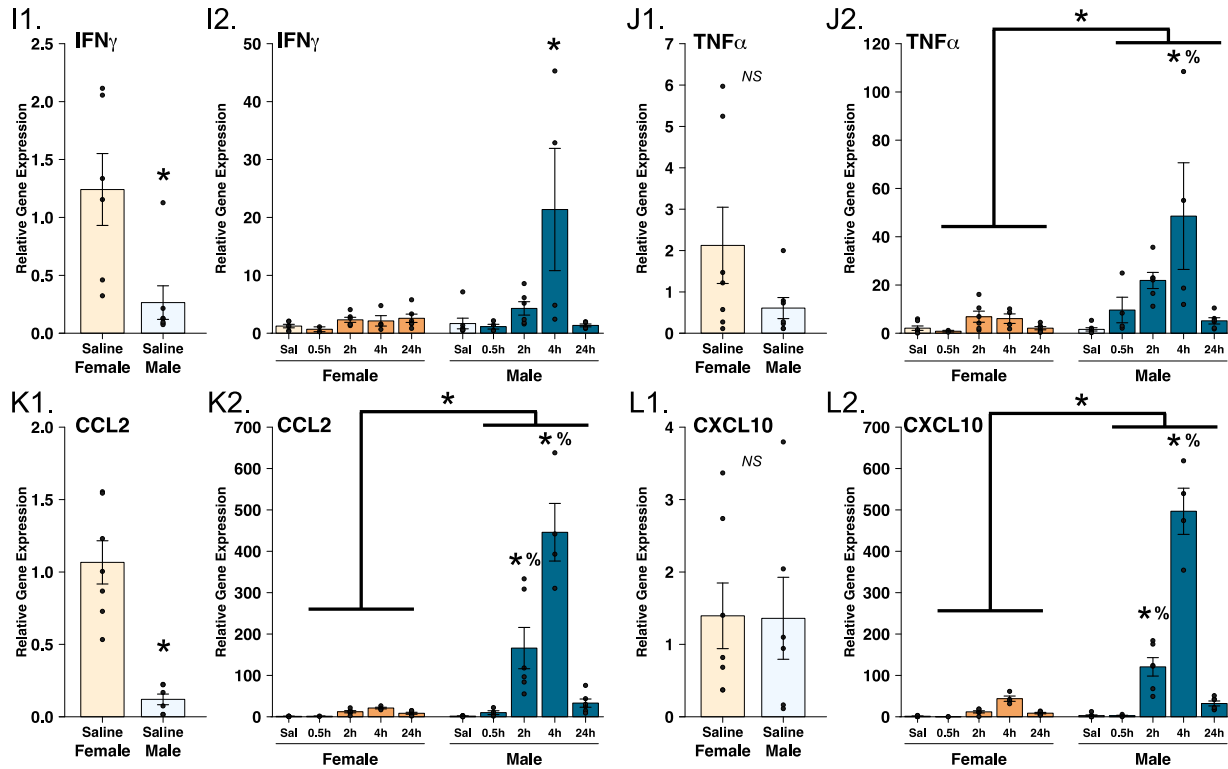
Figure 2.3 *mRNA gene expression of cytokines, chemokines, and markers of glial activation in the hippocampus*

Baseline gene expression was measured by normalizing the male saline-treated group to the female saline-treated group and analyzed using independent, two-sample t-tests. Baseline expression of **(A1)** CD11b, **(B1)** GFAP, **(C1)** IL-1α, **(D1)** IL-1β, **(E1)** IL-6, **(F1)** IL-10, **(G1)** IFNα, **(H1)** IFNβ, **(I1)** IFNγ, **(J1)** TNFα, **(K1)** CCL2, and **(L1)** CXCL10 are shown. Gene expression changes following poly I:C treatment were calculated by normalizing timepoints after poly I:C treatment to the saline-treated groups within sex to eliminate confounding variables of baseline sex differences. Gene expression was analyzed using two-way ANOVA tests for **(A2)** CD11b, **(B2)** GFAP, **(C2)** IL-1α, **(D2)** IL-1β, **(E2)** IL-6, **(F2)** IL-10, **(G2)** IFNα, **(H2)** IFNβ, **(I2)** IFNγ, **(J2)** TNFα, **(K2)** CCL2, and **(L2)** CXCL10. ***** above a bracket covering both sexes indicates a significant main effect of sex ($p < 0.05$); ***** above a horizontal line covering just one sex indicates a significant main effect of treatment ($p < 0.05$; A2, B2, H2); ***** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs the saline-treated group within sex (C2, D2, E2, F2, G2, I2, J2, K2, L2); **%** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs females at the same timepoint (C2, D2, E2, F2, G2, J2, K2, L2). *NS* indicates no statistical significance. Error bars represent standard error of the mean.
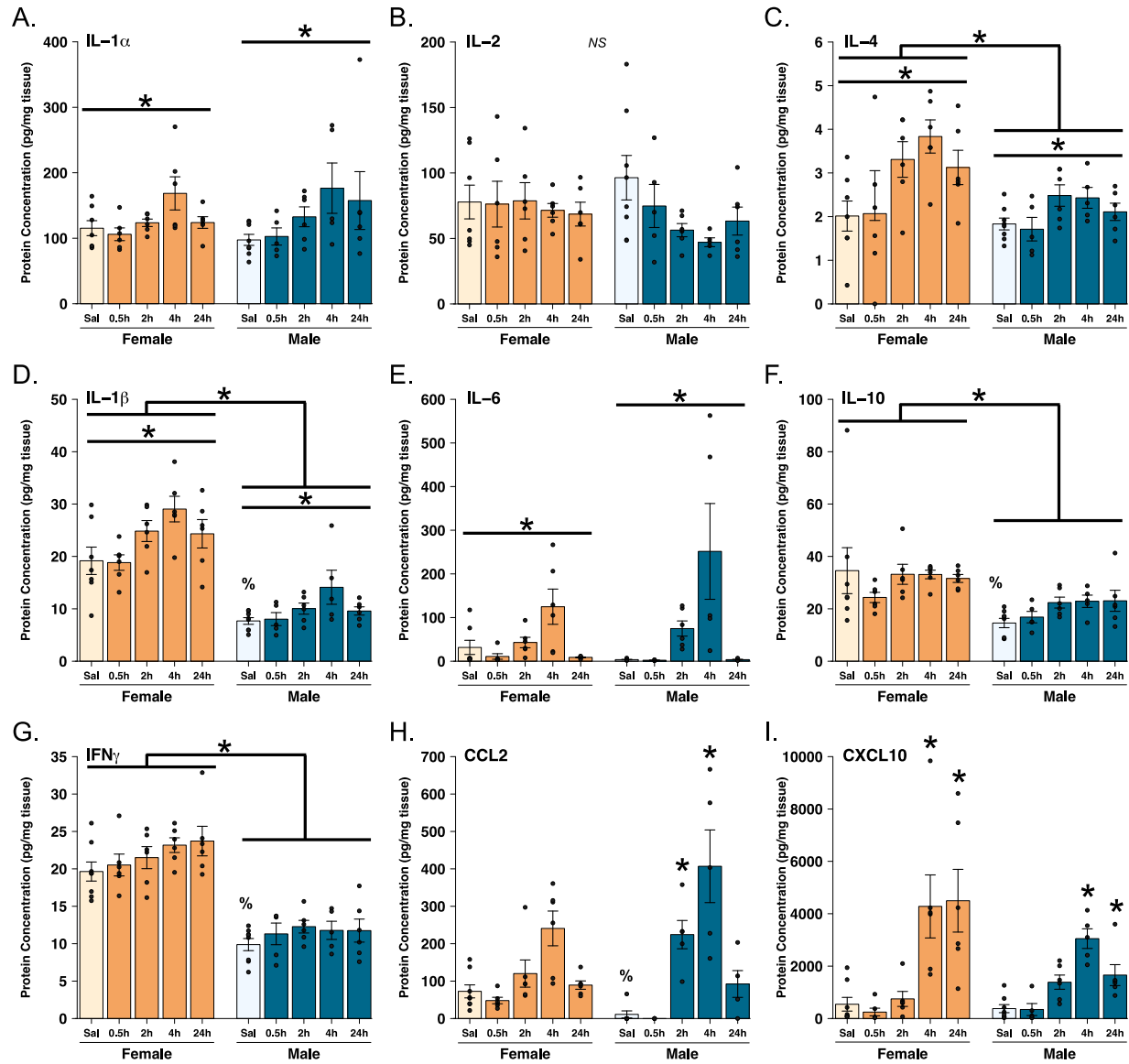
Figure 2.4 *Protein levels of cytokines and chemokines in the hippocampus*

Protein concentration (pg/mg) of **(A)** IL-1α, **(B)** IL-2, **(C)** IL-4, **(D)** IL-1β, **(E)** IL-6, **(F)** IL-10, **(G)** IFNγ, **(H)** CCL2, and **(I)** CXCL10 are shown. Two-way ANOVA tests were used to analyze these data. ***** above a bracket covering both sexes indicates a significant main effect of sex ($p < 0.05$); * above a horizontal line covering just one sex indicates a significant main effect of treatment ($p < 0.05$; A, C, D, E); * above a bracket indicates a significant main effect of sex (p < 0.05; C, D, F, G); * above a single bar indicates a significant post-hoc test ($p < 0.05$) vs the saline-treated group within sex (H, I); **%** above a single bar indicates a significant post-hoc test ($p < 0.05$) vs females at the same timepoint (D, F, G, H). *NS* indicates no statistical significance. Error bars represent standard error of the mean.
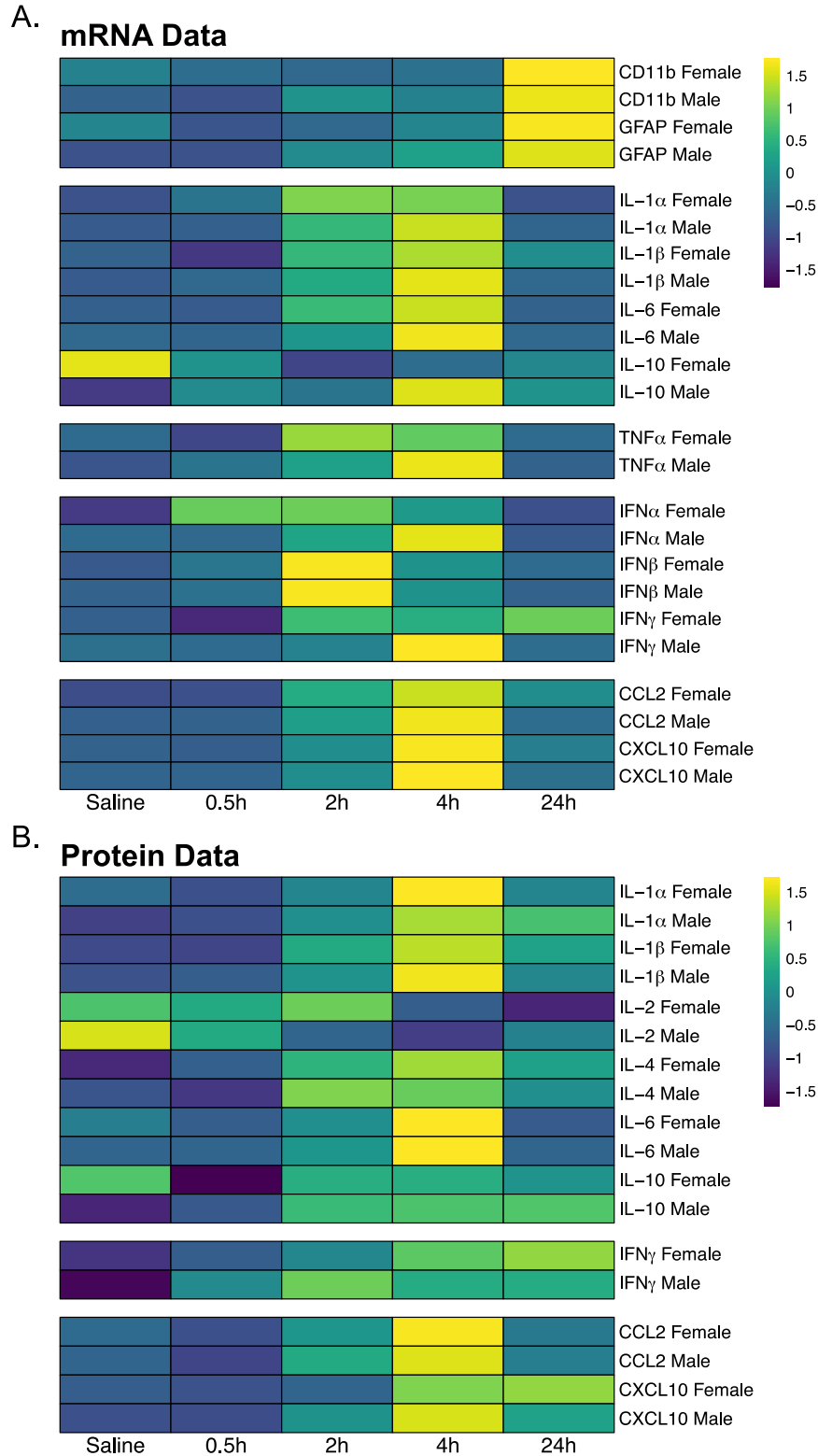
Figure 2.5 *Heatmaps of gene expression and protein levels in the hippocampus*

Changes in (**A**) mRNA gene expression and (**B**) protein levels for cytokines, chemokines, and markers of glial activation are shown. Values are centered and scaled across rows to highlight changes across the time course for each gene of interest; thus, differences in magnitude between gene expression and protein levels are not depicted.

Chapter 3:
Poly I:C Disruption of Hippocampal-Dependent Learning and Memory is Nuanced in Males and Females

3.1 Abstract

Neuroimmune system activation has been shown to disrupt cognitive processes including learning and memory, and neuroimmune dysregulation is also associated with disorders of memory such as dementia in Alzheimer's disease. We previously found that central administration of a viral mimic, polyinosinic:polycytidylic acid (poly I:C) induced inflammatory cytokines in the hippocampus of both males and females. Notably, males showed a greater magnitude of neuroimmune response for many of the cytokines we examined relative to females. Whether this sex difference in neuroimmune response corresponds to sex differences in neuroimmune modulation of cognitive processes, however, remains to be explored. Here, we used intracerebroventricular administration of poly I:C and hippocampal-dependent memory tests in both male and female C57BL/6N mice to determine how neuroinflammation disrupts learning and memory consolidation and whether biological sex plays a modulatory role. We found that pre-training poly I:C disrupted learning in a context fear conditioning task in both sexes, and post-training poly I:C disrupted memory consolidation in the same task in males only. We measured the immediate early gene cFos as a proxy of neuronal activation following training in context fear conditioning with or without poly I:C and found that pre-training poly I:C had both sex- and hippocampal subregion-specific effects on learning-induced cFos levels. We also tested whether pre-training poly I:C could disrupt a multiple memory systems task in an escape-

motivated T-maze. While poly I:C had no effect on learning ability or rate of learning in the task, poly I:C shifted the bias between place- and response-based learning strategies in both males and females, but in opposite directions. Collectively, these data suggest that poly I:C does disrupt learning and memory mechanisms in both sexes, but *via* sex-specific underlying mechanisms.

3.2 Introduction

Activating the neuroimmune system has been shown at length to disrupt cognition and molecular mechanisms underlying learning and memory (Dantzer et al., 2008; Donzis & Tronson, 2014; Tchessalova & Tronson, 2019; Yirmiya & Goshen, 2011). This is largely because neuroimmune cells and their related signaling are a necessary component and regulator of neural processes under healthy, homeostatic conditions (Marin & Kipnis, 2013). The hippocampus is particularly important for learning and memory consolidation, spatial navigation, and affect and, as such, is heavily implicated in debilitating memory- and mood-related disorders including Alzheimer's disease, Post-Traumatic Stress disorder, and major depressive disorder, among others (Acheson, Gresack, & Risbrough, 2012; Mufson et al., 2015; Rothman & Mattson, 2009; Sala et al., 2004). Notably, these diseases and disorders disproportionately affect women and present with significant neuroimmune dysregulation (Kronfol, 2000; Lynch, 2014; Miller, Maletic, & Raison, 2009; Z. Wang & Young, 2016). We previously found that central administration of poly I:C induced a greater magnitude of hippocampal cytokine response in males compared with females as well as a sex difference in the response of Type I interferons (Chapter 2; Posillico, Garcia-Hernandez, & Tronson, 2021). However, whether these sex differences in hippocampal neuroimmune activation are functionally relevant for hippocampal-dependent learning and memory is unknown and important for understanding the role of the neuroimmune system in health and disease.

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Learning, memory consolidation, and spatial navigation are essential cognitive processes for survival, and dysregulation of these processes is widespread in neurodegenerative diseases and memory-related disorders, many of which affect the hippocampus. Contextual fear conditioning paradigms require the hippocampus during learning, as animals must form conjunctive representations of a particular context with the experience of an aversive stimulus such as a foot shock (O'Reilly & Rudy, 2001; Rudy & O'Reilly, 2001). This is related to but distinct from hippocampal function during spatial learning and memory in which the hippocampus is responsible for forming spatial maps and encoding spatial relationships between features in a context (Moser, Kropff, & Moser, 2008; O'Keefe, 1976; O'Keefe & Dostrovsky, 1971). Learning in a T-maze task may require the hippocampus if the animal is using information about spatial cues to complete the task which is referred to as a "place-based" strategy (Kleinknecht et al., 2012; Morris, Garrud, Rawlins, & O'Keefe, 1982; Tolman, Ritchie, & Kalish, 1946). However, it is also possible that the animal is making associations between a stimulus, such as the goal of the maze, and a response, such as a left or right body turn, and this is termed a "response-based" strategy that does not require the hippocampus (Packard, 1999; Packard & McGaugh, 1996). Importantly, animals may be able to use either strategy to successfully learn in this memory task, and this cognitive flexibility requires the hippocampus (Kleinknecht et al., 2012) and is also an important component for survival and implicated in Alzheimer's disease (Albert, 1996; Hulshof et al., 2022), Post-Traumatic Stress disorder (Ben-Zion et al., 2018), and age-related cognitive decline (Guarino, Forte, Giovannoli, & Casagrande, 2020). As such, it is vital that we determine whether and how neuroimmune activation modulates various memory systems for a more holistic understanding of target mechanisms for disease treatment.

In-tact neuroimmune system function is required for hippocampal synaptic plasticity and memory processes. *In vitro* work has shown inhibition of IL-1 receptor activity under healthy, physiological conditions inhibits hippocampal long-term potentiation induction (Ross et al., 2003) and maintenance (Schneider et al., 1998). This effect has also been shown *in vivo* whereby IL-1 receptor inhibition results in impaired hippocampal-dependent spatial memory and fear conditioning (Goshen et al., 2007). However, this study also showed that lower levels of immune activation actually improved memory performance while much greater immune activation disrupted hippocampal-dependent memory. Given the significant sex difference in magnitude of neuroimmune activation following central administration of poly I:C (Chapter 2; Posillico, Garcia-Hernandez, & Tronson, 2021), it is important to determine whether this will translate to functional sex differences in memory.

Microglia are the resident immune cells of the brain. Under healthy conditions, microglia are responsible for surveying the microenvironment for invading pathogens or tissue damage. In addition, microglia are important for regulating the stability of neuronal synapses, which is important for both maintaining neural activity and cognition as well as allowing for experience-dependent plasticity processes important for learning and memory mechanisms (Badimon et al., 2020; Crapser et al., 2020; Nguyen et al., 2020). Neuroimmune activation from bacterial endotoxins and viruses impair microglial processes that support synaptic plasticity and can cause microglia to engulf synapses that result in hippocampal-dependent memory impairment in both sexes (Akiyoshi et al., 2018; Garber et al., 2019; Vasek et al., 2016; Wegrzyn, Freund, Faissner, & Juckel, 2021). Microglia can be activated by poly I:C as well as respond to and produce inflammatory cytokines (Carpentier et al., 2008; Kigerl et al., 2014; Ransohoff & Brown, 2012), including those that we previously found increased in the hippocampus following central

administration of poly I:C (Chapter 2; Posillico, Garcia-Hernandez, & Tronson, 2021). We did

not find increases in CD11b, a marker for microglial activation, until 24 hours after poly I:C

administration (Chapter 2, Figure 2.3A2). However, this does not absolve microglia in the acute

response to poly I:C, and it is still important to determine whether microglia mechanisms may be

at play in the neuroimmune modulation of learning and memory under poly I:C.

In this study, we aimed to determine whether central administration of poly I:C disrupts

learning and memory consolidation in males and females using multiple memory tasks. We

previously found that poly I:C resulted in a greater magnitude increase of hippocampal cytokines

in males compared with females and a sex difference in the anti-viral Type I interferons (Chapter

2). Here, we examined whether these sex differences resulted in functional differences in

learning and memory performance. We used both pre-training and post-training treatment of

poly I:C in a hippocampal-dependent context fear conditioning task to determine whether

learning- or memory consolidation-specific mechanisms may be differentially impacted by

neuroimmune activation and whether and how biological sex played a role in these processes.

We also used an escape-motivated T-maze task and a social memory task in females to assess

whether poly I:C modulated memory under different motivational states than that induced by

context fear conditioning. It is possible to find sex differences in behavior as a result of distinct

underlying neurobiological mechanisms in males and females, and it is also possible for males

and females to have similar behavioral outputs with sex differences in neural processes (Becker

& Koob, 2016; McCarthy, Arnold, Ball, Blaustein, & de Vries, 2012). Thus, we also analyzed

hippocampal cFos and microglia morphology after learning to address this possibility.

We found that pre-training poly I:C disrupts learning in context fear conditioning in both

sexes, and post-training poly I:C disrupts memory consolidation in males only. Interestingly, we

also found sex- and subregion-specific effects on training-induced cFos in the hippocampus. In the CA1, pre-training poly I:C blunted training-induced cFos in males but not females, and in the CA3, pre-training poly I:C enhanced training-induced cFos in females but not males. We did not find effects of either poly I:C treatment or training on two measures of microglia morphological analysis, but we did find sex differences in overall microglia morphology in the hippocampus. Pre-training poly I:C did not impact the ability to learn in the escape-motivated T-maze task, but it did shift the learning strategy used in both sexes, and in opposite directions. Collectively, these data show that poly I:C has similar effects on behavioral outputs of learning in males and females, but there are sex-specific underlying hippocampal mechanisms at play. Future studies will aim to determine intracellular mechanisms impacted by poly I:C during learning in both males and females.

3.3 Methods

3.3.1 Animals

173 male and female 8–9-week-old C57BL/6N mice were purchased from Envigo (Indianapolis, IN) and used in these experiments. All mice were individually housed in standard polypropylene mouse cages with *ad libitum* access to food and water in a room with maintained temperature, pressure, and humidity under a 12:12h light:dark cycle. Animals were given at least one week of acclimation to the colony room prior to any experimental manipulations. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

3.3.2 Stereotaxic Surgeries

Bilateral guide cannulae (PlasticsOne, Roanoke, VA) targeting the lateral ventricles were implanted as discussed in Chapter 2 using standard stereotaxic methods (KOPF, Tujunga, CA) at the following coordinates relative to Bregma: ML: +/- 1.00 mm, AP: 0.30 mm, DV: -2.50 mm. Briefly, animals were administered a pre-surgical analgesic (5 mg/kg Carprofen, subcutaneous) and anesthetized for surgery using an intraperitoneal injection of 250 mg/kg of Avertin (2,2,2-tribromoethanol). Guide cannulae were secured using dental cement. Animals were given a second dose of Carprofen (5 mg/kg, subcutaneous) 24 hours after surgery to maintain a total of 48 hours of analgesia. Mice were monitored daily for 10 days post-operative and were given at least 2 weeks to recover from surgery prior to use in experiments.

3.3.3 Poly I:C Administration

Polyinosinic:polycytidylic acid (poly I:C; Cat. No. P1530; Sigma-Aldrich, St. Louis, MO) was prepared according to the manufacturer's instructions and sterile-filtered using a 0.22 μm filter prior to administration. For intracerebroventricular (ICV) delivery, 20 μg of poly I:C (2 μL of 10 μg/μL poly I:C) (X. Zhu et al., 2016) or an equal volume of 0.9% sterile saline were infused *via* the implanted guide cannula under brief isoflurane anesthesia as described in Chapter 2.

3.3.4 Estrous Phasing

Visual assessment of the vaginal opening in conjunction with vaginal cytology were used to determine the estrous phase of females on the day of treatments when possible (Byers et al., 2012). For vaginal cytology, females were gently lavaged with 10 μL of sterile water, and samples were dispensed onto clean microscope slides. Once the samples were dried, they were imaged at 5X magnification under bright field microscopy, and the ratio of cells present was

used to classify the estrous phase. We did not find any patterns suggesting that estrous phase modulated behavior in females for any task.

3.3.5 Context Fear Conditioning

A context fear conditioning task was used to assess the effects of poly I:C on fear learning and memory. To determine whether poly I:C disrupted acquisition or learning in the task, mice were given a pre-training treatment of either ICV poly I:C ($n = 8$ male; $n = 10$ female) or sterile saline ($n = 9$ male; $n = 10$ female) and trained 4 hours later. During training, mice were placed in a 25 cm x 30.5 cm x 25 cm chamber with grid floors (MedAssociates, St. Albans, VT). Animals were given 3 minutes to explore the context before receiving a 2-second 0.8 mA foot shock and immediately removed and returned to their home cage. The apparatus was cleaned with 70% ethanol between each animal. 72 hours later, mice were brought back to the chamber for testing, during which mice had 3 minutes to explore the context. Their behavior was assessed during training and testing using Video Freeze software (MedAssociates).

To test whether poly I:C disrupted memory consolidation, mice were first trained in the task as described above. Immediately after being removed from the context, mice were given a post-training treatment of either ICV poly I:C ($n = 9$ male; $n = 10$ female) or sterile saline ($n = 9$ male; $n = 10$ female) and returned to their home cage. 72 hours later, mice were brought back to the context to assess their behavior during testing.

Statistical Analysis of Context Fear Conditioning

Context fear conditioning data were analyzed using two-way ANOVAs with treatment and sex as factors. Significant interactions were followed up using post-hoc tests with Bonferroni

corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

3.3.6 T-Maze

To determine whether poly I:C biased learning strategies employed during a memory task, we used an escape-motivated T-maze task. In this task, mice were placed at the base of a T-shaped maze (base arm: 69 cm x 7.5 cm L x W; each left and right arm: 44 cm x 7.5 cm L x W) with clear plexiglass walls (25 cm high) so that they could orient to distinct distal cues in a brightly lit behavior room. A 3 cm-diameter hole was cut into the floor at the end of each arm of the T from which a short ramp led to a small, dark chamber with clean bedding for escape from the maze. Only one escape entry was open during training, termed the "escape hole", while the other was blocked, termed the "dummy hole". Mice were given 3 minutes to complete the task during training trials. A trial ended when mice entered the escape box successfully and were returned to their home cage. If mice did not find or enter the escape box by the end of 3 minutes, the experimenter gently guided mice towards the hole where they successfully escaped the maze and were returned to their home cage. Mice were given 2 training trials separated by 2 hours each day for 3 training days and a total of 6 training trials. To determine whether poly I:C would bias the learning strategy employed during this task, males and females were administered either ICV poly I:C ($n = 9$ male; $n = 9$ female) or sterile saline ($n = 8$ male; $n = 9$ female) 2 hours prior to the first training trial on days 1 and 2 of training only. Latency to reach the entry holes and preference for the escape hole were measured across training trials to assess successful learning of the task throughout the 3 days of training. Preference for the escape hole was calculated as follows:

$$\left(\frac{time\ exploring\ escape\ hole\ (s)}{time\ exploring\ escape\ hole + dummy\ hole\ (s)}\right) \times 100\%$$

During the probe test on day 4, the T-maze was rotated 180° in the behavior room where

all distal cues remained stationary and both escape entries were blocked. Mice were placed at the

base of the maze and given 3 minutes to explore. If mice made the same body turn (left or right)

at the junction of the T as they would have to successfully reach the escape hole during training,

this was considered a response-based strategy. If mice made the opposite body turn at the

junction of the T, such that they ended up in the same spatial location relative to the distal cues in

the room as the escape hole during training, this was considered a place-based strategy. We

considered mice to have "made a decision" when they first reached one of the holes at the end of

the arms of the T during the probe test. Behavior during training trials and the probe test was

recorded using a video camera, and latency to make a decision and duration spent exploring each

of the holes in the maze were scored using EthoVision XT Ver. 19 computer software (Noldus

Information Technology Inc.; Leesburg, VA). Preference for each learning strategy was

calculated as a preference for the response-based arm, where scores above 50% preference

indicate preference for the response-based strategy and scores below 50% preference indicated

preference for the place-based strategy as follows:

$$\left(\frac{time\ spent\ in\ response\ arm\ (s)}{time\ spent\ in\ response\ arm + place\ arm\ (s)}\right) \times 100\%$$

Statistical Analysis of T-Maze Behaviors

Latency to reach escape entries and preference for the escape hole across training trials

were analyzed using mixed repeated-measures ANOVA tests using training trial as the within-

subjects factor and treatment and sex as the between-subjects factors with Greenhouse-Geisser

corrections for sphericity when necessary. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method. To specifically determine if groups showed a significant ($p < 0.05$) preference for the escape hole throughout training, we also used one-sample, two-tailed t-tests of each group against a mean of 50%.

To assess behavior during the probe test, preference for learning strategy was analyzed using a mixed repeated-measures ANOVA with strategy (response- or place-based) as the within-subjects factor and treatment and sex as the between-subjects factors. Latency to make a decision during the probe test was analyzed using a two-way ANOVA with treatment and sex as factors. Any significant interactions ($p < 0.05$) were followed up with post-hoc tests with Bonferroni corrections for multiple comparisons. Effect sizes were calculated using the partial eta squared method.

3.3.7 Social Memory

Given the low sociability of males during the social preference task (Chapter 2, Figure 2.2D), the social memory task was only done in female mice. We previously found that poly I:C significantly reduced social preference in females when administered four hours prior to the task (Chapter 2, Figure 2.2D). Thus, here we used post-training poly I:C treatment to determine whether poly I:C disrupted consolidation of a social memory and avoid the confounding variable that pre-training poly I:C disrupts sociability in females. For the social memory task, 15 females were habituated to a three-chamber apparatus (76 cm x 21.5 cm x 30.5 cm L x W x H) with empty wire corrals centered in the two end chambers for 30 minutes 24 hours prior to training in the task. On training day, mice were placed back in the chamber where one corral contained a novel mouse of the same strain, age, and sex, and the other corral was kept empty. Mice were

given 2 5-minute trials to freely explore the apparatus, each separated by 30 minutes. Immediately following the second training session, mice were treated with either ICV poly I:C (n = 8) or sterile saline (n = 7) and returned to their home cage. The mouse used in the corral was kept consistent across both training trials and termed the "familiar mouse". 24 hours after training, mice were tested for social memory under drug-free conditions. During the test, mice were placed back into the apparatus in which one corral contained the familiar mouse, and the other corral contained a novel mouse, termed the "stranger mouse". If animals had in-tact social memory, we predicted that they would prefer to spend more time with the stranger mouse than the familiar mouse. Animals were given 5 minutes to freely explore the apparatus, and behavior was recorded using a video camera and EthoVision XT Ver. 19 computer software (Noldus Information Technology Inc.; Leesburg, VA). Time spent actively interacting with the corrals was hand-scored by two individuals blind to treatment groups, and preference for the stranger mouse was calculated as follows:

$$\left(\frac{time\ exploring\ stranger\ mouse\ (s)}{time\ exploring\ stranger\ mouse + familiar\ mouse\ (s)} \right) \times 100\%$$

Statistical Analysis of Social Memory

Social memory data were analyzed using a one-way ANOVA with treatment as the factor. We expected that saline-treated females would show a significant preference for the stranger mouse, so we ran a one-sample, one-tailed t-test of the saline-treated group against a mean of 50% preference. We tested whether poly I:C-treated groups showed a preference for either the stranger or familiar mouse using a one-sample, two-tailed t-test of the poly I:C-treated group against a mean of 50%.

3.3.8 Immunohistochemistry

We used immunohistochemistry to assess neuronal activity and microglia morphology in the hippocampus during training of context fear conditioning. For this experiment, mice were given either ICV poly I:C ($n = 14$ male; $n = 10$ female) or sterile saline ($n = 14$ male; $n = 10$ female) and either trained in context fear conditioning 4 hours later ($n = 7$ males per treatment; $n = 5$ females per treatment) or left naïve in their home cage ($n = 7$ males per treatment; $n = 5$ females per treatment). 90 minutes later, animals were euthanized with an overdose of Avertin (2,2,2-tribromoethanol, intraperitoneal) and transcardially perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde as a fixative. Whole brains were collected and post-fixed in 4% paraformaldehyde for 48 hours and transferred to 10%, 20%, and 30% solutions of sucrose in phosphate buffer for 24 hours each. Brains were stored in 30% sucrose at 4°C until sectioned at 40 μm using a cryostat.

Sections of dorsal hippocampus tissue (between -1.8 and -2.0 mm relative to Bregma) were stained for cFos as a proxy for neuronal activation and Iba1 to assess microglia morphology using standard immunohistochemistry protocols for free-floating sections as previously described (Tronson et al., 2009). Briefly, sections were incubated in primary antibody (anti-cFos 1:2000, Abcam, Waltham, MA; anti-Iba1 1:10,000, Wako Chemicals, Richmond, VA), biotinylated secondary antibody (1:200, Vector Labs; Burlingame, CA), ABC peroxidase complex (Vector Labs) for signal amplification, and 3,3'-diaminobenzidine (DAB) chromogen (Sigma-Aldrich, St. Louis, MO) for signal visualization.

To quantify cFos, 10X magnified images of the CA1, CA3, and dentate gyrus subregions of the hippocampus were taken, and the number of cFos+ cells in each subregion were manually counted using the Cell Counter plugin with ImageJ software within consistent areas across samples (Schneider, Rasband, & Eliceiri, 2012).

To assess microglia morphology, 40X magnified images of the CA1 and CA3 subregions of the hippocampus were taken, and fractal dimension and lacunarity measures were calculated for individual representative microglial cells using the FracLac ImageJ plugin (Ver. 2.5 (Karperien, 2013). Morphology is commonly used to determine activation states of microglia, where more thin, ramified, and complex microglial processes are associated with resting or surveilling functions and more amoeboid shapes with thicker or more polarized branching is associated with immune activation or inflammatory functions (Karperien, Ahammer, & Jelinek, 2013; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Stence, Waite, & Dailey, 2001). Using calculated measures such as fractal dimension and lacunarity reduce human error and potential bias that may occur with simple visual assessment of microglia morphology.

Statistical Analysis of Immunohistochemistry

Counts of cFos+ cells were analyzed using three-way ANOVAs with treatment, context fear conditioning training, and sex as factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method. In order to account for baseline differences in cFos+ cells between naïve males and females and thus better capture possible differences in the effects of training and poly I:C treatment in both sexes, the data were normalized to naïve groups within treatment such that the average of each naïve group was set to 1. We analyzed fold changes from naïve groups using two-way ANOVA tests with sex and treatment as factors and followed up significant interactions ($p < 0.05$) using post-hoc tests with Bonferroni corrections for multiple comparisons.

Measures of microglia morphology were analyzed using three-way ANOVAs with treatment, context fear conditioning training, and sex as factors. Significant interactions ($p <$

0.05) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons. Effect sizes were calculated using the partial eta squared method.

3.3.9 Data Visualization and Statistical Software

Data visualization and statistical analyses were completed using R 3.6.2 (R Core Team, 2019) with the following packages: dplyr (v0.8.5; (Wickham et al., 2020)), tidyr (v1.0.2; (Wickham & Henry, 2020)), rstatix (v0.5.0; (Kassambara, 2020)), DescTools (v0.99.34; (Signorell et al., 2020)), sjstats (v0.17.9; (Ludecke, 2020)), and plotrix (v3.8.2; (Lemon, 2006)).

3.4 Results

3.4.1 Central Administration of Poly I:C Disrupts Learning in Both Sexes and Memory Consolidation in Only Males During Context Fear Conditioning

To test whether poly I:C disrupted learning in context fear conditioning, we treated males and females with either poly I:C or sterile saline 4 hours prior to being trained in the context and tested animals 3 days later without additional treatments (Figure 3.1A). When treated with poly I:C before training, both males and females showed learning impairments expressed as decreased freezing in the context during testing compared to saline-treated groups (Figure 3.1B; main effect of Treatment: $F(1, 29) = 5.887$, $p = 0.022$, $\eta^2_p = 0.169$). This was not due to any treatment effects on locomotor behavior (Figure 3.1C) or shock reactivity (Figure 3.1D) during training (no main effects of Treatment for locomotor behavior: $F(1, 28) = 2.106$, $p = 0.158$; shock reactivity: $F(1, 28) = 2.266$, $p = 0.143$). Interestingly, females showed overall higher levels of freezing in the context compared with males (Figure 3.1B; main effect of Sex: $F(1, 29) = 11.158$, $p = 0.002$, $\eta^2_p = 0.278$) despite males showing higher locomotor activity (Figure 3.1C) and shock reactivity

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(Figure 3.1D) during training (main effects of Sex for locomotor behavior: $F(1, 28) = 13.385$, p = 0.001, $\eta^2_p = 0.323$; shock reactivity: $F(1, 28) = 9.445$; $p = 0.005$, $\eta^2_p = 0.252$).

To test whether this effect was specific to disruptions in learning or in memory consolidation, we treated separate cohorts of mice with either poly I:C or sterile saline immediately after training in the context and tested animals 3 days later without additional treatments (Figure 3.1E). Here, we found a trend that poly I:C disrupted memory consolidation in males only (Figure 3.1F; Sex x Treatment interaction: $F(1, 32) = 4.041$, $p = 0.053$, $\eta^2_p = 0.112$). Post-hoc tests showed a trend that males treated with poly I:C during training froze less than saline-treated males ($p = 0.076$), and there was no difference between poly I:C- and saline-treated females ($p = 1.00$). There were no effects of sex or treatment on either locomotor behavior (Figure 3.1G) or shock reactivity (Figure 3.1H) during training in this experiment.

3.4.2 Pre-Training Poly I:C Has Sex-Specific Effects on cFos in the Hippocampus During Context Fear Conditioning

Pre-training treatment of poly I:C has sex- and subregion-specific effects on cFos-positive cells in the hippocampus. In the CA1 region, training in context fear conditioning significantly increased the number of cFos-positive cells in both males and females as expected (Figure 3.2A1; main effect of Training: $F(1, 40) = 97.069$, $p < 0.001$, $\eta^2_p = 0.708$). There were no main effects of treatment or sex on count data in the CA1. However, normalized data in the CA1 showed that poly I:C had different effects on training-induced cFos in males and females (Figure 3.2A2; Sex x Treatment interaction: $F(1, 20) = 4.779$, $p = 0.041$, $\eta^2_p = 0.193$). Saline-treated males trained in the context showed a significantly higher increase in cFos-positive cells compared to all other groups. Notably, poly I:C treatment did not affect training-induced cFos in females ($p = 1.00$), but pre-training poly I:C blunted training-induced cFos in males ($p = 0.043$).

In the CA3 subregion, training again caused an expected increase in cFos-positive cells in both sexes (Figure 3.2B1; main effect of Training: $F(1, 40) = 102.153$, $p < 0.001$, $\eta^2_p = 0.719$). Importantly, poly I:C had a sex-specific effect on training-induced cFos in the CA3 subregion (Figure 3.2B1; Treatment x Training x Sex interaction: $F(1, 40) = 5.316$, $p = 0.026$, $\eta^2_p = 0.117$) such that poly I:C-treated males trained in context fear conditioning had significantly lower levels of cFos compared with poly I:C-treated females ($p < 0.001$), but saline-treated males and females trained in the context did not differ ($p = 0.177$). This effect may be because males showed overall lower levels of cFos compared with females (Figure 3.2B1; main effect of Sex: $F(1, 40) = 48.162$, $p < 0.001$, $\eta^2_p = 0.546$). However, the data normalized to naïve groups within treatment confirm that poly I:C treatment had different effects on training in context fear conditioning between males and females (Figure 3.2B2; Treatment x Sex interaction: $F(1, 20) = 7.749$, $p = 0.012$, $\eta^2_p = 0.279$). Specifically, poly I:C treatment increased training-induced cFos levels in females ($p = 0.046$) but not males ($p = 0.107$).

In the dentate gyrus (DG), training in context fear conditioning again showed an expected increase in cFos-positive cells in both males and females, consistent with what was seen in the CA1 and CA3 subregions (Figure 3.2C1; main effect of Training: $F(1, 40) = 28.274$, $p < 0.001$, $\eta^2_p = 0.414$). Treatment alone appeared to differentially impact cFos levels in males and females (Figure 3.2C1; Treatment x Sex interaction: $F(1, 40) = 5.393$, $p = 0.025$, $\eta^2_p = 0.119$), though this may be driven by the large training-induced cFos in saline-treated males. Interestingly, poly I:C treatment significantly affected training-induced cFos in both sexes (Figure 3.2C1; Treatment x Training interaction: $F(1, 40) = 4.901$, $p = 0.033$, $\eta^2_p = 0.109$), such that saline-treated groups trained in the context had a significantly higher number of cFos-positive cells relative to saline-treated naïve groups ($p < 0.001$), but poly I:C-treated groups did not show differences between

trained and naïve cFos levels ($p = 0.204$). This effect is consistent with normalized cFos data in the dentate gyrus, where poly I:C treatment blunted training-induced cFos in both sexes (Figure 3.2C2; main effect of Treatment: $F(1, 20) = 5.857$, $p = 0.025$, $\eta^2_p = 0.227$). Here, we also found that males had greater training-induced cFos overall compared with females (Figure 3.2C2; main effect of Sex: $F(1, 20) = 4.989$, $p = 0.037$, $\eta^2_p = 0.200$).

3.4.3 Sex Differences in Hippocampal Microglia Morphology Regardless of Poly I:C or Training in Context Fear Conditioning

Analysis of microglia morphology is commonly used as a measure of microglial activation states. There were no effects of treatment or training in context fear conditioning on either fractal dimension or lacunarity measures in either sex in the CA1 or CA3 subregions (Figure 3.3). However, in both the CA1 and CA3, male microglia had significantly decreased fractal dimension values relative to microglia in females (Figure 3.3A and 3.3C; main effect of Sex in CA1: $F(1, 37) = 164.118$, $p < 0.001$, $\eta^2_p = 0.816$; CA3: $F(1, 37) = 150.988$, $p < 0.001$, $\eta^2_p = 0.807$). There was also a sex difference in microglia lacunarity in both the CA1 and CA3 subregions such that male microglia showed greater lacunarity compared with female microglia (Figure 3.3B and 3.3D; main effect of Sex in CA1: $F(1, 37) = 35.909$, $p < 0.001$, $\eta^2_p = 0.493$; CA3: $F(1, 37) = 35.144$, $p < 0.001$, $\eta^2_p = 0.494$).

3.4.4 Poly I:C Changes Learning Strategy in a T-Maze Task in a Sex-Specific Manner

We next used an escape-motivated T-maze task to determine if the effects of poly I:C on learning could be extended to other learning and memory tasks dependent on brain regions beyond the hippocampus. To determine whether males and females were able to learn the task, we measured latency to successfully reach the escape hole as well as preference for the escape

hole across 3 days of training. We found that latency to reach the escape hole changed across trials, though not in the expected direction (Figure 3.4A; main effect of Trial: $F(2.8, 84.13) = 4.05$, $p = 0.011$, $\eta^2_p = 0.119$). We predicted that animals would successfully find the correct escape hole more directly and therefore more quickly during later training trials. However, saline and poly I:C-treated groups in both sexes had increased latency by the final training trial on day 3. Interestingly, poly I:C-treated mice were faster at finding the escape hole compared with saline-treated groups throughout training, suggesting that pre-training poly I:C treatment did not impact the ability for males and females to learn or complete the task (Figure 3.4A; main effect of Treatment: $F(1, 30) = 5.653$, $p = 0.024$, $\eta^2_p = 0.159$). We did not find any significant effects of training trial, treatment, or sex in latency to reach the dummy hole of the maze (Figure 3.4B).

Preference for the escape hole also changed across training trials (Figure 3.4C; main effect of Trial: $F(5, 140) = 7.607$, $p < 0.001$, $\eta^2_p = 0.214$). Here, we expected that mice would spend more time at the escape hole in later training trials as animals learned the task. However, all groups of mice showed significant preference for the escape hole immediately during trial 1 of day 1 (Figure 3.4C; female saline: $t(8) = 5.185$, $p < 0.001$; male saline: $t(7) = 6.195$, $p < 0.001$; female poly I:C: $t(8) = 7.885$, $p < 0.001$; male poly I:C: $t(8) = 9.643$, $p < 0.001$). Interestingly, saline-treated males only showed a significant preference trial 1 of day 1 and did not differ from 50% preference for the remainder of training (Figure 3.4C). Similarly, saline-treated females only showed a significant preference for the escape hole on trial 1 of day 1 and trial 1 of day 2 (Figure 3.4C; $t(8) = 3.564$, $p = 0.007$) and not for any other trial of training. In contrast, poly I:C-treated males showed a significant preference for all trials on days 1 and 2 (trial 2, day 1: $t(8) = 6.162$, $p < 0.001$; trial 1, day 2: $t(8) = 3.105$, $p = 0.015$; trial 2, day 2: $t(8) = 2.340$, $p = 0.047$), a trend for preference on trial 1 of day 3 ($t(8) = 2.050$, $p = 0.074$), and only failed to show a

preference on the final training trial of day 3 ($t(8) = 0.062$, $p = 952$; Figure 3.4C). This pattern held true for poly I:C-treated females as well, with the exception of trial 2 of day 1 where they showed a significant preference for the dummy hole instead (Figure 3.4C; trial 2, day 1: $t(8) = -3.011$, $p = 0.017$; trial 1, day 2: $t(8) = 6.133$, $p < 0.001$; trial 2, day 2: $t(8) = 3.674$, $p = 0.006$; trial 1, day 3: $t(8) = 2.610$, $p = 0.031$; trial 2, day 3: $t(8) = 1.983$, $p = 0.083$).

We also found that males and females showed different preferences across trials (Figure 3.4C; Trial x Sex interaction: $F(5, 140) = 3.45$, $p = 0.006$, $\eta^2_p = 0.110$) and that treatment had sex-specific effects across training trials (Figure 3.4C; Trial x Treatment x Sex interaction: $F(5, 140) = 3.835$, $p = 0.003$, $\eta^2_p = 0.120$), though post-hoc tests revealed these effects were driven only by the behavior on trial 2 of day 1 in training.

Tests during the probe trial revealed a sex difference in the learning strategy used for saline-treated animals. A greater proportion of saline-treated females used a response-based strategy while a greater proportion of saline-treated males used a place-based strategy, suggesting that females may use more response-based learning and males may use more place-based learning (Figure 3.4D). Interestingly, poly I:C treatment during training in the T-maze did not result in a bias for learning strategy either sex in that there was a more even split between response- and place-based learning strategies for poly I:C-treated males and females (Figure 3.4D). These data suggest that pre-training poly I:C forces males and females to use a different learning strategy than what would be used naturally in order for both sexes to still successfully learn and perform in the task, as was seen during training (Figure 3.4A and 3.4C).

There were no significant differences in preference for learning strategy as measured by time spent exploring each arm of the T-maze during the probe test (Figure 3.4E). However, saline-treated females seemed to show a greater preference for the response-based goal (Figure

3.4E), in line with as was seen in their initial expression of learning strategy (Figure 3.4D). There were no significant differences in latency to reach the chosen goal arm for any group, and most animals took less than 10 seconds to do so (Figure 3.4F).

3.4.5 Poly I:C Disrupts Social Memory in Females

Given that saline-treated males did not show significant sociability in the social preference task (Chapter 2, Figure 2.2D), social memory was only tested in females. Saline-treated females showed a significant preference for the stranger mouse compared to the familiar mouse (Figure 3.5A; $t(6) = 2.061$, $p = 0.042$), and poly I:C-treated females did not show any preferences ($t(7) = 0.269$, $p = 0.796$) suggesting that poly I:C treatment disrupted social memory consolidation in females. In line with these findings, there was a trend towards a difference between poly I:C- and saline-treated groups when compared directly ($F(1, 13) = 3.779$, $p = 0.074$, $\eta^2_p = 0.225$). Importantly, these effects were not due to any differences in the time poly I:C-treated females spent interacting with the mice in the corrals, and this was expected because mice were trained in the social memory task prior to receiving any treatments (Figure 3.5B; $F(1, 13) = 0.157$, $p = 0.699$).

3.5 Discussion

Here, we showed that pre-training poly I:C treatment disrupted learning in context fear conditioning in both sexes *via* distinct underlying hippocampal mechanisms. We also showed that poly I:C during training of a T-maze task shifted learning strategies in both males and females but in opposite directions. Interestingly, post-training poly I:C disrupted context fear memory consolidation in males only and disrupted social memory in females. These data show that neuroimmune activation by central administration of poly I:C significantly impacts

hippocampal-dependent memory processes and mechanisms in specific ways for males and females.

Poly I:C administered 4 hours prior to training in context fear conditioning resulted in decreased freezing levels for both males and females during testing 3 days later (Figure 3.1A). We also found that overall, females showed higher levels of freezing for both poly I:C- and saline-treated groups compared with males. However, analysis of behavior during training showed that males had more exploration of the context and had higher shock reactivity than females. Strong levels of fear conditioning rely on sufficient time spent exploring and learning about the context in which animals receive the aversive foot shock stimulus (Fanselow, 1990; Keiser et al., 2017; Rudy & O'Reilly, 1999), and a strong conjunctive representation requires learning about both the context and the foot shock (Rudy & O'Reilly, 1999; Rudy & O'Reilly, 2001). Given the increased locomotor behavior during context exploration and increased shock reactivity during training in males regardless of treatment, it is interesting that this did not translate to stronger levels of freezing in males during the testing phase. Extant literature shows inconsistencies regarding sex differences in context fear conditioning. Some studies show that males have stronger fear conditioning than females (Pryce, Lehmann, & Feldon, 1999; Wiltgen, Sanders, Behne, & Fanselow, 2001), while others show the opposite (Moore et al., 2010; Ris et al., 2005). However, factors such as training and testing design may dictate the information that males and females learn and use in context fear conditioning and memory retrieval (Keiser et al., 2017; Tronson & Keiser, 2019), and as such it is inappropriate to state anything conclusive about sex differences in the "strength" of context fear conditioning in this particular experiment.

It is perhaps more important that pre-training treatment with poly I:C did not affect behavior during training in context fear conditioning for either males or females in this

experiment. Decreased locomotor activity is one of many adaptive sickness behaviors that have been previously defined and frequently used to assess the effects of immune stimulants on behavior (Hart, 1988; Kent, Bluthé, Kelley, & Dantzer, 1992). In fact, one study that specifically used intracerebroventricular administration of poly I:C found that poly I:C significantly decreased spontaneous home cage locomotor behavior (X. Zhu et al., 2016). In contrast, we assessed locomotor activity in a novel context while mice explored the fear conditioning apparatus prior to receiving the foot shock and did not find any effects of poly I:C. This means that the freezing deficits seen in poly I:C-treated males and females during testing were not simply due to a decreased opportunity or ability to explore the context during fear conditioning training. Rather, it suggests that poly I:C specifically disrupted mechanisms of learning and memory in both sexes.

Pre-training manipulations are often used to study the learning or acquisition phase of memory, but it is important to consider that these manipulations may also affect overlapping but distinct memory consolidation processes that begin immediately thereafter (Abel & Lattal, 2001; McGaugh & Roozendaal, 2009). For our pre-training paradigm, we trained males and females in context fear conditioning 4 hours after treatment with either poly I:C or saline because we previously found a peak in poly I:C-induced cytokine levels at this timepoint (Chapter 2, Figure 2.3 and Figure 2.4). However, we know that the acute effects of poly I:C last longer than 4 hours, as evidenced by lingering fever until 12 hours post-treatment (Figure 2.2), weight loss until 48 hours post-treatment (Figure 2.2), and elevated protein levels of some cytokines at 24 hours post-treatment (Figure 2.4). Therefore, it is possible that the deficits we saw in fear conditioning following pre-training treatment of poly I:C might be due to disruptions in memory consolidation processes either instead of or in addition to disruptions in learning mechanisms. To better parse

these apart, we also used a post-training treatment design (McGaugh & Roozendaal, 2009). Here, we found that only males showed disruptions in context fear conditioning when tested 3 days later, and females were completely unaffected (Figure 3.1F). These data suggest that while poly I:C treatment disrupts learning in both sexes, it only disrupts memory consolidation in males.

However, just as the inflammatory effects from poly I:C do not immediately dissipate after 4 hours, they do not immediately take effect either. After 30 minutes, none of the cytokines we previously measured showed increases in gene expression or protein levels (Chapter 2, Figure 2.3 and Figure 2.4), and only interferon (IFN) β showed peak expression after 2 hours (Figure 2.3H2). It is therefore possible that the learning mechanisms that pre-training poly I:C disrupt in females include memory consolidation mechanisms that fall within the first ~2 hours following training that post-training treatment with poly I:C did not capture. Taken together, these data still highlight important sex differences in the window for vulnerability to learning and memory disruptions by neuroinflammation such that males are vulnerable for a longer period of time during learning and memory consolidation relative to females.

Although pre-training poly I:C disrupted context fear conditioning in both sexes, cFos data in the hippocampus suggest this may be *via* distinct mechanisms in males and females. Normalized cFos levels in the dentate gyrus showed that pre-training poly I:C attenuated training-induced increases for both males and females, though the fold difference in males appears greater than that of females, and this treatment effect may be driven by training-induced cFos in saline-treated males specifically (Figure 3.2C2). Interestingly, in the CA1 poly I:C blunted training-induced cFos in males only, and in the CA3, poly I:C potentiated training-induced cFos in females only. The CA1, CA3, and dentate gyrus subregions have all been implicated in context fear conditioning in various ways. Evidence suggests that output from the

CA1 is involved in encoding contextual fear, and the CA1 is critical for context fear memory consolidation (Daumas, Halley, Francés, & Lassalle, 2005; Hunsaker & Kesner, 2008; Hunsaker, Tran, & Kesner, 2009; Tronson et al., 2009; M. Zhou, Conboy, Sandi, Joëls, & Krugers, 2009). The CA3 subregion has been shown to be required for rapid formation of the conjunctive representation during training in context fear conditioning (Daumas et al., 2005; Lee & Kesner, 2004). The dentate gyrus, and importantly the connection between dentate gyrus and CA3, are required for context fear acquisition and encoding specific features of the context important for context discrimination (Bernier et al., 2017; Hainmueller & Bartos, 2020; Hernández-Rabaza et al., 2008). The functional distinction between these subregions suggests that pre-training poly I:C has specific effects on individual processes required for learning in context fear conditioning, rather than simply causing general increases or decreases in neuronal activity, and these effects are different for males and females. Nevertheless, disruption of these processes during training in context fear conditioning produces similar behavioral deficits in both sexes.

Under healthy conditions, microglia help regulate mechanisms of synaptic plasticity through phagocytosis of the extracellular matrix (Nguyen et al., 2020). However, it is unclear whether functions related to synaptic plasticity similarly alter microglia morphology in the ways that are classically seen during neuroinflammation. We did not find that context fear training affected microglia morphology in either sex. Microglia are directed to neuronal synapses to facilitate plasticity by detecting neuronal activity (Badimon et al., 2020), and we did see training- and poly I:C-induced effects on cFos levels, a proxy for neuronal activation. In our experiment, representative microglia were chosen for morphological analyses at random, and we know that only select populations of neurons are recruited in the formation of an engram (Han et al., 2007; Park et al., 2016; Zhou et al., 2009). Thus, it is possible that the microglia chosen for analysis

were not at sites of neuronal plasticity to reveal any relevant changes. Future studies using fluorescent microscopy are needed to specifically identify microglia at active neuronal synapses to fully assess morphological changes during synaptic remodeling.

During neuroinflammation, microglia can be activated by stimulation of innate immune receptors by invading pathogens or cellular debris and by circulating cytokines increased during inflammatory events (Kigerl et al., 2014). As such, we predicted that poly I:C would activate microglia, and this would be captured *via* changes in morphology. Microglia with ramified, complex, and thin branching are associated with homeostatic surveillance functions while microglia more amoeboid in shape with retracted and thicker processes are associated with inflammation (Karperien et al., 2013). Surprisingly, we did not see any effect of poly I:C treatment on microglia morphology in either sex despite the robust cytokine response in the hippocampus induced by poly I:C that we found previously (Chapter 2). Interestingly, one study found that while microglia showed the classic amoeboid shape in response to the bacterial endotoxin lipopolysaccharide (LPS), microglia retained their processes and still had a somewhat "bushy" morphology in response to poly I:C *in vitro* while still releasing inflammatory cytokines (He, Taylor, Yao, & Bhattacharya, 2021). Together with our findings, these data suggest that not all forms of microglia activation result in drastic changes in morphology, and these morphological analyses may not be sufficient for studying the effects of various experimental manipulations on microglia moving forward.

Notably, we found sex differences in microglia morphology regardless of training or poly I:C treatment. Fractal dimension assesses self-similarity, where higher numbers indicate that microglia are made up of more complex patterns (i.e., more complex branching), and lower numbers indicate more simple patterns (i.e., retracted or thicker branching; (Karperien,

Ahammer, & Jelinek, 2013)). Lacunarity assesses heterogeneity or rotational variance, where higher numbers indicate that microglia have a more heterogeneous shape (i.e., more complex branching), and lower numbers indicate a more homogenous shape (i.e., more amoeboid shape). Typically, fractal dimension and lacunarity show a positive correlational relationship such that they either both increase or both decrease with changes in morphology (Jelinek, Karperien, Buchan, & Bossomaier, 2005). In contrast, we found that male microglia showed decreased fractal dimension and increased lacunarity compared to females in both the CA1 and CA3 subregions. However, this is still a reasonable finding. During microglial activation, processes are retracted, become thicker, and must be directed towards sites of stimulation for successful phagocytosis (Badimon et al., 2020). A lower fractal dimension is in line with this because branching becomes less complex. If processes are completely retracted such as with fully amoeboid microglia, there would be very low rotational variance and therefore lower lacunarity. However, for "intermediate" activated microglia with branches shifted to only one side or with only a few total branches, it is reasonable to imagine that rotational variance could be higher for these microglia than for microglia with evenly distributed branches and therefore show higher lacunarity. As such, our data suggest that male microglia have a more "activated" morphology relative to females in the way that microglia are typically discussed in the literature.

There are several reasons why researchers must be cautious when using the term "activated" when talking about microglia and in using morphological analyses. First, it is possible for microglia to be stimulated by immune challenge and produce inflammatory cytokines without shifting morphology to an amoeboid state that is classically considered "active" (He et al., 2021). While we did not directly measure microglia response to poly I:C, we also did not find major morphological changes to microglia despite showing robust increases in

inflammatory cytokines at even earlier timepoints than examined here (Chapter 2). Second, we found that male microglia in the hippocampus had less complex branching than female microglia which would suggest that male microglia are more "active" than female microglia. However, we previously found that baseline expression of several cytokines was higher in the female hippocampus, and there were no instances of males showing higher baseline levels of cytokines (Chapter 2, Figure 2.3). If more "active" phenotypes always correspond to more inflammatory profiles, we should have seen the opposite effect. Finally, we did not find fractal dimension and lacunarity measures to change in the same direction in our analyses despite the way these terms are used would suggest that they should (Karperien & Jelinek, 2015). More in-depth studies have also captured this divergence (Karperien, Jelinek, & Milošević, 2011). Collectively, these findings highlight the level of nuance that exists in microglia form and function. To truly understand what these data mean in the context of neuroinflammation in health and disease, it is imperative that we move away from using rudimentary measures to define microglia as either "active" or "resting/surveilling" and towards terms that more accurately represent specific functions.

In addition to context fear conditioning, we also tested whether poly I:C would disrupt learning in a T-maze task that may utilize multiple memory systems. One method requires the hippocampus to encode spatial cues during training and subsequently complete the task using a "place-based" strategy, while the other method requires the dorsal striatum to encode stimulus-response associations during training and complete the task using a "response-based" strategy (Goodman, 2020; Tolman et al., 1946). Pre-training poly I:C on days 1 and 2 of training significantly affected latency to reach the escape hole in both males and females. Interestingly, both poly I:C-treated groups tended to complete the task faster during each training trial

compared to the saline-treated groups. In fact, both saline-treated males and saline-treated females appeared to reach the dummy hole more quickly during most of the training trials than they did the escape hole. Additionally, both saline-treated groups tended to show a lower preference for the escape hole during training relative to the poly I:C-treated groups, though it was clear that all animals learned where the escape hole was and what its purpose was based on observation during behavioral scoring. In these ways, it is abundantly clear that poly I:C did not disrupt learning in the T-maze task. Rather, it is possible that poly I:C treatment increased motivation to escape the maze for both males and females relative to saline-treated groups, and this may be capturing some degree of sickness responses to the poly I:C that we did not see in other behavioral measures or visual assessments. It is noteworthy that we did not see significant improvements across the training trials for any of the groups, as it appeared that all animals showed significant preference for the trained escape hole and completed the task in the shortest amount of time on the very first training trial. It is possible that this task was not difficult enough to allow us to capture a learning curve to determine whether biological sex or poly I:C treatment had any effects on learning ability. However, it is still worth determining *how* mice were completing the task with the use of the probe trial.

Although poly I:C did not disrupt learning in the T-maze task, it did appear to affect the learning strategies used for both males and females, and in opposite directions. During the probe test, we found a sex difference in the proportion of saline-treated animals that expressed use of each type of learning strategy. Specifically, more saline-treated females exhibited a response-based learning strategy, and this was evident in both the initial probe decision as well as in preference for the response-based hole during the probe test. In contrast, more saline-treated males expressed using a place-based strategy in their initial decision. Interestingly, males did not

show the same split in preference for the place-based hole in time spent at each end of the T-maze, though that measure may not be the most appropriate way to assess learning strategy in the probe test. On the other hand, poly I:C-treated males and females did not show a strong split in the proportion of animals that used each type of strategy. This suggests that poly I:C removed the bias for a response-based strategy in females and removed the bias for a place-based strategy in males. Taken together, poly I:C can disrupt both hippocampal-dependent learning strategies and dorsal striatal-dependent learning strategies without preventing animals from learning overall, indicating that cognitive flexibility may be spared. Future experiments are required to better tease apart treatment group differences during training as well as the large variability in preference scores during the probe trial to fully understand these behaviors.

Lastly, we found that poly I:C disrupted social memory consolidation in females. We previously found that poly I:C did decrease sociability in females when given the choice between a novel conspecific and a novel object (Chapter 2, Figure 2.2). As such, we only administered poly I:C after training in the social memory task to prevent any decreases in social exploration. From an evolutionary perspective, it has been argued that decreased social interactions are important for preventing the spread of illness and increasing likelihood of survival for a group (Hart, 1991). However, whether decreases in social memory as a result of illness and infection are similarly important for increasing survival or simply a confound of decreased social interaction during illness is unclear. Previous studies implicate the hippocampus, and specifically the CA2 subregion, in the formation and consolidation of social memories (Hitti & Siegelbaum, 2014; Kogan et al., 2000; Montagrin, Saiote, & Schiller, 2018; Okuyama, 2018). While we did not specifically look at the hippocampus during social memory, we know that poly I:C induces significant fever and hippocampal cytokine responses in both sexes (Chapter 2). Thus, it is

possible that the disruption in social memory for females here is a result of this inflammation in the hippocampus, though other brain regions are likely to be involved as well.

Collectively, we found sex differences in neuroimmune modulation of learning and memory at multiple levels of analysis in these studies. While we found similar behavioral deficits from poly I:C on learning in context fear conditioning, there were sex- and subregion-specific effects on hippocampal neuronal activity that suggest sex differences in underlying neurobiological mechanisms of neuroimmune disruption of learning. Further, we found that poly I:C disrupted memory consolidation of context fear conditioning in males but not females. While this does not rule out the possibility that poly I:C spares immediate memory consolidation mechanisms in females, it does highlight a sex difference in the window of vulnerability for neuroimmune disruption of learning and memory mechanisms. Finally, we did not find disruptions of learning in an escape-motivated T-maze task from poly I:C in either males or females, but we did find a sex difference in learning strategy employed during this task and found that poly I:C eliminated learning strategy bias in both sexes. These experiments show that neuroimmune modulation of learning and memory in males and females is incredibly nuanced. We cannot simply use one measure of memory, one measure of neuroimmune activation, or one sex to fully understand how neuroimmune processes interact with mechanisms of learning and memory that may contribute to the etiology of memory-related diseases and disorders in both sexes, and these data highlight the importance of paying attention to such nuance moving forward.

3.6 Figures

A.

Training

Testing

20μg Poly I:C or Saline

4hrs

72hrs

B. Freezing Behavior

C. Locomotor Behavior

D. Shock Reactivity

E.

Training

Testing

20μg Poly I:C or Saline

72hrs

F. Freezing Behavior

G. Locomotor Behavior

H. Shock Reactivity
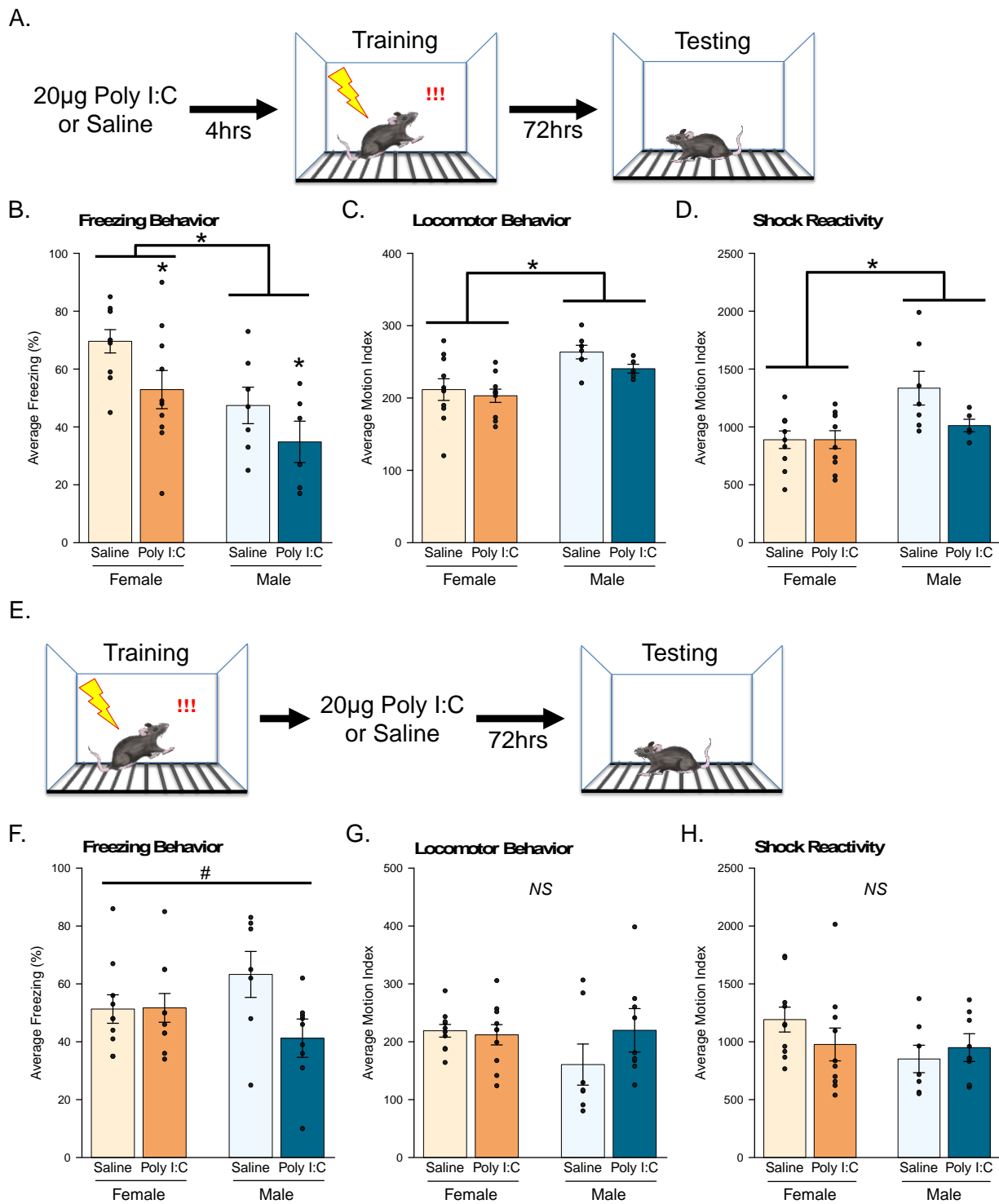
Figure 3.1 *Analysis of poly I:C on learning and memory consolidation in context fear conditioning*

Experimental design used to test the effects of poly I:C on **(A)** learning and **(E)** memory consolidation in context fear conditioning. Average percent time spent freezing during testing in **(B)** pre-training treatment design and **(F)**

post-training treatment design. Average exploration activity during training in **(C)** pre-training treatment design and **(G)** post-training treatment design. Average reactivity to foot shock during training in **(D)** pre-training treatment design and **(H)** post-training treatment design. ***** above a single bar (B) indicates main effect of treatment ($p < 0.05$) following two-way ANOVA; ***** above a bracket (B, C, D) indicates main effect of sex ($p < 0.05$) following two-way ANOVA; **#** above horizontal line (F) indicates trend ($p < 0.06$) of a sex x treatment interaction following two-way ANOVA; *NS* indicates no statistical significance. Error bars represent standard error of the mean.
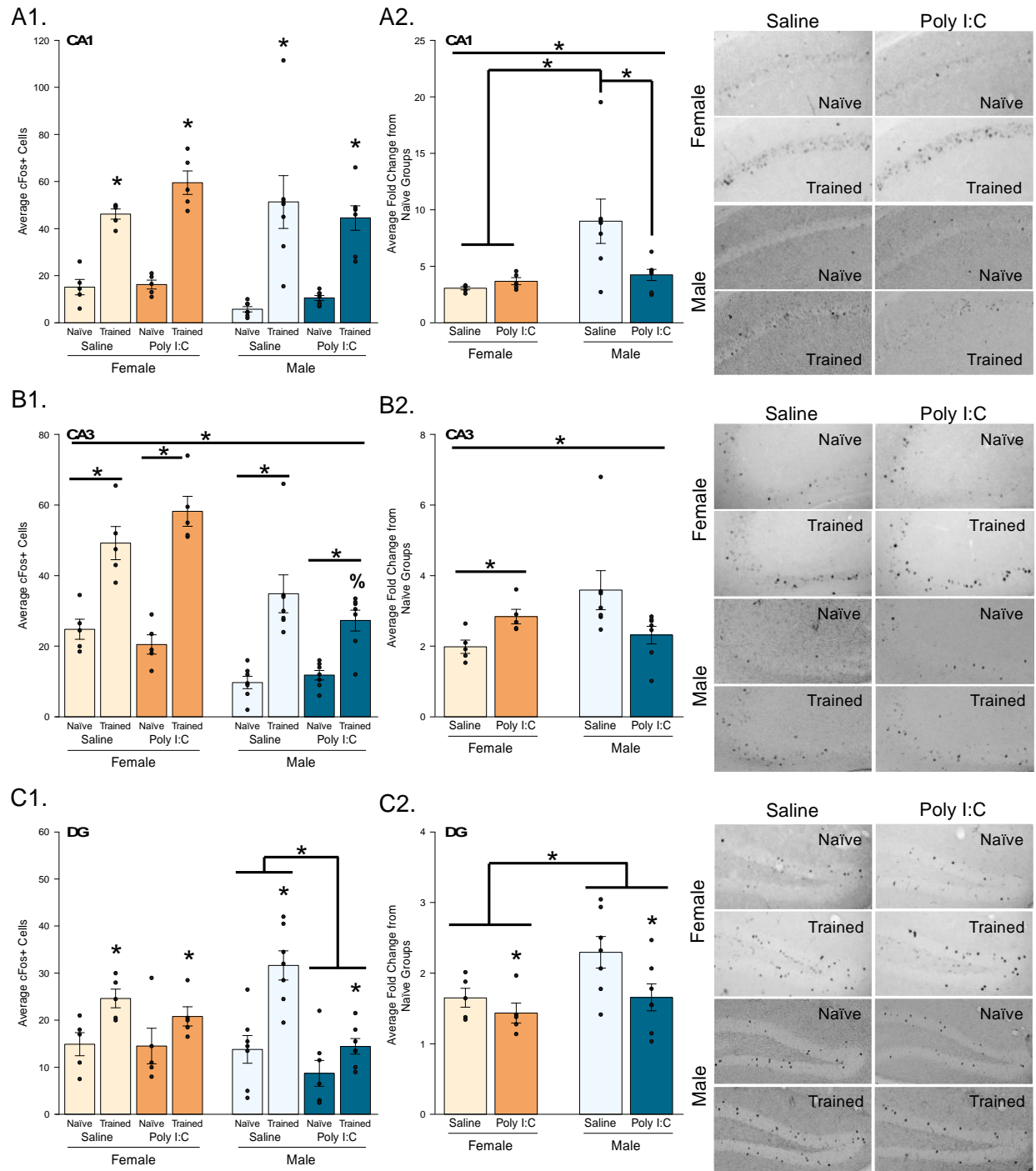
Figure 3.2 *Effects of pre-training poly I:C treatment and training in context fear conditioning on cFos levels in the hippocampus*

Average number of cFos-positive cells with associated representative images in the **(A1)** CA1, **(B1)** CA3, and **(C1)** dentate gyrus (DG) subregions of the hippocampus. Average fold change in number of cFos-positive cells from naïve groups within each treatment for **(A2)** CA1, **(B2)** CA3, and **(C2)** dentate gyrus (DG) subregions. * above a single bar indicates a main effect ($p < 0.05$) of training (A1, C1) or treatment (C2) following a three-way or two-way ANOVA, respectfully; * above bracket in (C2) indicates a main effect ($p < 0.05$) of sex following a two-way ANOVA; * above a horizontal line over all bar data (A2, B1, B2) indicates a two-way (A2, B2) or three-way (A1)

interaction ($p < 0.05$) following ANOVA tests; ***** elsewhere indicates a significant post-hoc test ($p < 0.05$) with Bonferroni correction following significant interactions. Error bars represent standard error of the mean.
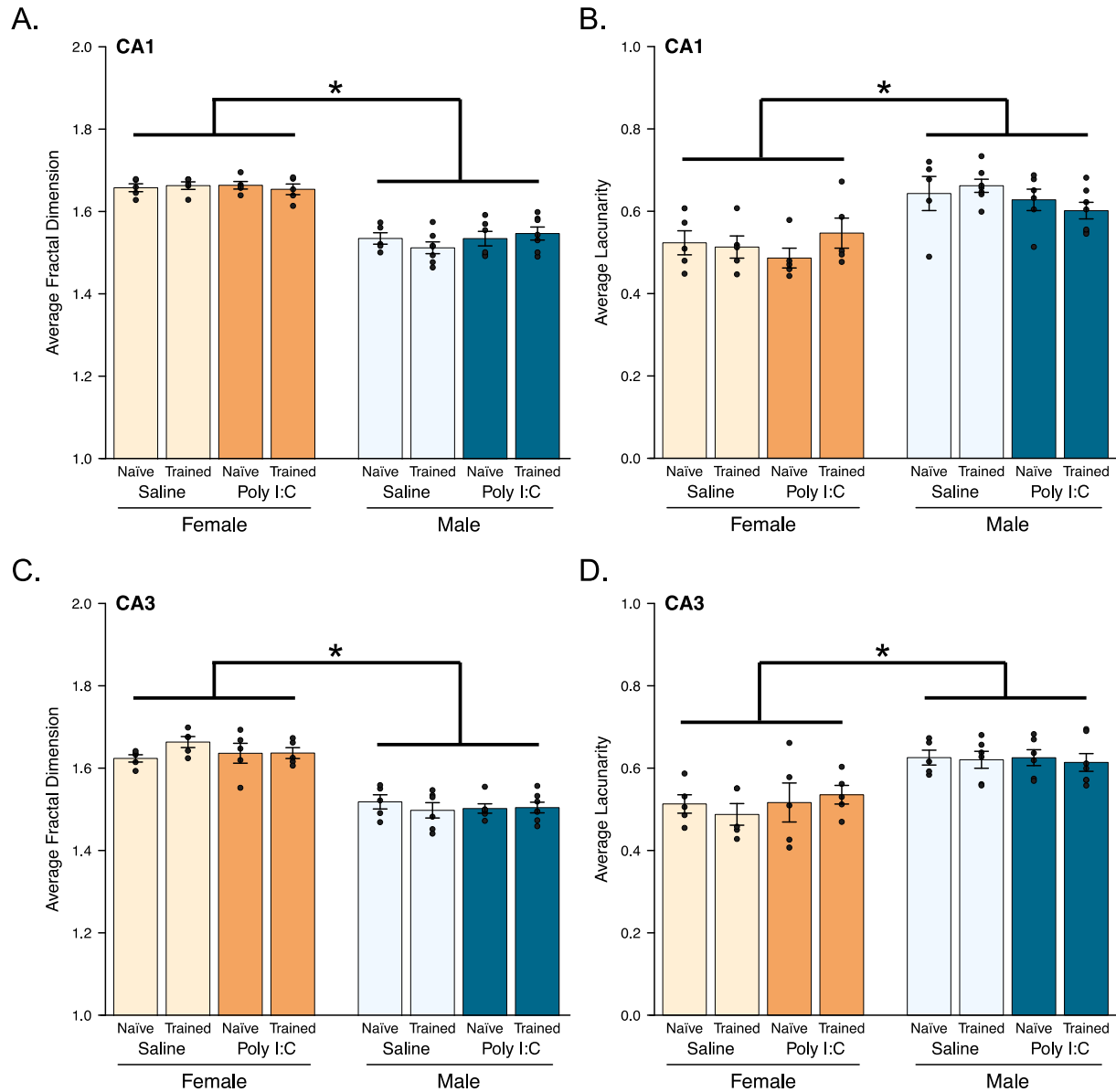
Figure 3.3 *Pre-training poly I:C and training in context fear conditioning on fractal dimension and lacunarity of hippocampal microglia*

Average fractal dimension of microglia in (**A**) CA1 and (**C**) CA3 subregions. Average lacunarity of microglia in (**B**) CA1 and (**D**) CA3 subregions. ***** above a bracket indicates main effect of sex ($p < 0.05$) following three-way ANOVA tests. Error bars represent standard error of the mean.
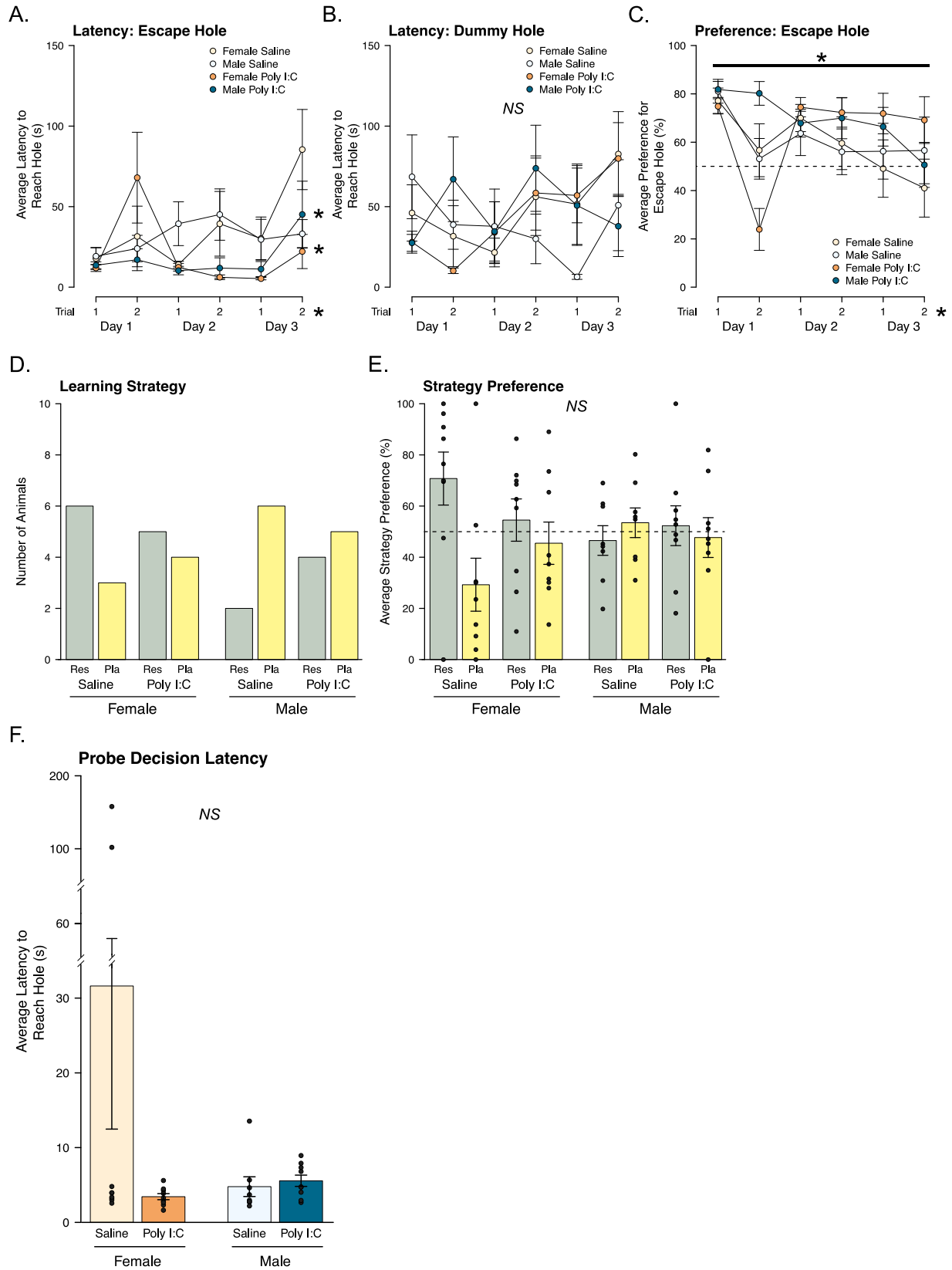
Figure 3.4 *Effects of pre-training poly I:C on behavior training and learning strategy in T-maze task*

Average latency to reach the **(A)** escape hole and **(B)** dummy hole across training days. **(C)** Average preference for the escape hole across training days. Dashed line drawn at 50%. **(D)** Number of animals expressing response- or place-based learning strategies during the probe test. **(E)** Preference for each learning strategy during the probe test. **(F)** Average latency to reach initial goal arm during probe test. ***** next to poly I:C-treated groups (A) indicates main effect of treatment ($p < 0.05$) following mixed repeated-measures ANOVA; ***** next to trial number (A, C) indicates main effect of trial ($p < 0.05$) following mixed repeated-measures ANOVAs; ***** above a horizontal line indicates three-way interaction ($p < 0.05$) following three-way ANOVA; *NS* indicates no statistical significance. Error bars represent standard error of the mean.
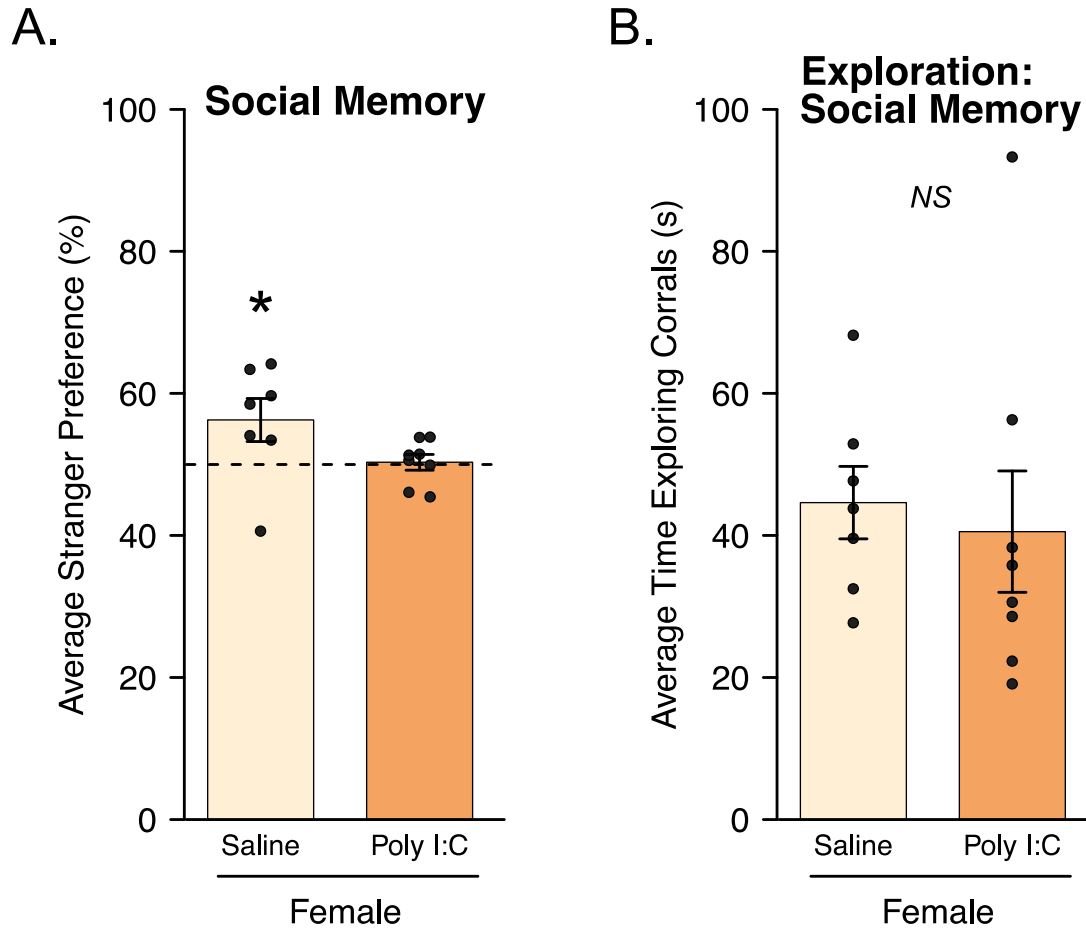
Figure 3.5 *Social memory in females*

(A) Average preference for a stranger mouse compared to a familiar mouse. Dashed line drawn at 50%. **(B)** Total time spent interacting with corrals during social memory test. ***** above a single bar (A) indicates a difference from 50% ($p < 0.05$) following a one-sample t-test; *NS* indicates no statistical significance. Error bars represent standard error of the mean.

Chapter 4:
Type I Interferons Contribute to Poly I:C-Induced Learning Deficits in Males

4.1 Abstract

Understanding specific underlying mechanisms by which neuroimmune activation or dysregulation modulates learning and memory mechanisms in both males and females is an important area of research for health and disease. We previously found that central administration of a viral mimic, polyinosinic:polycytidylic acid (poly I:C) induces a sex difference in the magnitude of cytokine response as well as a sex difference in the expression of anti-viral Type I interferons in the hippocampus. We also found that pre-training poly I:C disrupts hippocampal-dependent learning in both sexes but seemingly *via* distinct underlying mechanisms. Here, we tested whether sex differences in expression of the chemokine CXCL10 or Type I interferons from poly I:C specifically contributed to these learning deficits. Intracerebroventricular administration of recombinant CXCL10 protein did not induce sickness responses or learning and memory consolidation deficits in a context fear conditioning task in males. In contrast, inhibition of Type I interferon receptors prior to central poly I:C administration attenuated the poly I:C-induced learning deficits in males, but not females. We found that in both sexes, Type I interferon receptor inhibition was able to blunt the increases of IL-6 cytokine expression as well as of subsequently phosphorylated STAT3 protein, which shows that the inhibitor is effective in both males and females. Taken together, these data suggest that Type I interferons play a more important role in the neuroimmune disruption of learning mechanisms in males than they do in females, and a female-specific mechanism has yet

to be identified. Future research aimed at better understanding the nuance in sex-specific neuroimmune modulation of cognitive processes will have important implications for treatment development for memory-related disorders in both sexes.

4.2 Introduction

Experience-dependent synaptic plasticity in the hippocampus is a core feature of mechanisms of learning and memory, and glutamatergic signaling is required for hippocampal memory formation (Kandel & Schwartz, 1982; Katagiri et al., 2001; Omrani et al., 2009). The neuroimmune system is well-poised to both support and disrupt mechanisms of synaptic plasticity due to key neuroimmune cells – astrocytes and microglia – located at neuronal synapses that play important roles in regulating glutamate transmission (Anderson & Swanson, 2000; Haydon, 2001). Several mechanisms by which astrocytes, microglia, and inflammatory cytokines modulate synaptic plasticity and glutamatergic signaling depend on neuronal activation (Al Awabdh et al., 2016; Badimon et al., 2020; Nguyen et al., 2020). We previously found that central administration of poly I:C disrupted learning in both males and females, and cFos levels in the hippocampus used as a proxy for neuronal activation following training in context fear conditioning suggested that there are sex differences in the underlying mechanisms of memory impairment (Chapter 3). Separately, we found sex differences in the anti-viral Type I interferons induced by poly I:C as well as in the magnitude of cytokine response to poly I:C overall (Chapter 2; Posillico et al., 2021). As such, we aimed to identify underlying intracellular mechanisms by which the sex differences in neuroimmune activation from poly I:C may prime the hippocampus for learning disruptions in distinct ways in males and females.

Glutamate transporter 1 (GLT-1) is a high-affinity glutamate transporter and accounts for more than 95% of excess hippocampal glutamate uptake (K. Tanaka et al., 1997). While many

cell types express glutamate transporters, astrocyte-specific expression makes up approximately 80% of total GLT-1 expression (Furness et al., 2008; Petr et al., 2015). Studies have shown that GLT-1 expression is induced by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Li et al., 2006). Interestingly, this PI3K/Akt pathway can be activated by poly I:C-stimulation of Toll-like receptor 3 (TLR3; Chen et al., 2020), a receptor expressed on astrocytes (Kielian, 2006). Thus, it is possible that poly I:C directly modulates GLT-1 protein levels in astrocytes and significantly affects glutamatergic signaling in the hippocampus specifically, but this has yet to be explored.

Many inflammatory cytokines have been shown to modulate processes of synaptic plasticity. For example, previous studies found that during suppressed neuronal activity, tumor necrosis factor (TNF) α is produced by astrocytes and acts on neurons to increase surface expression of GluR1-containing AMPA receptors, an ionotropic glutamate receptor, to increase neuronal sensitivity (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen & Malenka, 2006). Interleukin (IL)-1β was also found to significantly increase AMPA receptor expression in neuronal membranes, though to a lesser extent than TNFα (Stellwagen et al., 2005). Furthermore, several studies have shown that pathophysiological levels of TNFα, IL-1β, and CXCL10 disrupt long-term potentiation and depression of neuronal synapses *in vitro* (Albensi & Mattson, 1999; A. J. Cunningham, Murray, O'Neill, Lynch, & O'Connor, 1996; Rizzo et al., 2018; Ross et al., 2003; H. Schneider et al., 1998; Tancredi et al., 1992; Vlkolinský, Siggins, Campbell, & Krucker, 2004), and substantial increases in IL-1β disrupt learning and memory *in vivo* (Goshen et al., 2007). Interestingly, the IL-1 receptor has been shown to colocalize with NR2B subunits of the NMDA receptor, another ionotropic glutamate receptor, in response to neuronal activation (Gardoni et al., 2011), and this connection has been implicated in

excitotoxicity-mediated memory impairment (Fogal & Hewett, 2008). Collectively, these data highlight important interactions between neuroimmune and glutamatergic signaling, specifically *via* changes to glutamate receptors in the hippocampus as a result of inflammation. We found that males had a significantly greater increase in IL-1β, TNFα, and CXCL10 in response to poly I:C compared with females, but whether this sex difference contributes to sex differences in changes to glutamate receptors and signaling remains unknown.

Type I interferons (IFNs), including IFNα and IFNβ, bind to the same Type I interferon receptor, which contains 2 subunits: IFNα receptor subunit 1 (IFNAR1) and IFNAR2 (Domanski & Colamonici, 1996; Mogensen, Lewerenz, Reboul, Lutfalla, & Uzé, 1999). IFNα and IFNβ are inflammatory cytokines important for reducing viral replication during early stages of infection (Basler & García-Sastre, 2002; Isaacs & Lindenmann, 1988). In addition to these neuroimmune-specific functions, Type I interferons are also important for neural activity during homeostatic conditions (Blank & Prinz, 2017; Hosseini et al., 2020). An extensive series of studies showed that under normal, healthy conditions, the Type I interferon receptor is required for in-tact long-term potentiation, dendritic spine morphology, and hippocampal-dependent spatial learning and memory compared with both Type I interferon receptor-deficient mice and mice with conditional inhibition of Type I interferon receptor (Hosseini et al., 2020). Interestingly, despite binding to the same receptor, studies have shown that significant increases in IFNα and IFNβ, such as those from neuroimmune activation, result in activation of similar but distinguishable intracellular activities (Grumbach et al., 1999; Marijanovic, Ragimbeau, Van Der Heyden, Uzé, & Pellegrini, 2007; Platanias, Uddin, Domanski, & Colamonici, 1996). For example, *in vitro* studies on hippocampal neurons have shown that IFNα disrupts long-term potentiation independent of NMDA receptors (Mendoza-Fernández et al., 2000). Conversely, while IFNβ has also been

shown to modulate neuronal excitability, studies found that this is associated with changes in NR2B subunit-containing NMDA receptors (Costello & Lynch, 2013; Di Filippo et al., 2016).

We previously found that males showed increased expression of both IFNα and IFNβ in response to the viral mimic poly I:C, and females only showed increased expression of IFNβ (Chapter 2; Posillico et al., 2021). Given the important role of Type I interferons in anti-viral responses and the specificity with which pathophysiological levels of Type I interferons modulate neuronal activity, we hypothesized that sex differences in Type I interferon induction by poly I:C may contribute to sex-specific effects of poly I:C on glutamatergic and intracellular signaling. As such, we predicted that Type I interferon receptor inhibition will prevent, at least in part, the effects of poly I:C on glutamate signaling and potentially unveil sex-specific mechanisms of poly I:C modulation of learning and memory.

In this study, we aimed to identify underlying mechanisms by which poly I:C may prime the hippocampus for disruptions in learning and memory in male and female C57BL/6N mice. First, we tested whether exogenous CXCL10 contributed to sickness responses and learning deficits induced by poly I:C given the massive increases in CXCL10 gene expression and protein levels we found previously. Next, we examined the role of Type I interferon signaling in poly I:C-induced learning deficits and in protein levels of astrocytic glutamate transporter, ionotropic and metabotropic glutamate receptors, and intracellular signaling molecules downstream of poly I:C and Type I interferon receptor activation. To do this, we treated males and females with an interferon alpha receptor subunit 1 (IFNAR1) antibody to inhibit the Type I interferon receptor 2 hours prior to administration of poly I:C. We hypothesized that Type I interferons contributed disruptions in learning and that the sex differences in Type I interferon induction from poly I:C

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(Chapter 2) would result in sex differences in the underlying glutamatergic and intracellular signaling mechanisms at play.

We did not find any effects of exogenous CXCL10 on sickness responses or learning and memory consolidation in context fear conditioning. In contrast, we found that pre-treatment with the IFNAR1 inhibitor partially rescued the poly I:C-induced learning deficit in males but not females. We did not find any effects of poly I:C on levels of GLT-1 or glutamate receptors, but we showed that poly I:C increases IL-6 and phosphorylated STAT3 in both sexes and that IFNAR1 pre-treatment significantly reduces both in each sex as expected. These data suggest that there is a Type I interferon-dependent mechanism of poly I:C-induced learning deficits in males, and a female-specific mechanism of neuroimmune disruption of learning and memory remains to be determined.

4.3 Methods

4.3.1 Animals

169 male and female 8–9-week-old C57BL/6N mice were purchased from Envigo (Indianapolis, IN) and used in these experiments. All mice were individually housed in standard polypropylene mouse cages with ad libitum access to food and water in a room with maintained temperature, pressure, and humidity under a 12:12h light:dark cycle. Animals were given at least one week of acclimation to the colony room prior to any experimental manipulations. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

4.3.2 Stereotaxic Surgeries

All mice were implanted with bilateral guide cannula (PlasticsOne, Roanoke, VA) targeting the lateral ventricles using standard stereotaxic methods as discussed in detail in Chapter 2 and used in Chapter 3. Briefly, a pre-surgical analgesic (5 mg/kg Carprofen, subcutaneous) was injected subcutaneously, and animals were anesthetized for surgery using an intraperitoneal injection of 250 mg/kg of Avertin (2,2,2-tribromoethanol). Guide cannulae were secured using dental cement and implanted at the following coordinates relative to Bregma: ML: +/- 1.00 mm, AP: 0.30 mm, DV: -2.50 mm. Animals were given a second dose of Carprofen (5 mg/kg, subcutaneous) 24 hours after surgery to maintain a total of 48 hours of analgesia. Mice were monitored daily for 10 days post-operative and were given at least 2 weeks to recover from surgery prior to use in experiments.

4.3.3 Experimental Treatments

Polyinosinic:polycytidylic acid (poly I:C; Cat. No. P1530; Sigma-Aldrich, St. Louis, MO) was prepared according to the manufacturer's instructions and sterile-filtered using a 0.22 μm filter prior to administration. For intracerebroventricular (ICV) delivery, 20 μg of poly I:C (2 μL of 10 μg/μL poly I:C) (X. Zhu et al., 2016) or an equal volume of 0.9% sterile saline were infused *via* the implanted guide cannula under brief isoflurane anesthesia.

Monoclonal mouse IFNAR1 antibody was used to block Type I interferon receptors (clone: MAR1-5A3; Cat. No. I-401; Leinco Technologies, Inc., St. Louis, MO). Under brief isoflurane anesthesia, mice were administered 20 μg of IFNAR1 (2 μL of 10 μg/μL) or an equal volume of mouse IgG$_1$ isotype control (IgG; clone: HKSP; Cat. No. I-536; Leinco Technologies, Inc.) *via* the implanted guide cannula for ICV delivery. To block Type I interferon receptors, IFNAR1 (or IgG) was always given 2 hours prior to poly I:C (or sterile saline) administration for these experiments.

Recombinant mouse CXCL10 was used to test the effects of CXCL10 on sickness behaviors and context fear conditioning (Cat. No. 466-CR-050/CF; R&D Systems, Minneapolis, MN). CXCL10 was reconstituted in sterile phosphate-buffered saline (PBS) per the manufacturer's instructions, and mice given 100 ng of CXCL10 (2 μL of 50 ng/μL) or an equal volume of sterile PBS ICV under brief isoflurane anesthesia.

4.3.4 Estrous Phasing

Visual assessment of the vaginal opening in conjunction with vaginal cytology were used to determine the estrous phase of females on the day of treatments, when possible, as detailed in Chapter 3 (Byers, Wiles, Dunn, & Taft, 2012). Briefly, vaginal lavage samples were dispensed onto clean microscope slides and imaged at 5X magnification under bright field microscopy where the ratio of cells present was used to classify the estrous phase. We did not find any patterns suggesting that estrous phase modulated behavior or molecular endpoints in females for any experiment.

4.3.5 Sickness Responses

To determine whether CXCL10 contributed to the fever and weight loss induced by poly I:C, we administered CXCL10 with or without poly I:C and measured sickness responses. For this experiment, we infused either 100 ng of CXCL10 or sterile PBS immediately followed by 20 μg of poly I:C or sterile saline. Body weights and rectal temperatures (RET-3; Physitemp, Clifton, NJ) were measured at 2, 4, 6, 12, 24, and 48 hours following ICV administration of PBS and Saline ($n = 4$), CXCL10 and saline ($n = 5$), PBS and poly I:C ($n = 5$), or CXCL10 and poly I:C ($n = 5$) in males. Visual measures of sickness (piloerections, squinted eyes, hunched posture,

and low responsivity) were assessed throughout (Hart, 1991). No changes in overt sickness behaviors were observed for any experiment (data not shown).

Statistical Analysis of Sickness Responses

Analyses of body weight and temperature changes were completed using mixed repeated-measures ANOVA, using time post-infusion as the within-subjects factor and treatment 1 (PBS or CXCL10), treatment 2 (sterile saline or poly I:C), and sex as the between-subjects factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

4.3.6 Context Fear Conditioning

We used a contextual fear conditioning task to test mechanisms of fear learning and memory disruption as described in detail in Chapter 3. To test whether CXCL10 modulates acquisition or memory consolidation in males during context fear conditioning, we administered 100 ng of CXCL10 ($n = 6$) or sterile PBS ($n = 6$) 15 minutes prior to training. During training, mice were given 3 minutes to explore the context of the apparatus before receiving a 2-second 0.8 mA foot shock, after which they were immediately removed and returned to their home cage. The apparatus was cleaned with 70% ethanol between each animal. 24 hours later, mice were brought back to the chamber for testing, during which mice had 3 minutes to explore the context. Their behavior was assessed during training and testing using Video Freeze software (MedAssociates).

To test whether pre-treatment with a Type I interferon receptor inhibitor rescued the poly I:C-induced learning deficits in males and females, we administered 20 μg of IFNAR1 or IgG 2

hours prior to administration of 20 μg of poly I:C or sterile saline. 4 hours following poly I:C or saline treatment, animals were trained in context fear conditioning as explained above. 72 hours after training, animals were brought back to the context for testing where mice again had 3 minutes to explore the context while their behavior was recorded and assessed using Video Freeze software. The treatment groups were as follows: IgG and saline ($n = 9$ male; $n = 10$ female), IFNAR1 and saline ($n = 10$ male; $n = 10$ female), IgG and poly I:C ($n = 10$ male; $n = 11$ female), IFNAR1 and poly I:C ($n = 8$ male; $n = 10$ female).

Statistical Analysis of Context Fear Conditioning

The effects of CXCL10 on behavior during context fear conditioning were analyzed using one-way ANOVA tests with treatment as the factor.

The effects of IFNAR1 and poly I:C on behavior during context fear conditioning were analyzed using three-way ANOVA tests with treatment 1 (IFNAR1 or IgG), treatment 2 (poly I:C or sterile saline), and sex as factors. Significant interactions ($p < 0.05$) were followed up with post-hoc tests using Bonferroni corrections for multiple *a priori* comparisons. Effect sizes were calculated using the partial eta squared method.

4.3.7 Quantitative Real-Time PCR

We used quantitative real-time PCR to test the effects of blocking Type I interferon receptors prior to poly I:C administration on cytokine and chemokine responses in the hippocampus. The treatment groups were as follows: IgG and saline ($n = 8$ male; $n = 10$ female), IFNAR1 and saline ($n = 9$ male; $n = 10$ female), IgG and poly I:C ($n = 9$ male; $n = 8$ female), IFNAR1 and poly I:C ($n = 7$ male; $n = 10$ female). We also tested whether pre-treatment with the IgG isotype control modulated the cytokine responses to poly I:C. For this experiment, animals

were treated with either sterile saline ($n = 6$ male, $n = 5$ female) or IgG ($n = 6$ male, $n = 6$ female), and 2 hours later, all animals were treated with poly I:C.

For both experiments, 4 hours following poly I:C (or sterile saline) administration, mice were transcardially perfused with 0.1 M phosphate buffer to remove circulating blood from the brain. The dorsal hippocampus was dissected out, placed in RNase-/DNase-free tubes, flash frozen, and stored at -80°C. Frozen samples were mechanically homogenized, and messenger RNA (mRNA) was extracted under sterile, RNase-free conditions using an extraction kit (PureLink RNA Mini Kit, Cat. No. 12183020; Invitrogen, Carlsbad, CA). RNA quality, purity (A260/280 > 1.80), and quantity were assessed using UV spectroscopy (BioSpectrometer Basic; Eppendorf, Hamburg, Germany). Genomic DNA present in the sample was removed using DNase treatment, and 800 ng of cDNA was synthesized from each mRNA sample using a cDNA synthesis kit (QuantiTect Reverse Transcriptase Kit, Cat. No. 205314; Qiagen, Hilden, Germany). Relative gene expression was measured using Power SYBR Green PCR Master Mix (Cat. No. 4368702; Applied Biosystems, Foster City, CA) in 10 µL reactions (ABI 7500 real-time PCR system; Cat. No. 4351105; Applied Biosystems).

Based on results from Chapter 2, we used the geometric mean of the quantification cycle (Cq) values from housekeeping genes *hprt1* (QuantiTect Primer Assay, Cat. No. QT00166768; Qiagen) and *rplp0* (Cat. No. QT00249375; Qiagen) in the $2^{-\Delta\Delta Cq}$ method for calculations of relative expression for our target genes (see Chapter 2, Figure 2.1). We analyzed the relative expression of the following genes of interest: *ccl2*, *cxcl10*, *ifnα*, *ifnβ*, *ifnγ*, *il-1β*, and *il-6*. The sequences for gene primers can be found in Table 1 (Chapter 2) and were ordered through Integrated DNA Technologies and diluted to 0.13 µM to be used for PCR. All Qiagen primers were diluted per the manufacturer's instructions.

Statistical Analysis of Real-Time PCR

For each PCR reaction, the quantification cycle (Cq) was determined, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression of each gene. Any samples with abnormal amplification curves, melt curves, and/or melt peaks across replicates or gene targets were removed from analyses ($n = 1$ male; $n = 2$ female). Any outliers were identified as samples outside the range of 2 standard deviations from the group mean and excluded from analyses.

To analyze the effects of poly I:C-induced cytokine expression with IFNAR1 pre-treatment, we normalized each group to its respective same-sex IgG/saline-treated group to control for any sex differences in gene expression at baseline. We used three-way ANOVA tests with treatment 1 (IFNAR1 or IgG), treatment 2 (poly I:C or sterile saline), and sex as factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

To analyze the effects of IgG pre-treatment on poly I:C-induced cytokine responses, we normalized each group to its respective same-sex saline pre-treatment counterpart. Here, we used two-way ANOVA tests with treatment 1 (IgG or sterile saline) and sex as factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

4.3.8 Western Blot

To determine whether poly I:C and pre-treatment with IFNAR1 modulated glutamate transmission and intracellular signaling in the hippocampus, we used western blot to measure protein levels of a glutamate transporter (GLT-1), glutamate receptors and subunit types (GluR1, GluR2, NR2A, NR2B, and mGluR5), and phosphorylated and total levels of transcription factors

(STAT1, STAT3, IRF3, and CREB). For this experiment, males and females were treated with either IFNAR1 or IgG 2 hours prior to treatment with either poly I:C or sterile saline. 4 hours after poly I:C (or sterile saline) administration, mice were euthanized *via* rapid decapitation, and the dorsal hippocampus was dissected out, flash frozen, and stored at -80°C until further processing. The treatment groups were as follows: IgG and saline (n = 6 male; n = 5 female), IFNAR1 and saline (n = 6 male; n = 5 female), IgG and poly I:C (n = 6 male; n = 6 female), IFNAR1 and poly I:C (n = 6 male; n = 5 female).

Frozen samples were lysed in high-detergent radioimmunoprecipitation assay (RIPA) buffer (1% NP-40 in tris-buffered saline (TBS) with the following protease inhibitors at a concentration of 1 mM: sodium fluoride, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), and Halt™ protease inhibitor cocktail (ThermoFisher)), centrifuged, and the supernatant containing whole cell lysates was collected and stored at -20°C. We used Bradford assays (Bio-Rad Laboratories, Hercules, CA) to determine the protein concentration of each sample.

Four separate gels and membranes were required to run all samples for each protein target due to space constraints of the gel boxes. Gels were set up such that poly I:C- and saline-treated comparisons could be directly measured within each pre-treatment (IgG or IFNAR1) and sex. Thus, gels were designed as follows: 1) female IgG/saline and IgG/poly I:C, 2) female IFNAR1/saline and IFNAR1/poly I:C, 3) male IgG/saline and IgG/poly I:C, 4) male IFNAR1/saline and IFNAR1/poly I:C.

To run western blots, 25 μg of whole cell lysates from each sample were reduced in loading buffer with β-mercaptoethanol, boiled for 5 minutes, subjected to SDS-PAGE gel electrophoresis (Bio-Rad Laboratories), and blotted to PVDF membranes (Millipore). Membranes were incubated in I-Block (ThermoFisher) for 1 hour, primary antibody in 1:1

wash:block buffer (wash buffer: 1X PBS with 0.1% Tween-20) overnight at 4°C, and secondary antibody in 1:1 wash:block buffer for 1 hour at room temperature the following day. All membranes were incubated in β-tubulin to control for possible differences in the total amount of protein loaded in each lane of the gel. Membranes were imaged and band signals were analyzed using a LI-COR Odyssey XF Imager and accompanying software (LI-COR Biosciences, Lincoln, NE). A list of the antibodies and dilutions used for this experiment can be found in Table 4.1.

Statistical Analysis of Western Blot

For each sample on each membrane, the signal for the protein of interest was normalized to the respective signal for β-tubulin to control for possible differences in protein concentrations between samples. The data were then normalized to the average signal from the saline-treated samples on each membrane to measure fold change in protein levels induced by poly I:C. Thus, the average signal from all saline-treated groups was set to 1. This was done to account for differences in protein levels that may have been a result of the samples being loaded in separate gels and blotted onto separate membranes rather than differences caused by the experimental manipulations directly. However, this prevents us from being able to determine whether the pre-treatments (IFNAR1 or IgG) alone induce changes in protein levels, and it also prevents us from being able to capture sex differences in protein levels at baseline.

To analyze these normalized data, we used three-way ANOVA tests with treatment 1 (IFNAR1 or IgG), treatment 2 (poly I:C or sterile saline), and sex as factors. Significant interactions ($p < 0.05$) were followed up using post-hoc tests with Bonferroni corrections for multiple comparisons, and effect sizes were calculated using the partial eta squared method.

Data visualization and statistical analyses were completed using R 3.6.2 (R Core Team,

2019) with the following packages: dplyr (v0.8.5; (Wickham et al., 2020)), tidyr (v1.0.2;

(Wickham & Henry, 2020)), rstatix (v0.5.0; (Kassambara, 2020)), DescTools (v0.99.34;

(Signorell et al., 2020)), and sjstats (v0.17.9; (Ludecke, 2020)).

4.4 Results

4.4.1 CXCL10 Does Not Contribute to or Exacerbate Sickness Responses Induced by Poly I:C in Males

We treated males with recombinant CXCL10 protein and poly I:C to determine whether

increases in this chemokine contributed and/or exacerbated sickness responses induced by poly

I:C. We found significant changes in body weight across the 48-hour monitoring period (Figure

4.1A; main effect of Time: $F(6, 84) = 49.881$, $p < 0.001$, $\eta^2_p = 0.781$). Notably, we found that

poly I:C, but not CXCL10, caused more weight loss in males relative to saline-treated groups

across the timepoints (no main effect of Treatment 1: $F(1, 14) = 0.049$, $p = 0.829$; main effect of

Treatment 2: $F(1, 14) = 16.363$, $p = 0.001$, $\eta^2_p = 0.539$; Treatment 2 x Time interaction: $F(6, 84)$

$= 5.038$, $p = 0.0002$, $\eta^2_p = 0.265$). Specifically, poly I:C-treated groups, regardless of CXCL10

treatment, weighed less at the 12- ($p = 0.0002$) and 24-hour ($p = 0.025$) timepoints compared to

saline-treated groups, and these weight discrepancies recovered by 48 hours ($p = 0.740$). These

findings are consistent with previous data that showed poly I:C induces significant weight loss in

both sexes (Chapter 2, Figure 2.2B).

Similarly, we found significant changes in body temperature over the course of 48 hours

(Figure 4.1B; main effect of Time: $F(6, 90) = 86.437$, $p < 0.001$, $\eta^2_p = 0.852$), and that poly I:C-

treated groups mounted an acute fever response relative to saline-treated groups (main effect of Treatment 2: $F(1, 15) = 19.045$, $p = 0.0006$, $\eta^2_p = 0.559$; Treatment 2 x Time interaction: $F(6, 90) = 4.565$, $p = 0.0004$, $\eta^2_p = 0.233$). Post-hoc tests showed that poly I:C resulted in significantly higher temperatures at the 2- ($p = 0.010$) and 4-hour ($p = 0.0002$) timepoints and did not differ from saline-treated groups by the 6-hour timepoint ($p = 1.00$). These results were consistent with poly I:C-induced fever that we found previously (Chapter 2, Figure 2.2C). Interestingly, we did find an interaction between treatment 1 (CXCL10 or PBS) and timepoint ($F(6, 90) = 3.079$, $p = 0.009$, $\eta^2_p = 0.170$), but post-hoc tests revealed that there was only a difference between PBS- and CXCL10-treated groups at the 12-hour timepoint ($p = 0.024$), and this appears to be driven by a higher body temperature in the PBS-treated group (Figure 4.1B).

4.4.2 CXCL10 Does Not Affect Learning and Memory Consolidation in Males

We treated males with CXCL10 just prior to training in context fear conditioning to determine if CXCL10 contributed to the learning or memory consolidation deficits induced by poly I:C that we found previously (Chapter 3, Figure 3.1B and Figure 3.1F). We did not find any effects of CXCL10 on context exploration (Figure 4.1D; $F(1, 10) = 0.016$, $p = 0.901$) or shock reactivity (Figure 4.1E; $F(1, 10) = 0.1$, $p = 0.758$) during training. Further, CXCL10 during training did not affect freezing levels during testing 24 hours later (Figure 4.1C; $F(1, 10) = 2.394$, $p = 0.153$).

4.4.3 Blocking Type I Interferon Receptors Attenuates Poly I:C-Induced Learning Deficits in Males but Not Females

To test whether the anti-viral Type I interferon receptors were involved in poly I:C-induced learning deficits in males and females, we blocked the receptors with IFNAR1, treated

mice with poly I:C 2 hours later, and trained them in context fear conditioning 4 hours following poly I:C or sterile saline administration (Figure 4.2A). Behavior during testing 72 hours later revealed that poly I:C significantly decreased freezing levels (Figure 4.2B; main effect of Treatment 2: $F(1, 69) = 18.822$, $p = 0.00005$; $\eta^2_p = 0.214$), and this was similar to what we found previously (Chapter 3, Figure 3.1B). We also found a trend that suggested pre-treatment with IFNAR1 modulated the effects of poly I:C on learning differently in males and females (Figure 4.2B; Sex x Treatment 1 x Treatment 2 interaction: $F(1, 69) = 3.862$, $p = 0.0534$, $\eta^2_p = 0.053$). Interestingly, post-hoc tests for *a priori* comparisons revealed that males given poly I:C in the absence of the receptor inhibitor had significantly lower levels of freezing than both saline-treated male groups (IgG/saline: $p = 0.001$; IFNAR1/saline: $p = 0.002$), and males given poly I:C with the interferon receptor inhibitor on board did not show significant differences between either the saline-treated male groups or the group given poly I:C alone (Figure 4.2B). These data suggest that pre-treatment with IFNAR1 attenuated the learning deficits induced by poly I:C in males. Notably, females treated with poly I:C in the absence of the inhibitor did not show decreased levels of freezing as we found previously, and there were no significant differences between females treated with IFNAR1 and poly I:C and any other female group.

In contrast to our previous results, we found that poly I:C treatment significantly decreased locomotor behavior during training (Figure 4.2C; main effect of Treatment 2: $F(1, 64) = 71.384$, $p < 0.0001$, $\eta^2_p = 0.527$), which may have played a role in the poly I:C-induced learning deficits during training. However, there was no effect of pre-treatment with IFNAR1 on locomotor behavior, suggesting that locomotor behavior during training is not solely responsible for freezing levels during testing in males. Neither IFNAR1 (no main effect of Treatment 1: $F(1, 64) = 2.072$, $p = 0.155$) nor poly I:C (no main effect of Treatment 2: $F(1, 64) = 3.187$, $p = 0.079$)

had any effects on shock reactivity during training, and we replicated our previous results that showed males had higher shock reactivity compared with females, though this effect was weak (Figure 4.2D; main effect of Sex: $F(1, 64) = 5.384$, $p = 0.0235$, $\eta^2_p = 0.078$).

4.4.4 Blocking Type I Interferon Receptors Selectively Modulates Cytokine Expression Following Poly I:C

We measured hippocampal cytokine expression 4 hours after poly I:C with and without pre-treatment with IFNAR1 to determine whether blocking Type I interferon receptors had specific and predicted effects on neuroinflammation.

Interleukins

As expected, poly I:C significantly increased expression of interleukin (IL)-1β (Figure 4.3A; main effect of Treatment 2: $F(1, 57) = 11.763$, $p = 0.001$, $\eta^2_p = 0.171$). We also found males had greater expression of *il-1β* overall relative to females (main effect of Sex: $F(1, 57) = 8.238$, $p = 0.006$, $\eta^2_p = 0.126$). Interestingly, pre-treatment with IFNAR1 had sex-specific effects on *il-1β* expression (Figure 4.3A; Sex x Treatment 1 interaction: $F(1, 57) = 4.362$, $p = 0.041$). Though this effect was relatively weak ($\eta^2_p = 0.071$), post-hoc tests revealed that males pre-treated with IFNAR1 had significantly greater *il-1β* expression than females pre-treated with IFNAR1 ($p = 0.005$). Overall, pre-treatment with IFNAR1 did not modulate poly I:C-induced expression of *il-1β* in either sex (no Treatment 1 x Treatment 2 interaction: $F(1, 57) = 0.629$, $p = 0.431$).

Analysis of *il-6* revealed that poly I:C significantly increased expression in both sexes as predicted (Figure 4.3B; main effect of Treatment 2: $F(1, 59) = 41.412$, $p < 0.0001$, $\eta^2_p = 0.412$). IFNAR1 pre-treatment also affected expression in both males and females (Figure 4.3B; main

effect of Treatment 1: $F(1, 59) = 4.727$, $p = 0.034$, $\eta^2_p = 0.074$), and interestingly, there was a trend that suggested IFNAR1 modulated poly I:C-induced *il-6* expression in both sexes (Treatment 1 x Treatment 2 interaction: $F(1, 59) = 3.056$, $p = 0.0856$, $\eta^2_p = 0.049$). Specifically, poly I:C treatment alone caused a marked increase in *il-6* expression relative to both saline-treated groups in each sex ($p < 0.001$ for both), and pre-treatment with IFNAR1 significantly attenuated this ($p = 0.028$) but did not completely reduce *il-6* expression to saline-treated levels (IgG/saline: $p = 0.037$; IFNAR1/saline: $p = 0.006$).

Interferons

Expression of interferon (IFN)α, a Type I interferon, was decreased in animals treated with the Type I interferon receptor inhibitor compared with those given IgG (Figure 4.3C; main effect of Treatment 1: $F(1, 60) = 4.233$, $p = 0.044$), and this was a relatively weak effect ($\eta^2_p = 0.066$). We did not find any effects of poly I:C treatment on *ifnα* expression in either sex, in contrast to what we found previously (Chapter 2) where poly I:C significantly increased *ifnα* expression in males but not females (see Figure 2.3G2).

Analysis of *ifnβ*, another Type I interferon, revealed that poly I:C significantly increased expression in both sexes (Figure 4.3D; main effect of Treatment 2: $F(1, 53) = 48.762$, $p < 0.0001$, $\eta^2_p = 0.479$). Interestingly, poly I:C increased *ifnβ* expression to a greater magnitude in females compared with males (Figure 4.3D; Sex x Treatment 2 interaction: $F(1, 53) = 4.22$, $p = 0.045$; post-hoc test comparing poly I:C-treated males and females: $p = 0.048$), and this was a relatively weak effect ($\eta^2_p = 0.074$). We did not find any effects of IFNAR1 pre-treatment alone or on poly I:C-induced expression of *ifnβ* in either sex.

There were no effects of IFNAR1 pre-treatment or poly I:C administration on expression of *ifnγ*, a Type II interferon, in this experiment (Figure 4.3E). This contrasted with what we found previously, where poly I:C significantly increased expression of *ifnγ* at the 4-hour timepoint in males, but not females (Chapter 2, Figure 2.3I2).

Chemokines

Poly I:C significantly increased expression of *ccl2* in both sexes as expected (Figure 4.3F; main effect of Treatment 2: $F(1, 59) = 34.531$, $p < 0.0001$, $\eta^2_p = 0.369$). We did not find any effects of IFNAR1 treatment alone or in conjunction with poly I:C on expression of *ccl2* here.

Poly I:C also significantly increased *cxcl10* expression in the hippocampus of both males and females (Figure 4.3G; main effect of Treatment 2: $F(1, 60) = 54.975$, $p < 0.0001$, $\eta^2_p = 0.478$). In addition, we found that females treated with poly I:C had a greater increase in *cxcl10* expression compared with males (Figure 4.3G; Sex x Treatment 2 interaction: $F(1, 60) = 5.627$, $p = 0.021$), similar to the effects we found with *ifnβ*, and again, this was a relatively weak effect ($\eta^2_p = 0.086$).

4.4.5 Effects of Isotype IgG Control on Poly I:C-Induced Cytokine Expression Are Minimal

We next determined whether the IgG isotype we used as an appropriate control for IFNAR1 pre-treatment had any effects on poly I:C-induced cytokine expression in the hippocampus. For this experiment, all animals received poly I:C, and the IgG pre-treatment was compared with a sterile saline pre-treatment because sterile saline is the vehicle used to dilute poly I:C and the control we use for poly I:C-specific experiments. Thus, any effects that may be

induced by sterile saline alone are accounted for in both saline- and poly I:C-treated groups, and this would not necessarily be the case for IgG and IFNAR1 treatment groups.

We did not find any differences between saline and IgG pre-treatments on poly I:C-induced cytokine expression in either sex for *il-1β* (Figure 4.4A), *il-6* (Figure 4.4B), *ifnβ* (Figure 4.4D), *ifnγ* (Figure 4.4E), *ccl2* (Figure 4.4F), or *cxcl10* (Figure 4.4G). However, we did find that IgG pre-treatment increased expression of *ifnα* induced by poly I:C in both sexes (Figure 4.4C; main effect of Treatment 1: $F(1, 18) = 5.485$, $p = 0.031$, $\eta^2_p = 0.234$).

4.4.6 Poly I:C and IFNAR1 Pre-Treatment on Protein Levels of Glutamate Transporter, Glutamate Receptors, and Intracellular Signaling

We measured protein levels of an important glutamate transporter, glutamate receptors, and intracellular signaling molecules after poly I:C administration with or without IFNAR1 pre-treatment to determine whether poly I:C modulated mechanisms important for learning and memory and whether pre-treatment with IFNAR1 prevented or attenuated those changes that might explain the effects we found on behavior.

Glutamate Transporter

We did not find any effects of poly I:C, Type I interferon receptor inhibition, or the combination of both on protein levels of glutamate transporter (GLT) 1 in either males or females (Figure 4.5A).

Glutamate Receptors

Protein levels of the ionotropic glutamate receptor AMPA type subunit 1 (GluR1) were not statistically significantly affected by IFNAR1 or poly I:C in either sex (Figure 4.5B). However, results showed a trend whereby IFNAR1 may have modulated levels affected by poly

I:C (trend towards a Treatment 1 x Treatment 2 interaction: $F(1, 35) = 3.036$, $p = 0.090$, $\eta^2_p = 0.080$). Visually, it appears that poly I:C may result in a small decrease in GluR1 levels, and pre-treatment with IFNAR1 may rescue or reverse this effect (Figure 4.5B).

Protein levels of the AMPA type subunit 2 receptor (GluR2) revealed a different pattern of effects. Here, we found that males had higher levels of GluR2 relative to females overall (Figure 4.5C; main effect of Sex: $F(1, 33) = 4.845$, $p = 0.035$, $\eta^2_p = 0.128$). However, this may be driven by the sex-specific effect of poly I:C (Figure 4.5C; Sex x Treatment 2 interaction: $F(1, 33) = 4.525$, $p = 0.041$, $\eta^2_p = 0.121$) where poly I:C-treated males, regardless of pre-treatment, had higher levels of GluR2 relative to poly I:C-treated females ($p = 0.028$).

Analysis of metabotropic glutamate receptor 5 (mGluR5) revealed a significant effect of IFNAR1 pre-treatment in both males and females (Figure 4.5D; main effect of Treatment 1: $F(1, 34) = 4.896$, $p = 0.034$, $\eta^2_p = 0.126$). Interestingly, there was a trend that suggested IFNAR1 pre-treatment modulated the effects of poly I:C on mGluR5 protein levels (Figure 4.5D; trend of Treatment 1 x Treatment 2 interaction: $F(1, 34) = 3.942$, $p = 0.055$, $\eta^2_p = 0.104$). Here, it appeared that poly I:C decreased mGluR5 levels in both sexes, and pre-treatment with IFNAR1 prevented and potentially reversed the effects of poly I:C such that mGluR5 levels were higher than for saline-treated groups (Figure 4.5D).

Next, we examined protein levels of ionotropic NMDA receptor subtypes 2A (NR2A) and 2B (NR2B). We did not find any significant effects of IFNAR1 or poly I:C on levels of either NR2A (Figure 4.6A) or NR2B (Figure 4.6B) in either males or females. In addition to the overall levels of NR2A and NR2B, previous studies have shown that the ratio of these subunits is particularly important for synaptic plasticity (Cui et al., 2013). Thus, we also calculated the ratio

of NR2A/NR2B here (Figure 4.6C); however, again, we did not find any effects of treatments or sex on the ratio of protein levels of these NMDA receptor subunits in this experiment.

Transcription Factors

STAT1 is a transcription factor that can be phosphorylated at tyrosine site 701 and activated by downstream signaling of activated Type I interferon receptors (Ramana, Chatterjee-Kishore, Nguyen, & Stark, 2000), among other notable pathways. We could not visualize any bands for phosphorylated STAT1 protein in any sample in this experiment (data not shown); thus, we only reported total levels of STAT1 protein here. We did not find any effects of treatments or sex on the total levels of STAT1 protein (Figure 4.7A).

STAT3 is a transcription factor that can be phosphorylated at tyrosine site 705 and activated in response to IL-6 and Type I interferon receptor activation (Ho & Ivashkiv, 2006). Here, we found that IFNAR1 pre-treatment modulated poly I:C-induced changes in the proportion of phosphorylated STAT3 (pSTAT3) relative to total STAT3 in both sexes (Figure 4.7B; Treatment 1 x Treatment 2 interaction: $F(1, 35) = 12.645$, $p = 0.001$, $\eta^2_p = 0.265$). Specifically, poly I:C significantly increased levels of pSTAT3, and pre-treatment with IFNAR1 significantly attenuated this increase in both sexes, albeit to a different extent (Sex x Treatment 1 x Treatment 2 interaction: $F(1, 35) = 4.699$, $p = 0.037$, $\eta^2_p = 0.118$). Post-hoc tests revealed that females treated with poly I:C without any inhibitor had greater levels of pSTAT3 than all other female groups ($p < 0.0001$ for all), while males treated with poly I:C alone had greater levels of pSTAT3 than only the IgG and saline-treated male group. However, this sex difference may be due to the overall lower magnitude change in pSTAT3 levels in males relative to females (Figure 4.7B; main effect of Sex: $F(1, 35) = 47.429$, $p < 0.0001$, $\eta^2_p = 0.575$).

Analysis of total levels of STAT3 protein showed a trend of an effect of poly I:C in both sexes suggesting that poly I:C treatment, regardless of IFNAR1 pre-treatment, decreased levels of STAT3 (Figure 4.7C; trend of an effect of Treatment 2: $F(1, 35) = 3.596$, $p = 0.066$, $\eta^2_p = 0.093$).

Interferon regulatory factor 3 (IRF3) is an interferon regulatory transcription factor that can be phosphorylated at serine site 396 and activated by viral stimulants, including poly I:C, that activate Toll-like receptor 3, among other pathways (Honda, Takaoka, & Taniguchi, 2006; Jefferies, 2019). We could not visualize bands for phosphorylated IRF3 for any samples (data not shown) and thus only reported total levels of IRF3 here. We did not find any effects of treatments or sex on the protein levels of IRF3 in this experiment (Figure 4.8A).

The cAMP-response element binding protein (CREB) is a transcription factor that can be phosphorylated at serine site 133 and activated by a wide variety of stimuli and signaling cascades, including Toll-like receptor 3 activation of the NF-κB pathway that poly I:C has been shown to initiate (Wen, Sakamoto, & Miller, 2010). Again, we did not visualize any bands of phosphorylated CREB for any samples in this experiment and only reported total levels of CREB here. Similar to total levels of STAT1 and IRF3, we did not find any effects of IFNAR1 or poly I:C treatments on levels of CREB in either males or females (Figure 4.8B).

4.5 Discussion

We showed that Type I interferon receptor activation is involved in poly I:C-induced learning deficits in males, but not females. Specifically, inhibiting IFNAR1 prior to poly I:C administration partially rescued the learning deficits in context fear conditioning in males only. We showed that pre-treatment with IFNAR1 effectively blunts the poly I:C-induced increase in *il-6* expression and phosphorylated STAT3 protein in the hippocampus of both sexes, indicating

that IFNAR1 pre-treatment worked as expected in both males and females. Taken together, these data suggest that Type I interferons are involved in a male-specific mechanism of learning deficits induced by poly I:C, and female-specific mechanisms remain to be determined.

Previously, we found massive increases in CXCL10 expression and protein levels 4 hours after poly I:C administration (Chapter 2). In fact, this chemokine showed the largest magnitude increase following poly I:C and one of the largest magnitude sex differences in expression than all other markers that we examined (Posillico et al., 2021). CXCL10 is implicated in cognitive impairments and disruption of synaptic plasticity mechanisms during inflammation and neurodegenerative disease (X. Liu et al., 2018; Rocha et al., 2014; Satrom et al., 2018; Vlkolinský et al., 2004). Because of this, we tested whether CXCL10 contributed to the sickness responses and learning deficits induced by poly I:C (Chapter 3). Central administration of CXCL10 had no effect on weight loss or fever response on its own or in combination with poly I:C in males, suggesting that CXCL10 does not play a role in these physiological measures of sickness induced by poly I:C in male mice. However, we did replicate our previous results (Chapter 2) that showed poly I:C caused significant weight loss and an acute fever response which recover within 48 hours (Posillico et al., 2021).

We previously showed that central administration of poly I:C disrupts both learning and memory consolidation of context fear conditioning in males (Chapter 3). As such, we treated males with CXCL10 just prior to training in context fear conditioning to test whether increased CXCL10 at the time of training or during memory consolidation played a role in learning and memory deficits caused by poly I:C in males. CXLC10 during training had no effect on locomotor behavior or shock reactivity during training, and it had no significant effect on freezing levels during testing in males. Thus, despite the massive increase in CXCL10 induced

by poly I:C at the 4-hour timepoint in males, our findings suggest that it does not play a role in weight loss, fever, or learning and memory impairments in these experiments. It is possible that the dose of CXCL10 we used here (100 ng) was simply not high enough to recapitulate the functional consequences, if any, that CXCL10 has on learning. Females showed a much smaller increase in CXCL10 from poly I:C compared with males (Chapter 2), but we did not test the role of CXCL10 on sickness or learning in females here. CXCL10 may indeed play a role in sickness or poly I:C-induced learning deficits for females (or males), but future experiments are needed to further test this question.

Another notable sex difference we previously found from poly I:C administration was a sex difference in Type I interferon induction. Specifically, we found that poly I:C increased expression of both *ifnα* and *ifnβ* in males and only increased expression of *ifnβ* in females (Chapter 2). We also previously found that while both males and females showed learning deficits from poly I:C, cFos data from the hippocampus suggested that there may be sex differences in the underlying mechanisms affected (Chapter 3). Type I interferons are important for the anti-viral immune response (Basler & García-Sastre, 2002; Isaacs & Lindenmann, 1988), and *ifnα* and *ifnβ* have individually been shown to disrupt mechanisms of synaptic plasticity and may also have distinct effects on glutamate receptors (Costello & Lynch, 2013; Di Filippo et al., 2016; Mendoza-Fernández et al., 2000). As such, we hypothesized that Type I interferons disrupt learning processes in both sexes, and sex differences in Type I interferon induction from the viral mimic poly I:C result in sex differences in the mechanisms of synaptic plasticity at play. Thus, we predicted that blocking activity of the Type I interferon receptor would prevent learning deficits and glutamatergic and intracellular signaling changes induced by poly I:C in both sexes and reveal the sex-specific mechanisms involved.

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Remarkably, we found that inhibiting Type I interferon receptors prior to poly I:C administration partially rescued the learning deficits from poly I:C in males. In females, we did not replicate our previous finding that pre-training poly I:C disrupted learning. It is possible that females are less vulnerable to cognitive impairments by poly I:C than males which would help to explain why we did not find consistent effects of pre-training poly I:C on learning. Alternatively, it is possible that methodological differences, including the fact that we administered an additional ICV infusion on the day of training, affected IgG/saline-treated females in such a way as to prevent a significant additional impairment by poly I:C. As such, we cannot conclude whether Type I interferon receptor inhibition had any specific effects on learning in females. However, it is interesting that in females, the IFNAR1/poly I:C treatment group had the lowest average freezing levels during testing compared with all other female treatment groups, suggesting that Type I interferon receptor inhibition prior to poly I:C may have the opposite effect on learning in females compared with males. Additional experiments are needed to increase sample size and statistical power to ensure the results in both sexes are not due to Type I or Type II errors.

Here, we found that poly I:C alone caused significant decreases in locomotor activity during training of context fear conditioning in both sexes. Reduced exploration of the apparatus prior to receiving the foot shock may impair the ability for animals to learn about the context, and a worse context representation could result in decreased fear expression and freezing levels during testing (Fanselow, 1990; Keiser et al., 2017; Rudy & O'Reilly, 1999). However, there are several reasons why this may not be the underlying cause of our results here. First, in females, significantly lower levels of locomotor activity during training did not result in significantly lower levels of freezing during testing. Further, in males, pre-treatment with IFNAR1 attenuated

the decrease in freezing levels from poly I:C during testing, but it did not affect locomotor activity during training. Together, these data suggest that A) decreased locomotor behavior during training is not sufficient to induce learning deficits expressed during testing in these experiments, and B) the mechanisms by which Type I interferon receptor inhibition partially rescues learning deficits in males are more specific than having global effects on locomotor activity.

We next tested how the Type I interferon receptor inhibition modulated poly I:C-induced cytokines in the hippocampus. First, we found that poly I:C alone increased expression of *il-1β*, *il-6*, *ifnβ*, *ccl2*, and *cxcl10* 4 hours after treatment in both sexes as expected given what we found previously (Posillico et al., 2021). The only cytokine for which IFNAR1 pre-treatment significantly modulated poly I:C-induced expression was *il-6*. In both males and females, IFNAR1 pre-treatment significantly attenuated the increase of *il-6* induced by poly I:C but did not completely abolish *il-6* expression completely. These data suggest Type I interferons at least partially contribute to the induction of hippocampal *il-6* specifically in both sexes, and this is supported by studies done by others as well (Murray et al., 2015). The experiments by Murray and colleagues only used female mice, and females with a genetic knockout of IFNAR1 also showed attenuated induction of *ifnβ* in the hippocampus following poly I:C treatment, though they also reported that the genetic mutation alone significantly affected *ifnβ* expression overall (Murray et al., 2015). We did not find any interaction of poly I:C and IFNAR1 on *ifnβ* expression in either males and females together or separately, and we did not find that IFNAR1 on its own significantly reduced *ifnβ* expression in the hippocampus, though differences in our methods for testing the effects of Type I interferon receptor could explain these contrasting findings. Interestingly, we found that IFNAR1 treatment alone significantly decreased expression

of *ifnα* for both sexes, which may have confounding effects on other measures from our experiments. However, this effect was relatively weak, and the variability of data within each group suggests that additional experiments are needed to determine the validity and replicability of these findings.

To ensure that the effects of IFNAR1 pre-treatment were specific to inhibition of the Type I interferon receptor and not simply non-specific effects of using a monoclonal antibody, we used an IgG isotype control from the same species, immunoglobulin class, and subclass as the IFNAR1 antibody for our control groups. However, because the isotype control is still an antibody itself that may interact with the immune system, we next tested whether pre-treatment with this IgG also modulated poly I:C-induced cytokine expression that could lead to misinterpretations of the data from IFNAR1 pre-treatment groups. Importantly, administration of the IgG isotype control prior to poly I:C treatment did not change *il-6* expression compared with animals that received a sterile saline treatment prior to poly I:C in either sex, suggesting that the effects of IFNAR1 on poly I:C-induced *il-6* expression are truly specific to Type I interferon receptor inhibition. We also did not find any differences between pre-treatments of IgG or sterile saline in the expression of *il-1β*, *ifnβ*, *ccl2*, and *cxcl10*. However, we did find that pre-treatment with IgG significantly increased the poly I:C-induced expression of *ifnα* in both males and females. In the previous experiment, we found that IFNAR1, regardless of poly I:C treatment, resulted in lower expression of *ifnα* in both sexes compared to animals treated with IgG (Figure 4.3C). However, given that IgG pre-treatment increased poly I:C-induced *ifnα* expression relative to a sterile saline pre-treatment (Figure 4.4C), it is possible that differences in *ifnα* expression between IFNAR1 and IgG pre-treatments were driven by IgG *increasing ifnα* expression, rather than IFNAR1 decreasing it. Whether these effects of IgG pre-treatment on

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ifnα specifically are functionally relevant for our behavioral or other molecular endpoints examined here, however, is an important question for immediate future experiments.

We also aimed to test whether poly I:C altered mechanisms of synaptic plasticity *via* changes in protein levels of glial glutamate transporter, various glutamate receptors, and intracellular signaling molecules and whether IFNAR1 pre-treatment modulated these effects in both sexes. We did not find any effects of poly I:C or IFNAR1 treatment on levels of glutamate transporter 1 (GLT-1) in either males or females. Similarly, there were no effects of either treatment – either alone or in combination – on levels of ionotropic glutamate receptor AMPA type subunit 1 (GluR1). In contrast, protein levels of ionotropic glutamate receptor AMPA type subunit 2 (GluR2) revealed a distinct pattern of effects. Here, we found that females treated with poly I:C, regardless of pre-treatment, had lower levels of GluR2 compared with males treated with poly I:C. Upon closer inspection of the data here, it appears as if poly I:C treatment in females, but not males, decreases GluR2 levels compared to saline-treated groups, though this is not something that statistical analyses found to be true. However, this may have important implications for sex differences in poly I:C-modulation of synaptic plasticity. Studies have shown that AMPA receptors that lack the GluR2 subunit are more likely to be calcium-permeable, and these calcium-permeable AMPA receptors play a role in both homeostatic synaptic plasticity as well as induction, but not maintenance, of long-term potentiation in hippocampal synapses (Isaac, Ashby, & McBain, 2007; Man, 2011). The animals used for our experiment were not subjected to any learning and memory tests where long-term potentiation-based mechanisms should be at play. Rather, it is more likely that changes in GluR2-containing AMPA receptors in females here may reflect synaptic scaling in which decreases in neuronal activity, perhaps from poly I:C treatment, result in increases in glutamate sensitivity *via*

increases in AMPA receptors into the cell membrane. If there is a sex difference in poly I:C modulation of GluR2 subunits, this would mean that there is a sex difference in how poly I:C primes the hippocampus for synaptic plasticity impairments during learning and memory tasks, though this remains to be confirmed.

We showed that levels of metabotropic glutamate receptor 5 (mGluR5) may be modulated by poly I:C and reversed with pre-treatment of IFNAR1. Specifically, we found a trend suggesting an interaction of IFNAR1 and poly I:C whereby poly I:C may be decreasing levels of mGluR5 and pre-treatment with IFNAR1 reverses this effect such that IFNAR1 with poly I:C increases mGluR5 levels beyond that of IgG/saline controls in both males and females. mGluR5 is a metabotropic glutamate receptor expressed both on the extracellular membrane as well as on intracellular membranes. mGluR5 expressed on the cell surface is important for both long-term potentiation and long-term depression in hippocampal slices (Purgert et al., 2014). Interestingly, studies have shown that most mGluR5 is expressed intracellularly (Hubert, Paquet, & Smith, 2001; O'Malley, Jong, Gonchar, Burkhalter, & Romano, 2003), and activation of intracellular mGluR5 specifically has been shown to mediate transcription, gene expression, and long-term depression in hippocampal slices (Jong, Kumar, & O'Malley, 2009; Kumar & Loane, 2012; Purgert et al., 2014). Thus, decreases in mGluR5 levels as a result of poly I:C may disrupt plasticity mechanisms required during learning and memory in both sexes, but it is also possible that IFNAR1 pre-treatment may overcorrect for this and subsequently result in disruption of plasticity mechanisms in the opposite direction. We did not find any sex differences in the effects of either poly I:C or IFNAR1 treatment on mGluR5 protein levels here, though it is still possible that there are sex-specific effects on extracellular versus intracellular expression of the receptor that the methods used here cannot detect.

Ionotropic NMDA glutamate receptors are also important for mechanisms of synaptic plasticity. Similar to AMPA glutamate receptors, the subunits that comprise NMDA receptors also have distinct functions. For example, NR2A-containing NMDA receptors can increase GluR1 trafficking into the cell surface membrane whereas NR2B-containing NMDA receptors inhibit GluR1 surface expression (Kim, Dunah, Wang, & Sheng, 2005). Additionally, studies have found that NR2A-containing NMDA receptors are required for long-term potentiation and NR2B-containing NMDA receptors are required for long-term depression induction in hippocampal slices, and this is true even for NMDA receptors outside of the synapse (Massey et al., 2004). Increases in IFNβ, such as that induced by poly I:C in both sexes, has been shown to modulate neuronal excitability *via* changes in NR2B subunit-containing NMDA receptors (Costello & Lynch, 2013; Di Filippo et al., 2016). Therefore, it is reasonable to test whether poly I:C affects protein levels of NMDA receptor subunits to contribute to disrupted synaptic plasticity during learning and memory processes. However, we did not find any effects of poly I:C or IFNAR1 treatments on levels of NR2A, NR2B, or the ratio of NR2A/NR2B in either males or females in these experiments.

We also measured protein levels of transcription factors that should be activated by the effects of poly I:C and Type I interferons. STAT1 can be activated by Type I interferon receptor activation (Ramana et al., 2000), and STAT3 can be strongly activated by Type I interferons as well as IL-6 (Ho & Ivashkiv, 2006). Interferon regulatory factor 3 (IRF3) can be activated by Toll-like receptor 3 stimulants including poly I:C (Honda et al., 2006; Jefferies, 2019), as can the cAMP-response element binding protein (CREB) *via* activation of the NF-κB pathway (Wen et al., 2010). Unfortunately, we could not visualize phosphorylated STAT1, IRF3, or CREB to determine how poly I:C or IFNAR1 treatment affected activation of these transcription factors,

and we did not find any effects of either treatment on total levels in either sex. However, we did find that poly I:C significantly increased phosphorylated STAT3 levels in both sexes, though to a lesser extent in males compared with females. We also found that pre-treatment with IFNAR1 significantly attenuated the phosphorylated STAT3 increase. Given that we found pre-treatment with INFAR1 blunted the poly I:C-induced increase in *il-6* expression, it is possible that phosphorylated STAT3 is specifically activated by IL-6 here. Importantly, these effects on both *il-6* and phosphorylated STAT3 were found in both males and females, indicating that both poly I:C and IFNAR1 pre-treatment are both acting as expected in these experiments, and the negative results from other protein endpoints are not simply due to the ineffectiveness of these treatments.

That we failed to see any effects of poly I:C or IFNAR1 treatment on levels of glial glutamate transporter and most glutamate receptors despite substantial evidence that would predict these changes is perhaps not surprising considering the methods we used in this experiment. Specifically, many of the studies that show neuroimmune modulation of these proteins discuss changes in their trafficking into and out of the cell membrane and not necessarily making or degrading total proteins. Our western blot studies used whole cell lysate samples, meaning that the samples included proteins from the membrane, cytoplasm, and nucleus. We expect that future experiments using subcellular fractionation to differentiate membrane-bound, cytoplasmic, and nuclear proteins would reveal more nuanced changes in glutamate transporter and receptors and help us to better understand the functional consequences of poly I:C on mechanisms of glutamatergic signaling and synaptic plasticity.

Taken together, we found that Type I interferons, and not CXCL10, play a significant role in the poly I:C-induced learning deficits in males. Inhibiting Type I interferon receptors prior to administration of poly I:C successfully attenuated poly I:C-induced *il-6* expression and

subsequent STAT3 activation in both sexes, but it only contributed to ameliorating poly I:C-induced learning deficits in context fear conditioning in males. We previously found that poly I:C treatment revealed sex differences in Type I interferon induction in the hippocampus (Posillico et al., 2021), and our data here suggest that Type I interferons may have sex-specific functions in learning and memory mechanisms as well. A female-specific mechanism for neuroimmune modulation of learning and memory continues to escape us, but these experiments strongly implicate Type I interferon signaling for males and highlight the need for future experiments to fully describe the precise mechanisms at play.

4.6 Table and Figures

Table 4.1 *Antibody information used for western blot*

| Antibody	Host Species	Dilution Factor	Brand/Manufacturer
Primary Antibodies			
β-Tubulin	Mouse	1:2000	Millipore
GLT-1	Guinea Pig	1:750	Millipore
GluR1	Mouse	1:250	NeuroMab/Antibodies Incorporated
GluR2	Mouse	1:250	NeuroMab/Antibodies Incorporated
mGluR5	Rabbit	1:1000	Millipore
NR2A	Rabbit	1:750	Millipore
NR2B	Rabbit	1:200	Chemicon/Millipore
pCREB (S133)	Rabbit	1:250	Cell Signaling
Total CREB	Rabbit	1:500	Cell Signaling
pIRF3 (S396)	Rabbit	1:250	Cell Signaling
Total IRF3	Rabbit	1:1000	Cell Signaling
pSTAT1 (Y701)	Rabbit	1:250	Cell Signaling
Total STAT1	Rabbit	1:500	Cell Signaling
pSTAT3 (Y705)	Rabbit	1:500	Cell Signaling
Total STAT3	Rabbit	1:1000	Cell Signaling
Secondary Antibodies			
Anti-Rabbit (IRDye 680RD)	Goat	1:15000	LI-COR
Anti-Mouse (IRDye 800CW)	Goat	1:15000	LI-COR
Anti-Guinea Pig (IRDye 680RD)	Donkey	1:15000	LI-COR
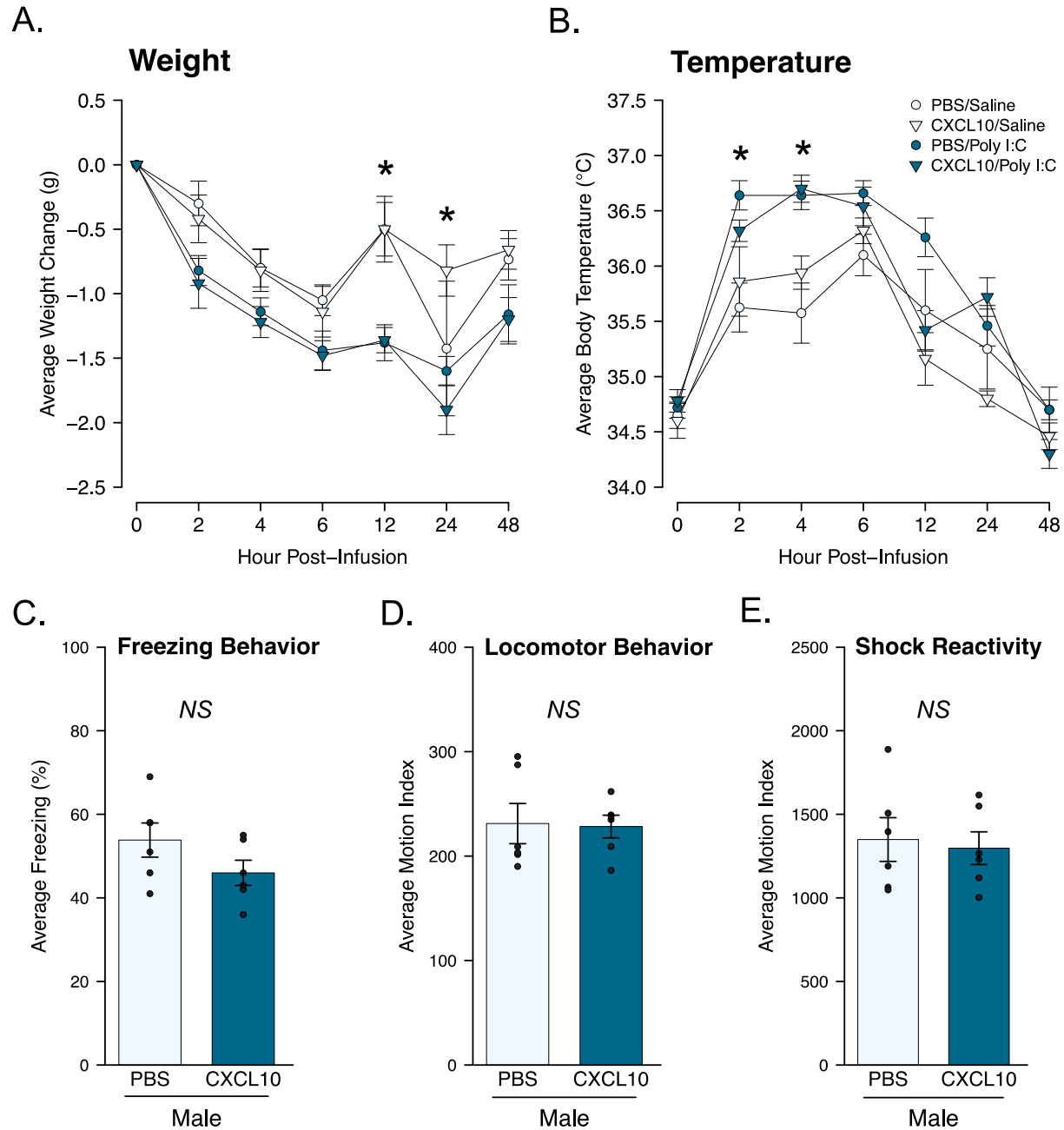
Figure 4.1 *Effects of CXCL10 on sickness responses and context fear conditioning*

(A) Average weight change from baseline (Hour Post-Infusion = 0) and **(B)** average body temperature change as measured *via* rectal thermometer following treatments of PBS or CXCL10 and saline or poly I:C. **(C)** Average percent time spent freezing during testing in context fear conditioning. **(D)** Average exploration activity and (E) shock reactivity during training of context fear conditioning. ***** above points on a line graph (A and B) indicate a significant difference ($p < 0.05$) between poly I:C- and saline-treated groups following an interaction in a mixed repeated-measures ANOVA; *NS* indicates no statistical significance. Error bars represent standard error of the mean.
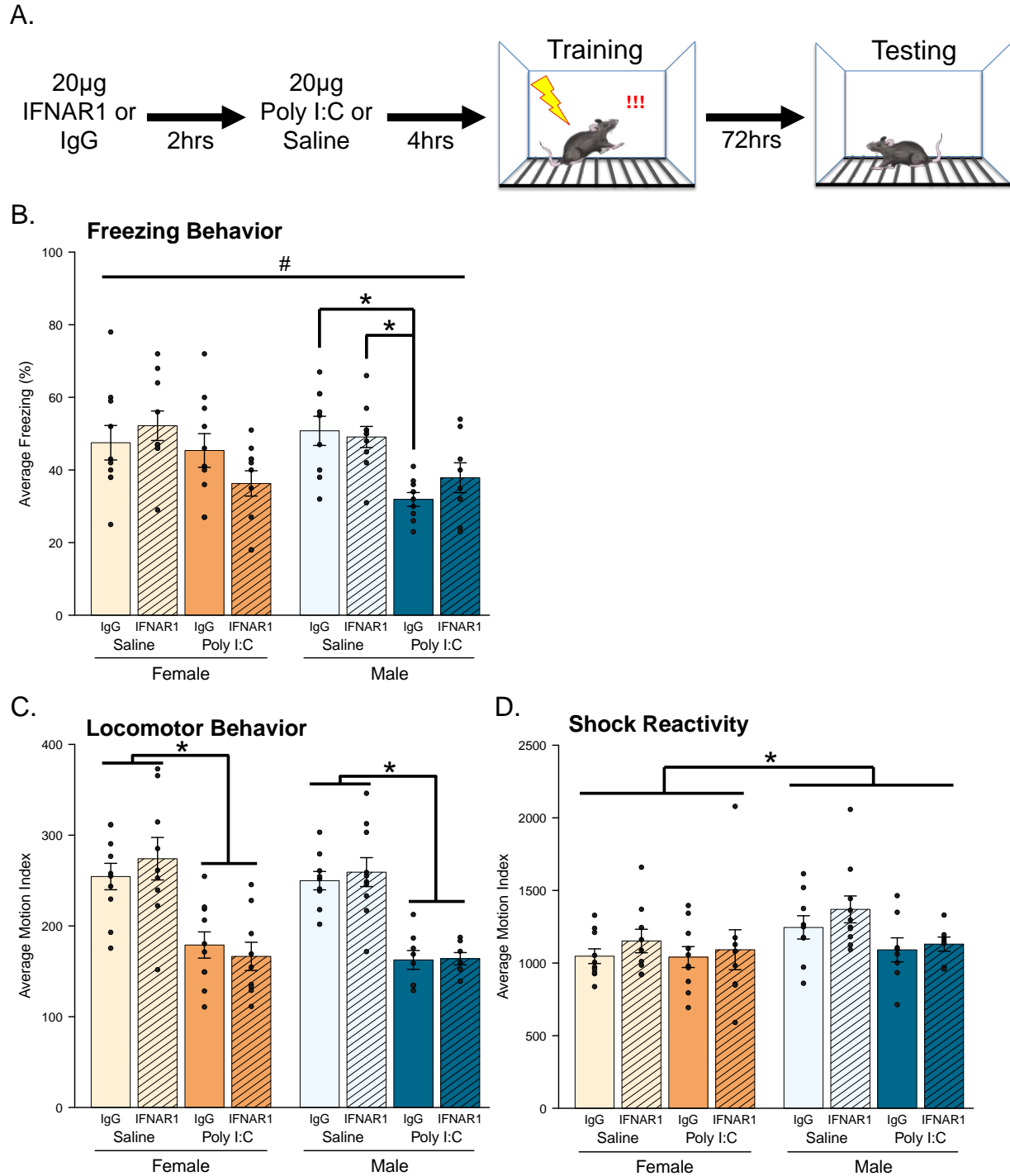
Figure 4.2 *Type I interferon receptor inhibition and poly I:C on context fear conditioning*

(A) Experimental design used to test the effects of Type I interferon receptor inhibition and poly I:C on learning in context fear conditioning. **(B)** Average percent freezing levels during testing of context fear conditioning. **(C)** Average exploration activity and **(D)** shock reactivity during training of context fear conditioning. * above a bracket (C, D) indicates a main effect ($p < 0.05$) of poly I:C (C) or sex (D) following a three-way ANOVA; * comparing two bars (B) indicates a significant post-hoc test ($p < 0.05$) following a three-way ANOVA interaction; # above horizontal line (B) indicates a trend ($p < 0.06$) of a sex x treatment 1 x treatment 2 interaction following a three-way ANOVA. Error bars represent standard error of the mean.
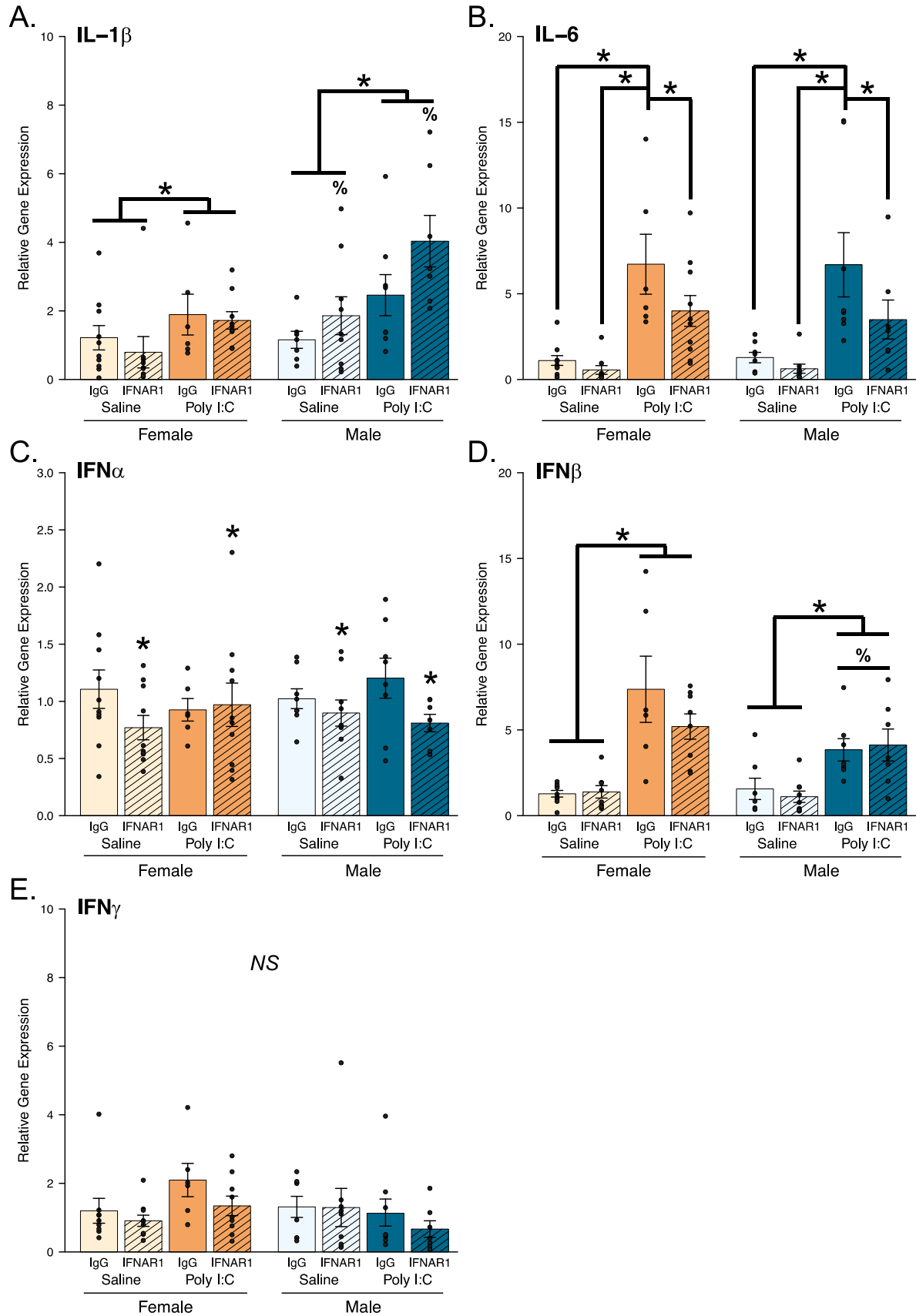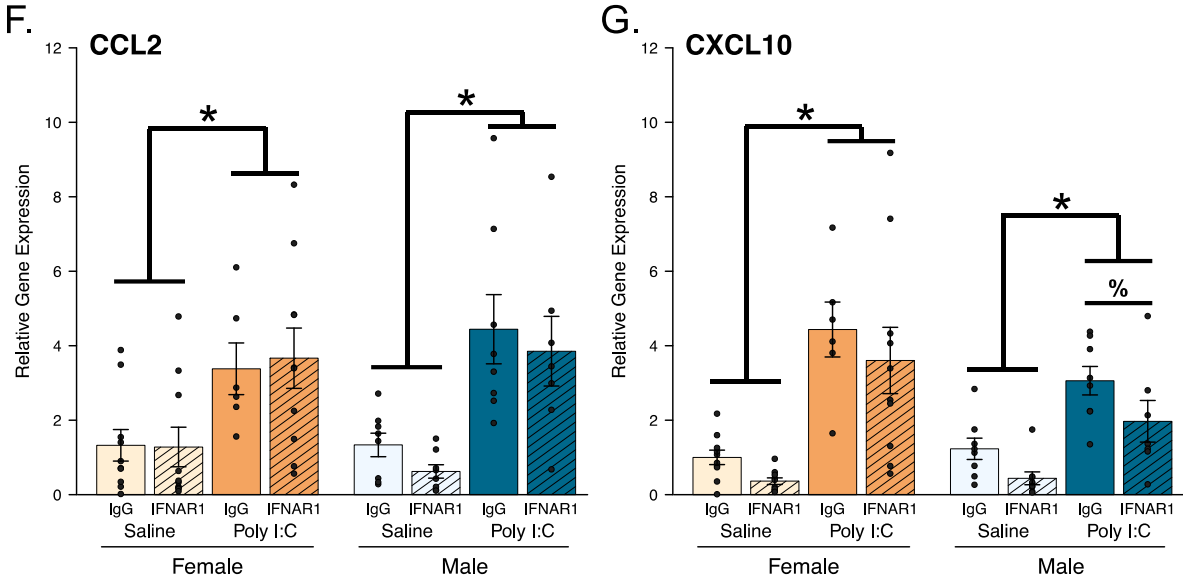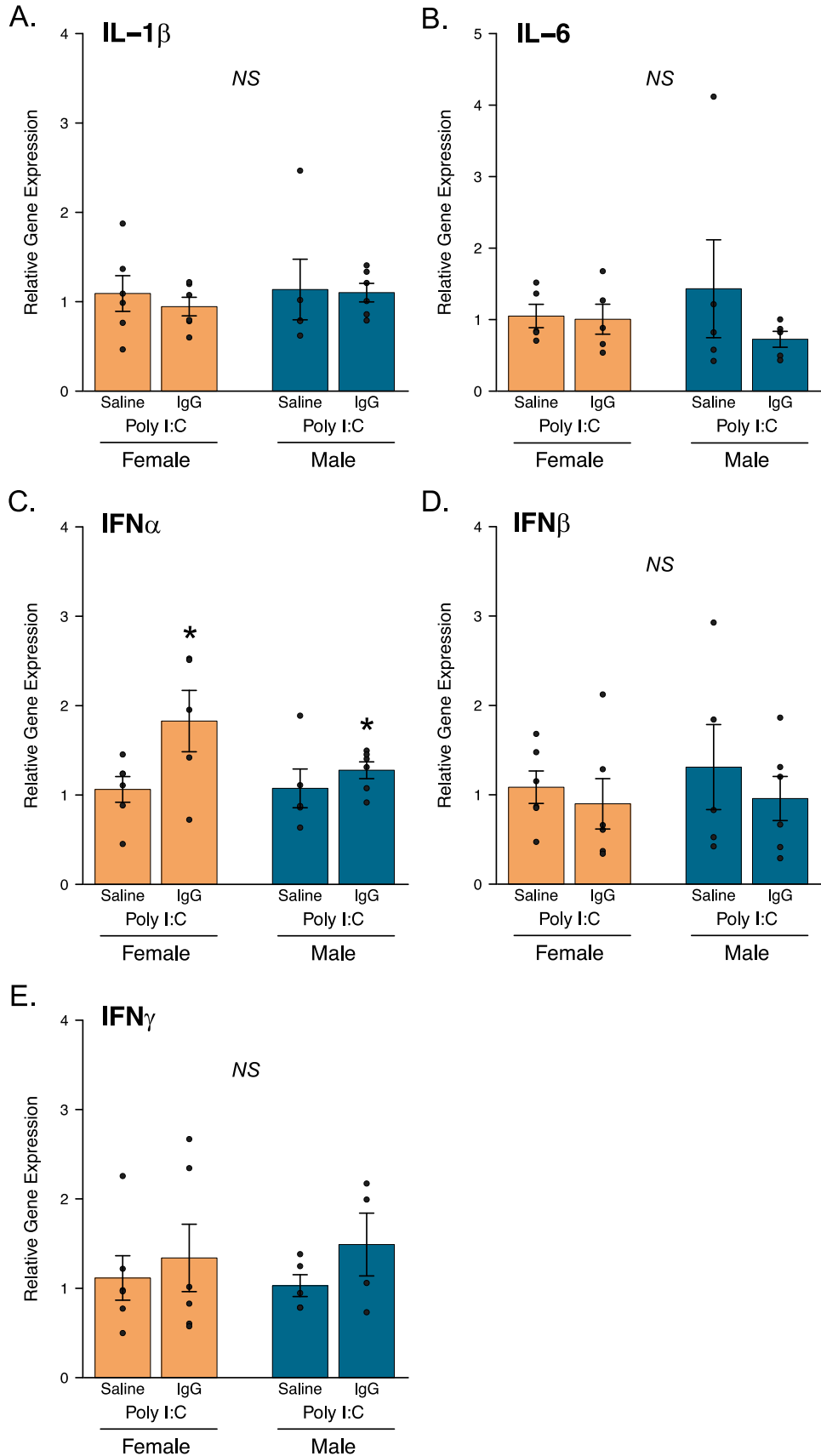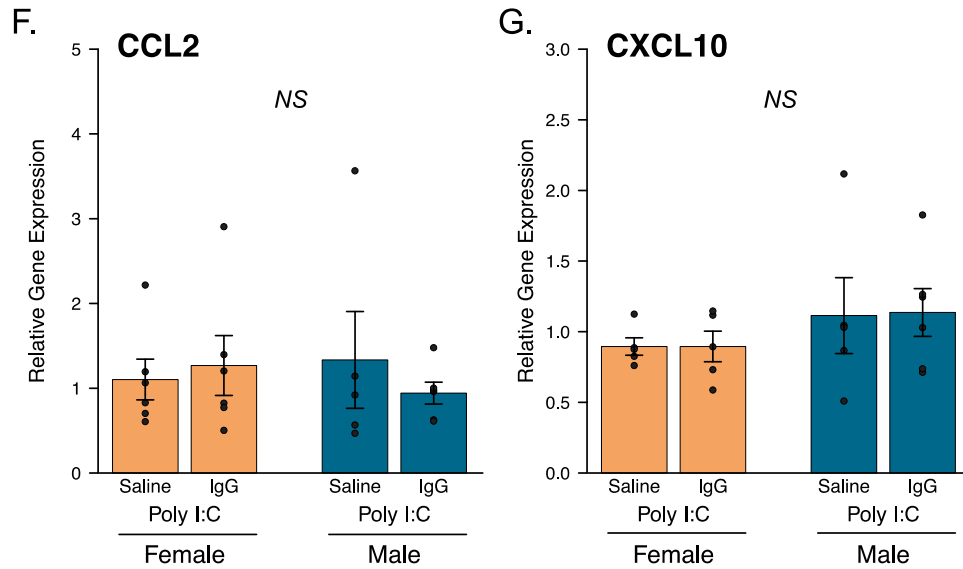
Figure 4.3 *Effects of IFNAR1 and poly I:C on hippocampal cytokine mRNA expression*

Gene expression changes from IgG/saline-treated groups within sex 4 hours following poly I:C or saline administration with either IgG or saline given 2 hours pre-poly I:C or saline treatment for **(A)** IL-1β, **(B)** IL-6, **(C)** IFNα, **(D)** IFNβ, **(E)** IFNγ, **(F)** CCL2, and **(G)** CXCL10. * above a single bar (C) indicates a main effect ($p < 0.05$) of IFNAR1 treatment following a three-way ANOVA; * above a bracket (A, D, F, G) indicates a main effect ($p < 0.05$) of poly I:C following a three-way ANOVA; **%** indicates a significant post-hoc test ($p < 0.05$) vs females in the same group(s) following a sex x treatment interaction in a three-way ANOVA; ***** comparing two bars (B) indicates a significant post-hoc test ($p < 0.05$) following a three-way ANOVA interaction. *NS* indicates no statistical significance. Error bars represent standard error of the mean.

Figure 4.4 *Effects of IgG isotype control on poly I:C-induced cytokine mRNA expression in the hippocampus*

Gene expression changes from saline/poly I:C-treated groups within sex 4 hours following poly I:C administration with either IgG or saline given 2 hours pre-poly I:C treatment for **(A)** IL-1β, **(B)** IL-6, **(C)** IFNα, **(D)** IFNβ, **(E)** IFNγ, **(F)** CCL2, and **(G)** CXCL10. ***** above a single bar (C) indicates a main effect ($p < 0.05$) of IgG treatment following a two-way ANOVA; *NS* indicates no statistical significance. Error bars represent standard error of the mean.
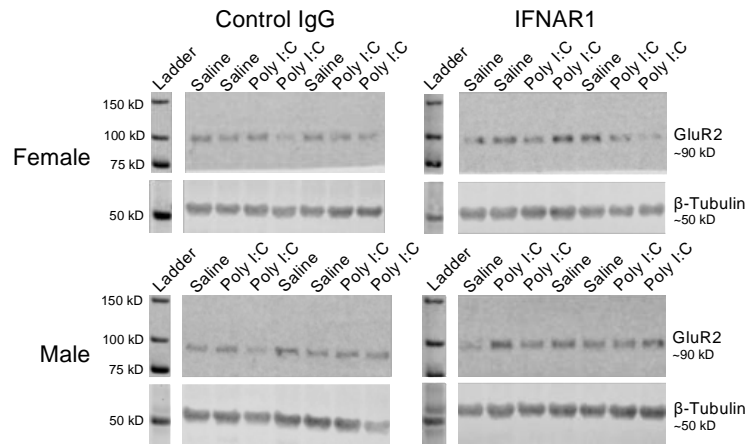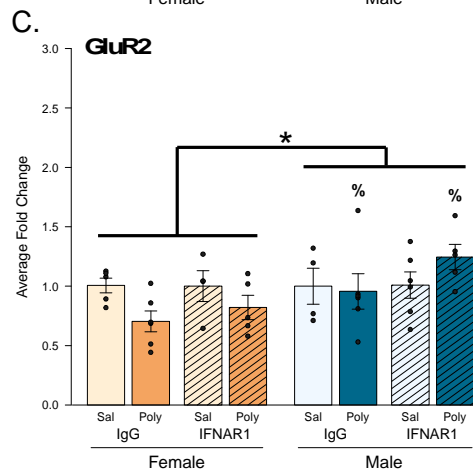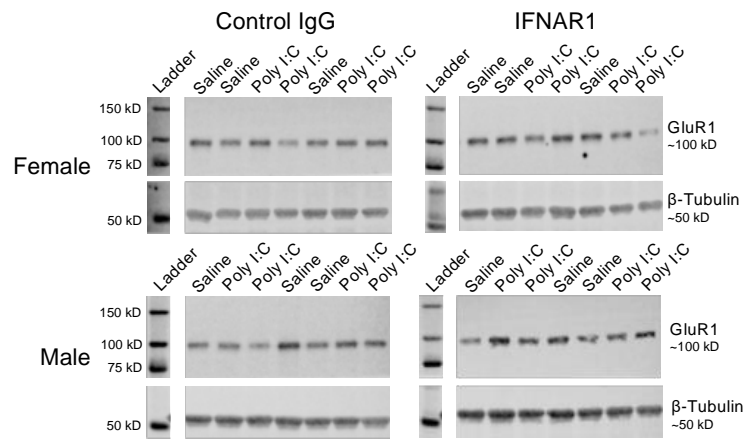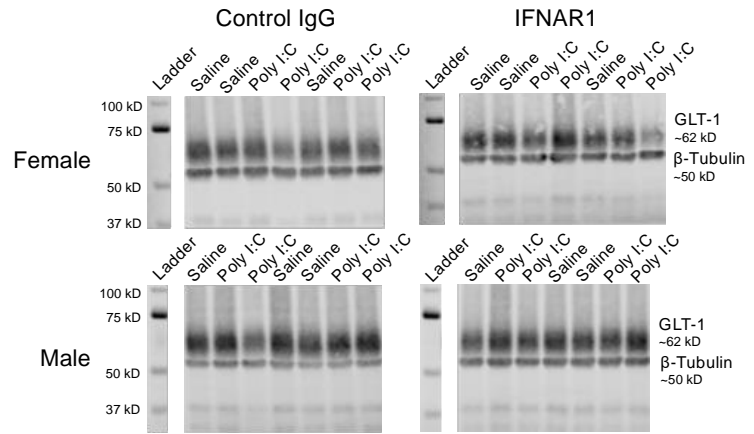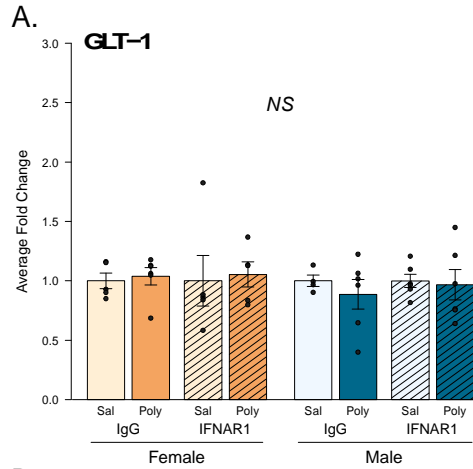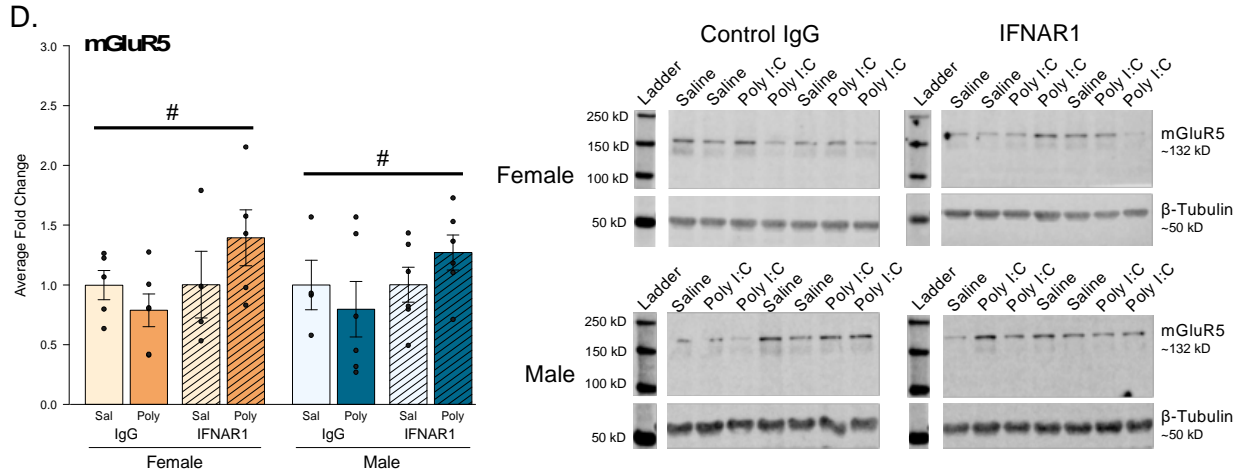
Figure 4.5 *Effects of IFNAR1 and poly I:C on glutamate transporter 1 and select glutamate receptors*

Average fold change in protein levels with associated representative western blot images for **(A)** GLT-1, **(B)** GluR1, **(C)** GluR2, and **(D)** mGluR5 4 hours following poly I:C administration with either IgG or saline given 2 hours pre-poly I:C treatment. ***** above a bracket (C) indicates a main effect ($p < 0.05$) of sex following a three-way ANOVA; **%** above a single bar (C) indicates a significant post-hoc test ($p < 0.05$) vs females in the same group(s) following a sex x treatment interaction in a three-way ANOVA; **#** above a horizontal line (D) indicates a trend ($p < 0.06$) of a treatment 1 x treatment 2 interaction; *NS* indicates no statistical significance. Error bars represent standard error of the mean.
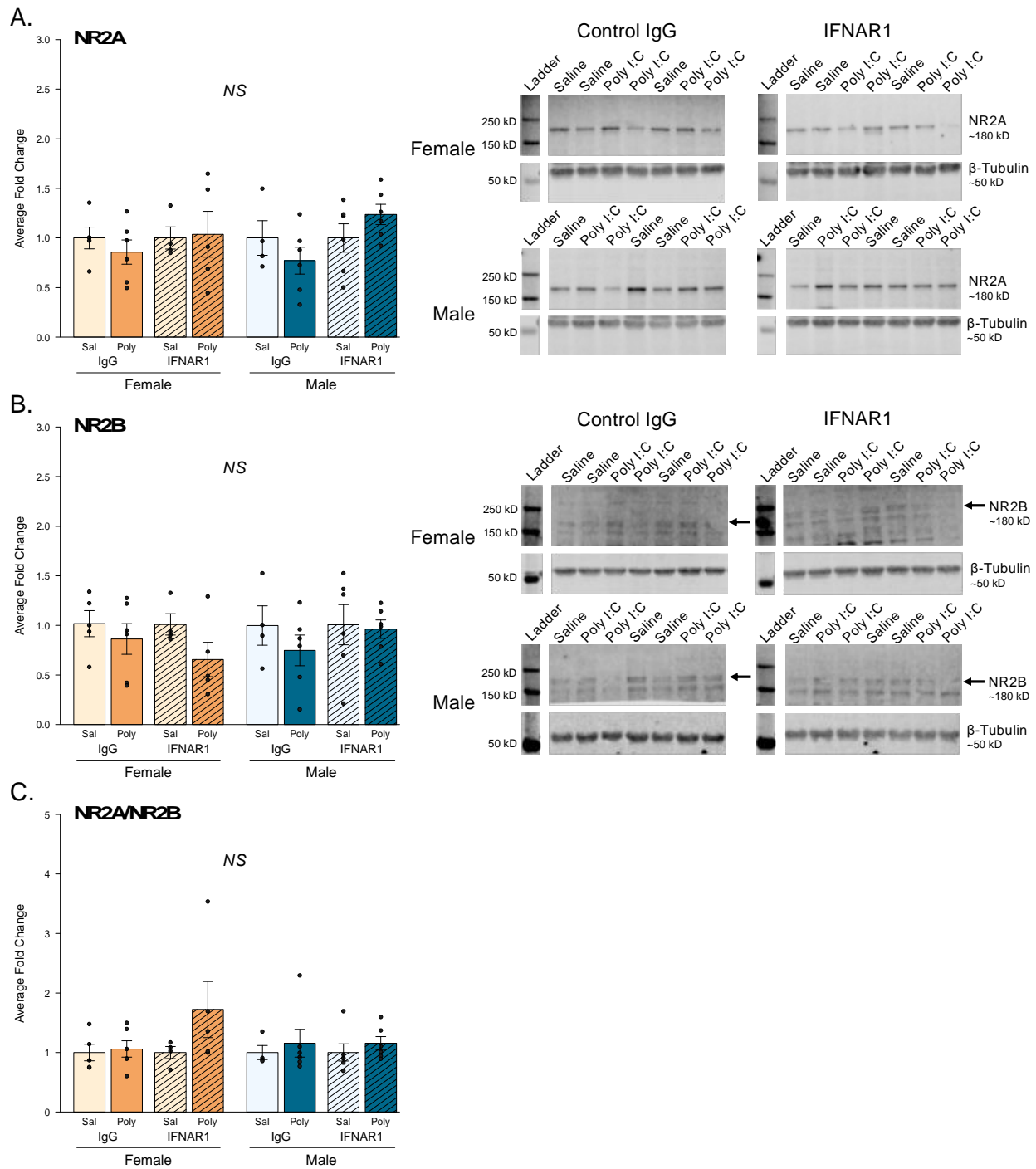
Figure 4.6 *Effects of IFNAR1 and poly I:C on NMDA receptor subtypes*

Average fold change in protein levels with associated representative western blot images for **(A)** NR2A, **(B)** NR2B, and **(C)** NR2A/NR2B ratio 4 hours following poly I:C administration with either IgG or saline given 2 hours pre-poly I:C treatment. *NS* indicates no statistical significance. Error bars represent standard error of the mean.
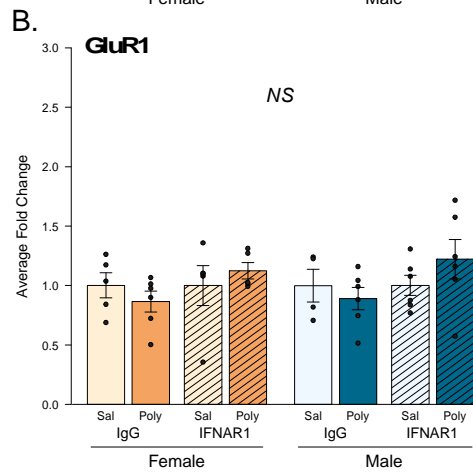
Figure 4.7 *Effects of IFNAR1 and poly I:C on STAT1 and STAT3*

Average fold change in protein levels with associated representative western blot images for **(A)** total STAT1, **(B)** phosphorylated STAT3/STAT3 ratio, and **(C)** total STAT3 4 hours following poly I:C administration with either IgG or saline given 2 hours pre-poly I:C treatment. ***** above a horizontal line (B) indicates a significant treatment 1 x treatment 2 interaction following a three-way ANOVA; ***** comparing two bars (B) indicates a significant post-hoc test ($p < 0.05$) following an interaction from a three-way ANOVA; **#** above a single bar (C) indicates a trend ($p < 0.07$) of an effect of poly I:C treatment in a three-way ANOVA; ***NS*** indicates no statistical significance. Error bars represent standard error of the mean.

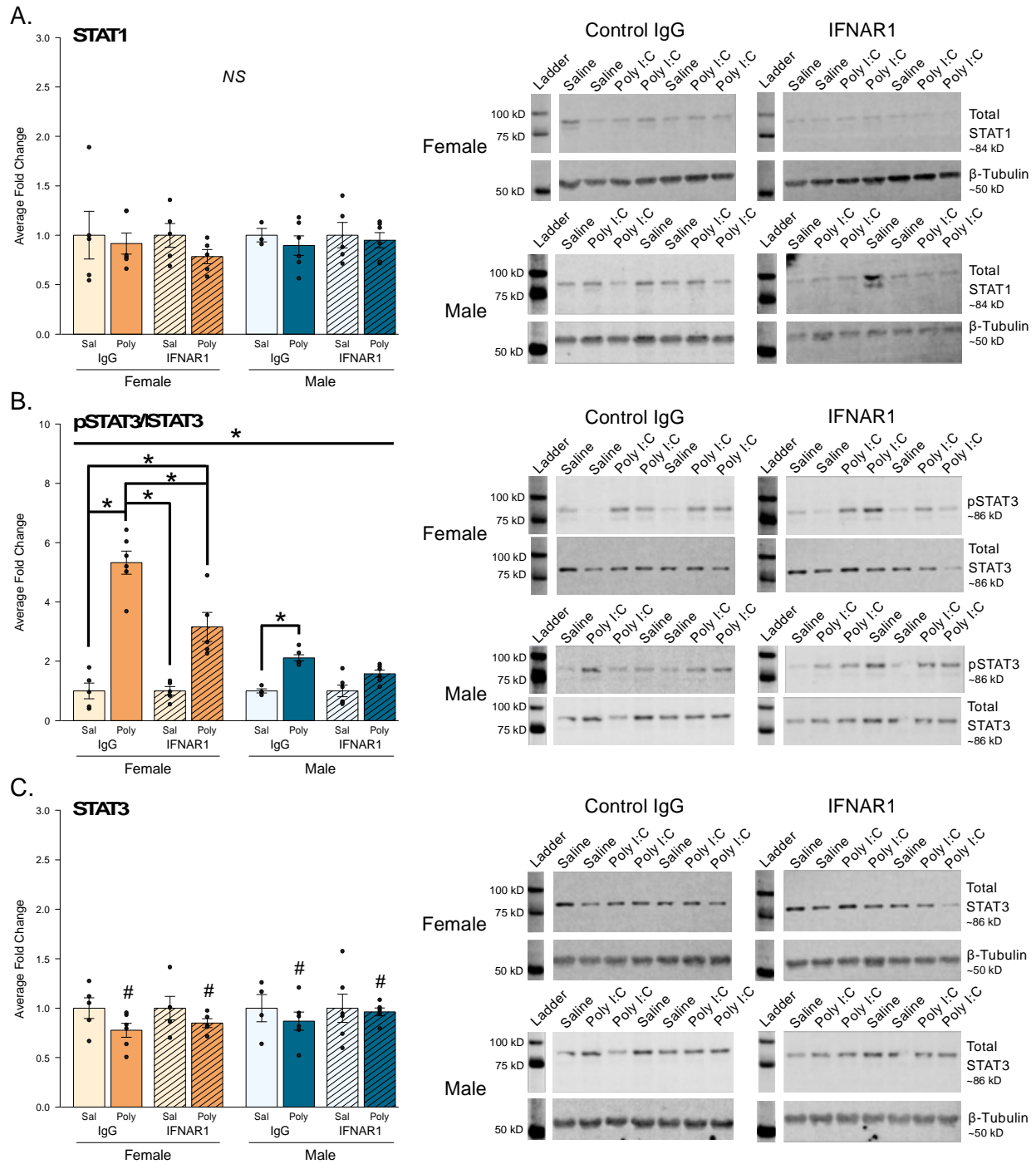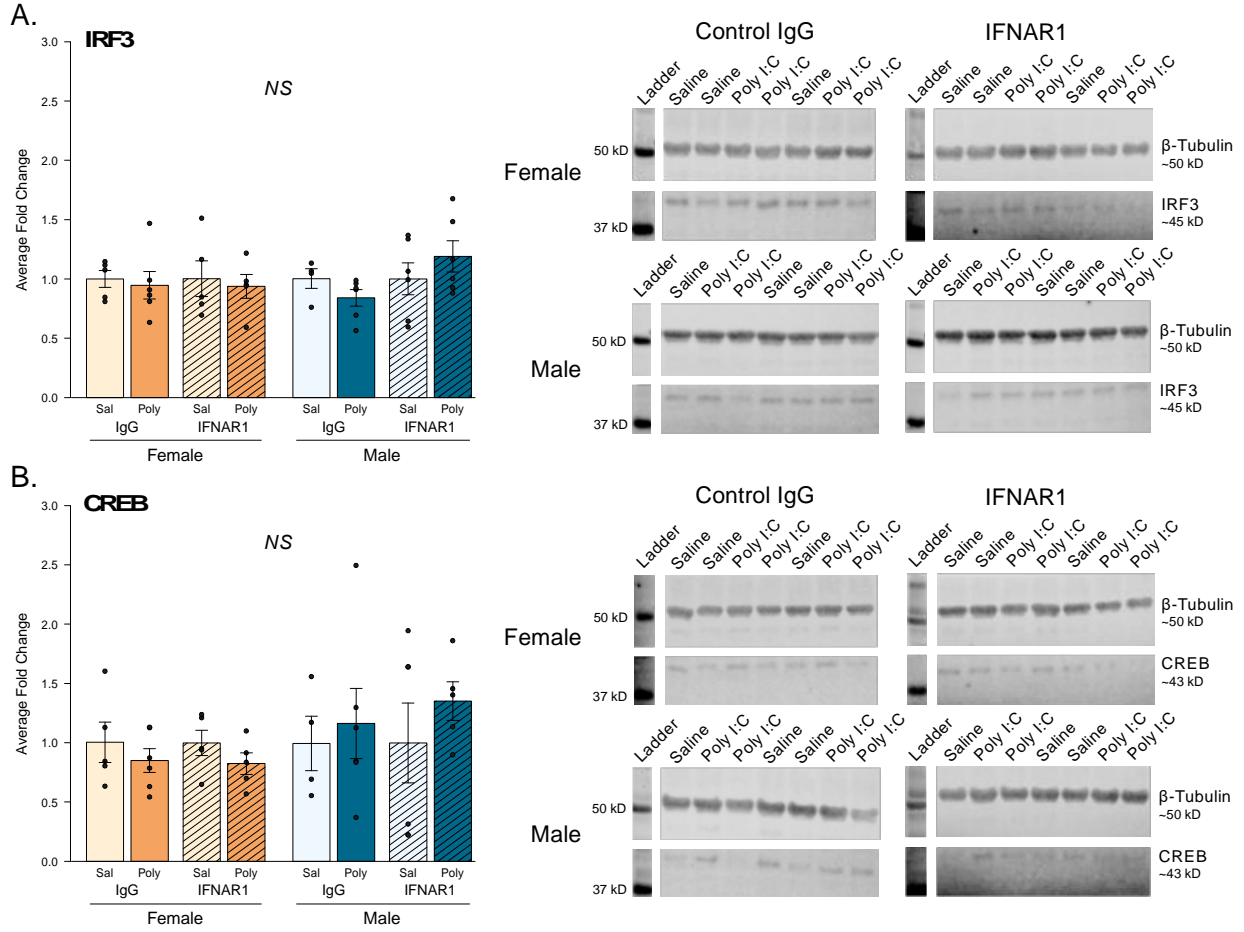**Figure 4.8** *Effects of IFNAR1 and poly I:C on IRF3 and CREB*

Average fold change in protein levels with associated representative western blot images for (**A**) total IRF3, and (**B**) total CREB 4 hours following poly I:C administration with either IgG or saline given 2 hours pre-poly I:C treatment. *NS* indicates no statistical significance. Error bars represent standard error of the mean.

Chapter 5:

Discussion

5.1 Summary of Experimental Findings

The work in this dissertation aimed to develop a better understanding of the interactions between neuroimmune activation and learning and memory processes in both males and females. To do this, we used central administration of a synthetic viral mimic, poly I:C, to stimulate the innate immune system in the brain and coupled it with hippocampal-dependent learning and memory tasks.

First, we characterized the sickness and hippocampal neuroimmune responses to central poly I:C in both males and females. There are several examples of sex differences in immune and neuroimmune function in the literature, and the direction and magnitude of those differences depends on the type of insult as well as route of administration (Coelho et al., 2021; Klein, 2012; Klein & Flanagan, 2016; Speirs & Tronson, 2018). Therefore, we needed to first define the response to poly I:C in both sexes to move forward with our experiments. We found that poly I:C caused an acute fever response and significant weight loss in males and females that recovered to saline-treated levels within 48 hours. We also found that poly I:C mounted a significant cytokine response in the hippocampus. Specifically, several inflammatory cytokines showed increased mRNA expression and protein levels shortly after treatment, with a peak in levels at the 4-hour timepoint and recovery to saline-treated levels by 24 hours in both sexes, with a few notable exceptions.

At baseline, females showed greater expression of several cytokines in the hippocampus compared with males, including *il-6*, *ifnα*, *ifnγ*, and *ccl2*. Importantly, none of the cytokines showed higher expression in males at baseline. Following stimulation with poly I:C, however, we found that males showed a much greater magnitude of mRNA gene expression response than females, and this was true for *il-1α*, *il-1β*, *il-6*, *il-10*, *ifnα*, *tnfα*, *ccl2*, and *cxcl10*. In fact, *il-10* and *ifnα* did not respond to poly I:C at all in females. Finally, while most cytokines showed peak expression 4 hours following poly I:C administration, this was not true for *cd11b*, *gfap*, or *ifnβ*. Rather, the markers for microglia and astrocyte activation, *cd11b* and *gfap*, only showed increased expression at the 24-hour timepoint, and one of the anti-viral Type I interferons, *ifnβ*, showed the earliest peak at 2 hours post-treatment. These data revealed both sex differences and similarities in the neuroimmune response to poly I:C (Posillico et al., 2021), so we next aimed to determine whether this had functional consequences on learning and memory.

We used both pre- and post-training treatments with poly I:C to test the effects of neuroinflammation on learning and memory consolidation mechanisms in males and females. First, we used a hippocampal-dependent contextual fear conditioning task and found that administration of poly I:C 4 hours prior to training in the context disrupted learning in both sexes. Because the effects of poly I:C last beyond this 4-hour timepoint, this finding could have been due to disruption of memory consolidation mechanisms instead of, or in addition to, learning or acquisition processes. When we administered poly I:C immediately after training, only males showed a deficit when they were tested 72 hours later. This design still does not allow us to capture the effects of neuroimmune activation in the immediate post-training window, as we did not see appreciable increases in fever, cytokine expression, or cytokine protein levels until the 2-hour timepoint. However, it does suggest that there is a sex difference

in the window of vulnerability for neuroimmune activation to disrupt learning and memory in that both sexes are sensitive during the learning and early consolidation period, but only males are vulnerable to disruptions that occur more than 2 hours following training. Our experiments did not further investigate the memory consolidation period to characterize this window of vulnerability in both sexes more specifically, though this is an interesting area of research for future studies.

To examine how the hippocampus responded to poly I:C alone and in combination with training in context fear conditioning, we measured levels of cFos in the CA1, CA3, and dentate gyrus subregions. In all three regions, training in the task caused a significant increase in the number of cFos-positive cells compared to naïve animals, as expected. Interestingly, poly I:C during training had both sex- and subregion-specific effects. In the CA1, pre-training poly I:C attenuated the training-induced cFos in males and had no effect in females. In the CA3, pre-training poly I:C potentiated the training-induced cFos in females, and again appeared to attenuate training-induced cFos in males, though this was not statistically significant. Finally, in the dentate gyrus, pre-training poly I:C attenuated training-induced cFos in both sexes. These data indicate that while pre-training poly I:C resulted in similar learning deficits in males and females during context fear conditioning, it appears to be *via* distinct underlying hippocampal mechanisms.

We did not find any effects of either training or poly I:C treatment on microglia morphology in the hippocampus of males or females as a proxy for changes in microglia functional states. However, we did find that in both the CA1 and CA3 subregions, male microglia had decreased measures of fractal dimension and increased lacunarity relative to female microglia. These measures suggest that there may be sex differences in microglia

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morphology in more homeostatic states. Whether these morphological changes correspond to inherent differences in function is not presently known, but it is certainly worth further investigation, particularly given that we found male cytokine responses to poly I:C to be much greater in magnitude compared to females in our experiments. Perhaps this is a result of different sensitives of neuroimmune cells such as microglia to particular insults that would result in sex differences in vulnerability of neuroimmune disruption of cognitive processes.

In addition to context fear conditioning, we also tested whether pre-training poly I:C disrupted a multiple memory systems task in an escape-motivated T-maze. For this task, mice can either rely on hippocampal encoding of spatial cues during training and use a "place-based" learning strategy or rely on the dorsal striatum to encode stimulus-response associations between the cross in the maze and a particular body turn and use a "response-based" strategy (Kleinknecht et al., 2012; Morris et al., 1982; Packard, 1999; Packard & McGaugh, 1996; Tolman et al., 1946). Given the flexible strategies available to the animal, it is perhaps not surprising that pre-training poly I:C did not prevent either males or females from learning the task or change the rate of learning. Rather, during the probe test where we assessed what type of learning strategy the mice employed, we found that poly I:C removed the bias for learning strategy seen in saline-treated animals. Specifically, a greater proportion of saline-treated females showed that they used a response-based strategy, and a greater proportion of saline-treated males showed that they used a place-based strategy. However, there was no clear bias for one strategy or the other in the poly I:C-treated groups. Again, these data uncovered nuance in the sex-specific neuroimmune modulation of learning and memory, where pre-training poly I:C does impact learning strategy in both sexes but *via* distinct neurobiological mechanisms.

The final set of experiments aimed to uncover the sex-specific mechanisms at play during neuroimmune modulation of memory. Our first target was signaling from the chemokine CXCL10, as this showed an astounding sex difference in magnitude of expression response to poly I:C. Specifically, females showed peak expression 4 hours post-treatment that was 40-fold greater than saline-treated female controls, and males showed peak expression at the 4-hour timepoint that was, on average, nearly 500 times greater than the saline-treated male controls. Given that CXCL10 has been shown to modulate mechanisms of synaptic plasticity (Kodangattil, Möddel, Müller, Weber, & Gorji, 2012; Nelson & Gruol, 2004; Vlkolinský et al., 2004), we hypothesized that sex differences in poly I:C-induced CXCL10 expression may result in sex-specific synaptic plasticity changes to disrupt memory. However, when we tested whether exogenous administration of CXCL10 impacted learning or memory consolidation during context fear conditioning in males, we did not find any effects. It is possible that the dose of CXCL10 we administered (100ng) was insufficient to match the massive increases in CXCL10 expression and protein levels induced by poly I:C, and it is possible that much greater levels of CXCL10 would be necessary to induce learning or memory consolidation disruptions. It is also possible that regardless of dose, CXCL10 alone is not sufficient to produce learning or memory consolidation deficits. Nevertheless, we moved forward in testing other targets that may be underlying the neuroimmune modulation of learning and memory in our mice.

Our next target of interest was in the anti-viral Type I interferon signaling. IFNα and IFNβ exhibit important anti-viral functions in acting to reduce viral replication during the acute stage of infection (Basler & García-Sastre, 2002; Isaacs & Lindenmann, 1988). Additionally, IFNα and IFNβ have individually been implicated in synaptic plasticity mechanisms including long-term potentiation, neuronal excitability, and interactions with glutamatergic NMDA

receptors (Costello & Lynch, 2013; Mendoza-Fernández et al., 2000). Given that we found sex differences in Type I interferon induction following poly I:C, where females only increased expression of IFNβ and males increased expression of both IFNα and IFNβ, we hypothesized that poly I:C would alter glutamatergic signaling in both sexes but in distinct ways that contribute to neuroimmune disruption of learning and memory.

Whole cell lysates from hippocampal tissue collected 4 hours after poly I:C administration did not reveal any changes in glutamate transporter 1 (GLT-1) protein and only two subtle changes to glutamate receptors. We found that levels of ionotropic glutamate receptor AMPA type subunit 2 (GluR2) were lower in poly I:C-treated females than they were in poly I:C-treated males, but we did not find any differences between poly I:C-treated and saline-treated groups within sex, so it is difficult to determine if this sex difference is functionally relevant. Further, we found a trend that suggested metabotropic glutamate receptor 5 (mGluR5) levels decreased in both sexes treated with poly I:C, but again, this may not translate in terms of functional consequences. These mostly negative results do not rule out the possibility that poly I:C is affecting glutamate transporter or receptor proteins in the hippocampus. It is likely that rather than affecting total amounts of these proteins, poly I:C is affecting how or whether they are trafficked into and out of the cellular membrane and therefore change their availability at the synapse, and whole cell lysates would not be able to capture these differences. Given the evidence that Type I interferons as well as other inflammatory cytokines, including IL-1β, IL-6, and TNFα, can modulate synaptic plasticity *via* changes in glutamatergic signaling (D'Arcangelo et al., 2000; Huang et al., 2011; Santello et al., 2011), it is worth exploring methods that can better examine changes in membrane-bound and cytosolic proteins separately

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to answer the question of whether poly I:C modulates glutamate receptors and whether this is similar or different in males and females.

Despite the limitations to our methods for studying glutamate receptors and glutamate transporter, we were able to detect significant increases in phosphorylated STAT3 protein 4 hours following poly I:C treatment in both males and females. STAT3 is a transcription factor that can be phosphorylated and activated by signaling from the inflammatory cytokine IL-6. We already found marked increases in IL-6 at this timepoint post-poly I:C, so it is not surprising that we saw increases in phosphorylated STAT3 as a result of poly I:C here. It does, however, verify that poly I:C was still having a significant inflammatory effect in this experiment, so this finding is an important positive control for these data.

We tested the role of Type I interferon signaling specifically in these effects using a Type I interferon receptor (IFNAR1) inhibitor prior to poly I:C treatment. Notably, inhibition of IFNAR1 attenuated the increases in IL-6 gene expression and also attenuated the phosphorylated STAT3 increases as a result of poly I:C. IL-6 has previously been shown to be modulated, in part, by Type I interferons (Murray et al., 2015), so again, these data are not necessarily surprising. However, they do indicate that poly I:C and the Type I interferon inhibitor were working as expected in these experiments.

Finally, to determine whether Type I interferon signaling contributed to the neuroimmune disruption of learning and memory in males and females, we administered the receptor inhibitor prior to treatment with poly I:C and trained animals in context fear conditioning. During testing, we found that only males showed attenuation of the poly I:C-induced learning deficits when pre-treated with the inhibitor. We did not find the same effects in females, suggesting that Type I

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interferons are more involved in the neuroimmune disruption of learning and memory consolidation in males than they are in females.

5.2 Microglia and Astrocytes: Where Are They Now?

Neuronal communication is modulated by two, separate, yet equally important cells: astrocytes that regulate and contribute extracellular glutamate, and microglia that can alter the stability and plasticity of synapses. Poly I:C is a synthetic viral mimic that has been shown to successfully activate the innate immune receptor Toll-like receptor 3 (TLR3) that is expressed on both microglia and astrocytes. However, we did not find acute activation of either cell type using the methods in these studies. How may they still be contributing to this system?

Markers of traditional microglia "activation" include increases in expression of the molecule CD11b and changes in morphology from a ramified shape with long, thin processes to an amoeboid shape with short, stout processes (Akiyama & McGeer, 1990; Karperien et al., 2013). CD11b has been shown to interact with intracellular adhesion molecules and is involved in the ability for microglia to actively move their processes to survey the microenvironment under homeostatic conditions. Upon microglia activation, increases in CD11b are correlated with significant changes in microglia morphology (Akiyama & McGeer, 1990; Roy, Fung, Liu, & Pahan, 2006). We looked at CD11b expression over the course of 24 hours following poly I:C administration, and we only found significant increases at the 24-hour timepoint in both sexes. In our study, we did not find any changes in microglia morphology in the hippocampus 5.5 hours following poly I:C treatment or 90 minutes following training in context fear conditioning. However, perhaps this is because we did not find associated increases in CD11b expression around this same timepoint. Our findings of delayed CD11b increases are consistent with a previous study in which microglia activation with lipopolysaccharide *in vitro* did not result in

increases of CD11b mRNA levels until 12 hours following treatment (Roy et al., 2006). While

this experiment did not examine microglia morphology, primary cultured microglia still

significantly increased expression of IL-1b within 6 hours of lipopolysaccharide treatment

despite not seeing increases in CD11b until 6 hours later. Similarly, a more recent study found

that following poly I:C administration, microglia *in vitro* retained a "bushy" (rather than

"amoeboid") morphology while still producing and releasing cytokines in response to treatment

(He et al., 2021). As such, neither increases in CD11b expression nor changes in morphology are

sufficient to determine whether microglia have been "activated" and are producing inflammatory

cytokines in response to innate immune receptor stimulation.

Morphology was originally used as a marker for "activation" because different

morphologies were associated with different functional states of microglia (Karperien et al.,

2013). For example, "resting" or "surveilling" microglia morphologies were associated with

more homeostatic functions and expression of cytokines that reduce inflammation, while

"active" morphologies were associated with active infections and inflammatory cytokines (Y.

Tang & Le, 2016). Although today's evidence suggests that microglia morphology is not

sufficient to determine its functional state, it is possible that stimulated microglia paired with

altered morphology still represents a unique functional state compared with both homeostatic

states and acute inflammation in the absence of morphological changes. We did not examine

morphology of microglia at the 24-hour timepoint when we saw increased CD11b expression in

both males and females, nor did we examine CD11b expression beyond 24 hours to determine

the point at which (if at all) it returned to baseline levels. However, we also did not test animals

in context fear conditioning until 72 hours following poly I:C administration, meaning this

increased CD11b and possible change in microglia functional state occurred during the memory

consolidation window for context fear conditioning. It is possible that extended microglia stimulation continues to affect mechanisms of synaptic plasticity required for proper memory consolidation and is playing a role in poly I:C-induced learning deficits that the endpoints we have examined here could not capture.

While we did not find any effects of poly I:C treatment on acute expression of CD11b or any effects of either poly I:C or training in context fear on microglia morphology, we did find evidence that suggested male microglia showed less complex branching and more self-similarity compared to female microglia in the hippocampus. Interestingly, this is not coupled with any baseline differences in CD11b expression between males and females. Rather, it is possible that these analyses reveal subtle sex differences in microglia morphology that may go undetected with more subjective and hand-scored measures of morphological analysis and, again, are not reflective of microglia "activation" in the traditional sense of the term (Karperien & Jelinek, 2015). Alternative measures must therefore be used to fully characterize microglia function in these experiments, and our data in combination with others suggests the need to move away from these measures of microglia "activation" and be more intentional and specific with what we mean by "activated" when referring to microglia moving forward.

Like microglia, there are measures for analyzing astrocyte activation that include both increases in molecular signals as well as morphological changes (Escartin et al., 2021; Liddelow & Barres, 2017; Pekny & Nilsson, 2005). In these experiments, we only examined gene expression of glial fibrillary acidic protein (GFAP), a known and commonly used marker of astrocyte activation following injury, infection, or stress (Brahmachari, Fung, & Pahan, 2006; Dubový, Klusáková, Hradilová-Svíženská, Joukal, & Boadas-Vaello, 2018; Pekny & Pekna, 2014; S. Zhang, Wu, Peng, Zhao, & Gu, 2017). Similar to the results we found for CD11b

expression, we also only found significant increases in GFAP expression 24 hours following poly I:C administration. However, when looking more closely at the data, it appears as though males showed on average a ~50-80% increases in GFAP expression at the 2- and 4-hour timepoints, respectively, compared to the saline-treated group. Post-hoc tests following this time x treatment interaction here did not show any significant differences, although it is highly likely that we did not have sufficient power to detect anything more subtle given the conservative nature of Bonferroni corrections and the high number of comparisons that were run in this analysis. If males did, in fact, have more activated astrocytes by the 4-hour timepoint following poly I:C treatment, this would support data in the literature that shows male-derived astrocytes have greater activation and inflammatory potential than astrocytes derived from female brains (Astiz et al., 2014; Loram et al., 2012; Santos-Galindo et al., 2011). It is also possible that a sex difference in the acute response of astrocytes to poly I:C contributes to the sex difference in the magnitude of cytokine response and/or the sex difference in Type I interferon expression following poly I:C.

Recent evidence shows that astrocytes are capable of *de novo* synaptic potentiation and memory enhancement (Adamsky et al., 2018), and there is significant evidence that supports astrocytic modulation of glutamatergic signaling (Feng et al., 2015; Xia et al., 2013). Perhaps sex differences in the recruitment of astrocytes during the acute phase of poly I:C infection result in astrocyte-driven changes in synaptic properties that differentially primes the hippocampus for disruption of learning and memory during acute neuroinflammation. As such, it is important that future experiments more explicitly and specifically examine both astrocytes and microglia function in the hippocampus during neuroimmune disruption of learning to better elucidate the possible mechanisms at play in both sexes.

5.3 Implications for Sex Differences in Neuroimmune Modulation of Training-Induced cFos

In the hippocampus, training in context fear conditioning with poly I:C on board had both sex- and subregion-specific effects on the number of cFos-positive cells used as a proxy for neuronal activation. In the dentate gyrus, poly I:C attenuated the training-induced cFos in both sexes. In the CA3, poly I:C potentiated training-induced cFos in females but not males, and in males, pre-training poly I:C may have even attenuated training-induced cFos. Lastly, in the CA1, poly I:C had no effect on cFos in females, but it significantly reduced training-induced cFos levels in males. Each of these subregions comprise the well-studied tri-synaptic hippocampal circuit in which information flows from the entorhinal cortex to the dentate gyrus, then from the dentate gyrus to the CA3 *via* mossy fibers, and then from the CA3 to the CA1 *via* Schaeffer collaterals. However, each individual subregion has been implicated in diverse aspects of learning and memory, particularly for contextual fear condition (Bernier et al., 2017; Daumas et al., 2005; Hernández-Rabaza et al., 2008; Tronson et al., 2009), and the snapshots of each subregion presented here cannot give us answers to whether or how communication through the hippocampal circuit may be modulated by poly I:C, nor can it tell us how specific information from the experience may be altered (or not) during encoding (Guzowski, Knierim, & Moser, 2004; Hunsaker, Mooy, Swift, & Kesner, 2007). This is particularly relevant considering that the tri-synaptic circuit is not the only mode of communication within the hippocampus (Dolleman-Van Der Weel & Witter, 1996; Ishizuka, Weber, & Amaral, 1990; Le Duigou, Simonnet, Teleñczuk, Fricker, & Miles, 2014; Soltesz & Losonczy, 2018; Treves & Rolls, 1992; Witter, Griffioen, Jorritsma-Byham, & Krijnen, 1988). That being said, the major output region of the hippocampus proper is the CA1 which projects, in large part, to the subiculum (Amaral, Dolorfo,

& Alvarez-Royo, 1991), and from there, the subiculum has connections to cortical and subcortical structures important for information processing during experience-dependent synaptic plasticity (N. Matsumoto, Kitanishi, & Mizuseki, 2019; O'Mara, 2005; L. W. Swanson & Cowan, 1977; Witter, Ostendorf, & Groenewegen, 1990). Therefore, it is possible that the attenuated training-induced cFos from poly I:C in the CA1 subregion in males only may have important implications for neuroimmune modulation of information processing in the subiculum that gives rise to the learning deficits we see here. Further investigation of CA1-subiculum connections and output from the subiculum during neuroinflammation would shed more light on this potential mechanism.

Given all of the ways that neuronal activation is sensed and used by microglia and astrocytes to modulate their functions and synaptic plasticity mechanisms, it is possible that the impact of sex- and subregion-specific differences in training-induced cFos might extend to changing functions of these important cells in different ways in each subregion. An additional layer of sex differences within these data is that males showed greater training-induced cFos relative to females in both the dentate gyrus and CA1, and this appeared to be driven by the saline-treated groups, suggesting the potential for baseline sex differences in neuronal sensitivity. This might be dictated by sex differences in baseline microglia or astrocyte function, and we have some evidence from microglia morphology data that suggests inherent differences in male and female microglia which may subsequently reflect differences in function. While speculative, an additional question we could be asking is, if they are true, whether these levels of sex differences are meaningful. Perhaps under normal, healthy, and homeostatic conditions, such nuanced sex differences do not play a significant role, but it is possible that these sex differences create conditions that precipitate significant and possibly detrimental differences in disease states

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that affect memory and cognition. Therefore, understanding the functional consequences, if any, of these findings is an important goal for future research to describe.

5.4 Sex-Specific Influences of Type I Interferons on Learning and Memory?

In Chapter 4, we did not replicate the same effect of poly I:C on learning in context fear conditioning in females that we saw in Chapter 3 (Figure 3.1 vs Figure 4.2). However, in comparing the data from these experiments, there is a noticeable difference in the freezing levels between the saline-treated females in Chapter 3 and the IgG/saline-treated females in Chapter 4, while the freezing levels in poly I:C-treated groups appears to be similar. In males, there are more comparable levels of freezing between the control and poly I:C-treated groups across experiments. This begs the question: did we fail to replicate the poly I:C-induced learning deficit in females in Chapter 4 because females are less sensitive to neuroimmune activation, or is there something unique about the IgG/saline-treated control group that prevented us from detecting an effect of poly I:C?

We tested whether the isotype IgG control modulated the neuroimmune response to poly I:C by comparing saline and IgG pre-treatments with poly I:C administration in both sexes. Surprisingly, we found that the IgG/poly I:C-treated groups showed higher expression of IFNα relative to saline/poly I:C-treated groups, though this main effect seemed to be driven more by the females than the males in that females pre-treated with IgG showed nearly twice as much expression of IFNα, on average, than the saline pre-treated females (Figure 4.4C). Unfortunately, we did not test whether isotype IgG control alone induced cytokine expression changes to determine whether this increase in IFNα is due to the poly I:C and IgG combination or just due to the IgG. This means it is possible that the IgG pre-treatment, particularly in females, induces IFNα expression that may modulate some synaptic plasticity mechanisms (Mendoza-Fernández

et al., 2000) and create a more unique brain state that affects learning even in the absence of an immune stimulant (Tchessalova, Posillico, & Tronson, 2018). This could be one potential reason why the two control groups between Chapters 3 and 4 show different freezing levels during testing. Alternatively, we previously showed that poly I:C treatment induces a greater magnitude of hippocampal cytokine expression in males than females. Perhaps this lower "level" of neuroimmune response provides females with more protection of necessary processes for learning and memory consolidation that poly I:C happened to break past in our experiments in Chapter 3 but not in Chapter 4.

How does this impact what we can say about the role of Type I interferons in neuroimmune modulation of learning in males and females? We found that inhibiting Type I interferon receptors prior to treatment with poly I:C attenuated the poly I:C-induced learning deficit in context fear conditioning in male mice. Due to the fact that we could not replicate the poly I:C-induced learning deficit in females, we cannot make any reliable conclusions about Type I interferon receptor signaling for them here. However, it is notable that the biggest group difference in freezing levels for the females of this experiment is between the IFNAR1/saline-treated group and IFNAR1/poly I:C-treated group. Likewise, is also interesting that the IFNAR1/poly I:C-treated group is freezing, on average, less than the IgG/poly I:C-treated group. This suggests that, if anything, pre-treatment with IFNAR1 might potentiate detrimental effects of poly I:C or play a role in inducing learning deficits in females. If this is true, perhaps these data uncover more intricate sex differences in the functional role of Type I interferons in synaptic plasticity and learning and memory.

We initially hypothesized that because males induced "more" Type I interferons (both IFNα and IFNβ) as a result of poly I:C, that Type I interferon signaling might simply produce

"more" changes to glutamatergic signaling and that both sexes would still show dysregulated signaling that would affect learning. However, if blocking Type I interferon receptors during neuroinflammation contributes to *worse* learning outcomes in females and *better* learning outcomes in males, maybe the IFNβ that is increased from poly I:C is actually helpful for females and hurtful for males. Alternatively, IFNβ could be helpful for both females and males but the IFNα induced in only males as a result of poly I:C is what is detrimental for males and why blocking signaling from both IFNα and IFNβ only produces marginal benefits in males. Thus, this would no longer be a question of "more" or "less" Type I interferon. Instead, these data highlight separate and distinct functions of IFNα and IFNβ that, rather than working in tandem, may actually be working in opposition in the context of neuroimmune modulation of learning and memory. Another way to conceptualize these seemingly contrasting effects of Type I interferon signaling on learning and memory is to imagine males and females along an inverted U-shaped curve with memory performance or ability on the y-axis and neuroinflammation on the x-axis. Perhaps poly I:C treatment pushes both males and females to the right side of the curve to decrease memory performance as neuroinflammation increases, with males further to the right than females because they showed a higher magnitude of cytokine response to poly I:C. Then, when Type I interferon signaling is inhibited, both sexes are pushed left along the curve, but females land past the peak and remain at sub-optimal memory performance and males land closer to the peak with increased memory performance. Whether or not this is truly the mechanism at play here remains to be determined, but this is an important question to further investigate in future experiments, as it may outline a potential therapeutic target for ameliorating cognitive impairments from dysregulated neuroimmune signaling.

5.5 The Pressing Hunt for Female-Specific Mechanisms

Collectively, these data implicate Type I interferon signaling in contributing to learning deficits induced by neuroinflammation in males. Unfortunately, we did not identify a female-specific mechanism by which poly I:C disrupted learning in context fear conditioning in females, and the elusive female-specific mechanisms of memory modulation pose a public health concern for the future (Tronson, 2018; Tronson & Keiser, 2019).

Neuroinflammation and dysregulated neuroimmune function have been proposed as an underlying causal mechanism of dementia, including that from Alzheimer's disease (Heneka et al., 2015), and we are now seeing increasing evidence of long-lasting and severe cognitive impairments following infection from SARS-CoV-2 that causes Coronavirus disease 2019 (COVID-19; Bucciarelli et al., 2022). Women are more than twice as likely to develop dementia from Alzheimer's disease compared with men and also suffer from worse cognitive outcomes as a result (Henderson & Buckwalter, 1994; Laws et al., 2018). While it seems that there is little evidence for sex differences in susceptibility for initial infection with COVID-19, there is mounting evidence that women suffer from more long-term and cognitive-related complications following infection than men do, a syndrome called "long COVID" (Bai et al., 2022; Bucciarelli et al., 2022; Fernández-de-Las-Peñas et al., 2022). Together, these data suggest that there is something unique about how dysregulated neuroinflammation affects cognitive processes including learning and memory that leave women at greater risk for poorer cognitive outcomes in these cases.

One recent study discovered significant increases in the presence of human herpesvirus 6A and 7 in subjects with Alzheimer's disease compared with controls (Readhead et al., 2018), and another recently showed that Epstein-Barr virus may be significantly associated with a

diagnoses of Multiple Sclerosis later in life (Bjornevik et al., 2022). If previous infection with certain viruses increases the risk for developing neurodegenerative diseases including Alzheimer's later in life, it is possible that prior infection with COVID-19 of today's pandemic may result in a similar fate. With Alzheimer's disease and long COVID both showing more severe cognitive outcomes in women already, we are running against the clock to identify female-specific mechanisms by which neuroimmune and immune dysfunction impact cognitive processes if we have hopes of being able to better ameliorate the symptoms of these debilitating disorders in both women and men.

5.6 Approaching Future Research

It is crucial that we not only consider, but integrate, the multitude of factors involved in long-lasting plasticity mechanisms in the hippocampus, including functions of astrocytes and microglia at the tripartite synapse during both healthy conditions and neuroinflammation, as well as the sex-specific mechanisms of hippocampal function in order to delineate mechanisms of neuroimmune disruption of learning and memory in males *and* females.

To understand the role of neuroimmune signaling in synaptic plasticity, and modulation of memory during inflammation, there are several questions that need to be answered. Where are the key points of signal integration at the tripartite synapse, and how is this signal integration expressed? Which mechanisms of neuroimmune modulation of neuronal activity supersede others during acute or chronic neuroinflammation and thus would dictate the net effects of glutamate transporter trafficking or gliotransmission? In studies of neuroimmune modulation of learning and memory specifically, are the signals that activate astrocytes and microglia from neuronal activation similarly impacting the synapse as activation of astrocytes and microglia from neuroinflammation? How do the signals from acute or chronic neuroinflammation affect

the ability for microglia and astrocytes to interpret activity from neurons during experience-dependent plasticity and learning? Since astrocytes are capable of *de novo* synaptic potentiation and enhancement of memory (Adamsky et al., 2018), does activation of astrocytes by neuroinflammation prime neuronal synapses in such a way to uniquely modulate synaptic plasticity during a learning event?

One way to greatly extend the studies presented here and begin to answer some of these important lingering questions for future research is to use more extensive tissue processing techniques that provide increased specificity of the samples being analyzed. For example, to better assess how poly I:C may modulate the trafficking of glutamate receptors into and out of the cellular membrane, we could use a subcellular fractionation protocol to isolate membrane-bound, cytosolic, and nuclear protein samples for western blot analyses of the same glutamate receptors examined here. In addition, we could use cell sorting techniques to isolate populations of neurons, microglia, and astrocytes in the hippocampus following treatment with poly I:C and Type I interferon receptor inhibitors to determine which cell types may be more important for some of the sex differences in cytokine responses we found as well as whether changes in trafficking of glutamate transporter 1 (GLT-1) is specifically occurring in astrocytes as we have predicted. Data from these experiments may elucidate additional targets of communication between microglia, astrocytes, and neurons that modulate mechanisms of synaptic plasticity and prove to be critical for learning and memory.

5.7 Conclusion

Here, I have presented sex differences in the expression of anti-viral Type I interferons following central administration of a viral mimic in mice (Posillico et al., 2021). Both IFNα and IFNβ have been shown to separately modulate glutamatergic signaling and transporter

expression (Costello & Lynch, 2013; Mendoza-Fernández et al., 2000). Thus, sex differences in these cytokines might result in sex-specific changes to glutamatergic signaling that explain, at least in part, why pre-training poly I:C disrupts context fear conditioning in both sexes, but seemingly *via* different hippocampal mechanisms. Evidence from these experiments suggests that Type I interferons contribute to the neuroimmune disruption of learning in males, but a female-specific mechanism continues to evade us. Signal integration and transduction at the synapse between experience-dependent plasticity mechanisms and neuroimmune activation is likely sex-specific. Characterizing this in both males and females must be at the forefront of our continued research if we hope to make meaningful sense of the dynamic interactions between the neuroimmune and memory systems in both males and females.

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